Developments in Hydrobiology 219

Manuel Maldonado
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Ancient Animals, New Challenges

Developments in Sponge Research

Springer

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Developments in Hydrobiology 219

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Ancient Animals, New Challenges

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SPONGE RESEARCH DEVELOPMENTS

Preface: Sponge research developments

M. Maldonado · X. Turon · M. A. Becerro · M. J. Uriz

Published online: 28 February 2012

Since 1970, world-wide experts on virtually every aspect of sponge biology have met together once every 4–8 years to present and discuss the latest developments in sponge research. The diverse contributions to each meeting have been published together as monographic proceedings, each book establishing a landmark reference (often more than 500 pages) on Sponge Science, and contributing collectively (7 books) to the establishment of a meaningful tradition. This current book "Ancient animals, new challenges: developments in sponge research," published as a special volume of the international journal *Hydrobiologia*, has attempted to continue this tradition by collecting contributions presented to the VIII World Sponge Conference, held by the Centre d'Estudis Avançats de Blanes (CEAB-CSIC) in Girona, Spain in September 2010.

The Conference hosted a total of 270 attendants from 36 countries, who presented 354 contributions. The Scientific Program included the topics of Evolution and Phylogeny, Organism and Cell Biology, Population Biology, Ecology, Natural Products, Sponges and Society, and Taxonomy. The present volume includes a subset of this research (27 articles), and hopefully will offer a window to the forefront of Sponge Science and its implications in Marine Life Science. The collection of articles reflects hot, ongoing debates in molecular research, such as the monophyletic versus paraphyletic nature of the sponge group, or the new awareness on pros and cons of standard barcodes and other markers in sponge taxonomy and phylogeny. It also features articles showing how the new sequencing technologies reveal the functional and phylogenetic complexity of the "microbial universe" associated to sponge tissues. The ecological interactions of sponges, the effects of nutrients and pollutants, the variability in reproductive patterns, and the processes generating genotypic and phenotypic variability in sponge populations are also covered in several contributions. Zoogeography, population structure and dynamics are also approached with both traditional and molecular tools. The effect of anthropogenic disturbance on the natural environment also finds its place in this volume, with papers dealing with metal accumulation and the potential role of sponges as biomonitors. Biodiversity data from unexplored tropical and deep sea areas are also presented.

Because the number of papers included in the volume is relatively low compared to the total of contributions to the Conference, it can be argued that we, the Guest Editors, missed the tradition of getting the bulk of the Conference published. Massive publication would only have been possible under the

Guest editors: M. Maldonado, X. Turon, M. A. Becerro & M. J. Uriz / Ancient animals, new challenges: developments in sponge research

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preceding format of monographic books, which has the potential drawback that all those relevant contributions are not indexed by conventional bibliographic databases, and are thus not readily available to interested readers. Publishing through *Hydrobiologia* guarantees wide diffusion and rapid accessibility. Nevertheless, we acknowledge a bias in the volume content relative to the wider richness and diversity of approaches presented to the VIII World Sponge Conference. The scope of the journal clearly focuses on molecular and experimental, hypothesis-driven studies, and this non-negotiable criterion has left out some solid descriptive work on biology, ecology, and taxonomy. We strived to find a compromise between our wish to see many of the papers presented in the Conference published and the serious limitations of space and scope inherent to an international journal such as *Hydrobiologia*. Honestly, we experienced a bittersweet feeling when seeing the book progressively growing with excellent contributions while we were aware that important work fell outside the scope of this journal and could not be accommodated in this volume. Our apologies if some "regular" contributors to the previous seven Sponge Conference Proceedings may have felt frustrated with the process of manuscript selection. Yet we hope the readers will enjoy the following selection of papers, which we believe represent collectively a significant contribution to our current understanding of sponges.

We would also like to give our explicit thanks to the sponsoring organizations, the people who helped in the organization of the Conference, keynote speakers and all participants, in particular those who submitted manuscripts to be considered in this volume, as well as the constructive reviewers who helped to get the best out of the manuscripts. We also thank Dr. Koen Martens, Editor-in-Chief of *Hydrobiologia*, who made an undeniable extra-effort to seek for excellence in all aspects of the manuscripts.

Finally, we want this volume to be a tribute to the several colleagues who passed away recently, having left behind outstanding pieces of work that have inspired several generations of students and researchers. Our homage to Dr. M[a]. Antonia Bibiloni and Professors Michelle Sara, Max Pavans de Ceccatty Patricia Bergquist, Solange Peixinho, Peter Murphy, and Lidia Scalera-Liaci. Their friendship, either if grown through close, daily work or emerged through sporadic mailing or conference contact, will always remain in our memories.

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 Short Research Note

No longer Demospongiae: Homoscleromorpha formal nomination as a fourth class of Porifera

Eve Gazave · Pascal Lapébie · Alexander V. Ereskovsky · Jean Vacelet · Emmanuelle Renard · Paco Cárdenas · Carole Borchiellini

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Abstract Over the past few years, there has been growing interest among the sponge community in the phylogenetic position of the Homoscleromorpha (i.e. within or outside the class Demospongiae). Recent molecular analyses clearly show that the Homoscleromorpha forms a distinct clade separated from the Demospongiae and is composed of two families, Oscarellidae and Plakinidae. Within the currently more widely accepted hypothesis of a monophyletic Porifera, we formally propose here to raise Homoscleromorpha to the class rank (the fourth one). We, therefore, provide a definition and a formal diagnosis. In the supplementary materials, we also present an alternative classification of the Homoscleromorpha, following the *PhyloCode*.

Keywords Porifera · New class · Homoscleromorpha · Oscarellidae · Plakinidae · *PhyloCode*

Electronic supplementary material The online version of this article (doi:10.1007/978-94-007-4688-6_2) contains supplementary material, which is available to authorized users.

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Past and current systematics

For two decades now, phylogenies using genetic and morphological data have provided crucial information toward resolving sponge systematics (Erpenbeck & Wörheide, 2007). While the phylogenetic status of Porifera (monophyly vs. paraphyly) is still debated (Philippe et al., 2009; Sperling et al., 2009), internal phylogenies for the major sponge groups (i.e. the three Recent classes: Demospongiae, Calcarea, and Hexactinellida) are becoming better resolved (Borchiellini et al., 2004; Dohrmann et al., 2006, 2008). However, the affinities and rank of Homoscleromorpha remain unresolved within the Linnaean classification (Muricy, 1999; Muricy & Diaz, 2002).

Traditionally, Homoscleromorpha has been classified as a family or a suborder of the subclass Tetractinellida, within the class Demospongiae, mainly due to the shared presence of siliceous tetractinal-like calthrop spicules (Lévi, 1956). Lévi (1973) later proposed classifying them as a distinct subclass of the Demospongiae. Until 1995, two families were recognized within the Homoscleromorpha, Plakinidae Schulze, 1880 and Oscarellidae Lendenfeld, 1887, distinguished by the presence or absence of the mineral skeleton, respectively. However, in 1990, the discovery of a skeleton-less *Corticium*-like species led Solé Cava et al. (1992) to propose the rejection of the family Oscarellidae. Later, when this species was described as a member of a new genus *Pseudocorticium* (Boury-Esnault et al., 1995), all homoscleromorph genera were merged into a single family, the Plakinidae. *Pseudocorticium* is indeed devoid of a mineral skeleton like the genus *Oscarella*, but is more similar in histological traits (notably the leuconoid aquiferous system and a well-developed ectosome with cortex) to the spiculate genus *Corticium*.

Nowadays, according to the two current synopses of the poriferan classification, *Systema Porifera* (Hooper et al., 2002) and the *World Porifera Database* (http://www.marinespecies.org/porifera/; Van Soest, 2011), Homoscleromorpha is a subclass of the class Demospongiae, containing one order Homosclerophorida Dendy, 1905, one family Plakinidae Schulze, 1880, and seven genera: *Oscarella* Vosmaer, 1884; *Plakina* Schulze, 1880; *Plakortis* Schulze, 1880; *Plakinastrella* Schulze, 1880; *Corticium* Schmidt, 1862; *Pseudocorticium* Boury-Esnault et al., 1995; *Placinolopha* Topsent, 1897.

New insights from molecular phylogenies/phylogenomics studies

Since these two synopses were compiled, molecular phylogenies have challenged these traditional classification schemes. Indeed, in 2004, the first molecular phylogeny of an extensive sampling of Demospongiae *sensu lato* (based on 18S and 28S rDNA) suggested that Homoscleromorpha should form a clade on its own, clearly separated from the rest of the Demospongiae (Borchiellini et al., 2004). Subsequent phylogenic/phylogenomic studies using several nuclear markers corroborated this hypothesis and suggested a sister-group relationship of Homoscleromorpha and calcareous sponges (Calcarea, also known as Calcispongia) (Dohrmann et al., 2008; Philippe et al., 2009; Pick et al., 2010) (Fig. 1). Alternatively, homoscleromorphs were recovered as the sister group of Eumetazoa, albeit with low statistical support (Sperling et al., 2009). Although analysis of complete mitochondrial genomes seemingly supported the traditional placement within the Demospongiae (Lavrov et al., 2008; Wang & Lavrov, 2008), these studies were hampered by a lack of data from Calcarea, preventing a true test of the phylogenetic position of Homoscleromorpha. In any case, the number of homoscleromorph species included in the above-mentioned studies was very low (one or two).

Recently, molecular phylogenetic taxon sampling of Homoscleromorpha was substantially improved to include 18S, 28S rDNA sequences and mitochondrial genomes of six of the seven presently described genera (Gazave et al., 2010a). This study greatly contributed to resolve internal relationships of the group, restored the supra-generic classification of Homoscleromorpha abandoned in 1995 (Boury-Esnault et al., 1995), and reinstated the families Oscarellidae and Plakinidae on the basis of molecular and morphological evidence (Fig. 1). Uncertainties remain concerning the monophyly of *Oscarella* but it clearly appears that *Corticium* and *Plakinastrella* are monophyletic genera. The *Plakina* issue is more challenging and calls for further detailed molecular investigations (Fig. 1).

Linnaean classification of Homoscleromorpha

Taking into account the recent molecular studies, we consider that it is now well-established that Homoscleromorpha is not closely related to other demosponges. We feel that these new insights should be reflected in the Linnaean classification. In this classification system, the rank of both Homoscleromorpha and Calcarea is directly dependent on the phylogenetic status of Porifera. The most complete and robust molecular study of Porifera to date clearly supports the hypothesis of its monophyly, a hypothesis that is also consistent with morphological characters (Philippe et al., 2009; Pick et al., 2010). We thus formally propose, in the present paper, to raise Homoscleromorpha from subclass within Demospongiae to a fourth Recent class of Porifera (there is also a fifth extinct class of Porifera, the Archaeocyatha e.g. Debrenne et al., 2002).

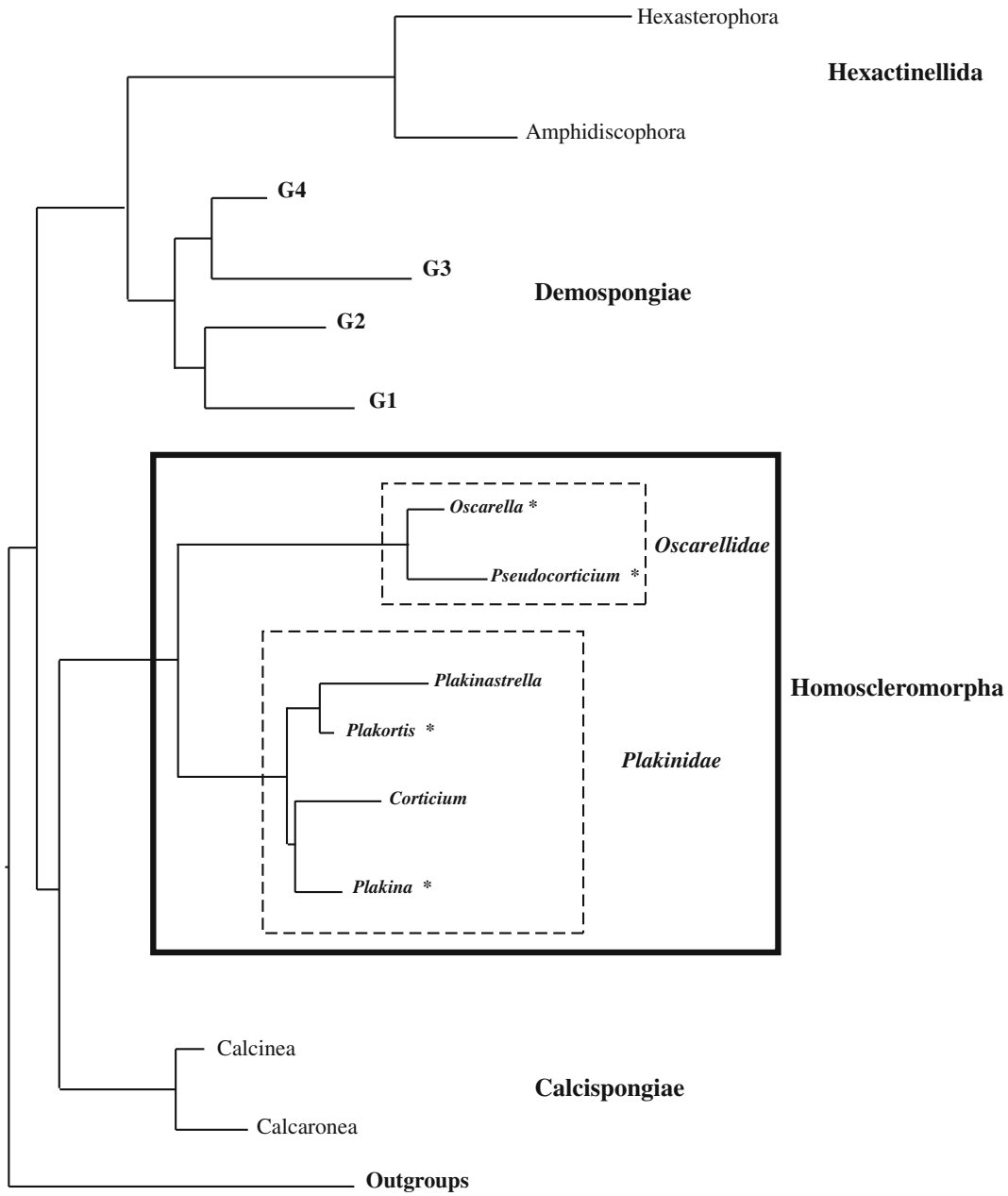

Fig. 1 Porifera simplified tree, following the monophyly hypothesis, mentioning the Linnaean names of the Homoscleromorpha families and genera. Homoscleromorpha genera that are not monophyletic or from which uncertainties subsist are identified with an *asterisk*

Definition (modified from Hooper & Van Soest, 2002)

Class Homoscleromorpha Bergquist, 1978

Other names. Microsclerophora Sollas, 1885. Carnosa Carter, 1875.

Definition

Porifera with cinctoblastula larvae and embryonic incubation; flagellated exo- and endopinacocytes; a basement membrane lining both choanoderm and pinacoderm; skeleton, if present, composed of

tetraxonic siliceous spicules–calthrops- and its derivatives with equal rays (diods, triods, and lophate spicules), arranged around oval to spherical choanocyte chambers reflecting the canal structure (sylleibid-like or leuconoid organization); no differentiation between megascleres and microscleres although size differences do occur between types of spicules; spicules usually small (100 μm or less), not localized in any particular region; choanocyte chambers with large choanocytes.

Remark

Order Homosclerophorida Dendy, 1905 has the same definition as the class Homoscleromorpha (except for the fact that it concerns the Demospongiae instead of the Porifera).

Formal diagnosis (in complement to Muricy & Diaz, 2002)

Diversity and area distribution

Homoscleromorphs are a small group of exclusively marine sponges. Among the 84 presently described species, they are 16 species of *Oscarella*, one of *Pseudocorticium*, 6 of *Corticium*, 6 of *Placinolopha*, 25 of *Plakina*, 11 of *Plakinastrella*, and 19 of *Plakortis*. They are encrusting or lumpy with a smooth surface, usually occurring at shallow depths. Recently, several new species have been described from various areas (from Brazil to Alaska, Africa and Indo-Australian coasts). They mainly pertain to the *Oscarella* and *Plakortis* genera (Moraes & Muricy, 2003; Muricy & Pearse, 2004; Ereskovsky, 2006; Ereskovsky et al., 2009a; Muricy, 2011; Pérez et al., 2011).

Skeleton

If present, it consists of small calthrops (peculiar type of tetractines spicules) and/or their derivatives (lophose calthrops, diodes and triodes). These spicules are evenly distributed in the sponge body and do not form a well-organized skeleton. Spicules are secreted by sclerocytes, pinacocytes of the external epithelium and also, to a lesser degree, by pinacocytes of the internal epithelia (Maldonado & Riesgo, 2007). In contrast to the axial filament of siliceous spicules in Demospongiae and Hexactinellida, the organic core of the spicules of Homoscleromorpha is amorphous, indicating a possible lack of a tertiary structure of the protein contained (Uriz, 2006).

Ultrastructure

The aquiferous system is sylleibid or leuconoid, with large choanocyte chambers (eurypilous, aphodal, or diploid). The homoscleromorphs are the only Porifera that have a true basement membrane with type IV collagen, tenascin and laminin, underlying the choanoderm and the pinacoderm (Boute et al., 1996) and larval ciliated epithelium (Boury-Esnault et al., 2003). Homoscleromorpha possess flagellated exopinacocytes and endopinacocytes, peculiar flagellated apopylar cells, and *zonula adhaerens* cell junctions in adults and larval epithelia (Ereskovsky et al., 2009a).

Development

Homoscleromorpha are sponges with embryos incubation (Ereskovsky, 2010). Spermatogenesis is asynchronous inside one spermatic cyst and a gradient in cell differentiation occurs along the spermatic cysts, with spermatocytes at one side and spermatozoids on the opposite side (Gaino et al., 1986; Ereskovsky, 2005). The spermatozoids have an acrosome (Baccetti et al., 1986; Riesgo et al., 2007). The hollow blastula is formed by means of multipolar egression (centrifugal migration of cells from the center to the periphery of the morula) and presents a central cavity (Ereskovsky & Boury-Esnault, 2002). The cinctoblastula larva possesses a belt of postero-lateral cells with an intranuclear crystalloid and ciliated cells with cross-striated rootlet (Boury-Esnault et al., 2003), which derives from the secondary centriole (Maldonado & Riesgo, 2008). All morphogenesis processes follow the epithelial type (Ereskovsky et al., 2009b).

Scope of the taxon (in complement of Muricy & Diaz, 2002)

Families Oscarellidae and Plakinidae, composed, respectively, of *Oscarella* and *Pseudocorticium* genera and *Plakortis*, *Plakinastrella*, *Corticium*, *Placinolopha,* and *Plakina* genera (Gazave et al., 2010a; Ivanisevic et al., 2010).

Discussion

Although molecular phylogenetic/phylogenomic studies have profoundly increased our understanding of this peculiar group of sponge, one main point is still uncertain: the evolutionary history of the basement membrane (Leys et al., 2009; Philippe et al., 2009). The appearance of a basement membrane as a histological barrier may have important implications concerning cell type specification systems and cell movement mechanisms. This baso-epithelial basement membrane present in both larvae and adult of homoscleromorphs but also in eumetazoans (1) may have been inherited from Urmetazoa (the last common ancestor of animals) and then subsequently lost in the three other sponge classes or (2) may have appeared independently twice in the course of evolution, in Homoscleromorpha sponges and in Eumetazoa. It may be noted that, alternatively, according to the sponge paraphyly hypothesis, this basement membrane may equally represent a synapomorphy of a clade containing Homoscleromorpha and Eumetazoa (named by some authors Epitheliozoa (Sperling et al., 2009)). To date, this issue has not yet been resolved but comparison of basement membrane molecular components between all Porifera classes and Eumetazoa may provide new evidence in the future.

In addition to molecular data, several morphological and developmental differences may be noticed between Homoscleromorpha and Demospongiae that support the molecular topology discussed above. The most remarkable morphological difference is the presence of a true epithelium (basement membrane and apical cell junctions) in Homoscleromorpha and its absence in Demospongiae (as well as in other sponge groups) (Ereskovsky, 2010). Another important cytological character is the flagellated exopinacoderm, which is absent in demosponges, calcareans and hexactinellids. An alveolar choanosomal skeleton, typical of many Plakinidae species, as well as diodes and triodes is also absent in demosponges (Muricy & Diaz, 2002). Concerning the developmental features that are different between those sponges classes, one can note: (1) asynchronous spermatogenesis, whereas it is synchronous in the Demospongiae (Gaino et al., 1986); (2) a multipolar egression during embryonic development (Ereskovsky & Boury-Esnault, 2002); (3) an epithelial invagination during metamorphosis (Ereskovsky et al., 2007); (4) a budding process by morphallactic morphogenesis (Ereskovsky & Tokina, 2007).

Morphological characters supporting the proposed sister-group relationship between Homoscleromorpha and Calcarea are more scarce. A remarkable resemblance is the presence of cross-striated rootlet in larval ciliated cells of both cinctoblastula (Homoscleromorpha) (Boury-Esnault et al., 2003) and amphiblastula (Calcaronea) and calciblastula (Calcinea) (Gallissian & Vacelet, 1992; Ereskovsky & Willenz, 2008). This type of rootlet is absent in other sponge groups and may represent a synapomorphy of a clade (Homoscleromorpha + Calcarea).

Relationships among Homoscleromorpha species also provide a basis for a new hypothesis regarding the evolution of morphological characters. Due to the restoration of the two Homoscleromorpha families (Oscarellidae and Plakinidae), the cortex, aquiferous system organization, and outer morphological similarities encountered between *Corticium* and *Pseudocorticium* (and previously proposed as synapomorphies) would appear to represent homoplasic characters.

Concerning the nonmonophyly of the genus *Plakina*, this may explain the wide variability in morphological characters previously observed in this genus (Muricy et al., 1998). Thus, the genus *Plakina* should be redefined in the future and, potentially subdivided into several genera on the basis of a comprehensive molecular and morphological analysis of extant species.

Conclusion

In this paper, we chose to follow the strongly supported sponge monophyly hypothesis and formally raised the Homoscleromorpha as a fourth poriferan class. Should the alternative hypothesis that Porifera is paraphyletic (Sperling et al., 2009) gain significant support in the future and the sponge monophyly hypothesis be convincingly refuted, Homoscleromorpha and Calcarea would need to change ranks and be reconsidered as potential distinct phyla. This example illustrates the constraints of a rank-based (hierarchical) nomenclatural system whereby molecular data may support a clade but without necessarily the support of morphological synapomorphies. We consider that the information obtained

from molecular (and other) datasets that do not necessarily fit into a hierarchical classification should not be lost (Manuel et al., 2003; Borchiellini et al., 2004; Cárdenas et al., 2010, 2011; Gazave et al., 2010b), and therefore, we provide here an alternative classification of Homoscleromorpha, using the draft recommendations and processes defined by the *PhyloCode* (http://www.ohio.edu/phylocode) (online resources 1 and 2).

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References

Baccetti, B., E. Gaino & M. Sara, 1986. A sponge with acrosome: *Oscarella lobularis*. Journal of Ultrastructure and molecular structure research 94: 195–198.

Bergquist, P. R., 1978. Sponges. Hutchinson & Co, London.

Borchiellini, C., C. Chombard, M. Manuel, E. Alivon, J. Vacelet & N. Boury-Esnault, 2004. Molecular phylogeny of Demospongiae: implications for classification and scenarios of character evolution. Molecular phylogenetics and evolution 32: 823–837.

Boury-Esnault, N., G. Muricy, M. F. Gallissian & J. Vacelet, 1995. Sponges without skeleton: a new Mediterranean genus of Homoscleromorpha (Porifera, Demospongiae). Ophelia 43: 25–43.

Boury-Esnault, N., A. V. Ereskovsky, C. Bezac & D. B. Tokina, 2003. Larval development in Homoscleromorpha (Porifera, Demospongiae). Invertebrate Biology 122: 187–202.

Boute, N., J. Y. Exposito, N. Boury-Esnault, J. Vacelet, N. Noro, K. Miyazaki, K. Yoshizato & R. Garrone, 1996. Type IV collagen in sponges, the missing link in basement membrane ubiquity. Biology of the Cell 88: 37–44.

Cárdenas, P., H. T. Rapp, C. Schander & O. S. Tender, 2010. Molecular taxonomy and phylogeny of the Geodiidae (Porifera, Demospongiae, Astrophorida)—combining phylogenetic and Linnaean classification. Zoologica scripta 39: 89–106.

Cárdenas, P., J. R. Xavier, J. Reveillaud, C. Schander & H. T. Rapp, 2011. Molecular phylogeny of the astrophorida (Porifera, Demospongiae[P]) reveals an unexpected high level of spicule homoplasy. PLoS ONE 6(4): e18318. doi:10.1371/journal.pone.0018318.

Carter, H. J., 1875. Notes introductory to the study and classification of the Spongida. The Annals and Magazine of Natural History 4: 1–40 (126–145, 177–200).

Debrenne, F., A. Y. Zhuravlev & P. D. Kruse, 2002. Class Archaeocyatha Bornemann, 1884. In Hooper, J. N. A. & R. W. M. Van Soest (eds), Systema Porifera: A Guide to the Classification of Sponges. Kluwer Academic/Plenum Publishers, New York: 1539–1699.

Dendy, A., 1905. Report on the sponges collected by Professor Herdman, at Ceylon, in 1902. Royal Society, London.

Dohrmann, M., O. Voigt, D. Erpenbeck & G. Wörheide, 2006. Non-monophyly of most supraspecific taxa of calcareous sponges (Porifera, Calcarea) revealed by increased taxon sampling and partitioned Bayesian analysis of ribosomal DNA. Molecular Phylogenetics and Evolution 40: 830–843.

Dohrmann, M., D. Janussen, J. Reitner, A. G. Collins & G. Wörheide, 2008. Phylogeny and evolution of glass sponges (Porifera, Hexactinellida). Systematic Biology 57: 388–405.

Ereskovsky, A. V., 2005. Comparative Embryology of Sponges (Porifera). Sanct-Petersburg University Press, Sanct-Petersburg.

Ereskovsky, A. V., 2006. A new species of *Oscarella* (Demospongiae: Plakinidae) from the Western Sea of Japan. Zootaxa 1376: 37–51.

Ereskovsky, A. V., 2010. The Comparative Embryology of Sponges. Springer-Verlag, Dordrecht, Heidelberg, London, New York.

Ereskovsky, A. V. & N. Boury-Esnault, 2002. Cleavage pattern in *Oscarella* species (Porifera, Demospongiae, Homoscleromorpha): transmission of maternal cells and symbiotic bacteria. Journal of Natural History 36: 1761–1775.

Ereskovsky, A. V. & D. B. Tokina, 2007. Asexual reproduction in homoscleromorph sponges (Porifera; Homoscleromorpha). Marine Biology 151: 425–434.

Ereskovsky, A. V. & P. Willenz, 2008. Larval development in *Guancha arnesenae* (Porifera, Calcispongiae, Calcinea). Zoomorphology 127: 175–187.

Ereskovsky, A. V., D. B. Tokina, C. Bezac & N. Boury-Esnault, 2007. Metamorphosis of Cinctoblastula Larvae (Homoscleromorpha, Porifera). Journal of Morphology 268: 518–528.

Ereskovsky, A. V., K. Sanamyan & A. E. Vishnyakov, 2009a. *Oscarella kamchatkensis* sp nov. from the North-West of Pacific. Cahiers de Biologie marine 50: 369–381.

Ereskovsky, A. V., C. Borchiellini, E. Gazave, J. Ivanisevic, P. Lapebie, T. Perez, E. Renard & J. Vacelet, 2009b. The Homoscleromorph sponge *Oscarella lobularis*, a promising sponge model in evolutionary and developmental biology. Bioessays 31: 89–97.

Erpenbeck, D. & G. Wörheide, 2007. On the molecular phylogeny of sponges. Zootaxa 1668: 107–126.

Gaino, E., B. Burlando, P. Buffa & M. Sara, 1986. Ultrastructural study of spermatogenesis in *Oscarella lobularis* (Porifera, Demospongiae). International Journal of Invertebrates Reproduction and Development 10: 297–305.

Gallissian, M. F. & J. Vacelet, 1992. Ultrastructure of the oocyte and embryo of the calcified sponge, *Petrobiona massiliana* (Porifera, Calcarea). Zoomorphology 112: 133–141.

Gazave, E., P. Lapébie, E. Renard, J. Vacelet, C. Rocher, A. V. Ereskovsky, D. V. Lavrov & C. Borchiellini, 2010a.

Molecular phylogeny restores the supra-generic subdivision of Homoscleromorph sponges (Porifera, Homoscleromorpha). PLoS ONE. doi:10.1371/journal.pone.0014290.

Gazave, E., S. Carteron, E. Richelle-Maurer, N. Boury-Esnault & C. Borchiellini, 2010b. Polyphyly of the genus *Axinella* and of the family Axinellidae (Porifera: Demospongiae). Molecular Phylogenetics and Evolution 57: 35–47.

Hooper JNA, van Soest RWM (2002) Systema Porifera: A Guide to the Classification of Sponges. New York: Kluwer Academic/Plenum Publishers.

Hooper, J. N., R. W. M. Van Soest & F. Debrenne (eds), 2002. Phylum Porifera Grant, 1836; Systema Porifera: A Guide to the Classification of Sponges. Kluwer Academic/Plenum Publishers, New York.

Ivanisevic, J., O. P. Thomas, C. Lejeusne, P. Chevaldonné & T. Pérez, 2010. Metabolic fingerprinting as an indicator of biodiversity: towards understanding inter-specific relationships among Homoscleromorpha sponges. Metabolomics. doi:10.1007/s11306-010-0239-2.

Lavrov, D. V., X. Wang & M. Kelly, 2008. Reconstructing ordinal relationships in the Demospongiae using mitochondrial genomic data. Molecular Phylogenetics and Evolution 49: 111–124.

Lendenfeld, R. V., 1887. On the systematic position and classification of sponges. Proceedings of the Zoological Society of London 1887: 558–662.

Lévi, C., 1956. Etude des Halisarca de Roscoff. Embryologie et systématique des démosponges. Archives de Zoology Experimentale et Generale 93: 1–184.

Lévi, C., 1973. Systématique de la classe des Demospongiaria (Démosponges). In Grassé, P. P. (ed.), Spongiaires. Masson & Compagnie, Paris: 577–632.

Leys, S. P., S. A. Nichols & E. D. M. Adams, 2009. Epithelia and integration in sponges. Integrative and Comparative Biology 49: 167–177.

Maldonado, M. & A. Riesgo, 2007. Intraepithelial spicules in a homosclerophorid sponge. Cell and Tissue Research 328: 639–650.

Maldonado, M. & A. Riesgo, 2008. Reproductive output in a Mediterranean population of the homosclerophorid *Corticium candelabrum* (Porifera, Demospongiae), with notes on the ultrastructure and behavior of the larva. Marine Ecology 29: 298–316.

Manuel, M., C. Borchiellini, E. Alivon, Y. Le Parco, J. Vacelet & N. Boury-Esnault, 2003. Phylogeny and evolution of calcareous sponges: monophyly of Calcinea and Calcaronea, high level of morphological homoplasy, and the primitive nature of axial symmetry. Systematic Biology 52: 311–333.

Moraes, F. C. & G. Muricy, 2003. Taxonomy of Plakortis and Plakinastrella (Demospongiae: Plakinidae) from oceanic islands off north-eastern Brazil, with description of three new species. Journal of the Marine Biological Association of the United Kingdom 83: 385–397.

Muricy, G., 1999. An evaluation of morphological and cytological data sets for the phylogeny of Homosclerophorida (Porifera: Demospongiae). Memoirs of the Queensland Museum 44: 399–409.

Muricy, G., 2011. Diversity of Indo-Australian *Plakortis* (Demospongiae: Plakinidae), with description of four new species. Journal of the Marine Biological Association of the United Kingdom 91: 303–319.

Muricy, G. & M. C. Diaz, 2002. Order Homosclerophorida Dendy, 1905, Family Plakinidae Schulze, 1880. In Systema Porifera. In Hooper, J. N. A. & R. W. M. Van Soest (eds), A Guide to the Classification of Sponges. Kluwer Academic/Plenum Publishers, New York: 71–82.

Muricy, G. & J. S. Pearse, 2004. A new species of *Oscarella* (Demospongiae: Plakinidae) from California. Proceedings of the California Academy of Sciences 55: 598–612.

Muricy, G., N. Boury-Esnault, C. Bézac & J. Vacelet, 1998. Taxonomic revision of the Mediterranean *Plakina* Schulze (Porifera, Demospongiae, Homoscleromorpha). Zoological Journal of the Linnean Society 124: 169–203.

Pérez, T., J. Ivanisevic, M. Dubois, L. Pedel, O. P. Thomas, D. Tokina & A. V. Ereskovsky, 2011. *Oscarella balibaloi*, a new sponge species (Homoscleromorpha: Plakinidae) from the Western Mediterranean Sea: cytological description, reproductive cycle and ecology. Marine Ecology 32: 174–187.

Philippe, H., R. Derelle, P. Lopez, K. Pick, C. Borchiellini, N. Boury-Esnault, J. Vacelet, E. Renard, E. Houliston, E. Queinnec, C. Da Silva, P. Wincker, H. Le Guyader, S. Leys, D. J. Jackson, F. Schreiber, D. Erpenbeck, B. Morgenstern, G. Wörheide & M. Manuel, 2009. Phylogenomics revives traditional views on deep animal relationships. Current Biology 19: 706–712.

Pick, K. S., H. Philippe, F. Schreiber, D. Erpenbeck, D. J. Jackson, P. Wrede, M. Wiens, A. Alié, B. Morgenstern, M. Manuel & G. Wörheide, 2010. Improved phylogenomic taxon sampling noticeably affects nonbilaterian relationships. Molecular Biology and Evolution 27: 1983–1987. doi:10.1093/molbev/msq089.

Riesgo, A., M. Maldonado & M. Durfort, 2007. Dynamics of gametogenesis, embryogenesis, and larval release in a Mediterranean homosclerophorid demosponge. Marine and Freshwater Research 58: 398–417.

Schmidt, O., 1862. Die Spongien des Adriatischen Meeres. Verlag Von Wilhelm Engelmann, Leipzig: 88.

Schulze, F. E., 1880. Untersuchungen über den Bau und die Entwicklung der Spongien. Neunte Mittheilung. Die Plakiniden. Zeitschrift fur Wissenschaftliche Zoologie Abt A 34: 407–451.

Solé Cava, A. M., N. Boury-Esnault, J. Vacelet & J. P. Thorpe, 1992. Biochemical genetic divergence and systematics in sponges of the genera *Corticium* and *Oscarella* (Demospongiae: Homoscleromorpha) in the Mediterranean Sea. Marine Biology 113: 299–304.

Sollas, W. J., 1885. A classification of the sponges. Annals and Magazine of Natural History 5: 395.

Sperling, E. A., K. J. Peterson & D. Pisani, 2009. Phylogenetic-signal dissection of nuclear housekeeping genes supports the paraphyly of sponges and the monophyly of Eumetazoa. Molecular Biology and Evolution 26: 2261–2274.

Topsent, E., 1897. Spongiaires de la Baie d'Amboine. (Voyage de MM. M. Bedot et C. Pictet dans l'Archipel Malais). Revue Suisse de Zoology 4: 421–487.

Uriz, M. J., 2006. Mineral skeletogenesis in sponges. Canadian Journal of Zoology 84: 322–356.

Van Soest, R., 2011. Homoscleromorpha. In: Van Soest, R. W. M., N. Boury-Esnault, J. N. A. Hooper, K. Rützler, N. J. de Voogd, B. Alvarez de Glasby, E. Hajdu, A. B. Pisera, R. Manconi, C. Schoenberg, D. Janussen, K. R. Tabachnick, M. Klautau, B. Picton, M. Kelly (eds), World Porifera database. http://www.marinespecies.org/porifera/porifera.php?p=taxdetails&id=131587, on April 26, 2011.

Vosmaer, G. C. J., 1884. Studies on sponges: II–IV. Mitteilungen aus der Zoologischen Station zu Neapel 5: 483–493.

Wang, X. & D. V. Lavrov, 2008. Seventeen new complete mtDNA sequences reveal extensive mitochondrial genome evolution within the Demospongiae. PLoS ONE 3: e2723.

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SPONGE RESEARCH DEVELOPMENTS

Molecular phylogeny of glass sponges (Porifera, Hexactinellida): increased taxon sampling and inclusion of the mitochondrial protein-coding gene, cytochrome oxidase subunit I

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Abstract Marine sponges of the class Hexactinellida (glass sponges) are among the most understudied groups of Porifera, and molecular approaches to investigating their evolution have only recently emerged. Although these first results appeared reliable as they largely corroborated morphology-based hypotheses, they were almost exclusively based on ribosomal RNA genes (rDNA) and should, therefore, be further tested with independent types of genetic data, such as protein-coding genes. To this end, we established the mitochondrial-encoded cytochrome oxidase subunit I gene (COI) as an additional marker, and conducted phylogenetic analyses on DNA- and amino-acid level, as well as a supermatrix analysis based on combined COI DNA and rDNA alignments. Furthermore, we increased taxon sampling compared to previous studies by adding seven additional species. The COI-based phylogenies were largely congruent with the rDNA-based phylogeny but suffered from poor bootstrap support for many nodes. However, addition of the COI sequences to the rDNA data set increased resolution of the overall molecular phylogeny. Thus, although obtaining COI sequences from glass sponges turned out to be quite challenging, this gene appears to be a valuable supplement to rDNA data for molecular evolutionary studies of this group. Some implications of our extended phylogeny for the evolution and systematics of Hexactinellida are discussed.

Keywords Porifera · Hexactinellida · Phylogeny · Cytochrome oxidase subunit I · Ribosomal DNA

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Introduction

A robust and comprehensive reconstruction of the poriferan Tree of Life is of prime importance for sponge science (and beyond), because all aspects of sponge biology can be best understood in light of the evolutionary context in which the past and current diversity of these animals emerged. Molecular phylogenetics certainly constitutes the most promising approach for attaining this goal, and progress in this field has been rapid over the last two decades or so (Erpenbeck & Wörheide, 2007). However, many gaps in phylogenetic knowledge remain to be filled, and it is a further, much greater challenge to fully reconcile morphology-based taxonomy with molecular phylogenies of Porifera.

With respect to the latter, one of the most understudied groups, the glass sponges (class Hexactinellida; see Leys et al., 2007 for a comprehensive review), may provide the best chances for establishing a systematics that integrates both morphological and molecular information. This is because the glass sponges appear to be an exceptional case, as compared to other sponges (e.g., Dohrmann et al., 2006), where the first published molecular systematic results were largely in line with morphological predictions (Dohrmann et al., 2008, 2009). Nonetheless, some results remain ambiguous, and the monophyly of certain taxa could not be tested due to the lack of sequence data for more than one species. Furthermore, these phylogenies were exclusively based on three ribosomal RNA genes (rDNA)—nuclear 18S, partial nuclear 28S, and partial mitochondrial 16S—and should, therefore, be tested with independent molecular markers.

While a number of protein-coding sequences have been published for a few hexactinellid species in nonphylogenetic studies (e.g., Gundacker et al., 2001; Bebenek et al., 2004; Manuel et al., 2004; Conejo et al., 2008; Rosengarten et al., 2008), molecular phylogenetic studies including this kind of data from glass sponges are scarce (e.g., Borchiellini et al., 1998; Rokas et al., 2003; Haen et al., 2007; Philippe et al., 2009; Sperling et al., 2009), and based on a very limited taxon sampling of Hexactinellida since they did not aim at reconstructing the internal relationships of this group. We, therefore, decided to establish the mitochondrial-encoded cytochrome oxidase subunit I gene (COI) as an additional marker, because (a) this gene is widely regarded as an easily amplifiable "standard" gene for molecular evolutionary studies, and (b) COI sequence data might be useful for other applications besides systematics, such as molecular species identification ("barcoding"; see Bucklin et al., 2011 for a recent review). We also increased taxonomic sampling of hexactinellids by including seven previously unsampled species, and discuss our new results in light of the current taxonomy of the group.

Materials and methods

We added nine additional specimens, seven of which belong to previously unsampled species (Table 1), to the taxon set reported in Dohrmann et al. (2009). 18S, 28S, and 16S rDNA sequences were obtained as previously described (Dohrmann et al., 2008). COI sequences spanning the "Folmer-" and the I3-M11 regions (cf. Erpenbeck et al., 2006; ca. 1.3 kb) were amplified using various combinations of mostly degenerate primers (Supplementary Table S1), Promega's GoTaq (reaction mixes as in Dohrmann et al., 2008), and "touch-down" thermal regimes with final annealing temperatures of 45 or 30°C. Since amplification of this complete region was only rarely successful, 5'- and 3'-halves had to be amplified separately in most cases. To obtain sequences, amplicons were further processed as described (Dohrmann et al., 2008). COI sequences from *Regadrella* sp., *Acanthascus dawsoni*, and *Oopsacas minuta* were taken from ongoing mitochondrial genome sequencing projects (Haen & Lavrov, in prep.); those of *Iphiteon panicea*, *Sympagella nux*, and *Aphrocallistes vastus* were downloaded from GenBank and served as initial templates for primer design (cf. Table S1). Supplementary Table S2 gives an overview of the data set and accession numbers for the newly generated sequences.

Ribosomal DNA sequences were manually aligned to previous alignments (Dohrmann et al., 2009), aided by RNA secondary structure in case of 18S and 28S (cf. Dohrmann et al., 2008); ambiguous regions were excluded from the phylogenetic analysis. COI sequences were pre-aligned in ClustalX 2.0 (Larkin et al., 2007), followed by manual refinement. The COI alignment was largely unambiguous, but contained several instances of single species, 1-bp insertions that were either sequencing errors or

Table 1 Newly sampled specimens

Family	Species	Collection region	Voucher
Rossellidae	***Sympagella nux***	Turks & Caicos Isl.[b]	See Haen et al. (2007)
	Acanthascus dawsoni	British Columbia	Gift of Sally Leys
Euplectellidae	***Regadrella* sp.**	Straits of Florida[b]	HBOI-8-VIII-09-2-001
Leucopsacidae	*Oopsacas minuta*	Mediterranean Sea	Gift of Jean Vacelet
Farreidae	***Aspidoscopulia* n. sp. 1**[c]	Coral Sea, Australia[a]	QM G332077
	***Aspidoscopulia* n. sp. 2**[c]	Coral Sea, Australia[a]	QM G332104
	Lonchiphora antarctica[d]	Antarctica	SMF 10772
	Sarostegia oculata[c]	Florida, W Atlantic[b]	HBOI 25-V-06-2-001
Tretodictyidae	***Psilocalyx wilsoni***[c]	Coral Sea, Australia[a]	QM G331821

Previously unsampled species are highlighted in bold. *HBOI* Harbor Branch Oceanographic Institution, *QM* Queensland Museum, *SMF* Senckenberg Museum Frankfurt

[a] Collected during Deep Down Under Expedition (http://www.deepdownunder.de/)

[b] Collected through HBOI by Johnson-Sea-Link II

[c] Morphological descriptions of these specimens are provided elsewhere (Dohrmann et al., 2011), [d] see Göcke & Janussen (2011)

putative +1 translational frameshifts (Haen et al., 2007; Rosengarten et al., 2008); these sites were removed.

Preliminary analyses recovered essentially the same relationships among nonbilaterian animals as reported in Dohrmann et al. (2008); however, topology and support values for Hexactinellida were not markedly affected when the outgroups were excluded (results not shown). Therefore, we did not include any nonhexactinellid sequences in the final analyses, instead designating the six amphidiscophorans as a multi-species outgroup. This is justified because monophyly of Hexactinellida and its two subclasses, Amphidiscophora and Hexasterophora, is beyond doubt (see Dohrmann et al., 2008), and the deep divergence between the latter two taxa makes them ideal outgroups for each other. Furthermore, investigating relationships between the major nonbilaterian animal lineages is beyond the scope of this article and should better be approached with different, e.g. phylogenomic, data sets (see Philippe et al., 2009; Pick et al., 2010).

Phylogenetic analyses of the COI DNA alignment, the concatenated rDNA alignment, and a supermatrix (cf. de Queiroz & Gatesy, 2007) of all four partitions were conducted in a maximum-likelihood (ML) framework as implemented in RAxML (Stamatakis, 2006) 7.2.6 (http://wwwkramer.in.tum.de/exelixis/software.html), using the Pthreads-parallelized version on a 64-bit Linux cluster at the Molecular Geo- and Palaeobiology Lab, LMU Munich. For the combined rDNA (3328 bp) and supermatrix (4582 bp) analyses, the markers were concatenated in SeaView 4.0 (Gouy et al., 2010) and analyzed under mixed substitution models. Because, in contrast to the previously used Bayesian Markov Chain Monte Carlo (BMCMC) application PHASE (see Dohrmann et al., 2008, 2009), computational limitations are not an issue with RAxML, the least simplifying models could be explored, namely the 16-state paired-sites model (cf. Savill et al., 2001) S16 for 18S + 28S double-stranded regions (stems), and independent GTR models (Lanave et al., 1984) for 18S single-stranded regions (loops), 28S loops, 16S, and COI. However, using the 7- and 6-state paired-sites models S7D and S6B, which do not fully account for mismatch pairs (see Savill et al., 2001), but were found best-fitting in the BMCMC framework among the models tested by Dohrmann et al. (2008, 2009), lead to essentially the same results (not shown). Among-site rate variation was modeled for each partition independently using discrete gamma distributions with four rate categories ($+G_4$; Yang, 1994). We also analyzed the COI data on the amino-acid (aa) level, with DNA sequences translated using the hexactinellid-specific mitochondrial genetic code (Haen et al., 2007), and employing the MtRev+F+G_4 model of aa replacement, as suggested by ProtTest 2.4 (Abascal et al., 2005) under the Akaike Information Criterion (AIC; Akaike, 1974).

In all analyses, clade stability was assessed by rapid bootstrapping (Felsenstein, 1985; Stamatakis et al., 2008) based on 1000 pseudoreplicates.

The final supermatrix and the associated structure-, partition-, and tree files are available at Open Data LMU (http://dx.doi.org/10.5282/ubm/data.40).

Results and discussion

Contrary to our expectations (see Introduction), and for reasons that remain somewhat elusive, obtaining COI sequence data from hexactinellid specimens turned out to be rather challenging. Extremely low annealing temperatures were required to obtain amplicons (see Materials and methods), and in many cases, only very faint bands of target sequences were observed or PCR failed completely for one or both of the fragments (cf. Table S2). Also, different primer combinations worked for different specimens, necessitating that PCRs be optimized individually and no standard protocol could be established after an initial optimization step. Further problems included amplification of nontarget DNA (e.g. prokaryotes; cf. Siddall et al., 2009), multiple

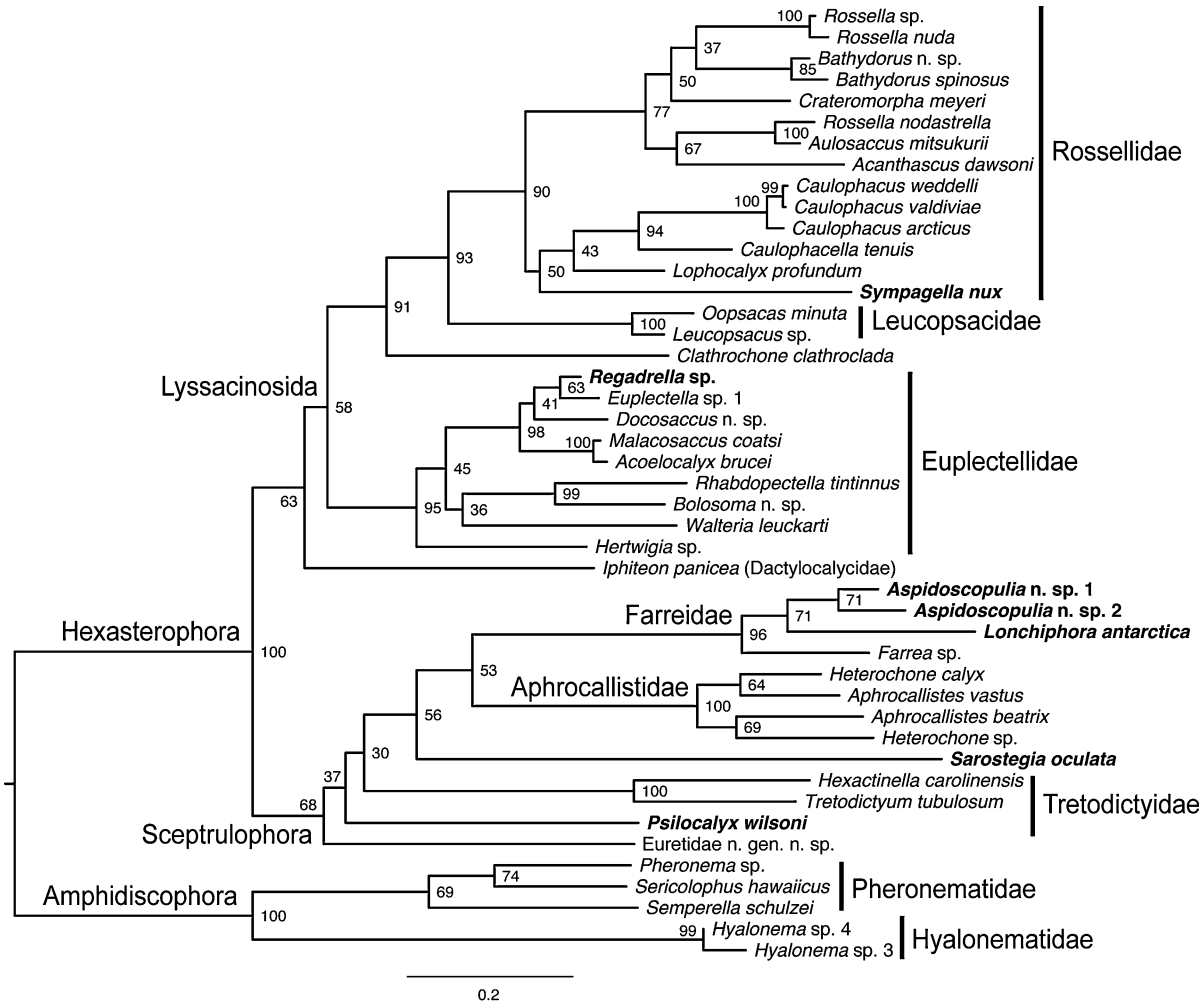

Fig. 1 Maximum likelihood phylogeny of Hexactinellida inferred from COI DNA sequence alignment (RAxML; GTR+G_4 substitution model). Bootstrap support values (1000 replicates) given at nodes. Previously unsampled species are highlighted in *bold*. Scale bar, expected number of substitutions per site. See "Materials and methods" section for further details

bands making gel extraction mandatory, and poor sequence reads leading to nonoverlapping of fragments (cf. Table S2) and requiring increased use of the IUPAC code for ambiguous base calls. Despite these practical difficulties, however, COI proved to be a useful addition to the three established rDNA markers (Dohrmann et al., 2008), as discussed below.

Phylogenies reconstructed from the COI alignments and the combined rDNA alignment, respectively, are largely congruent (Figs. 1, 2, 3), i.e. there are no conflicting clades with high bootstrap support (BS). Despite the overall congruence, many nodes are poorly (BS $< 70\%$) supported in the COI phylogenies, especially in the aa tree (Fig. 2). Strikingly, this is also the case to a lesser extent in the rDNA phylogeny, which appears less robust than the Bayesian trees presented in Dohrmann et al. (2009), even when the different significance levels of bootstrap versus posterior probability values (cf. Hillis & Bull, 1993; Huelsenbeck & Rannala, 2004) are taken into account. For example, support for order Lyssacinosida is very weak and the topology within family Rossellidae is less resolved compared to our previous study. We suspect that these results are due to further methodological and/or implementational differences between RAxML and PHASE, an issue that will be explored elsewhere.

Compared to the results of the separate analyses, robustness and resolution is increased when the two data sets are analyzed together (Fig. 4). For example, support for monophyly of Lyssacinosida is only 58 and 55% in the COI DNA and the rDNA phylogeny,

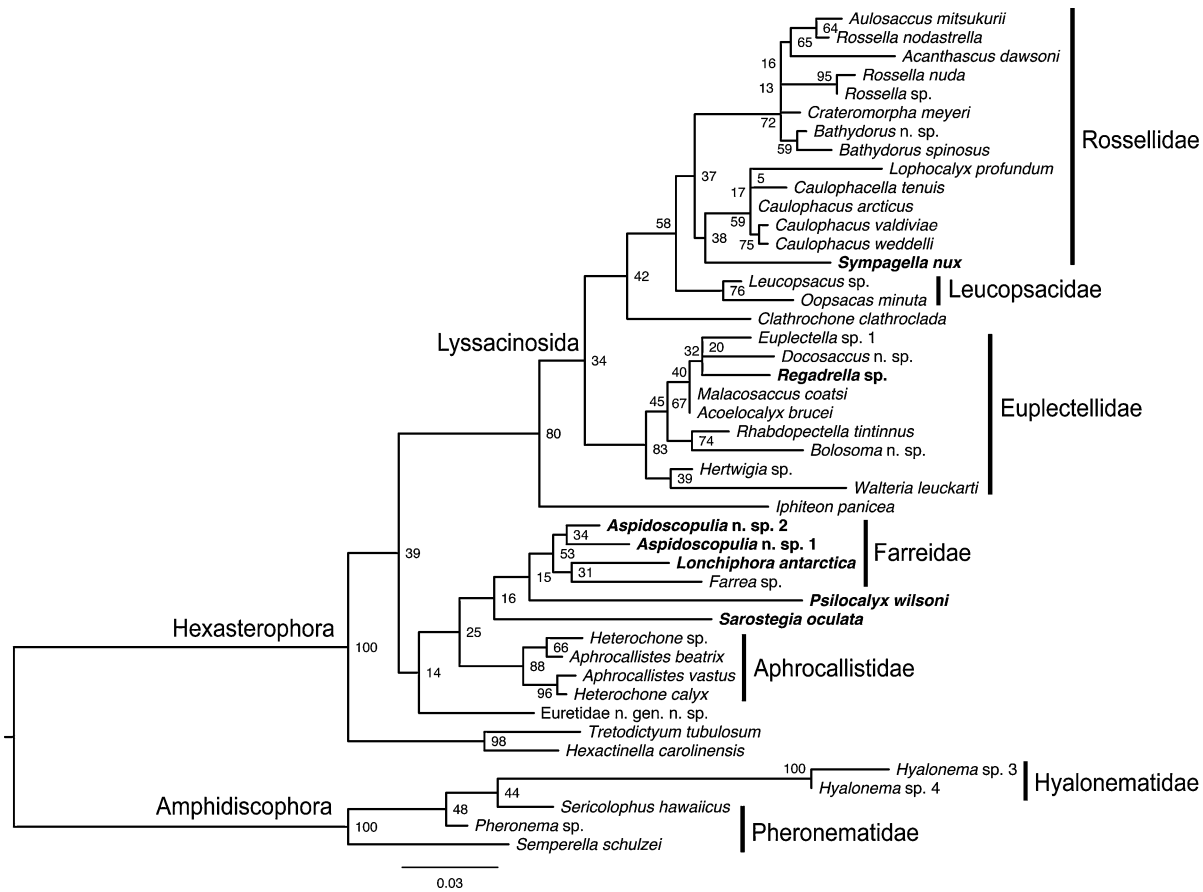

Fig. 2 Maximum likelihood phylogeny of Hexactinellida inferred from COI amino-acid sequence alignment (RAxML; MtRev+F+G_4 substitution model). Bootstrap support values (1000 replicates) given at nodes. Previously unsampled species highlighted in *bold*. Scale bar, expected number of substitutions per site. See "Materials and methods" section for further details

respectively, but rises to 75% in the combined tree, which is significant according to Hillis & Bull (1993). Below, we discuss the placement of the previously unsampled species, as well as some other new results, on the basis of the supermatrix tree (Fig. 4). A more in-depth discussion of the phylogeny of the dictyonal, sceptrule-bearing glass sponges (Sceptrulophora), and implications for spicule evolution is provided elsewhere (Dohrmann et al., 2011).

Within Sceptrulophora, we find that *Sarostegia oculata* does not group with the remaining Farreidae, which form a well-supported clade sister to Aphrocallistidae. Interestingly, *Sarostegia* is the only farreid with a euretoid dictyonal framework and lacks clavules, a spicule type that is typical for, and restricted to, Farreidae. Although this species does not group with the representative of Euretidae (but see Fig. 3), topology-tests indicate that our supermatrix data are consistent with such a placement, and we thus suggest resurrection of *Sarostegia*'s earlier classification in Euretidae (Dohrmann et al., 2011).

Although poorly supported here (BS = 64%), the position of *Psilocalyx wilsoni* as sister to the other two tretodictyids, *Hexactinella* and *Tretodictyum*, receives significant support (BS > 75%) when the taxon set is restricted to dictyonal sponges, allowing for the inclusion of additional rDNA positions (Dohrmann et al., 2011). Thus, monophyly of Tretodictyidae (Dohrmann et al., 2008) is further corroborated. It is particularly noteworthy that this morphologically well-characterized taxon (Mehl, 1992; Reiswig, 2002) was not resolved in the COI and rDNA trees, respectively (Figs. 1, 2, 3). These results indicate that considerable numbers of molecular characters may be required to support certain

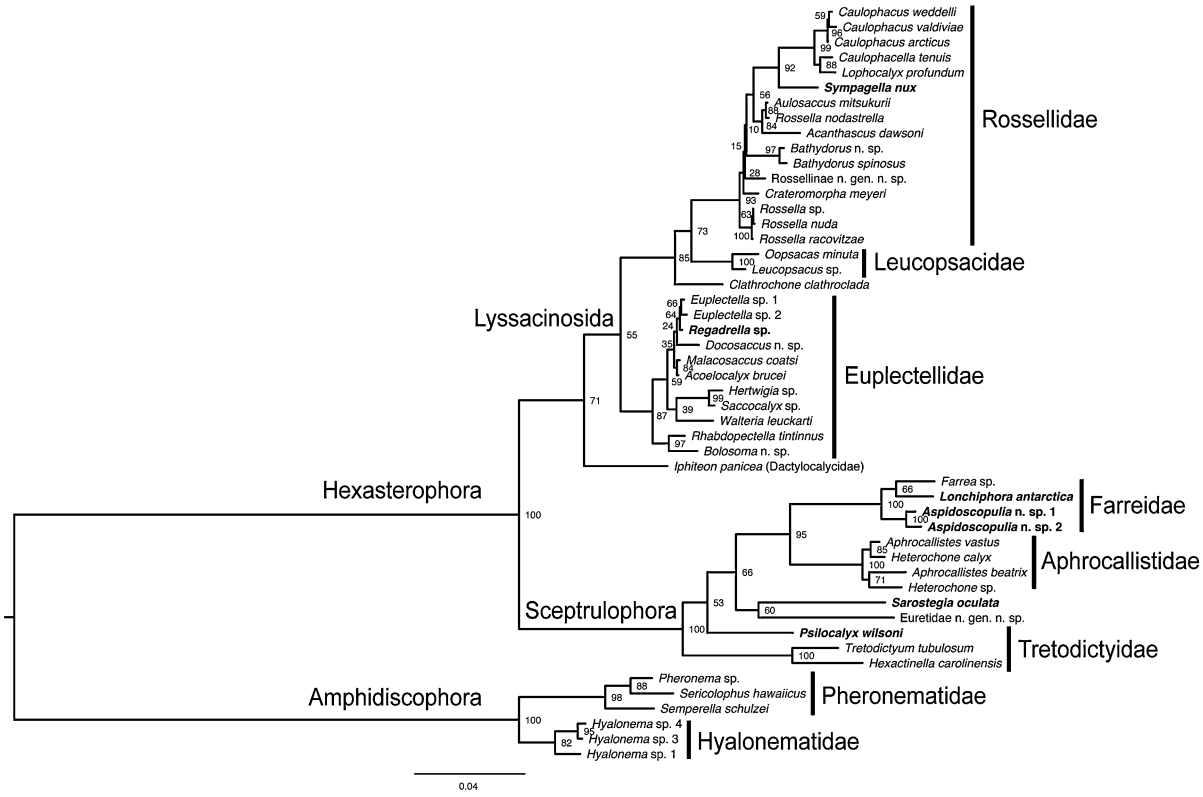

Fig. 3 Maximum likelihood phylogeny of Hexactinellida inferred from concatenated 18S, 28S, and 16S rDNA sequence alignments (RAxML; independent GTR+G_4 substitution models for 18S loops, 28S loops, and 16S; S16+G_4 paired-sites model for 18S+28S stems). Bootstrap support values (1000 replicates) given at nodes. Previously unsampled species highlighted in bold. Scale bar, expected number of substitutions per site. See "Materials and methods" section for further details

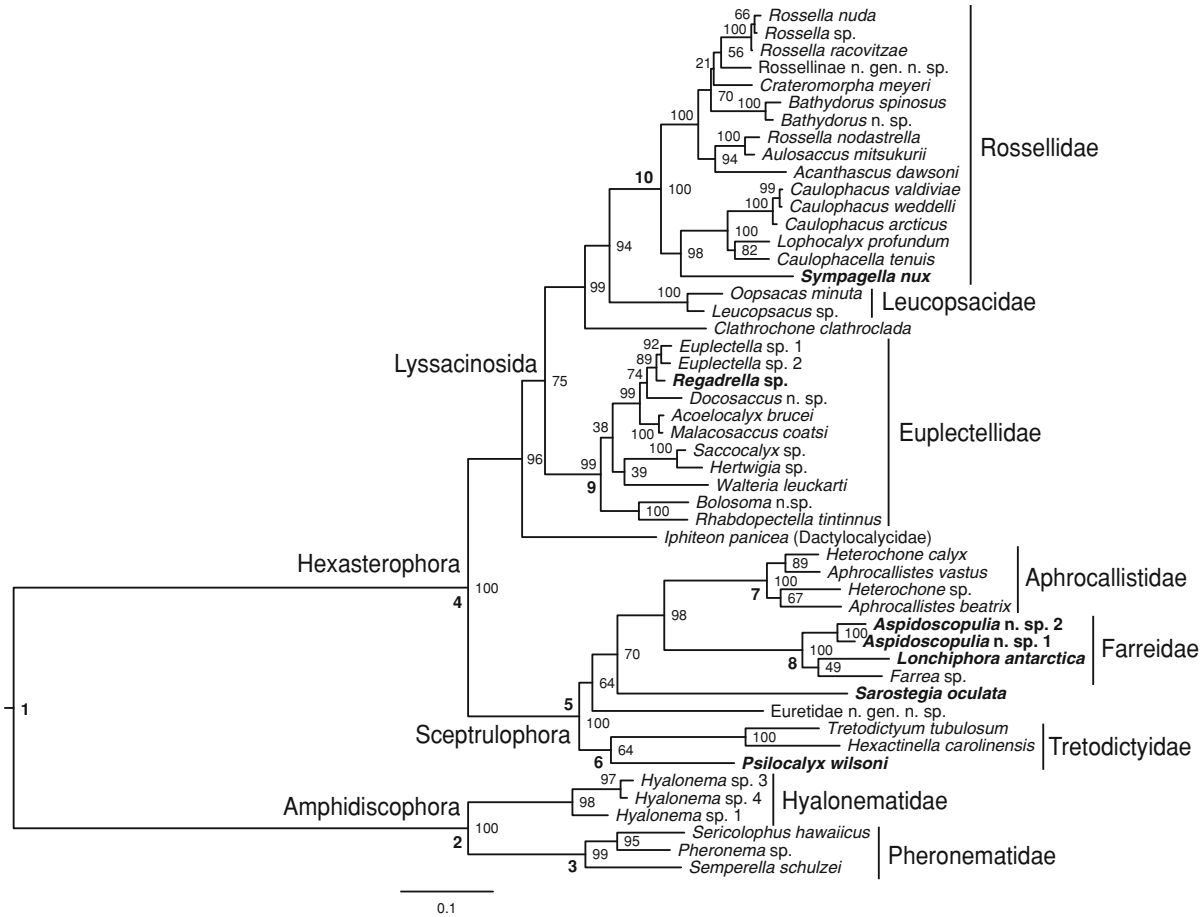

Fig. 4 Maximum likelihood phylogeny of Hexactinellida inferred from concatenated rDNA and COI DNA sequence alignments (RAxML; independent GTR+G_4 substitution models for 18S loops, 28S loops, 16S, and COI; S16+G_4 paired-sites model for 18S+28S stems). Bootstrap support values (1000 replicates) given at nodes. Previously unsampled species are highlighted in *bold*. Scale bar, expected number of substitutions per site. See "Materials and methods" section for further details. *Bold numbers* at nodes refer to the following putatively apomorphic morphological characters (for terminology, see Tabachnick & Reiswig, 2002). *1* triaxonic spicules, syncytial soft tissue, *2* amphidiscs, *3* sceptres, *4* hexasters, capability of spicule fusion, *5* sceptrules, euretoid dictyonal frameworks, *6* schizorhyses, bundled arrangement of dermal uncinates, *7* diarhyses, *8* clavules, farreoid dictyonal frameworks, *9* floricomes, *10* hypodermal pentactins

groups, and demonstrate the beneficial effect of supplementing rDNA evidence with COI sequence data.

Within order Lyssacinosida, we were able to resolve the phylogenetic position of *Clathrochone clathroclada* (see Dohrmann et al., 2009), as sister to Leucopsacidae+Rossellidae [note that this is also recovered in the rDNA tree (Fig. 3) with somewhat weaker support, but is strongly supported in the COI DNA tree (Fig. 1)]. This result rejects our earlier proposal that this species might belong to Leucopsacidae (Dohrmann et al., 2008), and corroborates the hypothesis that it represents an independent evolutionary lineage not belonging to any of the three described families of Lyssacinosida (Tabachnick, 2002a).

Within family Rossellidae, inclusion of *Sympagella nux* allowed us to test monophyly of subfamily Lanuginellinae, which was so far only represented by a single species, *Lophocalyx profundum*. Since Lanuginellinae is morphologically well defined by the presence of strobiloplumicomes (Tabachnick, 2002b), we expected that these two species would group together in the molecular phylogeny. Surprisingly, this hypothesis is not supported because *Sympagella* is resolved as sister to a *Caulophacus/*

Caulophacella/Lophocalyx clade. We consider the convergent evolution of strobiloplumicomes to be unlikely, and speculate that this spicule type was lost in *Caulophacus* and *Caulophacella*. Interestingly, a closer relationship of *Sympagella*, *Caulophacus*, and *Caulophacella* is consistent with earlier classification schemes of Rossellidae (see historical discussion in Tabachnick, 2002b; morphological characters supporting this grouping include the presence of a stalk and pinular hexactins or pentactins, with the latter also found among *Lophocalyx* spp.; however, these features are not unique to these genera). Thus, our results suggest that some abandoned taxonomic hypotheses have to be reconsidered.

Within family Euplectellidae, placement of *Regadrella* sp. (Corbitellinae) in a nested position within Euplectellinae (here: *Euplectella*, *Docosaccus*, *Acoelocalyx*, and *Malacosaccus*) again challenges monophyly of the latter subfamily (see Dohrmann et al., 2008, 2009). Thus, none of the three euplectellid subfamilies are currently supported by molecular data, which suggests that the features used to discriminate these taxa, namely the mode of attachment to the substrate (Tabachnick, 2002c), are highly plastic and of limited phylogenetic value (see also discussion in Dohrmann et al., 2009).

Conclusions

Given the technical difficulties we faced in generating COI sequence data from glass sponges, we consider it unlikely that this gene will play a major role in barcoding hexactinellids, since this approach to species identification relies heavily on easily applicable standard protocols that can be used in a high-throughput context (e.g., Ivanova et al., 2009). However, the additional sequence data proved a valuable supplement to rRNA genes for molecular phylogenetics, so the extra-effort that was required for most specimens certainly paid off. The good congruence between COI and rDNA phylogenies indicates that ribosomal RNA- and protein-coding genes harbor the same phylogenetic signal, thus increasing the reliability of molecular approaches for investigating organismal evolution of glass sponges. Combined analysis of the two data sets led to a more robust and resolved tree, providing a basis for further evolutionary studies such as reconstructing morphological character evolution or estimating divergence times. Finally, the increased taxon sampling of the present study provided some further hints as to where the current Linnean classification needs improvement; we are confident that continued addition of key taxa will ultimately help to resolve remaining taxonomic ambiguities, resulting in a system of Hexactinellida that is as natural as possible.

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References

Abascal, F., R. Zardoya & D. Posada, 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21: 2104–2105.

Akaike, H., 1974. A new look at the statistical model identifications. IEEE Transactions on Automatic Control 19: 716–723.

Bebenek, I. G., R. D. Gates, J. Morris, V. Hartenstein & D. K. Jacobs, 2004. *sine oculis* in basal Metazoa. Development Genes and Evolution 214: 342–351.

Borchiellini, C., N. Boury-Esnault, J. Vacelet & Y. Le Parco, 1998. Phylogenetic analysis of the Hsp70 sequences reveals the monophyly of Metazoa and specific phylogenetic relationships between animals and fungi. Molecular Biology and Evolution 15: 647–655.

Bucklin, A., D. Steinke & L. Blanco-Bercial, 2011. DNA barcoding of marine Metazoa. Annual Review of Marine Science 3: 471–508.

Conejo, M., M. Bertin, S. A. Pomponi & W. R. Ellington, 2008. The early evolution of the phosphagen kinases – insights from choanoflagellate and poriferan arginine kinases. Journal of Molecular Evolution 66: 11–20.

de Queiroz, A. & J. Gatesy, 2007. The supermatrix approach to systematics. Trends in Ecology and Evolution 22: 34–41.

Dohrmann, M., O. Voigt, D. Erpenbeck & G. Wörheide, 2006. Non-monophyly of most supraspecific taxa of calcareous sponges (Porifera, Calcarea) revealed by increased taxon sampling and partitioned Bayesian analysis of ribosomal

DNA. Molecular Phylogenetics and Evolution 40: 830–843.

Dohrmann, M., D. Janussen, J. Reitner, A. G. Collins & G. Wörheide, 2008. Phylogeny and evolution of glass sponges (Porifera, Hexactinellida). Systematic Biology 57: 388–405.

Dohrmann, M., A. G. Collins & G. Wörheide, 2009. New insights into the phylogeny of glass sponges (Porifera, Hexactinellida): monophyly of Lyssacinosida and Euplectellinae, and the phylogenetic position of Euretidae. Molecular Phylogenetics and Evolution 52: 257–262.

Dohrmann, M., C. Göcke, D. Janussen, J. Reitner, C. Lüter & G. Wörheide, 2011. Systematics and spicule evolution in dictyonal sponges (Hexactinellida: Sceptrulophora) with description of two new species. Zoological Journal of the Linnean Society (in press).

Erpenbeck, D. & G. Wörheide, 2007. On the molecular phylogeny of sponges (Porifera). Zootaxa 1668: 107–126.

Erpenbeck, D., J. N. A. Hooper & G. Wörheide, 2006. *CO1* phylogenies in diploblasts and the 'Barcoding of Life' – are we sequencing a suboptimal partition? Molecular Ecology Notes 6: 550–553.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Göcke, C. & D. Janussen, 2011. ANT XXIV/2 (SYSTCO) Hexactinellida (Porifera) and bathymetric traits of Antarctic glass sponges (incorporating ANDEEP-material); including an emendation of the rediscovered genus *Lonchiphora*. Deep-Sea Research Part II: in press.

Gouy, M., S. Guindon & O. Gascuel, 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27: 221–224.

Gundacker, D., S. P. Leys, H. C. Schröder, I. M. Müller & W. E. G. Müller, 2001. Isolation and cloning of a C-type lectin from the hexactinellid sponge *Aphrocallistes vastus*: a putative aggregation factor. Glycobiology 11: 21–29.

Haen, K. M., B. F. Lang, S. A. Pomponi & D. V. Lavrov, 2007. Glass sponges and bilaterian animals share derived mitochondrial genomic features: a common ancestry or parallel evolution? Molecular Biology and Evolution 24: 1518–1527.

Hillis, D. M. & J. J. Bull, 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42: 182–192.

Huelsenbeck, J. P. & B. Rannala, 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. Systematic Biology 53: 904–913.

Ivanova, N. V., A. V. Borisenko & P. D. N. Hebert, 2009. Express barcodes: racing from specimen to identification. Molecular Ecology Resources 9(Supplement 1): 35–41.

Lanave, C., G. Preparata, C. Saccone & G. Serio, 1984. A new method for calculating evolutionary substitution rates. Journal of Molecular Evolution 20: 86–93.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson & D. G. Higgins, 2007. ClustalW and ClustalX version 2.0. Bioinformatics 23: 2947–2948.

Leys, S. P. G. O., G. O. Mackie & H. M. Reiswig, 2007. The biology of glass sponges. Advances in Marine Biology 52: 1–145.

Manuel, M., Y. Le Parco & C. Borchiellini, 2004. Comparative analysis of Brachyury T-domains, with the characterization of two new sponge sequences, from a hexactinellid and a calcisponge. Gene 340: 291–301.

Mehl, D., 1992. Die Entwicklung der Hexactinellida seit dem Mesozoikum. Paläobiologie, Phylogenie und Evolutionsökologie. Berliner Geowissenschaftliche Abhandlungen Reihe E: Paläobiologie 2: 1–164.

Philippe, H., R. Derelle, P. Lopez, K. Pick, C. Borchiellini, N. Boury-Esnault, J. Vacelet, E. Renard, E. Houliston, E. Quéinnec, C. Da Silva, P. Wincker, H. Le Guyader, S. Leys, D. J. Jackson, F. Schreiber, D. Erpenbeck, B. Morgenstern, G. Wörheide & M. Manuel, 2009. Phylogenomics revives traditional views on deep animal relationships. Current Biology 19: 706–712.

Pick, K. S., H. Philippe, F. Schreiber, D. Erpenbeck, D. J. Jackson, P. Wrede, M. Wiens, A. Alié, B. Morgenstern, M. Manuel & G. Wörheide, 2010. Improved phylogenomic taxon sampling noticeably affects non-bilaterian relationships. Molecular Biology and Evolution 27: 1983–1987.

Reiswig, H. M., 2002. Family Tretodictyidae Schulze, 1886. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera. A Guide to the Classification of Sponges. Plenum, New York: 1341–1354.

Rokas, A., N. King, J. Finnerty & S. B. Carroll, 2003. Conflicting phylogenetic signals at the base of the metazoan tree. Evolution and Development 5: 346–359.

Rosengarten, R. D., E. A. Sperling, M. A. Moreno, S. P. Leys & S. L. Dellaporta, 2008. The mitochondrial genome of the hexactinellid sponge *Aphrocallistes vastus*: evidence for programmed translational frameshifting. BMC Genomics 9: 33.

Savill, N. J., D. C. Hoyle & P. G. Higgs, 2001. RNA sequence evolution with secondary structure constraints: comparison of substitution rate models using maximum-likelihood methods. Genetics 157: 399–411.

Siddall, M. E., F. M. Fontanella, S. C. Watson, S. Kvist & C. Erséus, 2009. Barcoding bamboozled by bacteria: convergence to metazoan mitochondrial primer targets by marine microbes. Systematic Biology 58: 445–451.

Sperling, E. A., K. J. Peterson & D. Pisani, 2009. Phylogenetic-signal dissection of nuclear housekeeping genes supports the paraphyly of sponges and the monophyly of Eumetazoa. Molecular Biology and Evolution 26: 2261–2274.

Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.

Stamatakis, A., P. Hoover & J. Rougemont, 2008. A rapid bootstrap algorithm for the RAxML web servers. Systematic Biology 57: 758–771.

Tabachnick, K. R., 2002a. Lyssacinosida *incertae sedis*. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera. A Guide to the Classification of Sponges. Plenum, New York: 1506–1509.

Tabachnick, K. R., 2002b. Family Rossellidae Schulze, 1885. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema

Porifera. A Guide to the Classification of Sponges. Plenum, New York: 1441–1505.
Tabachnick, K. R., 2002c. Family Euplectellidae Gray, 1867. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera. A Guide to the Classification of Sponges. Plenum, New York: 1388–1434.
Tabachnick, K. R. & H. M. Reiswig, 2002. Dictionary of Hexactinellida. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera. A Guide to the Classification of Sponges. Plenum, New York: 1224–1229.
Yang, Z., 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. Journal of Molecular Evolution 39: 306–314.

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SPONGE RESEARCH DEVELOPMENTS

Phylogenetic reconstruction of Polymastiidae (Demospongiae: Hadromerida) based on morphology

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Abstract Phylogeny of the sponge family Polymastiidae was reconstructed based on 25 morphological characters. Twenty-one polymastiid species and three suberitid species, *Suberites domuncula* as outgroup, *Aaptos aaptos* and *A. papillata* as sister groups, were included in the analyses. The reconstructions were done in PAUP* running heuristic search with the parsimony criterion. We analysed three possible evolutionary scenarios based on three alternative interpretations of the body plan of *Quasillina brevis* and *Ridleia oviformis*: first—*Ridleia* possesses aquiferous papillae whereas *Quasillina* lacks them, second—both genera lack papillae and third—the body in both genera is a single hyperdeveloped papilla. All three scenarios excluded the secondary loss of the papillae in the polymastiid evolution. Scenario 2 also excluded the secondary loss of the regular choanosomal skeleton, while scenario 1 assumed its loss in *Ridleia* and scenario 3 admitted its loss in both *Ridleia* and *Quasillina*. We prioritised scenario 2 due to its maximal parsimony and rescaled consistency index and subsequently favoured the clustering of *Ridleia* and *Quasillina* separately from the monophyletic polymastiid clade. In all three scenarios *Pseudotrachya hystrix* clustered separately from other polymastiids in agreement with the molecular evidence, and thus the exclusion of *Pseudotrachya* from Polymastiidae was proposed. The relationships between *A. papillata*, *Tentorium semisuberites*, *Polymastia uberrima*, the clade *Weberella bursa* + *Polymastia boletiformis* and the main polymastiid clade were ambiguous. Meanwhile, all scenarios showed the non-monophyly of *Polymastia* and *Aaptos*. Our hypotheses should be tested by reconstructions based on larger taxon sampling of hadromerid species and larger sets of morphological and molecular characters before any ultimate taxonomic decisions are taken.

Keywords Phylogeny · Morphology · Sponges · Polymastiidae

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Guest editors: M. Maldonado, X. Turon, M. A. Becerro & M. J. Uriz / Ancient animals, new challenges: developments in sponge research

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Introduction

Polymastiidae Gray, 1867 is a well-known, worldwide distributed demosponge family (Boury-Esnault, 2002). Twenty-four nominal genera with 133 nominal species and additional 10 subspecies and varieties have been allocated to Polymastiidae, but only 118 species plus 4 subspecies belonging to 15 genera are currently recognised as valid (van Soest et al., 2010). Taxonomy based on spicule shape has always been difficult in the case of polymastiids since these sponges possess quite uniform, simple spicules, which are in most cases smooth monactines varying from tylostyles to styles. Thus, the definition of polymastiid genera has been mainly based on the presence of any modified (acanthose, ornamented, etc.) monactines/diactines or more rarely on their peculiar body shapes (Boury-Esnault, 2002). However, in recent decades a number of other morphological characters including the skeleton and aquiferous system architecture of the choanosome, cortex and papillae as well as the number of spicule size categories have been actively used to classify polymastiid taxa (Boury-Esnault, 1987, 2002; Boury-Esnault et al., 1994; Kelly-Borges & Bergquist, 1997; Morrow & Boury-Esnault, 2000; Plotkin, 2004; Plotkin & Boury-Esnault, 2004; Boury-Esnault & Bézac, 2007; Plotkin & Janussen, 2008).

Despite the appearance of this useful morphological approach in the discriminating between the polymastiid taxa, almost 63% of polymastiid species are currently considered to belong to the genus *Polymastia* Bowerbank, 1864a (van Soest et al., 2010), which is in fact distinguished from other polymastiid genera exclusively by its lack of any unique features (Boury-Esnault, 1987, 2002). The evident essential differences between the type species of *Polymastia*, *P. mamillaris* (Müller, 1806), and a number of other *Polymastia* spp. are often ignored, and there is a practice to allocate the species that lack any diagnostic features of other polymastiid genera or possess combinations of the features of different genera to *Polymastia* without any proper argumentation. According to Boury-Esnault (2002) *Polymastia* always has papillae, its principal spicules are arranged in radial tracts, the ectosomal skeleton is composed of at least two layers—the superficial palisade of small tylostyles and the lower layer of intermediary spicules oriented tangential to the surface, the ectosomal spicules are always tylostyles. However, *P. boletiformis* (Lamarck, 1815), *P. zitteli* (von Lendenfeld, 1888) and *P. croceus* Kelly-Borges & Bergquist, 1997 have a reticulated arrangement of principal spicules that is similar to the skeleton of *Weberella* Vosmaer, 1885 (Plotkin & Janussen, 2008). Additionally, *P. boletiformis* has no intermediary spicules since the tylostyles constituting its inner cortical layer are of the same category as the principal choanosomal spicules (Boury-Esnault, 1987), which is again a feature of *Weberella*. *Polymastia invaginata* Kirkpatrick, 1907 lacks the lower ectosomal layer (Plotkin & Janussen, 2008). In *P. grimaldii* (Topsent, 1913) the cortical skeleton from the upper part is different from that of the lower part, which is a characteristic feature of *Radiella* Schmidt, 1870 (Plotkin, 2004). *P. tapetum* Kelly-Borges & Bergquist, 1997 and *P. umbraculum* Kelly-Borges & Bergquist, 1997 possess exotyles with umbrelliform distal extremities in addition to the usual tylostyles in the ectosome that are typical of *Proteleia* Dendy & Ridley, 1886. Furthermore, the choanosomal skeleton of *P. umbraculum* is reticulated (the feature of *Weberella* as stated above) and its body lacks papillae (that resembles suberitid species). There are many other examples of discrepancy, and such a practice has obviously made *Polymastia* a taxonomic dump.

Taxonomic problems exist in other polymastiid genera as well. Spherical distal knobs on the exotyles constitute the diagnostic character of *Sphaerotylus* Topsent, 1898 (Boury-Esnault, 2002). However, *S. borealis* (Swarczewsky, 1906) and *S. antarcticus* Kirkpatrick, 1907 have exotyles with umberilliform extremities, which make them similar to *Proteleia*, although the latter genus is distinguished by the additional cortical palisade (Koltun, 1966; Boury-Esnault, 2002; Plotkin, 2004). Another polymastiid genus with ornamented exotyles, *Tylexocladus* Topsent, 1898, shares the casual presence of centrotylote microxeas with *Atergia* Stephens, 1915 but the latter never possesses any exotyles (Boury-Esnault, 2002).

Finally there is still uncertainty about the relationships between Polymastiidae and the allied family Suberitidae Schmidt, 1870. The suberitid genus *Aaptos* Gray, 1867 possesses in fact the two main diagnostic features of Polymastiidae, the radial choanosomal skeleton and the ectosomal palisade of

small tylostyles. Moreover, *A. papillata* (Keller, 1880) has papillae, which was the reason for this suberitid being misidentified as a new polymastiid species, *Polymastia gleneni* Descatoire, 1966. Radial choanosomal skeletons and small papillae are also recorded in some species of *Suberites* Nardo, 1833, for example in *S. incrustans* Hansen, 1885, *S. caminatus* Ridley & Dendy, 1886 and *S. microstomus* Ridley & Dendy, 1887, despite the fact that, according to the generally accepted diagnosis of this genus, its choanosomal skeleton should be confused or alveolar, and most other *Suberites* spp. lack papillae (van Soest, 2002). It was for this reason that Topsent (1917) allocated *S. caminatus* var. *papillata* Kirkpatrick, 1908 to the polymastiid genus *Tentorium* Vosmaer, 1887. At the same time *Quasillina* Norman, 1869 with its confused choanosomal skeleton and *Ridleia* Dendy, 1888 with its choanosomal skeleton restricted to the tangentially arranged subcortical small tylostyles are both allocated to Polymastiidae by most authors (Topsent, 1898; Boury-Esnault et al., 1994; Boury-Esnault, 2002), although Dendy (1888) suggested that these genera were "the connecting link" between Suberitidae and Polymastiidae.

Evidently, a phylogenetic approach seems to be the only way to solve the taxonomic problems in Polymastiidae; but such methods have been never applied to this family. The aim of the present study was to summarise our knowledge of polymastiid morphology and to reconstruct the phylogeny of Polymastiidae based on these data.

Materials and methods

Selection of taxa

Our reconstruction was predominantly based on the type species of all currently accepted polymastiid genera including *Suberitechinus* de Laubenfels, 1949 but excluding *Trachyteleia* Topsent, 1928. These two monotypic genera were believed to be synonyms, *Trachyteleia* being subsequently considered as the senior name (Boury-Esnault, 2002). However, our re-examination of the type material revealed the differences between *Trachyteleia* and *Suberitechinus* and confirmed their validity. Insufficient data on *Trachyteleia* kept us from considering it in the analyses, whereas the ample data on *Suberitechinus* made it possible to involve the type species in our study (see also "Examined material" section for details).

Due to the considerable heterogeneity and complexity of *Polymastia* we found it reasonable to involve five species of this genus in addition to its type species *P. mamillaris* into our analyses. The species were chosen so that they represented well the morphological diversity of *Polymastia*.

For the similar reasons of heterogeneity and complexity of *Radiella* we considered its two species, *R. hemisphaerica* (Sars, 1872) and *R. sarsi* (Ridley & Dendy, 1886) in our study. The type species *R. sol* (Schmidt, 1870) could not be analysed due to the controversial data on it. On one hand the drawing by Schmidt resembled *R. sarsi*, his description being rather brief. On the other hand the re-description of *R. sol* by Boury-Esnault (2002) based on the assumed holotype considerably differed from Schmidt's drawing and resembled *R. hemisphaerica* (see Plotkin & Janussen, 2008 about the details).

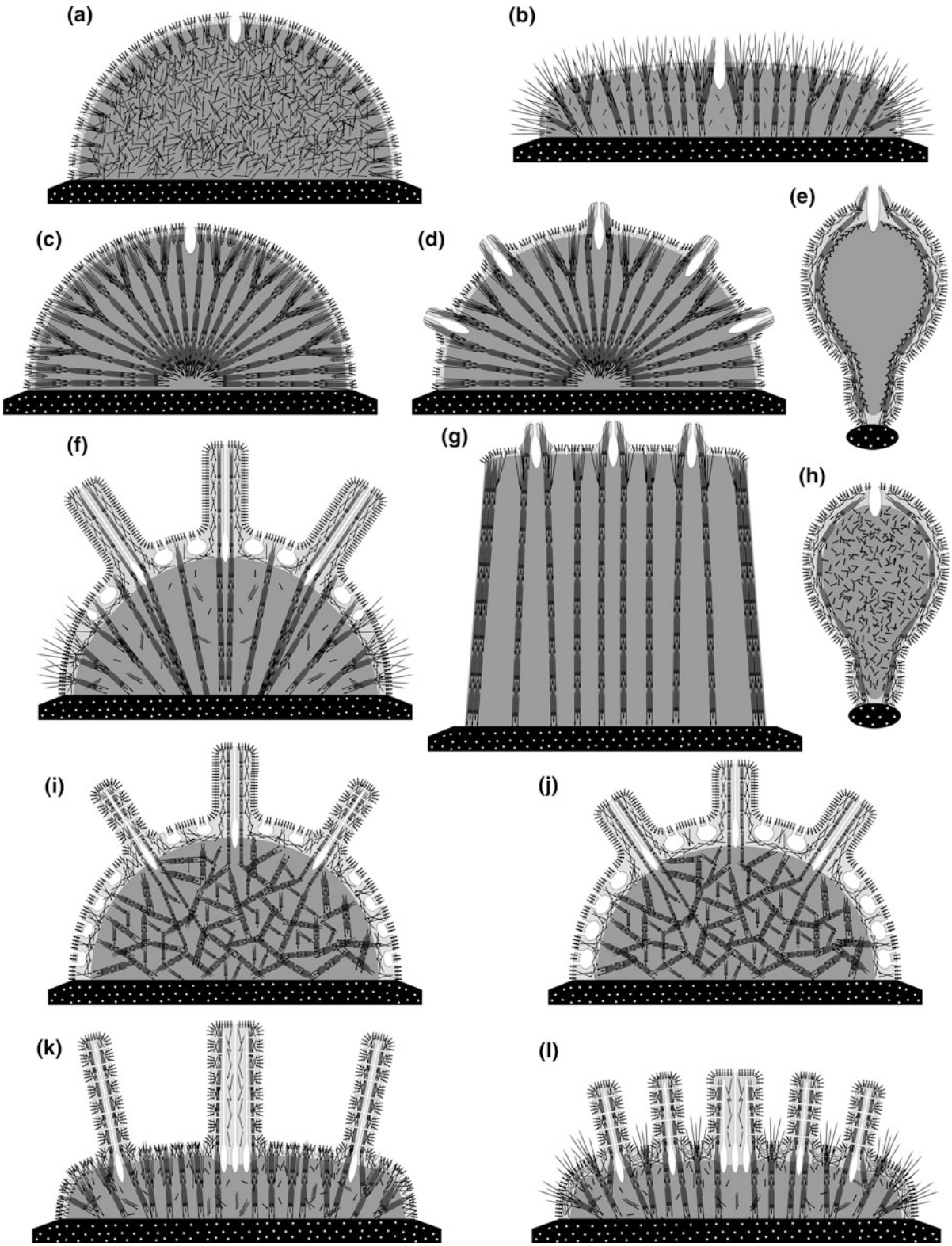
Two suberitid species were involved in the analyses. The type species of *Suberites*, *S. domuncula* (Olivi, 1792) was used as outgroup and two species of *Aaptos*, *A. aaptos* (Schmidt, 1864) and *Aaptos papillata* (Keller, 1880), were enrolled as sister groups.

Thus, altogether 24 species including 21 polymastiid species were involved in our reconstruction (Figs. 1, 2).

Examined material

Among the species treated in the analyses twelve species were studied from both type and comparative material, five species were studied only from the type material and seven species were studied only from the comparative material (Online Resource 1). Data from additional literature sources were taken into account in all cases as well. Two cases require detailed explanation.

The comparison of *Suberitechinus hispidus* (Bowerbank, 1864b) with *Trachyteleia stephensi* Topsent, 1928 based on the type material revealed that all spicules including the exotyles in the former species were uniformly smooth and much longer than the spicules of the respective categories in the latter species with its exotyles being finely spined on their distal parts. These spines were actually considered as the main feature distinguishing *Trachyteleia* from other polymastiid genera (Boury-Esnault, 2002). A


(a)
(b)
(c)
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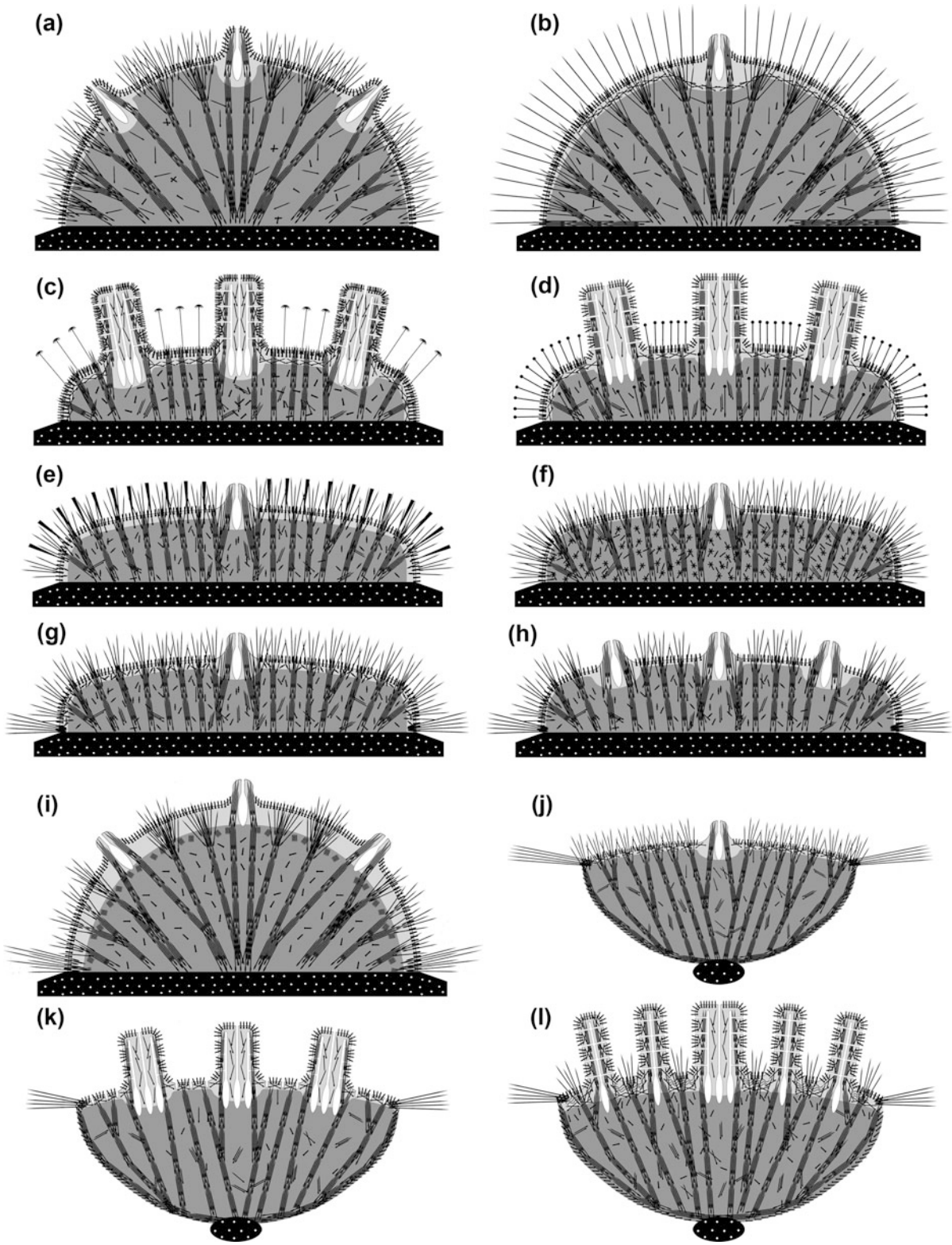
◀ **Fig. 1** Body plan schemes of the species considered in the phylogenetic reconstruction. *Black fields* with *white spots* indicate substrata. *Dark grey fields* indicate choanosome. *Light grey fields* indicate cortex. *Thick black straight lines* with or without swellings indicate spicules. *Thin black wavy lines* indicate collagen fibres.
a *Suberites domuncula*: globular growth pattern, papillae absent, oscula and ostia on smooth body surface, single-layered cortex (palisade or bouquets of small monactines), confused choanosomal skeleton of small and large monactines;
b *Pseudotrachya hystrix*: thickly encrusting growth pattern, papillae absent, oscula and ostia on hispid body surface, single-layered cortex (palisade of small diactines), radial main choanosomal skeleton of principal monactines echinating the surface, auxiliary choanosomal skeleton of small diactines;
c *Aaptos aaptos*: globular growth pattern, oscula and ostia on smooth body surface, single-layered cortex (palisade or bouquets of small monactines reinforced by intermediary monactines), radial main choanosomal skeleton of principal monactines;
d *Aaptos papillata*: globular growth pattern, ostia on smooth body surface, oscula on tops of well-developed exhalant papillae, single-layered cortex (palisade or bouquets of small monactines), radial main choanosomal skeleton of principal monactines;
e *Ridleia oviformis*: pedunculate growth pattern (scenario 1 and 2) or body is a hyper-developed exhalant papilla (scenario 3), ostia on smooth body surface, osculum on the body top (scenario 2) or on the top of papilla (scenarios 1 and 3), three-layered cortex (superficial palisade of small monactines, middle layer of criss-crossed intermediary monactines, inner layer of longitudinal tracts of principal monactines) in scenarios 1 and 2 or three-layered papilla wall in scenario 3, choanosomal skeleton reduced to subcortical tangential layer of small monactines;
f *Polymastia uberrima*: globular growth pattern, ostia on the upper smooth area of body surface, oscula on tops of well-developed exhalant papillae, upper cortex composed of superficial palisade of small monactines and inner layer of criss-crossed intermediary monactines with aquiferous cavities in between, lateral cortex lacks cavities, radial main choanosomal skeleton made of tracts of principal monactines echinating the lateral surface, auxiliary choanosomal skeleton of small and intermediary monactines;
g *Tentorium semisuberites*: columnar growth pattern, ostia on upper surface, oscula on tops of weakly developed exhalant papillae, single-layered upper cortex (palisade or bouquets of small monactines), choanosomal skeleton composed of longitudinal or radial tracts of principal monactines, the same tracts line lateral cortex;
h *Quasillina brevis*: pedunculate growth pattern (scenario 1 and 2) or body is a hyper-developed exhalant papilla (scenario 3), ostia on smooth body surface, osculum on the body top (scenarios 1 and 2) or on the top of papilla (scenario 3), three-layered cortex (superficial palisade of small monactines, middle layer of criss-crossed principal monactines, inner layer of longitudinal tracts of principal monactines) in scenarios 1 and 2 or three-layered papilla wall in scenario 3, choanosomal skeleton composed of free-scattered small monactines;
i *Polymastia boletiformis*: globular growth pattern, ostia on smooth body surface and on walls of inhalant papillae, oscula on tops of well-developed long exhalant papillae, three-layered cortex (superficial palisade of small monactines, middle layer of aquiferous cavities and inner layer of criss-crossed principal monactines), reticulated main choanosomal skeleton made of tracts of principal monactines, auxiliary choanosomal skeleton of small monactines;
j *Weberella bursa*: globular growth pattern, ostia on smooth body surface, oscula on tops of well-developed short exhalant papillae, three-layered cortex (superficial palisade of small monactines, middle layer of aquiferous cavities and inner layer of criss-crossed principal monactines), reticulated main choanosomal skeleton made of tracts of principal monactines, auxiliary choanosomal skeleton of small monactines;
k *Polymastia euplectella*: thickly encrusting growth pattern, ostia on smooth body surface and on walls of long inhalant papillae, oscula on tops of well-developed exhalant papillae, two-layered cortex (superficial palisade of small monactines and inner layer of criss-crossed intermediary monactines, the layers overlapping), radial main choanosomal skeleton made of tracts of principal monactines, auxiliary choanosomal skeleton of intermediary and small monactines;
l *Polymastia mamillaris*: thickly encrusting growth pattern, hispid surface, ostia on walls of papillae (some papillae are exclusively inhalant), oscula on tops of well-developed exhalant papillae, three-layered cortex (superficial palisade of small monactines, middle layer of collagen fibres and inner layer of criss-crossed intermediary monactines), radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, auxiliary choanosomal skeleton of intermediary and small monactines

more detailed comparative description of *Suberitechinus* and *Trachyteleia* will be come in a later study. For this study we accepted that both *Trachyteleia* and *Suberitechinus* are valid. Meanwhile, in the case of *T. stephensi*, which was known only from the type slide so far, we could not get enough data for the involving this species in our reconstruction. On the contrary, *S. hispidus* was treated in the analyses because a comprehensive comparative material was available.

Another difficulty concerned the widely distributed North Atlantic species *Polymastia boletiformis*. Topsent (1933) studied its type specimen from the unknown locality and concluded that it was conspecific with *P. robusta* (Bowerbank, 1861), although he did not examine the type of the latter. Most subsequent authors kept using the name *P. robusta* (Koltun, 1966; Borojevic, 1967; Cabioch, 1968; Boury-Esnault, 1987), although some favoured *P. boletiformis* as the senior synonym (Burton, 1959; van Soest et al., 1999). Nowadays the name *P. boletiformis* is prioritised in accordance with the nomenclature rules (van Soest et al., 2010). We could not re-examine the type of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◀ **Fig. 2** Body plan schemes of the species considered in the phylogenetic reconstruction (continued).
a *Polymastia invaginata*: thickly encrusting growth pattern, hispid surface, oscula on tops of well-developed exhalant papillae, ostia invisible, single-layered cortex (superficial palisade of small monactines), radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, echination is reinforced by single principal monactines, auxiliary choanosomal skeleton includes stellate bundles of small monactines and free-scattered monactines of various size;
b *Suberitechinus hispidus*: thickly encrusting growth pattern, hispid surface, osculum on the top of a single weakly developed exhalant papilla, ostia invisible, two-layered cortex (superficial palisade of small monactines and inner layer of criss-crossed intermediary monactines), radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, echination is reinforced by simply shaped exotyles, auxiliary choanosomal skeleton includes monactines of various size;
c *Proteleia sollasi*: thickly encrusting growth pattern, minutely hispid surface, oscula on tops of well-developed exhalant papillae, ostia on papillae walls, three-layered cortex (superficial palisade of small monactines, middle palisade of intermediary monactines and inner layer of criss-crossed intermediary monactines), exotyles with grapnel-like distal ornaments echinate the cortex, radial main choanosomal skeleton made of tracts of principal monactines, auxiliary choanosomal skeleton includes free-scattered monactines of various size;
d *Sphaerotylus capitatus*: thickly encrusting growth pattern, minutely hispid surface, oscula on tops of weakly developed exhalant papillae, ostia on papillae walls, two-layered cortex (superficial palisade of small monactines and inner layer of criss-crossed intermediary monactines), exotyles with spherical distal knobs echinate the cortex, radial main choanosomal skeleton made of tracts of principal monactines, auxiliary choanosomal skeleton includes free-scattered monactines of various size and exotyles;
e *Tylexocladus joubini*: thickly encrusting growth pattern, hispid surface, osculum on the top of a single weakly developed exhalant papilla, ostia invisible, single-layered cortex (superficial palisade of small monactines), exotyles with denticulate distal ornaments reinforce surface hispidation, radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, auxiliary choanosomal skeleton includes free-scattered monactines smaller than principal ones and smooth centrotylote microxeas;
f *Astrotylus astrotylus*: thickly encrusting growth pattern, hispid surface, osculum on the top of a single weakly developed exhalant papilla, ostia invisible, single-layered cortex (superficial palisade of small monactines), radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, auxiliary choanosomal skeleton includes free-scattered monactines of various size and astrotylostyle microscleres;
g *Acanthopolymastia acanthoxa*: thickly encrusting growth pattern, hispid surface, osculum on the top of a single weakly developed exhalant papilla, ostia invisible, two-layered cortex (superficial palisade of small monactines and inner confused mass of acanthose centrotylote microxeas), radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, auxiliary choanosomal skeleton includes free-scattered small monactines and acanthose centrotylote microxeas, marginal fringe of very long monactines;
h *Atergia corticata*: thickly encrusting growth pattern, hispid surface, oscula on tops of weakly developed exhalant papillae, ostia invisible, single-layered cortex (superficial palisade of small monactines), radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, auxiliary choanosomal skeleton includes free-scattered small monactines and smooth centrotylote microxeas, marginal fringe of very long monactines;
i *Spinularia spinularia*: thickly encrusting growth pattern, hispid surface, oscula on tops of weakly developed exhalant papillae, ostia invisible, single-layered cortex (superficial palisade of small monactines), radial main choanosomal skeleton made of tracts of principal monactines echinating the surface, auxiliary choanosomal skeleton of subcortical trichodragmata (packs of non-monactine rhaphids), marginal fringe of very long monactines;
j *Radiella sarsi*: radial growth pattern, hispid upper surface and smooth basal surface, osculum on the top of a single weakly developed exhalant papilla, ostia invisible, two-layered upper cortex (superficial palisade of small monactines and inner layer of criss-crossed intermediary monactines), basal cortex of oblique small monactines, radial main choanosomal skeleton made of tracts of principal monactines echinating the upper surface and lining the basal cortex, auxiliary choanosomal skeleton includes small and intermediary monactines, marginal fringe of very long monactines;
k *Radiella hemisphaerica*: radial growth pattern, smooth upper surface and hispid basal surface, oscula on tops of well-developed exhalant papillae, ostia on papillae walls and on the upper surface, two-layered upper cortex (superficial palisade of small monactines and inner layer of criss-crossed intermediary monactines), basal cortex of oblique small monactines, radial main choanosomal skeleton made of tracts of principal monactines which line the basal cortex and echinate both basal and upper surface, auxiliary choanosomal skeleton includes small and intermediary monactines, marginal fringe of very long monactines;
l *Polymastia grimaldii*: radial growth pattern, hispid upper surface and smooth basal surface, oscula on tops of well-developed exhalant papillae, ostia on papillae walls (some papillae are exclusively inhalant), three-layered upper cortex (superficial palisade of small monactines, middle layer of collagen fibres and inner layer of criss-crossed intermediary monactines), basal cortex of oblique small monactines, radial main choanosomal skeleton made of tracts of principal monactines echinating the upper surface and lining the basal cortex, auxiliary choanosomal skeleton includes small and intermediary monactines, marginal fringe of very long monactines

P. boletiformis because it seemed to be lost (reported by National History Museum in London and Muséum National d'Histoire Naturelle in Paris). However, we got several specimens from the Norwegian coast of the North Sea which fitted well the description of *P. robusta* from French coast by Boury-Esnault

(1987) and from the British Isles by van Soest et al. (1999). The data from these Norwegian sponges were treated in the analyses. Meanwhile, we examined one of the syntypes of *P. robusta* from England and found that its main choanosomal skeleton was radial, whereas our Norwegian sponges had a clearly reticulated skeleton. The arrangement of the choanosomal skeleton was one of the crucial points in our reconstruction, and one of the common species of *Polymastia* distinguished by a peculiar feature could not be ignored. Thus, in this study we kept using name *P. boletiformis* for this species taking in mind that it was not conspecific with *P. robusta*.

In the meantime, two other North Atlantic species, *P. radiosa* Bowerbank, 1866 and *P. euplectella* Rezvoj, 1927 were synonymised with *P. robusta* (Boury-Esnault, 1987), but later their validity was advocated by Boury-Esnault et al. (1994) and Plotkin (2004) respectively. Moreover, the synonymy of *P. robusta* still includes three other names—*P. bulbosa* Bowerbank, 1866, *P. ornata* Bowerbank, 1866 and *Reniera nivea* Hansen, 1885 (see van Soest et al., 2010). Thus, the final nomenclature decision can be only taken after a careful taxonomic investigation of all nominal species ever linked with *P. robusta*.

Selection of characters

Initially we considered 17 morphological characters including six binary characters and eleven multistage characters (Figs. 1, 2), the states of which were determined mostly from direct examination of specimens and partly from the species descriptions in literature (Rildey & Dendy, 1886, 1887; Kirkpatrick, 1907, 1908; Koltun, 1964a, b, 1966, 1970; Boury-Esnault, 1987, 2002; Boury-Esnault et al., 1994; Samaai & Gibbons, 2005). However, binary characters are usually preferred to ensure high accuracy when phylogenetic analyses are based on morphology (Hills, 2005), and the binary coding can also aid in increasing the number of characters. Therefore we coded each initial multistage character as several binary characters following the recommendations of Sarà & Burlando (1994) for sponges. This resulted in the appearance of four uninformative characters which took on one state only in one species whereas all other species possessed the alternative state: columnar growth pattern was observed only in *Tentorium semisuberites* (Schmidt, 1870); a hispid lateral surface was recorded only in *Polymastia uberrima* (Schmidt, 1870); the middle cortical layer is the additional palisade of small monactines only in *Proteleia sollasi* Dendy & Ridley, 1886; the inner cortical layer is the confused mass of acanthoxeas only in *Acanthopolymastia acanthoxa* (Koltun, 1964b). These four characters were excluded from the analyses.

Furthermore, binary coding of the characters led to an increase in the number of gaps, i.e. the cases of the inapplicability of some characters to the certain species. Among three options of dealing with the gaps, i.e. to exclude the inapplicable characters, to treat the gaps as missing data, or to designate the gaps as the additional character states, we favoured the last one. Such a choice meant coming back to the multistage (three-stage) characters in a few cases but minimised spurious taxonomic grouping. The final dataset included 25 characters (Table 1) and all characters were treated as unordered and weighted equally.

Alternative matrices

We faced some uncertainty in the interpretation of body plans of *Quasillina brevis* (Bowerbank, 1861) and *Ridleia oviformis* Dendy, 1888. The first problematic point is that *Quasillina* and *Ridleia* are characterised by a pedunculate growth pattern and the lack of tracts of principal spicules in the choanosome. However, these tracts constitute the inner layer of the cortex (Boury-Esnault, 2002), that is very similar to the skeleton of the papilla wall in other polymastiids (Fig. 3). In *Quasillina* the single osculum directly perforates the surface on the body summit, whereas in *Ridleia* several oscula are located on tiny elevations on the body summit (Topsent, 1898; Boury-Esnault et al., 1994; Boury-Esnault, 2002). Thus, the second problematic point is if these elevations can be considered as true papillae. We suggested three alternative interpretations: (1) *Ridleia* possesses weakly developed papillae whereas *Quasillina* lacks any papillae (this is the most traditional interpretation—see Boury-Esnault, 2002); (2) both *Ridleia* and *Quasillina* lack papillae; (3) the body in both genera is a single hyper-developed exhalant papilla. These gave rise to three alternative matrices (Table 2), and subsequently three different phylogenetic scenarios were reconstructed. We had to exclude character 17 (longitudinal tracts in the cortex) from the

Table 1 Morphological characters and their states used in the phylogenetic reconstruction of Polymastiidae

Character	States
1. Globular growth pattern	0. No/1. Yes
2. Thickly encrusting growth pattern	0. No/1. Yes
3. Radial growth pattern	0. No/1. Yes
4. Pedunculate growth pattern (scenario 3: body is a hyperdeveloped exhalant papilla)	0. No/1. Yes
5. Upper surface	0. Smooth/1. Hispid
6. Lateral surface	0. Not developed/1. Developed
7. Basal surface	0. Not developed/1. Developed
8. Presence of exhalant papillae	0. Absent/1. Present
9. Number of exhalant papillae	0. Always a single/1. May be several
10. Development of exhalant papillae	0. Weakly/1. Normally
11. Inhalant papillae	0. Absent/1. Present
12. Oscula on the body surface	0. Absent/1. Present
13. Ostia on the body surface	0. Absent/1. Present
14. Upper cortex: middle layer of collagen fibres	0. Absent/1. Present
15. Upper cortex: middle layer of aquiferous cavities	0. Absent/1. Present
16. Upper cortex: inner layer of criss-cross monactines	0. Absent/1. Present
17. Longitudinal tracts of principal monactines in the cortex	0. Absent/1. Present
18. Exotyles	0. Absent/1. Present
19. Specialised basal cortex	0. Absent/1. Present
20. Marginal spicule fringe	0. Absent/1. Present
21. Main choanosomal skeleton is regular	0. No/1. Yes
22. Arrangement of the regular choanosomal skeleton	0. Radial/1. Reticulated
23. Auxiliary choanosomal monactines	0. Absent/1. Present
24. Non-monactines in choanosome	0. Absent/1. Present
25. Total number of the size categories of monactines (excluding exotyles and spicules of the fringe)	0. Two/1. More than two

reconstruction of scenario 3 since in this case all taxa including *Q. brevis* and *R. oviformis* got the state "absent" of this character.

Analytic tools

The analyses were done in PAUP*, version 4b10 (Swofford, 2002) running heuristic search with the parsimony criterion. 50% majority rule consensus trees were calculated.

Results

Statistics of trees (Table 3)

Heuristic search produced 84 equally parsimonious trees of 60 steps from matrix 1, 462 trees of 57 steps from matrix 2 and 52 trees with 58 steps from matrix 3. The trees from matrix 2 had the highest overall consistency index (CI) and rescaled consistency index (RC). The lowest CI and RC were recorded for the trees from matrix 1.

Six characters (3—radial growth pattern, 7—basal surface, 8—presence of exhalant papillae, 12—oscula on the body surface, 15—middle cortical layer of aquiferous cavities and 19—specialised basal cortex) showed the best consistency in all three scenarios (RC = 1). Character 4 demonstrated the best consistency in scenario 2 (as "pedunculate growth pattern") and scenario 3 (as "body is a hyper-developed exhalant papilla"), whereas in scenario 1 it was homoplasious. Character 17 (presence of inner cortical layer of longitudinal tracts) showed the best consistency in scenario 2, but it was homoplasious in scenario 1, and in scenario 3 it was not considered.

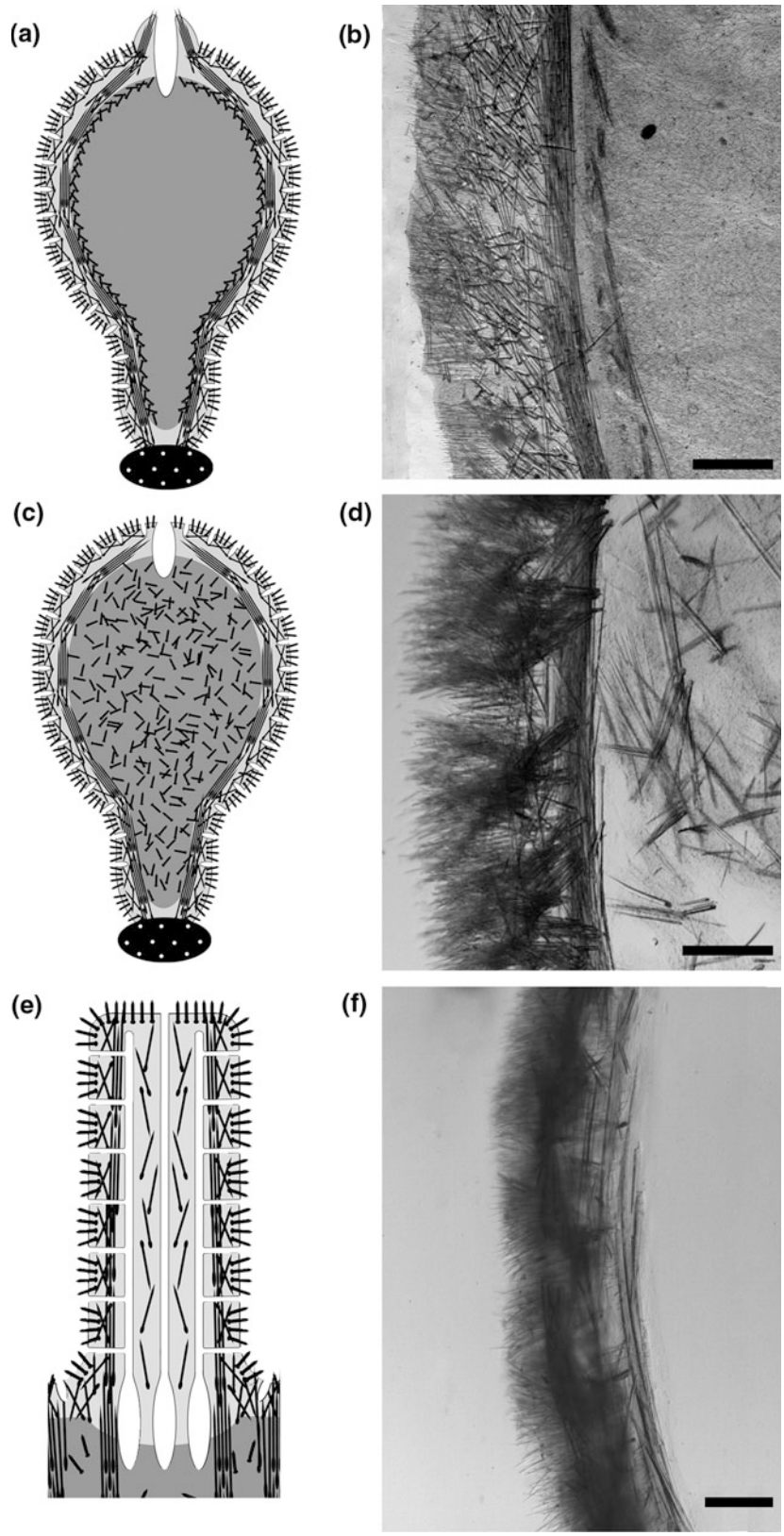


Fig. 3 Bodies of *Quasillina* and *Ridleia* in comparison with the papilla architecture of *Polymastia*. *Scale bars* 0.2 mm. **a** Body plan scheme of *Ridleia*; **b** longitudinal section through the body wall of *R. oviformis* (holotype BMNH 1883.12.13.69); **c** body plan scheme of *Quasillina*; **d** longitudinal section through the body wall of *Q. brevis* (uncatalogued specimen from ZMUB); **e** scheme of a papilla of *Polymastia*; **f** longitudinal section through the papilla wall of *P. euplectella* (specimen ZMUB 9667)

Table 2 Species/character data matrix

Species	Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Suberites domuncula		1	0	0	0	0	1	0	0	–	–	0	1	1	0	0	0	0	0	0	0	0	–	1	0	0
Aaptos aaptos		1	0	0	0	0	1	0	0	–	–	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1
Aaptos papillata		1	0	0	0	0	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Acanthopolymastia acanthoxa		0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
Astrotylus astrotylus		0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0
Atergia corticata		0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
Polymastia mamillaris		0	1	0	0	1	0	0	1	1	1	1	0	0	1	0	1	0	0	0	0	1	0	1	0	1
Polymastia euplectella		0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0	0	0	0	1	0	1	0	1
Polymastia uberrima		1	0	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1
Polymastia boletiformis		1	0	0	0	0	1	0	1	1	1	1	0	1	0	1	1	0	0	0	0	1	1	1	0	0
Polymastia invaginata		0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
Polymastia grimaldii		0	0	1	0	1	0	1	1	1	1	1	0	0	1	0	1	0	0	1	1	1	0	1	0	1
Proteleia sollasi		0	1	0	0	1	0	0	1	1	1	?	0	0	0	0	1	0	1	0	0	1	0	1	0	1
Pseudotrachya hystrix		0	1	0	0	1	0	0	0	–	–	0	1	1	0	0	0	0	0	0	0	1	0	0	1	–
Quasillina brevis	Matrix 1	0	0	0	1	0	1	0	0	–	–	0	1	1	0	0	1	1	0	0	0	0	–	1	0	0
	Matrix 2	0	0	0	1	0	1	0	0	–	–	0	1	1	0	0	1	1	0	0	0	0	–	1	0	0
	Matrix 3	0	0	0	1	–	0	0	1	0	1	0	0	0	0	0	0	ex	0	0	0	0	–	1	0	0
Radiella sarsi		0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	1
Radiella hemisphaerica		0	0	1	0	0	0	1	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	1	0	1
Ridleia oviformis	Matrix 1	0	0	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	0	0	0	–	1	0	1
	Matrix 2	0	0	0	1	0	1	0	0	–	–	0	1	1	0	0	1	1	0	0	0	0	–	1	0	1
	Matrix 3	0	0	0	1	–	0	0	1	0	1	0	0	0	0	0	0	ex	0	0	0	0	–	1	0	1
Sphaerotylus capitatus		0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1
Spinularia spinularia		0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
Suberitechinus hispidus		0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1
Tentorium semisuberites		0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1
Tylexocladus joubini		0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0
Weberella bursa		1	0	0	0	0	1	0	1	1	1	0	0	1	0	1	1	0	0	0	0	1	1	1	0	0

Numeration of the binary characters and their states are shown. Gap states are indicated by "–" (treated as additional states). Missing data are indicated by "?". States for three alternative interpretations (scenarios) of *Quasillina*/*Ridleia* body plan are given in separate rows. In scenario 3 character 17 is excluded for all taxa (indicated by "ex")

Table 3 Consistency indices of the most parsimonious trees derived from three alternative matrices

Characters	Consistency index			Rescaled consistency index		
	Matrix 1	Matrix 2	Matrix 3	Matrix 1	Matrix 2	Matrix 3
1. Globular growth pattern	0.200	0.250	0.333	0.040	0.100	0.200
2. Thickly encrusting growth pattern	0.500	0.500	0.333	0.455	0.455	0.273
3. Radial growth pattern	1.000	1.000	1.000	1.000	1.000	1.000
4. Pedunculate growth pattern (body is hyperdeveloped papilla)	0.500	1.000	1.000	0.000	1.000	1.000
5. Upper surface	0.333	0.333	0.500	0.267	0.267	0.389
6. Lateral surface	0.500	0.500	0.500	0.438	0.438	0.417
7. Basal surface	1.000	1.000	1.000	1.000	1.000	1.000
8. Presence of exhalant papillae	1.000	1.000	1.000	1.000	1.000	1.000
9. Number of exhalant papillae:	0.400	0.400	0.400	0.229	0.250	0.250
10. Development of exhalant papillae	0.333	0.400	0.400	0.222	0.300	0.280
11. Inhalant papillae	0.250	0.250	0.250	0.000	0.000	0.000
12. Oscula on the body surface	1.000	1.000	1.000	1.000	1.000	1.000
13. Ostia on the body surface	0.500	0.500	0.500	0.455	0.455	0.444
14. Upper cortex: middle layer of collagen fibres	0.500	0.500	0.500	0.000	0.000	0.000
15. Upper cortex: middle layer of aquifer. cavities	1.000	1.000	1.000	1.000	1.000	1.000
16. Upper cortex: inner layer of criss-cross monactines	0.200	0.333	0.500	0.120	0.267	0.450
17. Longitudinal tracts of principal monactines in the cortex	0.500	1.000		0.000	1.000	
18. Exotyles	0.500	0.500	0.500	0.333	0.333	0.333
19. Specialised basal cortex	1.000	1.000	1.000	1.000	1.000	1.000
20. Marginal spicule fringe	0.500	0.500	0.500	0.400	0.400	0.400
21. Main choanosomal skeleton is regular	0.500	1.000	0.500	0.250	1.000	0.250
22. Arrangement of the regular choanosomal skeleton	0.667	1.000	0.667	0.444	1.000	0.444
23. Auxiliary choanosomal monactines	0.250	0.500	0.500	0.000	0.333	0.333
24. Non-monactines in choanosome	0.500	0.500	0.500	0.400	0.400	0.400
25. Total number of the size categories of monactines	0.400	0.333	0.333	0.280	0.200	0.200
All characters	0.483	0.509	0.500	0.362	0.397	0.374

Character 21 (main choanosomal skeleton is regular) and character 22 (arrangement of the main choanosomal skeleton) had RC = 1 in scenario 2, whereas in scenarios 1 and 3 their homoplasy was high.

Phylogenetic scenarios (Fig. 4)

Three reconstructed scenarios demonstrated five main similarities.

(1) *Aaptos aaptos* and *Pseudotrachya hystrix* (Topsent, 1890) clustered separately from the clade formed by 20 polymastiid species and *Aaptos papillata* in all generated trees. The main apomorphies of this clade were the acquisition of exhalant papillae (character 8) and the loss of oscula on the body surface (character 12). No reversal of these two characters was recorded. The autopomorphy of *P. hystrix* was the replacement of monactines with diactines in the superficial cortical palisade.

(2) All trees also indicated the monophyly of the main polymastiid clade comprising 14 species (all polymastiids except *P. hystrix*, *Tentorium semisuberites*, *Quasillina brevis*, *Ridleia oviformis*, *Weberella bursa* (Müller, 1806), *Polymastia boletiformis* and *P. uberrima*). The apomorphies of this clade were the loss of the lateral surface (character 6) and the shift from globular to thickly encrusting or radial growth patterns (character 1). Meanwhile the same apomorphies were recorded outside of the main

polymastiid clade—the shift from globular to columnar growth pattern occurred in *T. semisuberites* (autapomorphy) and the shift from globular to thickly encrusting growth pattern together with the loss of the lateral surface took place in *P. hystrix*.

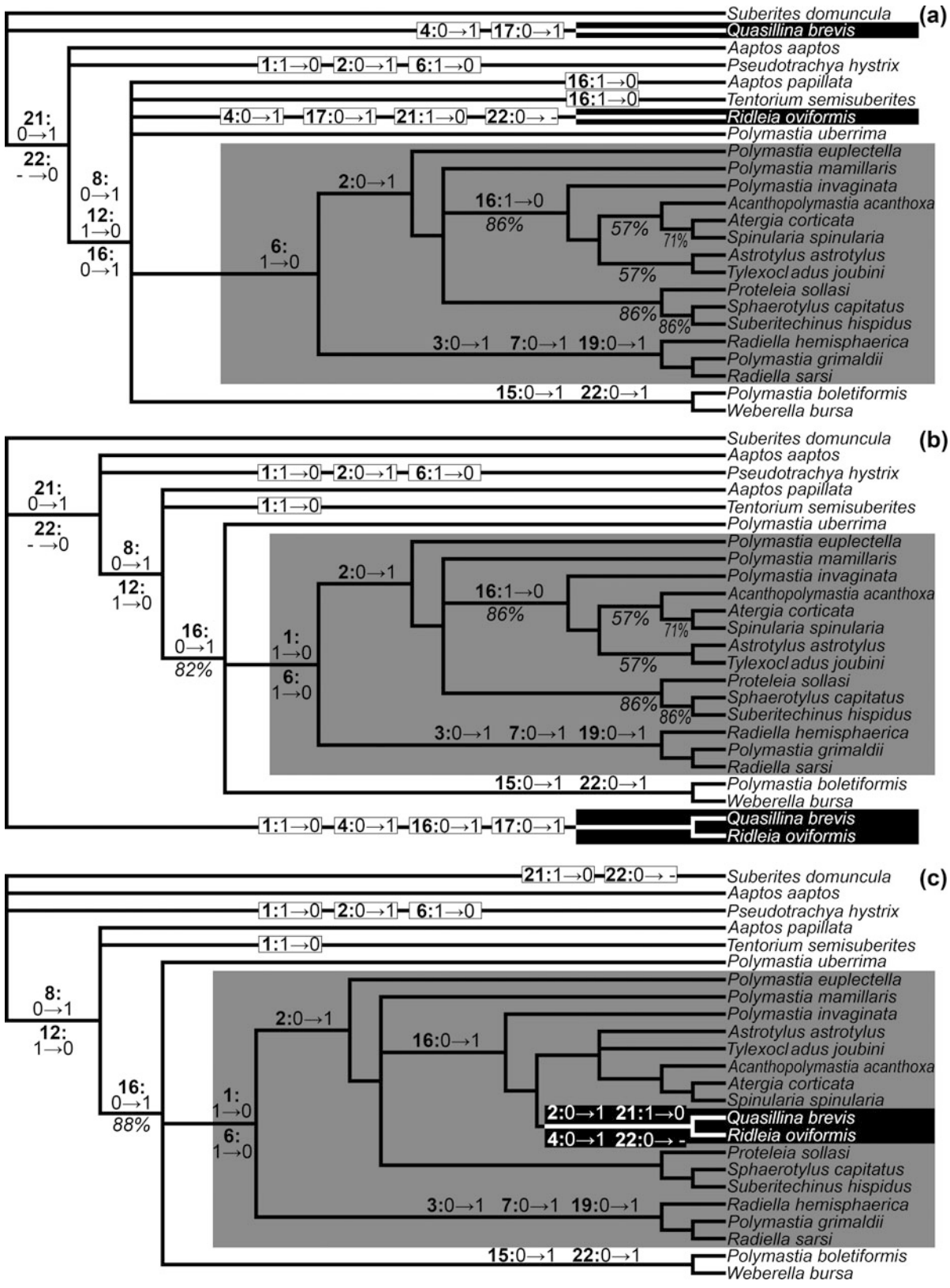
(3) *Weberella bursa* and *P. boletiformis* always clustered together due to three shared apomorphies—acquisition of an inner cortical layer of aquiferous cavities (character 15), transformation of the arrangement of the main choanosomal skeleton from radial to reticulated (character 22) and reduction in the number of main monactine categories from three to two (character 25), the latter character being extremely homoplasious with several reversals in all three scenarios. This clade formed uncertain trichotomy with the main polymastiid clade and *P. uberrima* in all trees.

(4) The main polymastiid clade always branched into two subclades. The first subclade (*Radiella hemisphaerica* + *R. sarsi* + *Polymastia grimaldii*) possessed three unique apomorphies—a shift to radial growth pattern (character 3) together with the acquisition of a basal surface (character 7) and the specialisation of the basal cortex (character 19). In the second subclade (eleven species) there was a shift to thickly encrusting growth pattern (character 2), the same apomorphy recorded earlier in *Pseudotrachya hystrix*. Within this subclade *Polymastia euplectella* clustered separately due to the acquisition of a hispid upper surface (character 5) and the loss of ostia on the surface (character 13) in other ten species. Meanwhile, the same two apomorphies took place in *R. sarsi* + *P. grimaldii* within the *Radiella*-subclade.

(5) *Polymastia mamillaris* formed uncertain trichotomy with two constellations which were supported in most trees, although some trees of scenarios 1 and 2 did not favour them. The first constellation (*Polymastia invaginata* + *Tylexocladus joubini* + *Astrotylus astotylus* + *Acanthopolymastia acanthoxa* + *Atergia corticata* + *Spinularia spinularia*) was characterised by two apomorphies—the loss of an inner cortical layer of criss-cross monactines (character 16) and the reduction in the number of monactine categories (character 25). In the meantime the same reduction also took place in *W. bursa* + *P. boletiformis* and in some other species outside the main polymastiid clade. *P. invaginata* clustered apart from other five species which acquired choanosomal non-monactines (character 24), but these spicules were also acquired by *Pseudotrachya hystrix* (see above). Furthermore, the acquisition of a marginal spicule fringe (character 20) which discriminated *A. acanthoxa*, *A. corticata* Stephens, 1915 and *S. spinularia* (Bowerbank, 1866) from *T. joubini* Topsent, 1898 and *A. astrotylus* Plotkin & Janussen, 2007 was also shared by the *Radiella*-subclade. The only apomorphy of the second constellation (*Proteleia sollasi* + *Sphaerotylus borealis* + *Suberitechinus hispidus*) was the acquisition of exotyles (character 18), although they also appeared by *T. joubini* from the other constellation.

As expected the differences between three reconstructed scenarios mainly concerned the clustering of *Quasillina brevis* and *Ridleia oviformis*, and this also influenced the clustering of *A. papillata* and *T. semisuberites*.

In all trees of scenario 1 there was an uncertain polytomy formed by *A. papillata*, *T. semisuberites*, *R. oviformis*, *P. uberrima*, the clade *W. bursa* + *P. boletiformis* and the main polymastiid clade of 14 species, whereas *Q. brevis* clustered separately from both species of *Aaptos* and all polymastiid species including *P. hystrix*. On the contrary, in most trees of scenarios 2 and 3 *A. papillata* and *T. semisuberites* clustered separately from the superclade formed by *P. uberrima*, *W. bursa* + *P. boletiformis* and the main polymastiid clade. The apomorphies of this superclade were the acquisition of an inner cortical layer of criss-cross monactines (character 16) and the auxiliary monactines in the choanosome (character 23), but both these characters were homoplasious. The layer of criss-cross monactines was lost in the constellation *P. invaginata* + *T. joubini* + *A. astrotylus* + *A. acanthoxa* + *A. corticata* + *S. spinularia* (see above). The auxiliary choanosomal monactines existed in the outgroup (*S. domuncula* possessed both large and small monactines in the choanosome), and both scenarios 2 and 3 suggested the loss of them (inherited by *P. hystrix*, both *Aaptos* spp. and *T. semisuberites*) before their appearance in the polymastiid superclade.


(a)
Suberites domuncula
Quasillina brevis
Aaptos aaptos
Pseudotrachya hystrix
Aaptos papillata
Tentorium semisuberites
Ridleia oviformis
Polymastia uberrima
Polymastia euplectella
Polymastia mamillaris
Polymastia invaginata
Acanthopolymastia acanthoxa
Atergia corticata
Spinularia spinularia
Astrotylus astrotylus
Tylexocladus joubini
Proteleia sollasi
Sphaerotylus capitatus
Suberitechinus hispidus
Radiella hemisphaerica
Polymastia grimaldii
Radiella sarsi
Polymastia boletiformis
Weberella bursa
4:0→1 17:0→1
1:1→0 2:0→1 6:1→0
16:1→0
16:1→0
4:0→1 17:0→1 21:1→0 22:0→ -
21: 0→1
22: - →0
8: 0→1
12: 1→0
16: 0→1
2:0→1
16:1→0
86%
57%
71%
57%
6: 1→0
86%
86%
3:0→1 7:0→1 19:0→1
15:0→1 22:0→1
(b)
Suberites domuncula
Aaptos aaptos
Pseudotrachya hystrix
Aaptos papillata
Tentorium semisuberites
Polymastia uberrima
Polymastia euplectella
Polymastia mamillaris
Polymastia invaginata
Acanthopolymastia acanthoxa
Atergia corticata
Spinularia spinularia
Astrotylus astrotylus
Tylexocladus joubini
Proteleia sollasi
Sphaerotylus capitatus
Suberitechinus hispidus
Radiella hemisphaerica
Polymastia grimaldii
Radiella sarsi
Polymastia boletiformis
Weberella bursa
Quasillina brevis
Ridleia oviformis
1:1→0 2:0→1 6:1→0
1:1→0
1:1→0 4:0→1 16:0→1 17:0→1
21: 0→1
22: - →0
8: 0→1
12: 1→0
16: 0→1
82%
1: 1→0
6: 1→0
2:0→1
16:1→0
86%
57%
71%
57%
86%
86%
3:0→1 7:0→1 19:0→1
15:0→1 22:0→1
(c)
21:1→0 22:0→ -
Suberites domuncula
Aaptos aaptos
Pseudotrachya hystrix
Aaptos papillata
Tentorium semisuberites
Polymastia uberrima
Polymastia euplectella
Polymastia mamillaris
Polymastia invaginata
Astrotylus astrotylus
Tylexocladus joubini
Acanthopolymastia acanthoxa
Atergia corticata
Spinularia spinularia
2:0→1 21:1→0
Quasillina brevis
4:0→1 22:0→ -
Ridleia oviformis
Proteleia sollasi
Sphaerotylus capitatus
Suberitechinus hispidus
Radiella hemisphaerica
Polymastia grimaldii
Radiella sarsi
Polymastia boletiformis
Weberella bursa
1:1→0 2:0→1 6:1→0
1:1→0
8: 0→1
12: 1→0
16: 0→1
88%
1: 1→0
6: 1→0
2:0→1
16:0→1
3:0→1 7:0→1 19:0→1
15:0→1 22:0→1

◀ **Fig. 4** 50% Majority rule consensus cladograms of the trees reconstructed in the heuristic search with the parsimony criterion. Percentage of the trees (if less than 100%) in which each clade appears is given under the respective branch. Main apomorphies are given along the branches and indicated as number of a character, initial state of the character, *arrow*, derived state of the character. For numeration of the characters and their states see the Table 1. *Grey field* shows the main polymastiid clade; *black fields* with white branches and fonts emphasise *Quasillina brevis* and *Ridleia oviformis*. **a** Scenario 1, **b** scenario 2, **c** scenario 3

Scenarios 2 and 3 supported the clustering of *R. oviformis* with *Q. brevis*, but the only unique apomorphy of this clade recorded in both scenarios was the shift of growth pattern—in scenario 2 the pedunculate pattern replaced the globular one (characters 1 and 4), whilst in scenario 3 the body as a hyper-developed papilla appeared instead of thickly encrusting pattern (characters 2 and 4) with the subsequent loss of the upper surface (character 5). The principal difference between scenarios 2 and 3 was the explanation of the absence of a regular choanosomal skeleton (characters 21 and 22) in *R. oviformis* and *Q. brevis*. Scenario 2 suggested that the common ancestor of *Ridleia*, *Quasillina* and other polymastiids lacked a regular choanosomal skeleton, and thus put *Ridleia* and *Quasillina* separately from all other polymastiid species including *P. hystrix*, and from both species of *Aaptos*. This scenario also considered the acquisition of the longitudinal tracts of large monactines in the cortex (character 17) as the unique apomorphy of *Ridleia* + *Quasillina*. On the contrary, scenario 3 favoured the secondary loss of the radial choanosomal skeleton in *Ridleia* and *Quasillina* and subsequently placed *R. oviformis* + *Q. brevis* as the sister group to the cluster of five polymastiids with choanosomal non-monactines (*T. joubini*, etc., see above) inside the main polymastiid clade. The peripheral longitudinal tracts of monactines in the *Ridleia* and *Quasillina* were interpreted as homologous to the tracts in the papillae walls of other polymastiids and thus character 17 was not considered in scenario 3.

Discussion

Phylogeny

Our study raised up at least four general problems of phylogenetic reconstructions based on morphology—the lack of knowledge about the ancestral state of a character, the possibility of secondary loss of some character states, the lack of knowledge about the homology of characters and the inapplicability of some characters to certain taxa. The crucial point in the polymastiid phylogeny is the acquisition of papillae and a radial choanosomal skeleton. Our reconstruction was based on the suggestion that the ancestor of Polymastiidae lacked papillae and regular choanosomal skeleton. The radial skeleton of most polymastiids and the reticulated skeleton of *W. bursa* and *P. boletiformis* were considered as two variations of the regular skeleton. However, these assumptions can be disputed, and in order to test them a more comprehensive reconstruction involving much more species from Suberitidae and other hadromerid families is required.

All three reconstructed scenarios prioritised the hypothesis that the papillae were acquired only once and were never lost during the evolution of Polymastiidae, although the degree of papillae development and their number varied considerably. Meanwhile, only scenario 2 suggested that the regular choanosomal skeleton being radial or reticulated was never lost in the polymastiid evolution, while scenario 1 assumed that it was lost in *Ridleia* and scenario 3 admitted that both *Ridleia* and *Quasillina* have lost the regular skeleton. We favoured scenario 2 because it was most parsimonious and had the highest RC, and also because scenario 3 was based on rather speculative assumptions about the hyper-development of papilla. Scenario 1 was excluded due to its minimal parsimony and consistency and because it did not support the clustering of *Ridleia* with *Quasillina* that contradicted generally accepted morphological conception about these genera (Dendy, 1888; Boury-Esnault, 2002). However, our conclusion about the position of *Ridleia* and *Quasillina* outside the monophyletic polymastiid clade may be challenged if new morphological and molecular data come to hand.

The clustering of *Pseudotrachya* Hallmann, 1914 separately from other polymastiids demonstrated in our study has good congruence with the data of Nichols (2005) who involved the partial 28S rDNA of 15 suberitid species (including two species of *Suberites* and two species of *Aaptos*) and six polymastiid species in a general phylogenetic reconstruction of Demospongiae. In his trees four polymastiid species

clustered together and separately from Suberitidae, whereas *Pseudotrachya* sp. clustered neither with other polymastiids nor with suberitids. Thus, we can suggest that the acquisition of the non-monactines in the choanosomal skeleton occurred in parallel in *Pseudotrachya* and the subclade *T. joubini* + *A. astrotylus* + *A. acanthoxa* + *A. corticata* + *S. spinularia*. The choanosomal non-monactines were also acquired by some species of *Suberites*, e.g. by *S. ficus* (van Soest, 2002). However, it remains unclear whether *Pseudotrachya* inherited the non-monactines from a suberitid ancestor or acquired them anew, and the true taxonomic position of this genus can not be determined until a larger set of hadromerid taxa is analysed.

Our study indicated the monophyly of the genuine polymastiid clade which included 14 species, although no strict synapomorphies of this clade have been found so far. Meanwhile, the relationships between *Tentorium*, *P. uberrima*, the clade with the reticulated choanosomal skeleton (*Weberella* + *P. boletiformis*) and the genuine polymastiid clade remain ambiguous. Favouring scenario 2 and also taking account of scenario 3 we suggest that *Weberella* + *P. boletiformis* and *P. uberrima* are more closely related to other polymastiids than to *Tentorium*, and subsequently they can be considered as true polymastiids. In any case the non-monophyly of *Polymastia* becomes quite evident. This conclusion is in good agreement with the data of Nichols (2005) in whose trees *Polymastia* sp. 1 clustered separately from three other *Polymastia* spp., the latter forming the genuine polymastiid clade together with *S. spinularia*. Furthermore, within this polymastiid clade there were two subclades, one composed of *P. invaginata* and *S. spinularia*, and the other of *P. pachymastia* and *Polymastia* sp. 2. However, Nichols did not carry out a careful morphological study of his specimens, and therefore there was a high risk of misidentification of some other polymastiid or even suberitid genera as *Polymastia* sp. 1 and sp. 2.

The question about whether *Tentorium* is a polymastiid taxon cannot be solved without solving the same question about *A. papillata* because in all our trees the latter species and *T. semisuberites* appeared to be sister groups of the main polymastiid clade. Moreover, all species of *Aaptos* which possess papillae obviously have many affinities with *Tentorium* spp. that cannot be ignored (e.g. see Plotkin & Janussen, 2008). On the other hand the clustering of the type species of *Aaptos* separately from *A. papillata* undoubtedly indicates the non-monophyly of this genus.

Within the genuine polymastiid clade the strongest criticism can target the clustering of the *Radiella*-subclade apart from other species. We were based on the assumption that the radial growth pattern was homologous in both *Radiella* spp. and *P. grimaldii*. At the same time we suggested that the specification of a basal cortex was not strictly correlated with the acquisition of the radial growth pattern, and some species might have the same skeletal architecture in the upper and basal cortex. However, our assumptions may be disputed, and more significance should be attached to the similarities between *P. grimaldii* and *P. mamillaris* (in character 14, collagen layer in the cortex and character 11, presence of inhalant papillae) as it has already been suggested by Koltun (1966) and partly by Boury-Esnault (2002).

In general the most disputable point of our study is the treatment of the gaps for inapplicable characters as additional states and the subsequent favouring of the composite coding instead of the binary coding in a number of cases. The composite coding was advocated by Maddison (1993) as the only effective procedure to eliminate the inapplicable cells within the character matrices. However, such an approach can lead to false grouping because the state "inapplicability" is unconditionally interpreted as synapomorphy by the search algorithm (Strong & Lipscomb, 1999). Non-additive binary coding that splits the potentially composite inapplicable characters into several binary characters has been thought to be a better alternative (Pleijel, 1995) and prioritised in the phylogenetic reconstructions of several sponge groups (Sarà & Burlando, 1994). However, this method may group taxa based on non-homologous absence of evidence, rather than on shared derived character states (Strong & Lipscomb, 1999). A third option is reductive coding (Wilkinson, 1995) which codes inapplicable characters as gaps and interprets these states as missing data (the default set in PAUP* 4b10). It means that the treatment of these characters exclusively follows mathematical expediency, while the morphological evidence is completely ignored. In the case of Polymastiidae the attempt to use non-additive binary coding or reductive coding led to spurious clustering (*Pseudotrachya* appeared deeply inside the main polymastiid clade that contradicted

with present molecular evidence, *Quasillina* and *Ridleia* clustered apart from each other, strongly contradicting the commonly accepted assumption) as well as to an increasing number of uncertain polytomies and poorly supported clades. On the contrary, composite coding produced well resolved trees in all three scenarios.

All assumptions about the ancestral states and homologues in polymastiid morphology as well as the hypotheses about polymastiid evolution elaborated in our study should be tested by comprehensive phylogenetic analyses based on larger set of taxa and characters including both molecular and morphological data before any ultimate taxonomic decisions can be made.

Affiliations and grouping within *Polymastia*

Due to the considerable heterogeneity and complexity of *Polymastia* five species representing the great morphological variation within the genus as well as the type species were included in our phylogenetic analysis. The analysis resulted in a very sound basis for further grouping of known species within the genus. Thirty-eight species of *Polymastia* which were not considered in our analyses may be grouped with analysed species (Online Resource 2). The judgment upon the character states for these additional species was based partly on literature data and partly on our own observations of the specimens.

Eleven species may be grouped with *P. mamillaris*, the type species of the genus. They share thickly encrusting growth pattern, a hispid upper surface without ostia, numerous papillae, radial choanosomal skeleton, the cortex is at least two-layered (superficial palisade and inner criss-cross layer), three categories of monactines are present altogether. Among them *P. arctica* (Merejkowsky, 1878) and *P. harmelini* Boury-Esnault & Bézac, 2007 share also a middle cortical layer of collagen fibres with *P. mamillaris*, the differences between these three species being rather few and concerning mostly the peculiarities in spicule shape and size and some details of cortical and subcortical skeleton (Plotkin & Boury-Esnault, 2004; Boury-Esnault & Bézac, 2007). Six other species differ from *P. mamillaris* mainly by the absence of the collagen layer. At the same time this layer is found in *P. grimaldii* which clustered far away from *P. mamillaris*. Thus, it seems that it is a rather variable character and is not useful for future phylogenetic reconstructions.

Eleven species may be grouped with *P. euplectella* due to the combination of thickly encrusting growth pattern, a smooth surface bearing ostia, numerous papillae, radial choanosomal skeleton, the cortex is usually two-layered and three categories of monactines are present. This is a diverse group with many differences between the species, which mainly concern the acquisition of various middle layers in the cortex, such as additional palisades, layers of aquiferous cavities, etc. Among this diversity only *P. spinula* Bowerbank, 1866 strongly resembles *P. euplectella* with its inner cortical layer of criss-cross monactines slightly overlapping the superficial palisade, and extremely long papillae of simple architecture, most of them being inhalant (Boury-Esnault, 1987). Two species, *P. lorum* Kelly-Borges & Bergquist, 1997 and *P. pepo* Kelly-Borges & Bergquist, 1997, are included with large doubt in the *euplectella*-group because they possessed smooth centrotylote microoxeas scattered in the cortex, the feature recalling acanthose cortical microxeas in *Acanthopolymastia* Kelly-Borges & Bergquist, 1997 or smooth choanosomal microxeas in *Atergia* and *Tylexocladus* Topsent, 1898.

Two species, *P. hirsuta* Bergquist, 1968 and *P. tissieri* (Vacelet, 1961), are grouped with *P. grimaldii* and subsequently with *Radiella* spp. *P. hirsuta* demonstrates radial growth pattern with specialisation of the basal surface and cortex (Kelly-Borges & Bergquist, 1997). At the same time it has additional long monactines reinforcing the hispidation over the entire surface, whereas in *P. grimaldii* and *Radiella* spp. such spicules are concentrated in a marginal fringe. On the contrary, *P. tissieri* possesses such a fringe, but this species is thickly encrusting and lacks basal surface as most *Polymastia* spp. (Boury-Esnault et al., 1994). The marginal fringe is also shared by *Acanthopolymastia*, *Atergia* and *Spinularia* Gray, 1867, and thus it may be a convergent feature.

One species, *P. hispidissima* Koltun, 1966, may be grouped with *P. invaginata* mainly due to a single-layered cortex (Koltun, 1966). These species are rather similar externally with the shaggy surface and usually a sole papilla, although *P. invaginata* may have several papillae.

Three species are logically grouped with *P. uberrima*. The autapomorphy of this group is the

diversification of the surface and cortex, the upper surface being smooth, bearing ostia and exhalant papillae, and the lateral surface being hispid and imperforated. Among these species *P. infrapilosa* Topsent, 1927 has almost no essential differences from *P. uberrima*, whereas *P. actinioides* Koltun, 1966 and *P.* cf. *pacifica* Koltun, 1966 are distinguished by reinforcement of their lateral hispidation with additional large monactines which can be considered simple exotyles like in *Suberitechinus hispidus*.

Eight species are characterised by non-radial choanosomal skeleton which is often reticulated or rarely meandering. This feature groups them with *Weberella bursa* and *P. boletiformis*, although there are a lot of differences. The main difference is three size categories of monactines in all grouped species, whereas *W. bursa* and *P. boletiformis* possess only two categories. Other differences concern the complication of the cortex which acquires additional layers in some species. Two species, *P. corticata* Ridley & Dendy, 1886 and *P. zitteli*, are grouped with *P. boletiformis* due to large papillae, whereas other six species seem to be closer to *W. bursa*. Among the latter *P. rubens* Kelly-Borges & Bergquist, 1997 is distinguished by the centrotylote microxeas in the cortex, the feature already discussed above for *P. lorum* and *P. pepo*.

The removal to other genera is proposed for two species: *P. tapetum* is allocated to *Proteleia* and *P. isidis* Thiele, 1905 is placed to *Sphaerotylus*, based on the re-examination of the type-material (details will come in a later study). *P. tapetum* possesses exotyles with the umbrelliform distal ornaments, the diagnostic feature of *Proteleia sollasi*. In fact the difference between these two species concerns only the additional cortical palisade which is present in *P. sollasi* but absent in *P. tapetum*. Exotyles with spherical distal knobs were discovered in *P. isidis*, and this feature together with some others is shared by *Sphaerotylus capitatus* (Vosmaer, 1885).

A unique species *P. umbraculum* possesses a combination of features of different genera: it shares a reticulated choanosomal skeleton with *Weberella*, exotyles bearing umbrelliform ornaments with *Proteleia*, three size categories of monactines and smooth centrotylote microxeas with a large number of polymastiid species. Thus, it cannot be grouped based on available morphological characters.

Conclusions

Both morphological and molecular evidence supports the exclusion of *Pseudotrachya* from Polymastiidae.

Quasillina and *Ridleia* are the next possible candidates to be excluded from Polymastiidae based on morphological evidence, but this assumption needs to be tested by molecular data.

Non-monophyly of *Polymastia* is evident from the morphological data and has some support from molecular data, although more genetic studies are required to confirm this.

Based on morphological data *Aaptos* is also non-monophyletic, but this has not been tested by molecular methods so far.

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References

Bergquist, P. R., 1961. A collection of Porifera from Northern New Zealand, with descriptions of seventeen new species. Pacific Science 15(1): 33–48.

Bergquist, P. R., 1968. The marine fauna of New Zealand: Porifera, Demospongiae, Part 1 (Tetractinomorpha and Lithistida). New Zealand Department of scientific and industrial research bulletin [New Zealand Oceanographic Institute Memoir 37] 188: 1–105.

Borojevic, R., 1967. La ponte et le développement de *Polymastia robusta* (Démosponges). Cahiers de Biologie Marine 8(1): 1–6.

Boury-Esnault, N., 1973. Résultats Scientifiques des Campagnes de la 'Calypso'. Campagne de la 'Calypso' au large des côtes atlantiques de l'Amérique du Sud (1961–1962). I. 29. Spongiaires. Annales de l'Institut océanographique 49(Supplement 10): 263–295.

Boury-Esnault, N., 1987. The *Polymastia* species (Demosponges, Hadromerida) of the Atlantic area. In Vacelet, J. & N. Boury-Esnault (eds), Taxonomy of Porifera from the N.E. Atlantic and Mediterranean Sea, NATO ASI Series, G 13. Springer, Berlin, Heidelberg: 29–66.

Boury-Esnault, N., 2002. Family Polymastiidae Gray, 1867. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera. A Guide to the Classification of Sponges, Vol. 1. Kluwer Academic/Plenum Publishers, New York: 201–219.

Boury-Esnault, N. & C. Bézac, 2007. Morphological and cytological descriptions of a new *Polymastia* species (Hadromerida, Demospongiae) from the North-West Mediterranean Sea. In Custódio, M. R., G. Lôbo-Hajdu, E. Hajdu & G. Muricy (eds), Porifera Research: Biodiversity, Innovation and Sustainability, Museu Nacional. Rio de Janeiro, Brazil: 23–30.

Boury-Esnault, N., M. Pansini & M. J. Uriz, 1994. Spongiaires bathyaux de la mer d'Alboran et du golfe ibéro-marocain. Mémoires du Muséum national d'Histoire naturelle 160: 1–174.

Bowerbank, J. S., 1861 (1860). List of British sponges. In McAndrew, R. (ed.), List of the British marine invertebrate fauna. Report of the British Association for the Advancement of Science 30: 279, 830–836.

Bowerbank, J. S., 1864a. A Monograph of the British Spongiadae, Vol. 1. Ray Society, London.

Bowerbank, J. S., 1864b. Description of two American sponges. The Canadian Naturalist and Geologist, New Series (Series 2) 1: 304–307.

Bowerbank, J. S., 1866. A Monograph of the British Spongiadae, Vol. 2. Ray Society, London.

Bowerbank, J. S., 1874. A Monograph of the British Spongiadae, Vol. 3. Ray Society, London.

Brøndsted, H. V., 1924 [1923]. Papers from Dr. Th. Mortensen's Pacific Expedition 1914–16. XV. Sponges from the Auckland and Campbell Islands. Videnskabelige Meddelelser fra Dansk naturhistorisk Forening i Kjøbenhavn 75: 117–167.

Burton, M., 1934. Sponges. Scientific reports of the Great Barrier Reef Expedition 1928–29 4(14): 513–621.

Burton, M., 1959. Sponges. In Scientific Reports, Vol. 10. John Murray Expedition, 1933–34. British Museum (Natural History), London: 151–281.

Cabioch, L., 1968. Contribution à la connaissance de la faune des Spongiaires de la Manche occidentale. Démosponges de la région de Roscoff. Cahiers de Biologie Marine 9(2): 211–246.

Carter, H. J., 1886. Descriptions of sponges from the neighbourhood of Port Phillip Heads, South Australia, continued. Annals and Magazine of Natural History (5) 17(97, 98, 101, 102): 40–53, 112–127, 431–441, 502–516.

De Laubenfels, M. W., 1949. The sponges of Woods Hole and adjacent waters. Bulletin of the Museum of Comparative Zoology at Harvard College 103: 1–55.

Dendy, A., 1888. Studies on the comparative anatomy of sponges. I. On the genera Ridleia, n. gen., and Quasillina, Norman. Quarterly Journal of Microscopical Science 282: 513–529.

Dendy, A. & S. O. Ridley, 1886. On *Proteleia sollasi*, a new genus and species of Monaxonid sponges allied to *Polymastia*. Annals and Magazine of Natural History 18: 152–159.

Descatoire, A., 1966. Sur quelques Démosponges de l'Archipel de Glénan. Cahiers de biologie marine 7: 231–246.

Gray, J. E., 1867. Notes on the arrangement of sponges, with the descriptions of some new genera. Proceedings of the Zoological Society of London 1867: 492–558.

Hallmann, E. F., 1914. A revision of the monaxonid species described as new in Lendenfeld's 'Catalogue of the sponges in the Australian museum'. Part I, II, III. Proceedings of the Linnean Society of New South Wales 39: 263–315, 327–376, 398–446.

Hansen, G. A., 1885. Spongiadae. The Norwegian North-Atlantic Expedition 1876–1878. Zoology 13: 1–26.

Hentschel, E., 1914. Monaxone Kieselschwämme und Hornschwämme der Deutschen Südpolar-Expedition 1901–1903. Deutsche Südpolar-Expedition, 1901–1903 15(1): 35–141.

Hills, R. V., 2005. Integration of morphological data Sets for phylogenetic analysis of Amniota: the importance of integumentary characters and increased taxonomic sampling. Systematic Biology 54(4): 530–547.

Keller, C., 1880. Neue Coelenteraten aus dem Golf von Neapel. Archiv für mikroskopische Anatomie und Entwicklungsmechanik 18: 271–280.

Kelly-Borges, M. & P. R. Bergquist, 1994. A redescription of *Aaptos aaptos* with descriptions of new species of *Aaptos* (Hadromerida: Suberitidae) from northern New Zealand. Journal of Zoology 234(2): 301–323.

Kelly-Borges, M. & P. R. Bergquist, 1997. Revision of Southwest Pacific Polymastiidae (Porifera: Demospongiae: Hadromerida) with descriptions of new species of *Polymastia* Bowerbank, *Tylexocladus* Topsent, and *Acanthopolymastia* gen nov. from New Zealand and the Norfolk Ridge, New Caledonia. New Zealand Journal of Marine and Freshwater Research 31: 367–402.

Kirkpatrick, R., 1907. Preliminary Report on the Monaxonellida of the National Antarctic Expedition. Annals and Magazine of Natural History 20: 271–291.

Kirkpatrick, R., 1908. Porifera (Sponges). II. Tetraxonida, Dendy. National Antarctic Expedition, 1901–1904, Natural History, 4, Zoology: 1–56.

Koltun, V. M., 1964a. Sponges (Porifera), collected in the Greenland Sea and in the North area off Spitzbergen and Frantz Josef Land by r/v "F. Litke" in 1955, r/v "Ob" in 1956 and r/v "Lena" in 1957-1958. Oceanographic Expeditions to the northern part of the Greenland Sea and the adjacent arctic basin. Publications Arctic and Antarctic Scientific Institute 259: 143–166.

Koltun, V. M., 1964b. Sponges of the Antarctic. Part 1. Tetraxonida and Cornacuspongida. In Pavlovskii, E. P., A. P. Andriyashev & P. V. Ushakov (eds), Biological Reports of the Soviet Antarctic Expedition (1955–1958), Explorations of the fauna of the seas, 2. Academy of Sciences of the USSR, Nauka, Moscow–Leningrad: 6–133, 443–448.

Koltun, V. M., 1966. Four-rayed sponges (order Tetraxonida) of the Northern and Far Eastern Seas of the USSR. Identifiers of the USSR fauna issued by the Zoological Institute of the Academy of Sciences of the USSR, 90. Academy of Sciences of the USSR, Nauka, Moscow–Leningrad.

Koltun, V. M., 1970. Sponge fauna of the north-western Pacific from the shallows to the ultra-abyssal depths. Report 1. Fauna of the Kurile-Kamchatka Trench and its environment (on the materials of the 39th cruise of the R/V "Vityaz"), Proceedings of the Institute of Oceanology, Academy of Sciences of the USSR 86: 165–221.

Lamarck, J. B. P. De Monet, Comte De, 1815 [1814]. Suite des polypiers empâtés. Mémoires du Muséum d'Histoire naturelle, Paris 1: 69–80, 162–168, 331–340.

Lambe, L. M. 1896. Sponges from the Atlantic coast of Canada. Transactions of the Royal Society of Canada, section 2 (2) 2: 181–211.

Lehnert, H., Stone, R. & W. Heimler, 2005. A new species of Polymastia (Porifera, Hadromerida, Polymastiidae) from the Aleutian Islands, Alaska, USA. Facies 51(1–4): 53–56.

Maddison, W. P., 1993. Missing data versus missing characters in phylogenetic analysis. Systematic Biology 42: 576–581.

Merejkowsky, C., 1878. Étude sur les Éponges de la Mer Blanche. Mémoires de l'Académie Impériale des sciences de St. Pétersbourg Series 7 26(7): 1–51.

Montagu, G., 1818. An essay on sponges, with descriptions of all the species that have been discovered on the coast of Great Britain. Memoirs of the Wernerian Natural History Society 2(1): 67–122.

Morrow, C. & N. Boury-Esnault, 2000. Redescription of the type species of the genus *Polymastia* Bowerbank, 1864 (Porifera, Demospongiae, Hadromerida). Zoosystema 22: 327–335.

Müller, O. F. 1806. Zoologia danica seu animalium Daniae et Norvegiae rariorum ac minus notorum. Descriptiones et Historia. 4. (N. Christensen: Hauniae): 1–46.

Nardo, G. D., 1833. Auszug aus einem neuen System der Spongiarien, wonach bereits die Aufstellung in der Universitäts-Sammlung zu Padua gemacht ist. In Isis, oder Encyclopädische Zeitung Coll. Oken, Jena: 519–523.

Nichols, S. A., 2005. An evaluation of support for order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. Molecular Phylogenetics and Evolution 34: 81–96.

Norman, A. M., 1869. Notes on a few Hebridean Sponges, and on a new *Desmacidon* from Jersey. Annals and Magazine of Natural History 3: 296–299.

Olivi, G. 1792. Zoologia Adriatica, ossia Catalogo ragionato degli Animali del Golfo e delle Lagune di Venezia: preceduto da una dissertazione sulla storia fisica e naturale del Golfo; e accompagnato da memorie, ed osservazioni di fisica storia naturale ed economia. (Lettera del signor G. Stange contenente la descrizione di alcune Spugne assai curiose dei lidi del Mare Mediterraneo in Italia, inserita nelle Transazioni Filosofische…1770. Della natura delle Spongie di Mare, e particolarmente, delle piu rare, che alligano nel Golfo di Smirne. Lettera del…G. Vio). (Bassano:): i–xxxiip.

Pleijel, P., 1995. On character coding for phylogenetic analysis. Cladistics 11: 309–315.

Plotkin, A., 2004. Sponge sciences in new millennium. In Pansini, M., R. Pronzato, G. Bavestrello & R. Manconi (eds), Biodiversity and Distribution of Polymastiidae (Demospongiae, Hadromerida) in the Arctic Area, Vol. 68. Bollettino dei Musei e degli Instituti Biologici dell'Universita di Genova, Genova: 535–547.

Plotkin, A. & N. Boury-Esnault, 2004. Alleged cosmopolitanism in sponges: the example of a common Arctic *Polymastia* (Porifera, Demospongiae, Hadromerida). Zoosystema 26(1): 13–20.

Plotkin, A. S. & D. Janussen, 2007. New genus and species of Polymastiidae (Demospongiae: Hadromerida) from the Antarctic deep sea. Journal of the Marine Biological Association of the United Kingdom 87(6): 1395–1401.

Plotkin, A. & D. Janussen, 2008. Polymastiidae and Suberitidae (Porifera: Demospongiae: Hadromerida) of the deep Weddell Sea, Antarctic. In Martínez Arbizu, P. & S. Brix (eds), Bringing Light into Deep-Sea Biodiversity. Zootaxa 1866: 95–135.

Pulitzer-Finali, G., 1986. A collection of West Indian Demospongiae (Porifera). In appendix, a list of the Demospongiae hitherto recorded from the West Indies. Annali del Museo civico di storia naturale Giacomo Doria 86: 65–216.

Rezvoj, P. D., 1927. A new species of sponges, *Polymastia euplectella*, from the Murman Coast. Comptes Rendus de l'Académie des Sciences USSR 18: 301–302.

Ridley, S. O. & A. Dendy, 1886. Preliminary Report on the Monaxonida collected by H.M.S. "Challenger". Annals and Magazine of Natural History 18(325–351): 470–493.

Ridley, S. O. & A. Dendy, 1887. Report on the Monaxonida collected by H.M.S. "Challenger" during the years 1873–1876. Report on the Scientific Results of the Voyage of H.M.S. "Challenger", 1873–1876, Zoology 20: 1–275.

Samaai, T. & M. J. Gibbons, 2005. Demospongiae taxonomy and biodiversity of the Benguela region on the west coast of South Africa. African Natural History 1: 1–96.

Sarà, M. & B. Burlando, 1994. Phylogenetic reconstruction and evolutionary hypotheses in the family Tethyidae (Demospongiae). In van Soest, R. W. M., Th. M. G. van Kempen & J.-C. Braekman (eds), Sponges in Time and Space. Balkema, Rotterdam: 111–116.

Sars, G. O., 1872. Spongiae. In Kongelige Norske Universitet (ed.), On some remarkable forms of animal life from the great depths off the Norwegian coast. I. Partly from posthumous manuscripts of the late professor Dr. Michael Sars. Brøgger & Christie. Christiania, Norway: 62–82.

Schmidt, O., 1864. Supplement der Spongien des adriatischen Meeres. Enthaltend die Histologie und systematische Ergänzungen. Wilhelm Engelmann, Leipzig: 1–48.

Schmidt, O., 1870. Grundzüge einer spongien-fauna des Atlantischen gebietes. Wilhelm Engelmann, Leipzig.

Stephens, J., 1915. Sponges of the coasts of Ireland. I. The Triaxonia and part of the Tetraxonida. Scientific investigations of the Fisheries Branch of the Department of Agriculture for Ireland 4: 1–43.

Strong, E. E. & D. Lipscomb, 1999. Character coding and inapplicable data. Cladistics 15: 363–371.

Swarczewsky, B. A., 1906. Beiträge zur Spongien-Fauna des Weissen Meeres. Memoires de la Société des Naturalistes de Kiew 20: 307–371.

Swofford, D. L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Sinauer Associates, Sunderland, MA.

Thiele, J., 1898. Studien über pazifische Spongien. I. Japanische Demospongien. Zoologica. Original-Abhandlungen aus dem Gesamtgebiete der Zoologie. Stuttgart 24(1): 1–72.

Thiele, J., 1905. Die Kiesel- und Hornschwämme der Sammlung Plate. Zoologische Jahrbücher Supplement 6 (Fauna Chiliensis III): 407–496.

Topsent, E., 1890. Notice préliminaire sur les spongiaires recueillis durant les campagnes de l'Hirondelle. Bulletin de la Société zoologique de France 15: 26–32, 65–71.

Topsent, E., 1898. Eponges nouvelles des Açores. Première serie. Mémoires de la Société zoologique de France 11: 225–255.

Topsent, E., 1913. Spongiaires provenant des campagnes scientifiques de la Princesse "Alice" dans les Mers du Nord (1898–1899, 1906–1907). Résultats des campagnes scientifiques accomplies par le Prince Albert I. Monaco 45: 1–67.

Topsent, E., 1917. Spongiaires. In Joubin, L. (ed.), Deuxième Expédition Antarctique Française (1908–1910) Commandée par le Dr. Jean Charcot. Sciences Physiques: Documents Scientifiques, 4. Masson & Cie, Paris: 1–88.

Topsent, E., 1927. Diagnoses d'Éponges nouvelles recueillies par le Prince Albert ler de Monaco. Bulletin de l'Institut océanographique Monaco 502: 1–19.

Topsent, E., 1928. Spongiaires de l'Atlantique et de la Méditerranée provenant des croisières du Prince Albert ler de Monaco. Résultats des campagnes scientifiques accomplies par le Prince Albert I de Monaco 74: 1–376.

Topsent, E. 1933. Eponges de Lamarck conservées au Muséum de Paris. Fin. Archives du Muséum national d'histoire naturelle. Paris (6) 10: 1–60.

Vacelet, J., 1961. Quelques Eponges remarquables de Méditerranée. Revue des Travaux de l'Institut des Pêches maritimes 25(3): 351–354.

van Soest, R. W. M., 2002. Family Suberitidae Schmidt, 1870. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera. A Guide to the Classification of Sponges, Vol. 1. Kluwer Academic/Plenum Publishers, New York: 227–244.

van Soest, R. W. M. & N. Stentoft, 1988. Barbados deep-water sponges. In Hummelinck, P. W. & L. J. Van der Steen (eds), Uitgaven van de Natuurwetenschappelijke Studiekring voor Suriname en de Nederlandse Antillen. No. 122. Studies on the Fauna of Curaçao and other Caribbean Islands 70(215): 1–175.

van Soest, R. W. M., B. Picton & C. Morrow, 1999. Sponges of the North East Atlantic. World Biodiversity Database CD-ROM Series. ETI, Amsterdam.

van Soest, R. W. M, N. Boury-Esnault, J. N. A. Hooper, K. Rützler, N. J. de Voogd, B. Alvarez de Glasby, E. Hajdu, A. B. Pisera, R. Manconi, C. Schoenberg, D. Janussen, K. R. Tabachnick, M. Klautau, B. Picton & M. Kelly, 2010. World Porifera database. Available online at http://www.marinespecies.org/porifera, consulted on 2010-12-21.

von Lendenfeld, R., 1888. Descriptive catalogue of the sponges in the Australian Museum, Sidney. Taylor & Francis, London.

Vosmaer, G. C. J., 1885. The Sponges of the "Willem Barents" Expedition 1880 and 1881. Bijdragen tot de Dierkunde 12: 1–47.

Vosmaer, G. C. J., 1887. Klassen und Ordnungen der Spongien (Porifera). In Bronn, H.G. (ed.), Die Klassen und Ordnungen des Thierreichs, 2, Leipzig–Heidelberg: 1–496.

Voultsiadou, E. & D. Vafidis, 2004. Rare sponge (Porifera: Demospongiae) species from the Mediterranean Sea. Journal of the Marine Biological Association of the United Kingdom 84: 593–598.

Wilkinson, M., 1995. A comparison of two methods of character construction. Cladistics 11: 297–308.

Wilson, H. V., 1904. Reports on an exploration off the West coasts of Mexico, Central and South America, and off the Galapagos Islands, in charge of Alexander Agassiz, by the U.S. Fish Commission Steamer 'Albatross' during 1891, Lieut. Commander Z.L. Tanner, U.S.S., commanding. XXX. The Sponges. Memoirs of the Museum of Comparative Zoology at Harvard College 30(1): 1–164.

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 Short Research Note

First evidence of miniature transposable elements in sponges (Porifera)

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Abstract Transposable elements play a vital role in genome evolution and may have been important for the formation of the early metazoan genome, but only little is known about transposons at this interface between unicellular opisthokonts and Metazoa. Here, we describe the first miniature transposable elements (MITEs, *Queen1* and *Queen2*) in sponges. *Queen1* and *Queen2* are probably derived from Tc1/mariner-like MITE families and are represented in more than 3,800 and 1,700 copies, respectively, in the *Amphimedon queenslandica* genome. *Queen* elements are located in intergenic regions as well as in introns, providing the potential to induce new splicing sites and termination signals in the genes. Further possible impacts of MITEs on the evolution of the metazoan genome are discussed.

Keywords Early diverging Metazoa · Porifera · Transposable element · MITE · *Amphimedon queenslandica* · *Queen* elements

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The origin of animals is among the greatest enigmas in evolutionary biology. In particular, genomic evolution during the transition of unicellular protists to multicellular metazoans is still largely speculative

(Hoenigsberg et al., 2008). Most comparative approaches in genomics focus on shared genes and gene families; however, other genomic components, such as transposable elements, which have been important in genome formation (Brosius, 1999; Batzer & Deininger, 2002; Kazazian, 2004), remain understudied.

Transposable elements encompass mobile DNA sequences that can integrate into the genome at new positions. They are represented in most eukaryotes where they account for up to 90% of the genome (Bailey et al., 2003). Strand breaks during transposition and insertion can trigger various genomic rearrangements (Lim & Simmons, 1994; Zhang & Peterson, 2004). Furthermore, transposons occasionally evolve into new genes or parts of genes, including regulatory elements (Brosius & Gould, 1992; Deininger & Batzer, 1999; Feschotte et al., 2002; Krull et al., 2007; Kuang et al., 2008). Consequently, transposable elements have an enormous evolutionary potential by directly influencing phenotypes encoded in genomes, ranging from subtle regulatory perturbations to the complete loss of gene function (Feschotte & Pritham, 2007).

For the evolution of multidomain proteins in Metazoa (animals), transposable element-mediated exon shuffling may be an important mechanism to move encoded motifs and domains (Tordai et al., 2005; Feschotte, 2008). Comparative genomics of basal metazoans and choanoflagellates, the closest living relatives of animals, has recently revealed uniquely arranged combinations of metazoan domains in the choanoflagellate sister group (King et al., 2008). Novel domain architectures, for example in cnidarians and sponges (Adamska et al., 2007; Putnam et al., 2007; Larroux et al., 2008), both basal metazoan phyletic lineages, might be transposable element-mediated exon rearrangements that occurred during early metazoan evolution. Indeed, transposable elements may have played a pivotal role in the genesis of many metazoan-specific gene families prior to the divergence of Porifera (possibly the earliest diverging extant Metazoa, e.g., Philippe et al., 2009) and eumetazoan lineages. Novel domain architectures emerging prior to animal cladogenesis appear to underpin metazoan-specific regulatory and protein networks that comprise cellular, developmental, and morphological synapomorphies, as revealed by the recently published genome of the demosponge *Amphimedon queenslandica* (Srivastava et al., 2010).

In the case of sponges, physiology and evolution affords a rapidly shifting capacity to produce complex secondary metabolites, which may be modulated by transposable element-mediated changes in the genome.

While 25% of the anthozoan *Nematostella vectensis* genome consists of transposable elements (Putnam et al., 2007), only little is known about transposable elements in *Amphimedon queenslandica,* the first sponge genome to be sequenced (Srivastava et al., 2010). Our own estimations also point to a 20-30% contribution of transposable elements to the *Amphimedon queenslandica* genome (unpublished observations), but information on elements in other sponges is scarce (e.g., Arkhipova, 2001; Wiens et al., 2009). Today, the only sponge element with a published sequence is a long terminal repeat-retrotransposon from the freshwater sponge *Lubomirskia baicalensis* (Wiens et al., 2009).

In the assembled genome of the demosponge *Amphimedon queenslandica* we have identified the first miniature transposable elements (MITEs) that we call "*Queen1*" and "*Queen2*" (see Supplementary Methods). MITEs are short, nonautonomous DNA transposons with high copy numbers and homogeneous lengths (Bureau & Wessler, 1992; Zhang et al., 2000).

Queen1 is 210 bp long with an inverted terminal repeat (TIR) of 28 bp, including a potential dinucleotide (TA) target site duplication (TSD), a 3-bp linker, and 14 bp of a sub-TIR sequence (Fig. 1). We estimated that there are more than 3,800 *Queen1* elements in the *A. queenslandica* genome (0.28% of the genome). Thirty-four percent of full-length *Queen1* elements display 90% identical inverted repeats (IRs).

Queen2 comprises more than 1,700 elements of 245 bp with a 25-bp TIR, a 2-bp linker, and 11 bp of sub-TIR (0.15% of the genome). Fifty-seven percent of full-length elements display 90% perfect IRs, indicating a relatively recent activity period. *Queen1* and *2* appear to be novel MITEs, probably deriving from the widespread Tc1/mariner transposase superfamily, as they also possess characteristic 5′-TA-3′ target sites (Fig. 2). Except for this target site preference, there is no sequence similarity between *Queen1* and *2*. Adjacent *Queen* elements are significantly often of the same type ($P < 0.01$).

Queen elements have not been detected in other organisms thus far, including in EST data of other sponges, and the high copy numbers of these MITEs

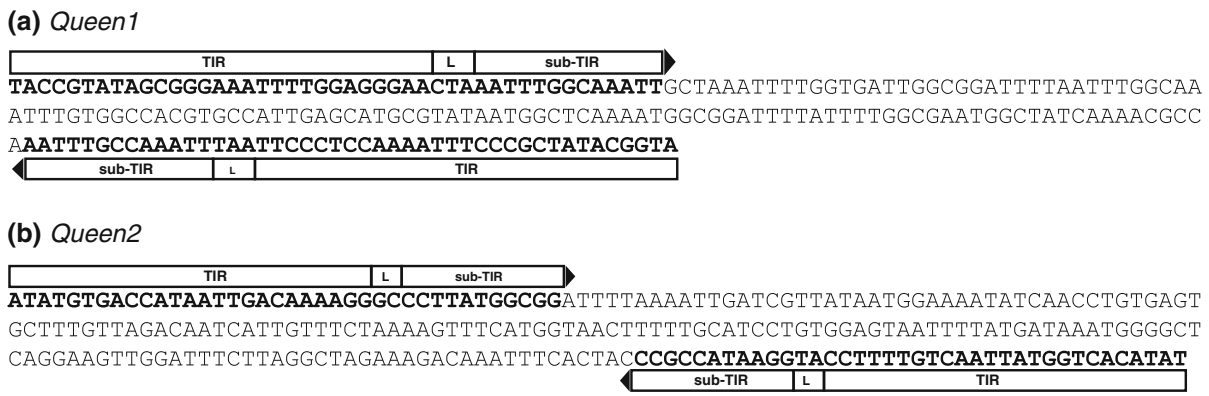

Fig. 1 *Queen1* and *Queen2* as detected in *A. queenslandica*. Arrows indicate location and direction of terminal inverted repeat (TIR), linker (L), and sub-TIR (sub-TIR) sequences

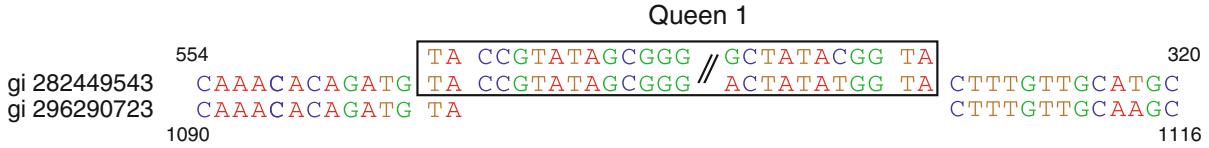

Fig. 2 Presence of *Queen1* (boxed region) in an EST sequence (gi282449543) and its absence at the genomic level (gi296290723). The discrepancy between EST and genome is probably caused by different experimental sources for the cDNAs and DNA extraction, and is also an indication that this specific MITE element inserted recently and/or is polymorphic. The coordinates of the sequences are indicated. The upper sequence in the box represents the *Queen1* consensus sequence (65%). The slashes replace 196 nucleotides of the MITE element

is unusual for nonbilaterian Metazoa. In the Placozoa *Trichoplax adhaerens*, MITEs are very rare; genome-wide, only a single family with about 20 copies is represented (Wang et al., 2010b). Likewise, the number of *CMITE* elements found in two stony coral genomes (Wang et al., 2010a) is far less than the number of *Queen* copies in *A. queenslandica*.

Generally, MITEs are located in low-copy-number genomic regions and in gene-rich environments (Bureau & Wessler, 1992; Zhang et al., 2000). Their frequent insertions close to genes indicates a significant potential for generating allelic and genomic diversity (Feschotte & Pritham, 2007). Consequently, regulatory and coding mutations are a frequent side effect of MITE insertion (Bureau & Wessler, 1992; Nakazaki et al., 2003; Xu et al., 2007; Kuang et al., 2008). We detected several *Queen* elements located in introns, where they might potentially influence the splicing of a pre-mRNA by introducing new splice sites, resulting in intron retention, exon skipping, or the creation of new exon/intron boundaries (Feschotte, 2008). This may result in new protein isoforms with different functions and fitness advantages; especially, because *Queen* elements might induce new splicing sites.

In this context, we detected what appeared to be *Queen* elements in the transcriptome of *A. queenslandica*; *Queen2* elements were present in many ESTs. However, currently the amount of EST data for *A. queenslandica* is very limited (63,542 EST sequences compared to the half million such sequences in *C. elegans* or several millions in human). So far no case of MITE inclusion in protein-coding sequence regions (in the sense orientation to genes) could be determined. In contrast, a detailed analysis of the predominantly computationally predicted annotation of the *A. queenslandica* genome did indicate such cases. However, careful inspections and RT-PCR analyses could not confirm any of the potential protein-coding MITE cassettes (data not shown).

Our results shed light on the occurrence and abundance of miniature transposable elements in sponges. The genetic features of *Queen1* and *Queen2* corroborate hypotheses that such transposable elements might have contributed to the evolution of early

Metazoa and the formation of the early-branching metazoan genome (Hoenigsberg et al., 2008). MITE insertions in introns and exons of (vital) genes, such as *Queen* element insertions in *A. queenslandica*, might have a wide range of effects at the transcriptional and posttranscriptional levels, as summarized by Feschotte (2008): (1) *Queen* insertions in the untranscribed region of genes might disrupt existing promoters, transcription start sites, and regulatory elements. (2) An intronic insertion of a *Queen* element might trigger antisense transcription and inhibition of sense transcription, as was shown for MITEs in Solanaceae (Kuang et al., 2008). (3) Once inserted into an intron, *Queen* elements might trigger formation of heterochromatin, leading to transcriptional inhibition of adjacent genes (Cam et al., 2008). (4) An intronic *Queen* element may interfere with the normal splicing pattern of a pre-mRNA, leading to various forms of alternative splicing (e.g., intron retention and exon skipping). (5) A *Queen* element that has inserted into an intron and contains cryptic splice sites may be incorporated as an alternative exon, which may result in the translation of a new protein isoform or in the destabilization or degradation of the mRNA by the nonsense-mediated decay (NMD) pathway.

The sequence divergence observed among *Queen1* and *Queen2* elements indicates that MITE integrations may be an ongoing process shaping the *A. queenslandica* genome. The full gambit of evolutionary effects caused by MITEs and other transposable elements will be evident with the discovery and characterization of a more complete set of TEs in *A. queenslandica* and other sponge classes and a comprehensive transcriptome analysis. Genome sequencing of calcareous, hexactinellid, and homoscleromorph sponges is therefore eagerly needed.

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References

Adamska, M., D. Matus, M. Adamski, K. Green, D. Rokhsar, M. Martindale & B. Degnan, 2007. The evolutionary origin of hedgehog proteins. Current Biology 17: R836–R837.

Arkhipova, I. R., 2001. Transposable elements in the animal kingdom. Molecular Biology 35: 157–167.

Bailey, J. A., G. Liu & E. E. Eichler, 2003. An *Alu* transposition model for the origin and expansion of human segmental duplications. American Journal of Human Genetics 73: 823–834.

Batzer, M. A. & P. L. Deininger, 2002. *Alu* repeats and human genomic diversity. Nature Reviews Genetics 3: 370–379.

Brosius, J. & S. Gould, 1992. On "genomenclature": a comprehensive (and respectful) taxonomy for pseudogenes and other" junk DNA". Proceedings of the National Academy of Sciences of the United States of America 89: 10706–10710.

Brosius, J., 1999. Transmutation of tRNA over time. Nature Genetics 22: 8–9.

Bureau, T. E. & S. R. Wessler, 1992. *Tourist* – a large family of small inverted repeat elements frequently associated with maize genes. Plant Cell 4: 1283–1294.

Cam, H. P., K. Noma, H. Ebina, H. L. Levin & S. I. S. Grewal, 2008. Host genome surveillance for retrotransposons by transposon-derived proteins. Nature 451: 431. U2.

Deininger, P. L. & M. A. Batzer, 1999. *Alu* repeats and human disease. Molecular Genetics and Metabolism 67: 183–193.

Feschotte, C., 2008. Opinion – transposable elements and the evolution of regulatory networks. Nature Reviews Genetics 9: 397–405.

Feschotte, C. & E. J. Pritham, 2007. DNA transposons and the evolution of eukaryotic genomes. Annual Review of Genetics 41: 331–368.

Feschotte, C., N. Jiang & S. R. Wessler, 2002. Plant transposable elements: where genetics meets genomics. Nature Reviews Genetics 3: 329–341.

Hoenigsberg, H. F., M. H. Tijaro & C. Sanabria, 2008. From unicellularity to multicellularity – molecular speculations about early animal evolution. Genetics and Molecular Research 7: 50–59.

Kazazian, H. H., 2004. Mobile elements: drivers of genome evolution. Science 303: 1626–1632.

King, N., M. Westbrook, S. Young, A. Kuo, M. Abedin, J. Chapman, S. Fairclough, U. Hellsten, Y. Isogai, I. Letunic, M. Marr, D. Pincus, N. Putnam, A. Rokas, K. Wright, R. Zuzow, W. Dirks, M. Good, D. Goodstein, D. Lemons, W. Li, J. Lyons, A. Morris, S. Nichols, D. Richter, A. Salamov, J. Sequencing, P. Bork, W. Lim, G. Manning, W. Miller, W. Mcginnis, H. Shapiro, R. Tjian, I. Grigoriev & D. Rokhsar, 2008. The genome of the choanoflagellate *Monosiga brevicollis* and the origin of metazoans. Nature 451: 783–788.

Krull, M., M. Petrusma, W. Makalowski, J. Brosius & J. Schmitz, 2007. Functional persistence of exonized mammalian-wide interspersed repeat elements (MIRs). Genome Research 17: 1139–1145.

Kuang, H., C. Padmanabhan, F. Li, A. Kamei, P. B. Bhaskar, S. Ouyang, J. Jiang, C. R. Buell & B. Baker, 2008. Identification of miniature inverted-repeat transposable elements

(MITEs) and biogenesis of their siRNAs in the Solanaceae: new functional implications for MITEs. Genome Research 19: 42–56.

Larroux, C., G. N. Luke, P. Koopman, D. S. Rokhsar, S. M. Shimeld & B. M. Degnan, 2008. Genesis and expansion of metazoan transcription factor gene classes. Molecular Biology and Evolution 25: 980–996.

Lim, J. K. & M. J. Simmons, 1994. Gross chromosome rearrangements mediated by transposable elements in *Drosophila melanogaster*. Bioessays 16: 269–275.

Nakazaki, T., Y. Okumoto, A. Horibata, S. Yamahira, M. Teraishi, H. Nishida, H. Inoue & T. Tanisaka, 2003. Mobilization of a transposon in the rice genome. Nature 421: 170–172.

Philippe, H., R. Derelle, P. Lopez, K. Pick, C. Borchiellini, N. Boury-Esnault, J. Vacelet, E. Deniel, E. Houliston, E. Quéinnec, C. Dasilva, P. Wincker, H. Le Guyader, S. Leys, D. J. Jackson, F. Schreiber, D. Erpenbeck, B. Morgenstern, G. Wörheide & M. Manuel, 2009. Phylogenomics restores traditional views on deep animal relationships. Current Biology 19: 706–712.

Putnam, N. H., M. Srivastava, U. Hellsten, B. Dirks, J. Chapman, A. Salamov, A. Terry, H. Shapiro, E. Lindquist, V. V. Kapitonov, J. Jurka, G. Genikhovich, I. V. Grigoriev, S. M. Lucas, R. E. Steele, J. R. Finnerty, U. Technau, M. Q. Martindale & D. S. Rokhsar, 2007. Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. Science 317: 86–94.

Srivastava, M., O. Simakov, J. Chapman, B. Fahey, M. E. A. Gauthier, T. Mitros, G. S. Richards, C. Conaco, M. Dacre, U. Hellsten, C. Larroux, N. H. Putnam, M. Stanke, M. Adamska, A. Darling, S. M. Degnan, T. H. Oakley, D. C. Plachetzki, Y. Zhai, M. Adamski, A. Calcino, S. F. Cummins, D. M. Goodstein, C. Harris, D. J. Jackson, S. P. Leys, S. Shu, B. J. Woodcroft, M. Vervoort, K. S. Kosik, G. Manning, B. M. Degnan & D. S. Rokhsar, 2010. The *Amphimedon queenslandica* genome and the evolution of animal complexity. Nature 466: 720–726.

Tordai, H., A. Nagy, K. Farkas, L. Banyai & L. Patthy, 2005. Modules, multidomain proteins and organismic complexity. FEBS Journal 272: 5064–5078.

Wang, S., L. Zhang, E. Meyer & M. V. Matz, 2010a. Characterization of a group of MITEs with unusual features from two coral genomes. PLoS ONE 5: e10700.

Wang, S., L .L. Zhang, E. Meyer & Z. M. Bao, 2010b. Genome-wide analysis of transposable elements and tandem repeats in the compact placozoan genome. Biology Direct 5.

Wiens, M., V. A. Grebenjuk, H. C. Schröder, I. M. Müller & W. E. G. Müller, 2009. Identification and isolation of a retrotransposon from the freshwater sponge *Lubomirskia baicalensis*: implication in rapid evolution of endemic sponges. Progress in Molecular and Subcellular Biology 47: 207–234.

Xu, L., L. J. Wang, T. Liu, W. Q. Qlan, Y. Gao & C. C. An, 2007. *Triton*, a novel family of miniature inverted-repeat transposable elements (MITEs) in *Trichosanthes kirilowii* Maximowicz and its effect on gene regulation. Biochemical and Biophysical Research Communications 364: 668–674.

Zhang, J. B. & T. Peterson, 2004. Transposition of reversed *Ac* element ends generates chromosome rearrangements in maize. Genetics 167: 1929–1937.

Zhang, Q., J. Arbuckle & S.R. Wessler, 2000. Recent, extensive, and preferential insertion of members of the miniature inverted-repeat transposable element family *Heartbreaker* into genic regions of maize. Proceedings of the National Academy of Sciences of the United States of America 97: 1160–1165.

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SPONGE RESEARCH DEVELOPMENTS

The mitochondrial genome of stygobitic sponge *Eunapius subterraneus*: mtDNA is highly conserved in freshwater sponges

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Abstract The complete mitochondrial DNA (mtDNA) genome of the *Eunapius subterraneus* (Porifera, Demospongiae), a unique stygobitic sponge, was analyzed and compared with previously published mitochondrial genomes from this group. The 24,850 bp long mtDNA genome is circular with the same gene composition as found in other metazoans. Intergenic regions (IGRs) comprise 24.7% of mtDNA and are abundant with direct and inverted repeats and palindromic elements as well as with open reading fames (ORFs) whose distribution and homology was compared with other available mt genomes with a special focus on freshwater sponges. Phylogenetic analyses based on concatenated amino acid sequences from 12 mt protein genes placed *E. subterraneus* in a well-supported monophyletic clade with the freshwater sponges, *Ephydatia muelleri* and *Lubomirskia baicalensis*. Our study showed high homology of mtDNA genomes among freshwater sponges, implying their recent split.

Keywords Porifera · Spongillidae · Stygobitic · Mitochondrial evolution

Introduction

Sponges (Porifera) are ancient metazoans that mainly inhabit marine ecosystems, with the notable exception of the suborder Spongillina, the group of extant freshwater demosponges whose fossil record begins in the Cretaceous. Only a small proportion of over 8,000 described species of sponges live in freshwater (Hooper & Van Soest, 2002). The cosmopolitan suborder Spongillina comprises more than 150 species included in 21 genera (Manconi & Pronzato,

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2002). Minimum evolution (ME) and maximum likelihood (ML) molecular clocks showed that freshwater sponges branched off between 183 and 141 MYa (Peterson & Butterfield, 2005). *Eunapius subterraneus* (Sket & Velikonja, 1984) is the only stygobitic member of the suborder Spongillina known from just few caves near Ogulin, Croatia (Bilandžija et al., 2007). This endemic sponge is classified in the IUCN category EN (endangered species) as an organism which is at high risk of becoming extinct (Bilandžija et al., 2009). Cave fauna stands out among all other freshwater biota owning peculiar species which are distinct from those in surrounding surface freshwater habitats.

The phylogenetic relationships between the freshwater sponge families are not fully resolved yet. Regardless of intensive morphology and molecular biology-based research, relationships within freshwater sponge taxa are very problematic and remain unclear. It has been suggested that the genus *Ephydatia* is paraphyletic and that endemic sponge species might have originated from such cosmopolitan founder species (Addis & Peterson, 2005; Meixner et al., 2007). Phylogenetic study of *E. subterraneus* based on three molecular markers (18S rDNA, ITS2 and COI), showed that it does not group with *Eunapius* species, but rather with other freshwater sponge genera, thus raising the question of true taxonomical designation of *E. subterraneus* (Harcet et al., 2010). Recent trends in molecular evolutionary studies pointed out sponges as excellent model organisms. The fact that sponges exhibit an interesting intermediate state of mitochondrial genome size between the large 76 kbp choanoflagellate (Burger et al., 2003) and the typical bilaterian mitochondrial (mt) genomes is important for investigating ancient animal relationships and mitochondrial genome evolution. The typical bilaterian mt genome is a compact molecule of 14–18 kb that contains 13 protein-coding genes, 22 transfer RNA (tRNA) genes and 2 ribosomal RNA (rRNA) genes (Boore, 1999). Sponges, as the first diverging animal phyla, have larger mt genomes (18–29 kbp) than typical of bilaterians (Lavrov, 2007; Lavrov, 2009). Demosponge mtDNA has a well conserved gene order and compact organization (Wang & Lavrov, 2008). At the same time it harbors additional rare genomic characters, such as the presence of additional genes including *atp9* (except *Amphimedon queenslandica*) (Erpenbeck et al., 2007), *trnI(cau)*, *trnR(ucu)*, multiple non-coding regions, a minimally derived genetics code and bacteria-like rRNA and tRNA genes (Lavrov et al., 2005). The larger size of mtDNA is mainly outcome of larger non-coding regions that are abundant with palindroms and repetitive elements (Lavrov, 2007; Lavrov, 2009). In this work, we report the complete mt genome of *E. subterraneus*, thus increasing the total number of mt genomes of sponges, especially within the family Spongillidae, available for phylogenetic analyses. Mt genome was analyzed and sequences were characterized regarding their length, gene content, and organization. These features were compared with mt sequences from other sponges with a special focus on freshwater sponges. Furthermore, analysis of intergenic regions provided significant information that could improve phylogenetic determination at the taxonomic level of order and/or genus. Non coding regions retained some ancestral features and are abundant with palindroms, direct and inverted repeats as previously reported (Lavrov, 2009; Erpenbeck et al., 2009). Moreover, numerous open reading frames (ORFs) were found and their distribution and homology was compared with other available mt genomes.

Materials and methods

Taxon sampling, DNA isolation, mtDNA PCR amplification, and sequencing

Live specimens of *E. subterraneus* were collected by cave diving near Ogulin (north-western Croatia). Material was kept at −80°C before use in the experiments. Total DNA was extracted from 0.2 g of tissue with Qiagen Genomic DNA kit. Degenerative primer sets were designed in our laboratory on the basis of multiple alignments with sequences available in GenBank and used for amplification of conserved regions of protein coding genes. Complete mtDNA of *E. subterraneus* was directly sequenced by primer walking using the ABI PRISM 3100 automatic sequencer. Sequencing reads were assembled using Lasergene processing software (DNASTAR Inc., Madison, USA) and checked manually for sequencing errors. Complete mitochondrial sequence is available in NCBI's GenBank under the accession number: GU086203.

Gene identification and sequence analysis

tRNA genes were identified by the tRNAscan-SE program (Lowe & Eddy, 1997) and secondary structures were drawn using CorelDraw12. Other genes were identified by homology searches in GenBank using BLAST (Benson et al., 2003). The secondary structures of rRNA genes were manually folded by analogy to published rRNA structures and drawn with CorelDraw12.

The Palindrome program from the EMBOSS software package (Rice et al., 2000) was used to search for closely spaced perfect inverted repeats with parameters as follows: minimum length of palindromic elements—7 nt; maximum length of palindromes—100 nt; maximum gap between repeat elements—10 nt. Secondary structures were predicted with mfold-server server http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi.

Phylogenetic analysis

Concatenated amino acid sequences of mitochondrial protein-coding genes were aligned under the default parameters in ClustalW 1.7 (Thompson et al., 1994). Ambiguously aligned regions were determined and excluded from further analyses using the program Gblocks 0.91b (Castresana, 2000), under the following conditions: minimum number of sequences for a conserved position: 15; minimum number of sequences for a flanking position: 23; maximum number of contiguous nonconserved positions: 4; minimum length of a block: 10; allowed gap positions: with half, use similarity matrices: yes. Final alignment was 3,599 aa long (available upon request). Akaike Information Criterion (AIC) implemented in ProtTest v. 2.4 (Drummond & Strimmer, 2001; Guindon & Gascuel, 2003; Abascal et al., 2005) was used to select the best-fit model of protein evolution. Phylogenetic analyses were performed under the AIC best-fit model (JTT + I + G + F) by maximum likelihood (ML) in PhyML-aLRT program (Guindon & Gascuel, 2003; Anisimova & Gascuel, 2006) and by Bayesian MCMC analysis in MrBayes v. 3.1.2. (Huelsenbeck & Ronquist, 2001) (two parallel runs each with one hot and three cold Markov chains; 1,500,000 generations; sampling frequency one in every hundred trees; consensus tree constructed based on the trees sampled after burn-in; the convergence of Markov chains was checked through standard deviations of split frequencies and log-likelihood scores for each run). Maximum parsimony (MP) heuristic search (1,000 random taxon-addition replicates with tree-bisection reconnection (TBR) branch swapping) was performed in PAUP v4.0b10 (Swofford, 2000). Support for the nodes in trees was estimated by bootstraping (1,000 bootstrap replicates in MP and 100 in ML) and by posteriror probabilities in MrBayes.

Divergence estimate

Time of divergence of *E. subterraneus* from other freshwater species was estimated on Bayesian tree by relaxed-clock method with non-parametric rate smoothing (NPRS) in program r8s (Sanderson, 2003). This method accounts for rate variation across lineages, thus providing more reliable estimates of divergence times on various timescales.

Results

Genome organisation

The mt genome of *E. subterraneus* [GenBank: GU086203] is circular and consists of 24,850 bp (Fig. 1) containing the same 25 tRNA genes as found in other sponges, including 3 additional tRNA genes (*trnI2(cau)*, *trnR2(ucu)*, *trnMe(cau)*), 2 rRNA, and 14 protein coding genes, including the *atp9* gene. All genes are positioned on the heavy strand and are transcribed clockwise. No introns or extra genes were found. Only one gene pair overlaps: nad5/trnA(ucg) (23 bp), as in *E. muelleri* and *Lubomirskia baicalensis*. Downstream of *cox1*, tRNA-like structure named *trnX* (Wang & Lavrov, 2008) was located. It has well-conserved primary structure with 95%/100% nucleotide identity with *E. muelleri/L. baicalensis.* Secondary structure, including the putative anticodon arm, is well conserved between three freshwater sponge species (Supplementary Fig. 1). Although *trnX* showed 54% identity with $tRNAMet^{(CAU)}$, the question remains whether this sequence is a functional tRNA.

The A + T content of *E. subterraneus* mt-genome is 59.18%. All types of sequences showed positive

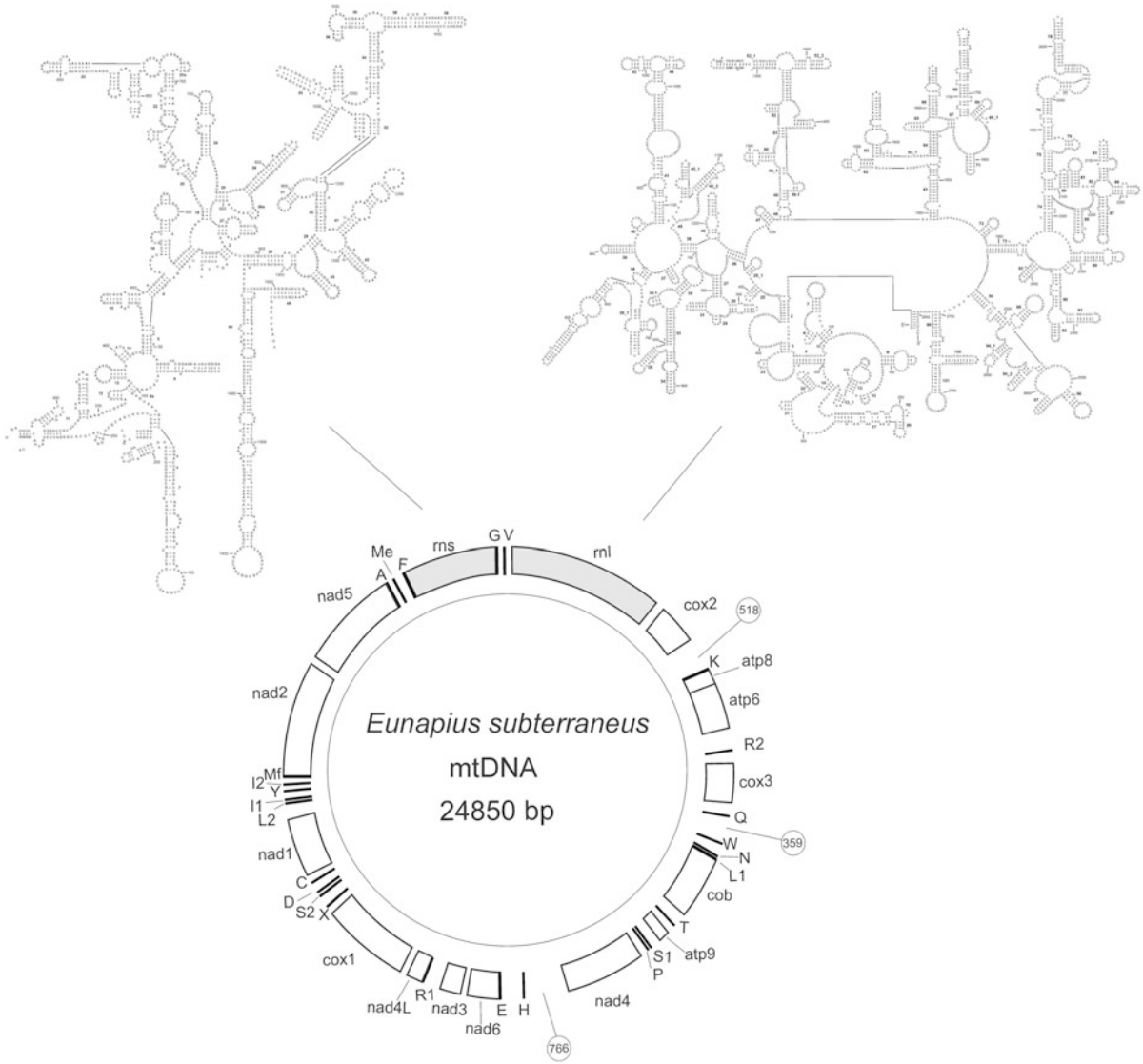

Fig. 1 Genetic map of *Eunapius subterraneus* mtDNA. Protein coding genes are in *white*, rRNA genes are in grey and tRNA genes are in *black* and are labeled by the one-letter code for their corresponding amino acid. The largest non-coding regions are indicated by circles with corresponding lengths. Schematic drawings of the predicted secondary structures of the rRNA genes are given on the side as indicated by *arrows*

GC-skew except non-coding regions, as previously noted only for *E. muelleri* and *Aplysina fistularis* (Wang & Lavrov, 2008). AT-skew was negative for all sequences except for the sense strand of rRNA genes. Mt genomes of all analyzed freshwater sponges are identical in gene order, content and codon usage table. Among existing codons those ending with A or T are preferred (38–40%). These data are consistent within so far described mitochondrial genomes (Wang & Lavrov, 2008). ATG was the most frequent initiation codon, GTG was inferred for *nad6*, and TTG start codon was inferred for *nad2*. The stop codons were either TAA or TAG for all protein coding sequences. Six of 14 protein coding genes were of the same length in all three freshwater sponge species, with well conserved primary structure and with identity between 98 and 100%. Although 8 genes slightly varied in length (3–54 bp) between the three species, their

sequence identity was high (94–99%). Even *atp8* and *nad6*, known to be the least conserved genes among demosponges (Wang & Lavrov, 2008), showed identity of 95 and 94%, respectively.

RNA genes

tRNA genes have well conserved primary and secondary structure and D- and T-loops. Presence of G18-U55 and G19-C56 showed a potential to form tertiary structures. A11-T24 pair in $tRNA^{Pro(UGG)}$, characteristic for demosponges, glass sponges, and placozoans as well as G11-C23 pair in $tRNA^{Trp\ (UCA)}$ characteristic for all bilaterian animals, were also present in *E. subterraneu*s mt-genome. Among individual tRNA genes, 21 showed 100% identity with *E. muelleri* tRNAswhile remaining 4 tRNAs (Leu1, Pro, Thr and Leu2) differed in just 1 or 2 nucleotides. Moreover, all three mt genomes display an identical tRNA gene order. This is surprisingly high similarity knowing that tRNA gene order is highly variable among sponges (Belinky et al., 2008).

Genes for small and large subunit ribosomal RNAs (*rns* and *rnl*) are arranged in the most common gene order $+rns + trnG + trnV + rnl$ (Wang & Lavrov, 2008). The 5′ and 3′ end nucleotides of *E. subterraneus* rRNAs were determined by similarity with *E. muelleri*, as well as secondary structure modeling (Fig. 1), indicating a length of *rns* as 1578 bp and *rnl* 2807 bp. rRNAs are abundant with repetitive elements (short dyads, dyad repeats and direct repeats). The longest direct repeats in *rnl* is 14 and in *rns* 24 nucleotides long Comparison between *E. subterraneus* and *E. muelleri* rRNAs revealed identical repetitive elements in *rns*, while in *E. subterraneus rnl*, palindromes and inverted repeats were not found. Furthermore, one long (22 bp—GGAAGCTA**CGCT TCCATGCCGC**) direct repeat found in *E. muelleri* was substituted with two shorter: 12 bp—GGGTTATAATGA and 14 bp—**CGCTTCCATGCCGC** repeats. Generally, in both *rnl* and *rns*, repeats are identical and presumably genealogically related in these freshwater sponges. Their abundance is correlated with length. The primary sequences of *rns* and *rnl* are well conserved sharing a highest sequence identity with homologous genes in *E. muelleri* (94%/93%), *L. baicalensis* (87%/88%), *Topsentia ophiraphidites* (66%/63%), *Axinella corrugata* (62%/57%) and *Suberites domuncula* (55%/61%).

Intergenic regions (IGRs) of *E. subterraneus*

Intergenic regions constitute 24.4% (6070 bp) of the mt genome of *E. subterraneus*, and are distributed among 38 segments with the largest of them downstream of *nad4* (766 bp). IGRs show length differences among freshwater sponges, which result in mtDNA size variation. Furthermore, non-coding regions of *E. muelleri* and *E. subterraneus* share a considerable similarity which may suggest a very recent split between the two taxa. In 59% of the whole non-coding regions unknown small open reading frames (sORFs) were found encoding for putative proteins of 33–66 amino acids. Deduced amino acid sequences are proline, arginine and serine rich and possess tryptophan which is encoded only by UGG as previously reported for *S. domuncula* (Lukić-Bilela et al., 2008). Despite their doubtful significance and the possibility of being biologically meaningless one small ORF of 55 codons (IGR between *nad4* and *trnH*) with two trypthophans, both of them encoded by UGA, has shown similarity with a conserved putative protein from ascomycetes *Talaromyces stipitatus*. While comparison of ORFs in Demospongiae, mt genomes revealed very little conservation in either their sequence or location, in freshwater sponges this was not the case. Two ORFs in the non coding region between *nad4*-tRNAH and *atp6*-tRNAR2 showed significant nucleotide identity (from 64 to 97%) (Fig. 2), whereas the IGRs of *E. subterraneus* in general are significantly less conserved showing 53 and 34% identity with *E. muelleri* and *L. baicalensis,* respectively.

Intergenic regions of *E. subterraneus* encompass various types of repeat motifs which are spread throughout the mt-genome. Direct repeats (12–24 bp long), inverted repeats (12–32 bp long), and palindromes (12–16 bp long) have been identified. Recently, repetitive palindromic elements with potential to form hairpin structures were found in mtDNA of several sponges (Lavrov, 2009; Erpenbeck et al., 2009). In this study, we searched for perfect copies of inverted repeats in the whole mt genome of *E. subterraneus.* We identified more than 82 short repetitive elements that range in size from 7 to 18 bp. According to previous classification (Lavrov, 2009), these repetitive elements were subdivided into 5 distinct families (Fig. 3). The most abundant family H7 (GACCGAC) and H8 (GATATC) are present in

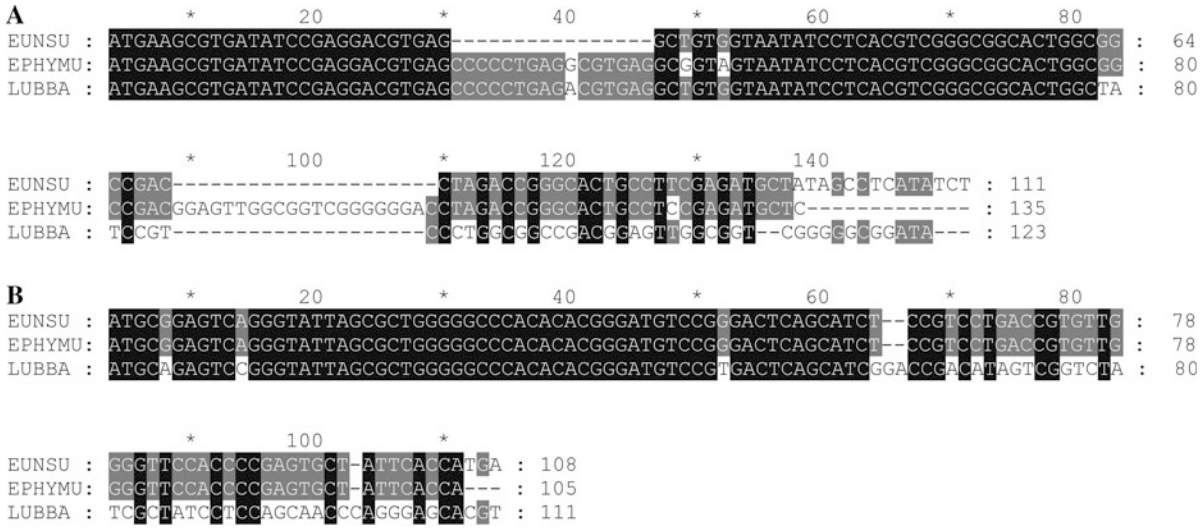

Fig. 2 Alignment of ORFs in the non coding region between *nad4*-tRNAH (**A**) and *atp6*-tRNAR2 (**B**) showed significant homology between mt genomes of freshwater sponges

all analyzed genomes. H9 (GGTGAGAC) reported to be specific for *E. muelleri* can be found in *E. subterraneus* as well. One new family, named H10 (TGAGGCT) is species specific for *E. subterraneus*. These elements are distributed throughout mitochondrial genome: 7/82 were located in rRNA (5 in *rns* and 2 in *rnl*), 2/82 were located in tRNA (S1 and I2) and 17/82 in coding regions (6 of them located in *nad2*). The remaining 56 were found in non-coding regions. GC-rich repetitive sequences are overrepresented in comparison with AT-rich counterparts. When the palindromic elements are folded into hairpin structures, the stem component of the hairpin varies from 5–10 nt in length, and the loop portion is usually 3–4 nt long.

Phylogenetic analyses and divergence time estimate

ML, Bayesian and MP analyses of concatenated amino acid sequences of 12 mt protein-coding genes resulted in a robust tree topology congruent with previous analyses of sponge mitochondrial genes and genomes (Fig. 4). *E. subterraneus* is placed in a well-supported monophyletic clade with the freshwater sponges, *E. muelleri* and *L. baicalensis*, thus placing the mt genome of *E. subterraneus* in the G4 group (Borchiellini et al., 2004). Based on available calibration points for Demospongiae (node constrained to min. 723—max. 867 MY (Peterson & Butterfield, 2005)) and *Ephydatia–Lubomirskia* split (node constrained to be min. 3—max. 10 MY old, (Weinberg et al., 2003; Veynberg, 2009; Lavrov, 2009), the divergence time of *E. subterraneus* was estimated to be around 7–7.5 MYA.

Discussion

Spongillidae, with more than half of the existing species of the suborder Spongillina, are the largest freshwater sponge family (Manconi & Pronzato, 2002). The origin of freshwater sponges and their phylogenetic relationships are still a matter of debate and remain unresolved. Lack of gemmules in some species, as one of the morphological characters on which freshwater sponge classification relies, as well as simple morphology and phenotypical plasticity, make classification of Spongillina species very problematic. Our study demonstrated the remarkable similarity of three freshwater mtDNA genomes in view of identical gene content, gene arrangement and sequence conservation, which is further emphasized when compared to similarity of *S. domuncula* (Lukić-Bilela et al., 2008) and *T. actinia* (Lavrov et al., 2005) mt genomes which group in the G4 clade as well. Although molecular data does not show any particularly close relationship between Suberitidae

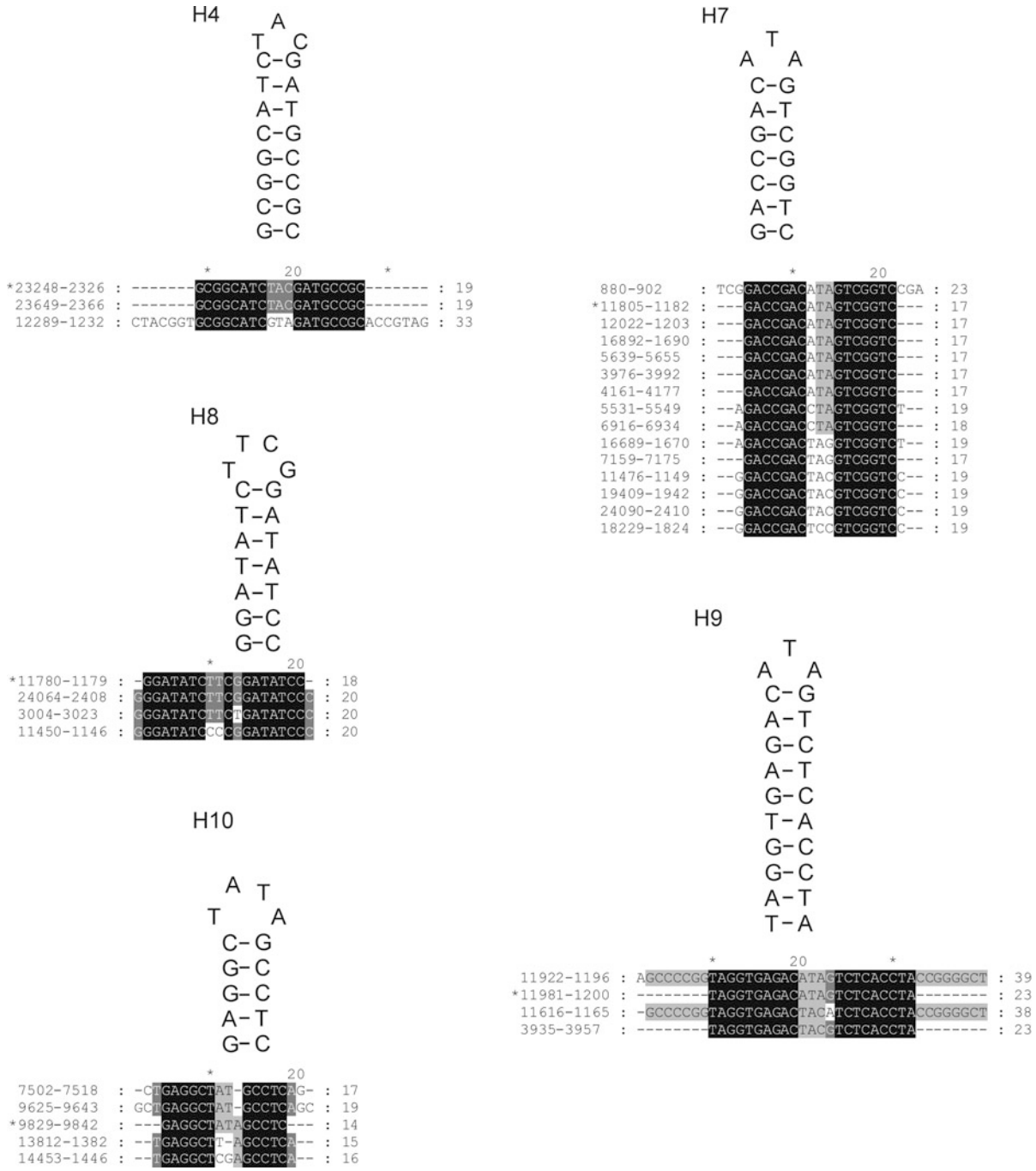

Fig. 3 Secondary structures and corresponding alignments of palindromic repetitive elements in *Eunapius subterraneus*. Families present in *E. subterraneus* are shown (H4-H10). The numbers in the alignment refer to their position in the mt genome. The sequence for which the secondary structure is given is marked with an *asterisk*

and Tethyidae these two species, belonging to the same order and the closest species available for comparison, have a surprisingly low level of mtDNA primary sequence conservation: 55% for the whole mtDNA, 47–83% for coding regions, 62–91% for tRNAs and 12% for non-coding regions. Whereas,

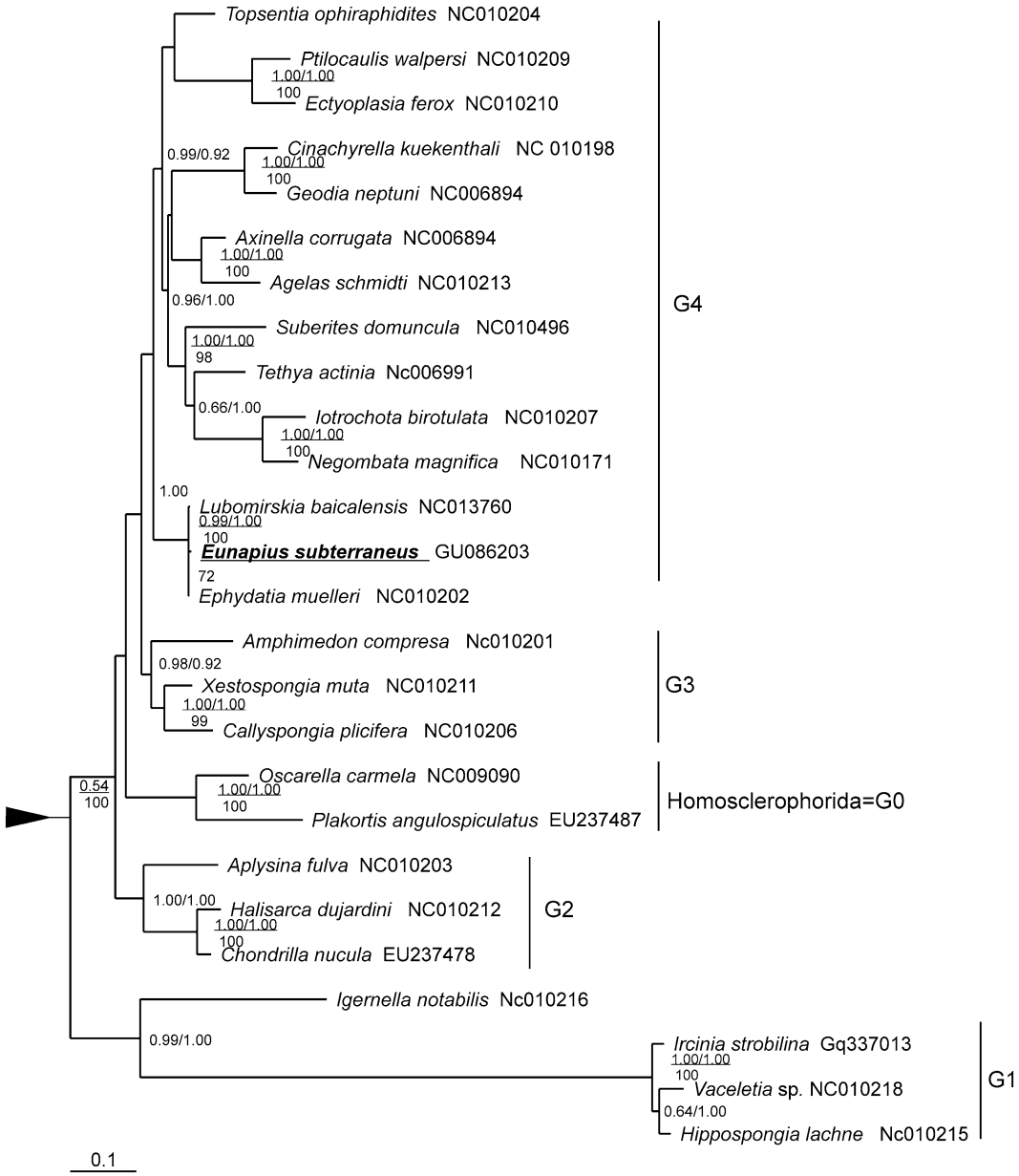

Fig. 4 Phylogenetic position of *Eunapius subterraneus* based on concatenated protein sequences of mitochondrial genes—PhyML-aLRT tree with SH-like branch support/Bayesian PP support (*above nodes*) and MP bootstrap support (*below nodes*). Five major clades within Demospongiae (G0–G4) are indicated

analysis between three freshwater sponge mtDNA sequence showed identity of 75–84, 91–100, 98–100, and 34–53%, respectively. Furthermore, *atp8* and *nad6*, the least conserved genes, showed high identity (more then 94%) between three freshwater sponges, while nucleotide analysis of the same genes between *S. domuncula* and *T. actinia* showed 57 and 73% identity. Unfortunately, the lack of mt genomes of closely related species prevents more accurate comparison and conclusion in this matter.

These results were further highlighted with phylogenetic analysis (Fig. 4). Short terminal branches of three sponge species in phylogenetic trees suggest their close relationships and probably their recent

diversification from a common ancestor. This is additionally emphasized by quite recent divergence time. Origin of Baikal sponge is estimated to be around 3–10 MYA (Lavrov, 2009), while split between *E. subterraneus* and globally distributed freshwater sponge *E. muelleri*, is estimated in this study around 7 MYA. This is in accordance with the scenario of origin of this cave sponge previously proposed (Harcet et al., 2010). Our results support the hypothesis that endemic freshwater sponge species in this case *E. subterraneus* and *L. baicalensis* might have originated from a cosmopolitan founder species such as *Ephydatia* (Meixner et al., 2007), but the question remains whether this phenomenon is common to other endemic sponges. *L. baicalensis* and *E. subterraneus* have several things in common. Both are endemic sponges living in similar type of habitats which is characterized by constant conditions with little fluctuations during the year. Smaller amounts of gemmules or their complete absence is caused by stable ecological conditions (Harcet et al., 2010). Furthermore, *L. baicalensis* is restricted to the ancient Lake Baikal (Siberia) and *E. subterraneus* is found only in the caves of Croatian Karst (Bilandžija et al., 2007). The close relationship of *Ephydatia* with above mentioned species analyzed in this study is in accordance with the results of the recent study based on phylogenetic analyses of three genes in a large number of freshwater sponge taxa (Harcet et al., 2010). In this study, *E. subterraneus* was positioned within the group of Spongillidae to the exclusion of all other *Eunapius* species. Although we could not obtain mt sequences for other species of the genus *Eunapius*, the work of Harcet et al. (2010) in combination with ours indicates that *E. subterraneus* is closer to the genus *Ephydatia* than to other *Eunapius* species. However, morphological characters used in current taxonomical practice are in collision with this finding suggesting the need for thorough revision of entire suborder.

Considering high degree of conservation of mtDNA among analyzed freshwater sponges, non-coding regions provide additional valuable information. So far, with a few exceptions, freshwater sponges possess larger non coding regions and display a higher abundance of ORFs in comparison to other mt genomes, although the putative ORFs from *E. subterraneus* show no significant similarity with any known protein. Small proteins in the nuclear genome of *Saccharomyces cerevisiae* include a number of important classes, such as transcriptional regulators and factors, transporters and metal ion chelators (Basrai et al., 1997). For instance, 184 sORFs of the *S. cerevisiae* are related to sequences in other eukaryotes, suggesting the evolutionary conservation of structure and perhaps function in key cellular processes (Kastenmayer et al., 2006). Interestingly, some demosponge protein-coding genes, such as *atp9* for ATP synthase subunit 9, are absent in all other metazoan phyla. However, their presence in some demosponges as well as choanoflagellates and fungi (Lavrov, 2007) suggests that this is probably an ancient premetazoan feature. ORFs without any significant similarity to any known protein have been found in the Cnidaria and Porifera (Shao et al., 2006; Flot & Tillier, 2007; Wang & Lavrov, 2008). Due to the highly divergent sequences of ORFs, it will be difficult to pin point their origin and nature.

In on-going approach to resolve the problematic class-level relationships among the sponges, comparative analysis of mitochondrial genomes (mtDNA) is preferably in use. In that light IGRs, abundant with palindromic elements and repetitive hairpin-forming elements, provide higher degree of diversity and more phylogenetic signal. Repetitive sequences in the mtDNA of *E. subterraneus* share primary sequence similarity with those in *E. muelleri* and *L. baicalensis* only for H7–8 family. In addition, every species has species-specific repetitive elements which are scattered through the whole mt genome. *L. baicalensis* has the largest number of these elements and the longest intergenic regions. Therefore, it was postulated that proliferation of repetitive elements can result in expanded intergenic regions. The potential importance of repetitive hairpin elements in mtDNA evolution was suggested, but their origin and function is still not known (Lavrov, 2009). These elements lacking obvious cellular function could introduce genetic variability and potentially be used in order to address unresolved taxonomic questions within Porifera. Data from more closely related taxa are needed to test this hypothesis. Previous research demonstrated positive correlation between the abundance of repetitive elements and the length of non-coding regions (and therefore total genome size) in sponge mitochondria (Lavrov, 2009). Although repetitive elements are numerous in *E. subterraneus* mtDNA, we found no evidence of their rapid proliferation as

described for *L. baicalensis*. The lack of sequence similarity between repetitive elements suggests that evolution of these elements occurred independently after the divergence of different phyla leading to their variations (Nedelcu & Lee, 1998; Lukić-Bilela et al., 2008). Several studies have reported the involvement of palindromic elements in biological processes (Arunkumar & Nagaraju, 2006), DNA replication and gene regulation (Wagner, 1991; Kornberg & Baker, 1992). Moreover, these elements were found in high concentration close to the replication origins in viruses (Arunkumar & Nagaraju, 2006). So far mtDNA control regions including the origin of replication are still unknown in sponges. However, in *Aphrocallistes vastus* (Rosengarten et al., 2008) putative control region abundant with repetitive elements was determined. Palindromic elements in a control region was also reported for Insecta (Saito et al., 2005), where it is indicated that regulatory systems have changed through the evolution of animals. In humans inverted repeats are recognition motifs for mtDNA primase (Hixson et al., 1986). Therefore, palindromic elements could be part or control regions of mtDNA. Another hypothesis is that small palindromic sequences are mobile elements (Paquin et al., 2000; Lavrov, 2009), as reported for *Volvox carteri* (Smith & Lee, 2009). Palindromic elements are spread through the whole mtDNA including the protein coding regions where they could influence molecular evolution of proteins (Aono et al., 2002). Recent studies suggest that repetitive elements present in mt genomes indicate either an early origin or multiple independent invasions (Erpenbeck et al., 2009). Considering the diversity of these repetitive elements in freshwater sponges, their sparse distribution, short evolutionary split for Spongillidae and the fact that most repetitive families are species specific, it is unlikely that they were present in ancestral mt genomes. Therefore, insights into mt IGRs may play an important role in the evolution of the metazoan mt genome, although more mtDNA data of freshwater sponges are necessary before these events can be adequately understood.

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References

Abascal, F., R. Zardoya & D. Posada, 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21: 2104–2105.

Addis, J. S. & K. J. Peterson, 2005. Phylogenetic relationships of freshwater sponges (Porifera, Spongillina) inferred from analyses of 18S rDNA, COI mtDNA, and ITS2 rDNA sequences. Zoologica scripta 34: 549–557.

Anisimova, M. & O. Gascuel, 2006. Approximate likelihood ratio test for branches: a fast, accurate and powerful alternative. Systematic Biology 55: 539–552.

Aono, N., T. Shimizu, T. Inoue & H. Shiraishi, 2002. Palindromic repetitive elements in the mitochondrial genome of *Volvox*. FEBS Letters 521: 95–99.

Arunkumar, K. P. & J. Nagaraju, 2006. Unusually long palindromes are abundant in mitochondrial control regions of insects and nematodes. Plos ONE 1: e110.

Basrai, M. A., P. Hieter & J. D. Boeke, 1997. Small open reading frames: beautiful needles in the haystack. Genome Research 7: 768–771.

Belinky, F., C. Rot, M. Ilan & D. Huchon, 2008. The complete mitochondrial genome of the demosponge *Negombata magnifica* (Poecilosclerida). Molecular Phylogenetics and Evolution 47: 1238–1243.

Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell & D. L. Wheeler, 2003. GenBank. Nucleic Acids Research 31: 23–27.

Bilandžija, H., J. Bedek, B. Jalžić & S. Gottstein, 2007. The morphological variability, distribution patterns and endangerment in the Ogulin cave sponge *Eunapius subterraneus*. Natura Croatica 16: 1–17.

Bilandžija, H., J. Bedek & B. Jalžić, 2009. Ogulin cave sponge. In Ozimec, R., et al. (eds), Red Book of Croatia Cave Dwelling Fauna. Ministry of culture, State Institute for Nature Protection, Zagreb: 176–177.

Boore, J. L., 1999. Animal mitochondrial genomes. Nucleic Acids Research 27: 1767–1780.

Borchiellini, C., C. Chombard, M. Manuel, E. Alivon, J. Vacelet & N. Boury-Esnault, 2004. Molecular phylogeny of Demospongiae: implications for classification and scenarios of character evolution. Molecular Phylogenetics and Evolution 32: 823–837.

Burger, G., L. Forget, Y. Zhu, M. W. Gray & B. F. Lang, 2003. Unique mitochondrial genome architecture in unicellular relatives of animals. Proceedings of the National Academy of Sciences USA 100: 892–897.

Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540–552.

Drummond, A. & K. Strimmer, 2001. PAL: An object-oriented programming library for molecular evolution and phylogenetics. Bioinformatics 17: 662–663.

Erpenbeck, D., O. Voigt, M. Adamski, M. Adamska, J. N. A. Hooper, G. Wörheide & B. M. Degnan, 2007. Mitochondrial diversity of early-branching metazoa is revealed by the complete mt genome of a Haplosclerid Demosponge. Molecular Biology and Evolution 24: 19–22.

Erpenbeck, D., O. Voigt, G. Wörheide & D. V. Lavrov, 2009. The mitochondrial genomes of sponges provide evidence

for multiple invasions by repetitive hairpin-forming elements (RHE). BMC Genomics 10: 591.
Flot, J. F. & S. Tillier, 2007. The mitochondrial genome of Pocillopora (Cnidaria: Scleractinia) contains two variable regions: the putative D-loop and a novel ORF of unknown function. Gene 401: 80–87.
Guindon, S. & O. Gascuel, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704.
Harcet, M., H. Bilandžija, B. Bruvo-Mađarić & H. Ćetković, 2010. Taxonomic position of *Eunapius subterraneus* (Porifera, Spongillidae) inferred from molecular data—a revised classification needed? Molecular Phylogenetics and Evolution 54: 1021–1027.
Hixson, J. E., T. W. Wong & D. A. Clayton, 1986. Both the conserved stem-loop and divergent 59-flanking sequences are required for initiation at the human mitochondrial origin of light-strand DNA replication. The Journal of Biological Chemistry 261: 2384–2390.
Hooper, J. N. A. & R. W. M. van Soest, 2002. Class Demospongiae Sollas, 1885. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera: A guide to the Classification of Sponges. Kluwer, New York: 15–18.
Huelsenbeck, J. P. & F. Ronquist, 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754–755.
Kastenmayer, J. P., L. Ni, A. Chu, L. E. Kitchen, W. C. Au, H. Yang, C. D. Carter, D. Wheeler, R. W. Davis, J. D. Boeke, M. A. Snyder & M. A. Basrai, 2006. Functional genomics of genes with small open reading frames (sORFs) in *S. cerevisiae*. Genome Research 16: 365–373.
Kornberg, A. & T. Baker, 1992. DNA Replication. W. Freeman & Co, New York.
Lavrov, D. V., 2007. Key transitions in animal evolution: a mitochondrial DNA perspective. Integrative and Comparative Biology 47: 734–743.
Lavrov, D. V., 2009. Rapid proliferation of repetitive palindromic elements in mtDNA of the endemic Baikalian sponge *Lubomirskia baicalensis.* Molecular Biology and Evolution. doi:10.1093/molbev/msp317.
Lavrov, D. V., L. Forget, M. Kelly & B. F. Lang, 2005. Mitochondrial genomes of two demosponges provide insights into an early stage of animal evolution. Molecular Biology and Evolution 22: 1231–1239.
Lowe, T. M. & S. R. Eddy, 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Research 31: 176–178.
Lukić-Bilela, L., D. Brandt, N. Pojskić, M. Wiens, V. Gamulin & W. E. G. Müller, 2008. Mitochondrial genome of *Suberites domuncula*: Palindromes and inverted repeats are abundant in non-coding regions. Gene 412: 1–11.
Manconi, R. & R. Pronzato, 2002. Suborder *Spongillina* subord. nov: Freshwater sponges. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera: A Guide to the Classification of Sponges. Kluwer, New York: 921–1019.
Meixner, M. J., C. Lüter, C. Eckert, V. Itskovich, D. Janussen, T. von Rintelen, A. V. Bohne, J. M. Meixner & W. R. Hess, 2007. Phylogenetic analysis of freshwater sponges provide evidence for endemism and radiation in ancient lakes. Molecular Phylogenetics and Evolution 45: 875–886.
Nedelcu, A. M. & R. W. Lee, 1998. Short repetitive sequences in green algal mitochondrial genomes: potential roles in mitochondrial genome evolution. Molecular Biology and Evolution 15: 690–701.
Paquin, B., M. J. Laforest & B. F. Lang, 2000. Double-hairpin elements in the mitochondrial DNA of allomyces. Molecular Biology and Evolution 17: 1760–1768.
Peterson, K. J. & N. J. Butterfield, 2005. Origin of the Eumetazoa: testing ecological predictions of molecular clocks against the Proterozoic fossil record. Proceedings of the National Academy of Sciences USA 102: 9547–9552.
Rice, P., I. Longden & A. Bleasby, 2000. EMBOSS: the European Molecular Biology Open Software Suite. Trends in Genetics 16: 276–277.
Rosengarten, R. D., E. A. Sperling, M. A. Moreno, S. P. Leys & S. L. Dellaporta, 2008. The mitochondrial genome of the hexactinellid sponge *Aphrocallistes vastus*: evidence for programmed translational frameshifting. BMC Genomics 9: 33.
Saito, S., K. Tamura & T. Aotsuka, 2005. Replication origin of mitochondrial DNA in insects. Genetics 171: 1695–1705.
Sanderson, M. J., 2003. r8 s; inferring absolute rates of evolution and divergence times in the absence of a molecular clock. Bioinformatics 19: 301–302.
Shao, Z., S. Graf, O. Y. Chaga & D. V. Lavrov, 2006. Mitochondrial genome of the moon jelly Aurelia aurita (Cnidaria, Scyphozoa): a linear DNA molecule encoding a putative DNA-dependent DNA polymerase. Gene 381: 92–101.
Sket, B. & M. Velikonja, 1984. Prethodni izvještaj o nalazima slatkovodnih spužvi (Porifera, Spongillidae) u spiljama Jugoslavije, In Deveti jugoslavenski speleološki kongres: 553–557.
Smith, D. R. & R. W. Lee, 2009. The mitochondrial and plastid genomes of *Volvox carteri*: bloated molecules rich in repetitive DNA. BMC Genomics 10: 132.
Swofford, D. L., 2000. PAUP: Phylogenetic Analysis Using Parsimony (and Other Methods), Version 4.0. Sinauer, Sunderland.
Thompson, J. D., D. G. Higgins & T. J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.
Veynberg, E., 2009. Fossil sponge fauna in lake baikal region. Progress in Molecular and Subcellular Biology 47: 185–205.
Wagner, E., 1991. Herpesvirus Transcription and its Regulation. CRC Press, Boca Raton.
Wang, X. & D. V. Lavrov, 2008. Seventeen new complete mtDNA sequences reveal extensive mitochondrial genome evolution within the Demospongiae. Plos ONE 3(7): e2723.
Weinberg, E., I. Weinberg, S. Efremova, A. Tanichev & Y. Masuda, 2003. Late Pliocene spongial fauna in Lake Baikal (from material from the Deep Drilling Core BDP-96–1). In Kashiwaya, K. (ed.), Long Continental Records from Lake Baikal. Springer-Verlag, Tokyo: 283–293.

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SPONGE RESEARCH DEVELOPMENTS

The complete mitochondrial genome of the verongid sponge *Aplysina cauliformis*: implications for DNA barcoding in demosponges

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Abstract DNA "barcoding," the determination of taxon-specific genetic variation typically within a fragment of the mitochondrial cytochrome oxidase 1 (*cox1*) gene, has emerged as a useful complement to morphological studies, and is routinely used by expert taxonomists to identify cryptic species and by non-experts to better identify samples collected during field surveys. The rate of molecular evolution in the mitochondrial genomes (mtDNA) of nonbilaterian animals (sponges, cnidarians, and placozoans) is much slower than in bilaterian animals for which DNA barcoding strategies were developed. If sequence divergence among nonbilaterian mtDNA and specifically *cox1* is too slow to generate diagnostic variation, alternative genes for DNA barcoding and species-level phylogenies should be considered. Previous study across the Aplysinidae (Demospongiae, Verongida) family of sponges demonstrated no nucleotide substitutions in the traditional *cox1* barcoding fragment among the Caribbean species of *Aplysina*. As the mitochondrial genome of *Aplysina fulva* has previously been sequenced, we are now able to make the first comparisons between complete mtDNA of congeneric demosponges to assess whether potentially informative variation exists in genes other than *cox1*. In this article, we present the complete mitochondrial genome of *Aplysina cauliformis,* a circular molecule 19620 bp in size. The mitochondrial genome of *A. cauliformis* is the same length as is *A. fulva* and shows six confirmed nucleotide differences and an additional 11 potential SNPs. Of the six confirmed SNPs, NADH dehydrogenase subunit 5 (*nad5*) and *nad2* each contain two, and in *nad2* both yield amino acid substitutions, suggesting balancing selection may act on this gene. Thus, while the low nucleotide diversity in Caribbean aplysinid *cox1* extends to the entire mitochondrial genome, some genes do display variation. If these represent interspecific differences,

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then they may be useful alternative markers for studies in recently diverged sponge clades.

Keywords mtDNA · Porifera · Demospongiae · Verongida

Abbreviations

mtDNA	Mitochondrial genome
atp6, *8*, *9*	ATP synthase F0 subunit #
cob	Apocytochrome b
cox1-3	Cytochrome *c* oxidase #
nad1-6, *4L*	NADH dehydrogenase subunit #
rnS	Small ribosomal RNA
rnL	Large ribosomal RNA

Introduction

"DNA barcoding," whereby the nucleotide sequence of a genomic fragment is utilized to aid the identification of an organism (Hebert et al., 2003), has emerged as a complementary approach to purely morphologically based taxonomic studies, and has proven especially useful in helping experienced taxonomists uncover cryptic species and for generalist researchers, such as community ecologists, to better identify organisms during broad surveys of biodiversity. DNA barcoding traditionally utilizes a 710-bp stretch at the 5′ end of the mitochondrial cytochrome oxidase I (*cox1*) gene. This fragment is relatively easy to amplify by PCR because of primer regions conserved across metazoan phyla (Folmer et al., 1994) and the abundance of mitochondrial DNA in eukaryotic cells. Mitochondrial genes are also preferred for such studies because of the relatively high rate of sequence evolution in bilaterian animals (especially at third-codon positions) which allows for discrimination even between recently diverged species, rapid coalescence, and because mitochondrial genomes (mtDNA) are typically maternally inherited and do not recombine (Boore & Brown, 1998; but see Galtier et al., 2009). The utility of DNA barcoding promises to advance organismal and ecological studies across the metazoan tree as the catalogs of taxon-specific variation are further expanded (Miller, 2007).

The Sponge Barcoding Project (Wörheide & Erpenbeck, 2007) has made the case for implementing DNA barcoding in poriferan research. From a morphological perspective, sponges are generally a character-poor group of organisms. Most taxonomy is based on the types and arrangements of siliceous and calcareous spicules, although the differences between species are often subtle, or relate to factors such as spicule size that could potentially be affected by the environment (e.g., Maldonado et al., 1999). The lack of characters has led, in part, to the presence of long-ranging "cosmopolitan" species that likely represent a number of cryptic species (Klautau et al., 1999). Further, the paucity and plasticity of characters make correct identification difficult for non-specialists. Indeed, in a survey of 138 faunal inventories from the North Atlantic, Schander & Willassen (2005) found that sponges were identified to the species level in less than half the cases, in contrast to groups like annelids and mollusks for which more than 75% of specimens were identified to species level. Given the importance of sponges to the discovery of new bioactive compounds (Sipkema et al., 2005) and the ecological importance of sponges in essentially every marine and aquatic environment (Bell, 2008), a tool for the rapid identification of specimens by natural products researchers and community ecologists would be of high value.

Initial studies using DNA barcoding in sponges, however, have seen mixed results. *Cox1* successfully discriminates between species in the genus *Tethya* (Heim et al., 2007a) and *Scopalina* (Blanquer & Uriz, 2007). In other cases, though, species that are clearly discriminated on the basis of morphology show the same *cox1* haplotype (Schroder et al., 2003; Heim et al., 2007b; Pöppe et al., 2010). Studies of *cox1* intraspecific variation in sponges also show extremely low rates compared to those in bilaterian species-level populations (Duran et al., 2004; Wörheide, 2006). Xavier et al. (2010) found high intraspecific divergences within the species *Cliona celata* but suggested this was evidence for cryptic speciation. The low variation in species-level *cox1* sequences is likely a direct reflection of the overall low rates of molecular evolution in sponge (and more generally nonbilaterian) mitochondrial genomes (Shearer et al., 2002; Lavrov et al., 2005; Huang et al., 2008). This rate difference between the nonbilaterian animals (sponges, cnidarians, and placozoans) and bilaterians is so drastic that phylogenetic trees built from complete mitochondrial genomes result in an artifactual (see Sperling et al., 2009) "Diploblastica," where the fast-evolving bilaterians are attracted toward outgroups and the

metazoan root is mis-placed between the slow-evolving sponges, placozoans, and cnidarians and the fast-evolving bilaterians (reviewed by Lavrov, 2007). The slow rate of mitochondrial sequence evolution in sponges and the inability to discriminate between species has led to the suggestion that the standard 5′ *cox1* barcode may not be suitable for DNA barcoding in many sponge taxa (Pöppe et al., 2010), as well as to a search for alternative markers, such as the 3′ end of *cox1* which may have more phylogenetic signal (Erpenbeck et al., 2005). It is worth noting that although DNA barcoding as a tool (e.g., specimen identification by non-specialists) can be used in a non-phylogenetic context, slow mitochondrial evolution (and consequent lack of signal) is a problem for resolution of species- and genus-level phylogenies using standard mitochondrial markers as well.

Even though nonbilaterian mitochondrial genomes exhibit low rates of molecular evolution, mitochondrial genes remain prime targets for barcoding markers and species-level phylogenetic questions given their clonal inheritance, lack of recombination, and perhaps most importantly the abundance of mtDNA in a cell, leading to relatively easy amplification even from sub-optimal specimens, for instance, those in museum collections. The question remains whether faster-evolving gene regions can be identified for use in conjunction with the standard 5′ *cox1* barcode. To test whether the strong conservation of *cox1* extends over the entire mitochondrial genome, and to aid the search for other potential mitochondrial markers, we sought to examine variability in the mitochondrial genomes of two closely related congeneric species of Demospongiae. We focused on the genus *Aplysina* Nardo 1834 (Demospongiae, Verongida, Aplysinidae), which is a genus of aspiculate sponges within the Myxospongiae or "G2" clade (Borchiellini et al., 2004; Nichols, 2005; Lavrov et al., 2008; Sperling et al., 2009). The taxonomy and phylogenetic relationships of the Aplysinidae have been well studied from both a morphological and molecular perspective in recent years, making it in some sense a "test case" for species discrimination within Porifera (Schmitt et al., 2005; Erwin & Thacker, 2007; Heim et al., 2007b; Kloppell et al., 2009; Lamarao et al., 2010). Importantly, despite the Caribbean *Aplysina* species showing clear morphological differences (e.g., Erwin & Thacker, 2007), they show no variation in the standard 5′ *cox1* fragment at the nucleotide level (Heim et al., 2007b).

The complete mitochondrial genome of one species of *Aplysina*, *A. fulva*, has already been sequenced (Lavrov et al., 2008), and thus we sequenced the mitochondrial genome of its likely sister species, *A. cauliformis* (Erwin & Thacker, 2007). The mitochondrial genomes of congeneric species of homoscleromorph sponges (classically considered demosponges) have been sequenced, but this sponge clade is now regarded as a separate sponge lineage (Borchiellini et al., 2004; Sperling et al., 2007, 2009; Lavrov et al., 2008). Congeneric species of the demosponge genus *Amphimedon* also have sequenced mitochondrial genomes, but that genus is unlikely to be monophyletic (Lavrov et al., 2008). Thus, this study on *Aplysina* represents the first comparison of mitochondrial genomes from closely related species in a monophyletic demosponge genus. Given the extremely recent divergence of Caribbean *Aplysina* species inferred by Heim et al. (2007b), this probably represents one of the most severe possible cases for molecular discrimination of species. Consistent with other reports of mitochondrial DNA variation in other sponges, we observe only six confirmed nucleotide substitutions (and 11 other sites with potential variation) between these two species. Of the six SNPs that can be confidently assigned, two are found in *nad5* and two in *nad2*. Both *nad2* variants result in amino acid changes, suggesting that exploration of that gene as a marker for species-level discrimination may be warranted.

Materials and methods

Collection and amplification

Aplysina cauliformis was collected using SCUBA from the M1 reef at the Discovery Bay Marine Station, Jamaica at 15 m depth. Tissue from one individual was lysed in 8 M urea buffer (Chen & Dellaporta, 1994), incubated at 65°C for 20 min, and total DNA was prepared by extraction in phenol–chloroform (1:1) and precipitation in 0.7 volumes isopropanol. To confirm the identity of the sponge, the 28S-ITS2 fragment used by Erwin & Thacker (2007) was also sequenced using the protocol outlined in their article, and submitted to GenBank (HQ730891). Phylogenetic analysis of that fragment demonstrated that the sponge was correctly identified (Supplemental Figure 1). A partial fragment of *cox1* was then amplified by PCR using the primers

LCO 1490–HCO 2198 (Folmer et al., 1994), and a partial fragment of *rnL* with custom primers CGAGA AGACCCCATTGAGCTTTACTA and TACGCTGT Sequence-specific primers pairs (CGAGAAGACC CCATTGAGCTTTACTG/CATATCTACCGAACC CCCAGAATGTG and CGCCCCAACTAAACTGT CTGCTTTAC/TATCTGCACCAGGCTCAATGTT AGGA) were then designed and used with Takara LA Taq for long-range PCR. Cycling conditions were as follows: a 1-min initial denaturation at 94°C, 30 cycles of 98°C for 10 s, and 62°C for 12 min but adding 10 s to the extension time every cycle after the first ten cycles, and a final extension step at 72°C for 10 min. This produced two overlapping fragments of 8 and 12 kilobases (kb), respectively. These products were gel-purified, sheared by sonication, and end-repaired with the DNA Terminator kit (Lucigen). Two–four kb fragments were size-selected by gel electrophoresis, blunt-end cloned into the pSmart LC-Kan vector (Lucigen), and transformed into *E. cloni* Supreme cells (Lucigen). Colonies were screened by PCR for the presence of inserts using flanking vector primers SL1 and SR2 (Lucigen). Sixty PCR products were purified by poly-ethylene glycol–NaCl (PEG:-NaCl) (Lis & Schleif, 1975) and sequenced by BigDye® Terminator v3.1 cycle sequencing on ABI PRISM® 3730 DNA Analyzers (Applied Biosystems, Inc.) at the DNA Analysis Facility on Science Hill, Yale University.

Sequence assembly and annotation

Sequences were assembled from chromatography data using the Phred Phrap Consed software package release 15.0 (Ewing & Green, 1998; Gordon et al., 1998). Regions of lower quality data were sequenced by direct PCR on genomic DNA, or additional sequencing of select gap spanning clones. The resulting 19620-bp contig had a minimum of 2–8× coverage on both strands with high phred values (40 or greater), with the exception of 2.6% of the genome that had 1× coverage with high phred values (GenBank: EU518938). The suite of tRNA genes were identified by tRNAscan-SE (Lowe & Eddy, 1997) using the program's default parameters for mitochondria or chloroplast sequences and the Mold and Protozoan mitochondrial translation code. Protein- and RNA-coding genes were initially identified using the program DOGMA (Wyman et al., 2004), and confirmed and annotated by Blast2 (Tatusova & Madden, 1999) and ClustalW (Thompson et al., 1994).

Results

Gene content

The complete mitochondrial genome (mtDNA) of *A. cauliformis* was shotgun-sequenced and assembled as a 19620-bp circular molecule. This sequence was nearly identical to that of *A. fulva* reported by Lavrov et al. (2008), but owing to the number of mitochondrial genomes reported in that study, detailed features of each individual mtDNA could not be reported, and thus we provide an in-depth examination of the *A. cauliformis* genome here. Analysis of the complete mitochondrial genome sequence predicted 14 protein-coding genes, 2 ribosomal RNA genes, and 25 transfer RNA genes. Seven of the protein-coding genes, both rRNAs, and 12 of the tRNAs were coded on one strand (top), while the remaining genes and tRNAs were arranged in a contiguous inverted block on the reverse strand. The protein-coding gene complement included 11 of the respiratory genes (*cob*, *cox1-3*, *nad1-4*, *4L*, *5*, *6*) canonical to metazoan mtDNA, as well as the ATP synthase F0 subunits 6, 8, and 9 (*atp6*, *atp8*, *atp9*) (Fig. 1) found in most demosponge mitochondrial genomes. As seen in most other demosponge mitochondrial genomes (Lavrov, 2007) *A. cauliformis* has no additional genes, large introns, or open reading frames of unknown function, or programmed translational frameshifting as seen in hexactinellid sponge mitochondrial genomes (Rosengarten et al., 2008). All of the protein-coding genes were found to contain a canonical AUG start codon, except *nad6* which appeared to employ a GUG codon 54 bases upstream of the first AUG, and all coding sequences terminated translation with the UAA codon except for *atp8* and *nad4L*, which use UAG.

The 25 tRNAs of *A. cauliformis* (Supplemental Figure 2) included two isoacceptors for methionine, the initiator $trn^{Met}(cau)i$ and the elongator $trn^{Met}(cau)e$ (*Mi* and *Me*, respectively, on Fig. 1). The following criteria were used to distinguish between the initiator and elongator *trnM* genes: Initiator $tRNA^{Met}$ molecules lack Watson–Crick base-pairing at the 1:7# position, have a R11:Y2# pair, and have a series of three guanines in the anticodon stem (Drabkin et al.,

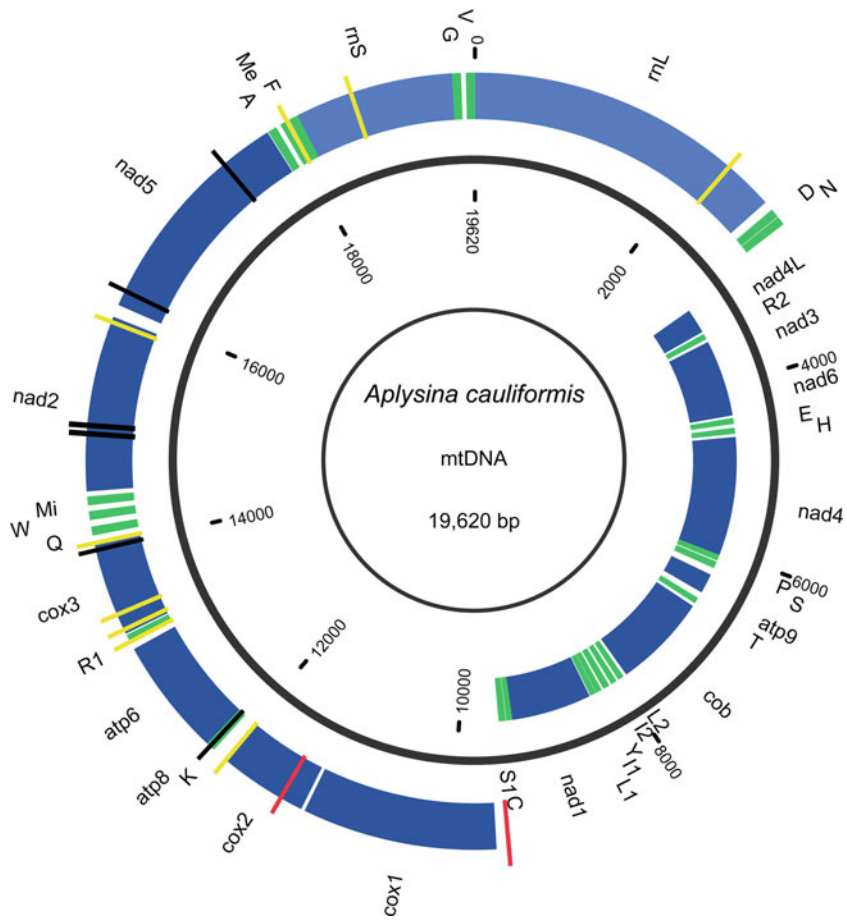

Fig. 1 *Aplysina cauliformis* mtDNA. The mitochondrial genome of the demosponge *Aplysina cauliformis* was determined to be a 19620-bp circular molecule encoding 14 respiratory genes, two rRNAs and 25 tRNAs. Protein-coding genes are represented with dark blue boxes, ribosomal RNAs in light blue, and tRNAs in green. The tRNAs are labeled with their one letter IUPAC amino acid abbreviation. Genes on the outside of the circle are transcribed clockwise, on the top strand. Genes inside the circle are transcribed counter-clockwise, on the reverse strand. The two isoacceptors for methionine are labeled *Mi* and *Me* for the initiator and elongator *trnM*, respectively. All other tRNA isoacceptors are enumerated 1 and 2. Confirmed nucleotide substitutions are indicated by black bars, the unresolved *A. fulva* positions (base N) are indicated by yellow bars, and the possible SNPs with conflicting sequence reads are red. The mtDNA map was generated with Circos (Krzywinski et al., 2009)

1998; Stortchevoi et al., 2003). Based on these motifs, the *trnM* at position 14363 was designated as the initiator, and the one at position 17987 was designated as the elongator. A third *trnM* gene was predicted by tRNAscan-SE on the reverse strand at position 8096, but was annotated as an isoacceptor for isoleucine according to the convention established for demosponge mtDNA (Lavrov et al., 2005). This annotation strategy assumes the *trnM* undergoes secondary modification of the C34 base in the anticodon to lysidine (2-lysyl-cytidine), thus gaining specificity for isoleucine as $tRNA^{Ile}(cau)$ (*I2* on Fig. 1). Evidence for conversion of *trnM* to *trnI* has been obtained for bacteria lacking a *trnI*, as well as in the potato mitochondrion, by both molecular and biochemical assays (Weber et al., 1990; Muramatsu et al., 1998). However, such experimental confirmation of lysidinylation is still lacking in sponges, and thus this annotation should be taken with some degree of caution.

In addition to methionine and isoleucine, leucine, arginine, and serine were also found to have two isoacceptor tRNAs (*L1*, *L2*, *R1*, *R2*, *S1*, and *S2* in Fig. 1). Several tRNAs had remarkable structural

features. Both *trnS* genes and the *trnY* gene revealed long variable arms, diagnostic for type-2 tRNAs (Lavrov et al., 2005). *trnA* had a mismatch in its anticodon arm, while *trnC*, *F*, *H*, *I1*, *P*, and *R1* all revealed mismatches in their acceptor arm. *trnL2* had a two pair mismatch—U1:U71 and U2:U72—at the top of its acceptor arm (Supplemental Figure 2). These mismatches are frequently seen in the primary sequence of animal mitochondrial tRNAs, and are thought to be corrected by an RNA editing process (Lavrov et al., 2000).

Comparison to *A. fulva*

The mitochondrial genome of *A. cauliformis* is nearly identical to that of *A. fulva*, on both a structural level and at the nucleotide and amino acid level. Indeed, both genomes are the same length (19620 bp) and only six single nucleotide differences were confirmed between them (Fig. 1; Table 1). Of these six, three represented transitions and three transversions. One resided in the trnK but did not result in a change in isoacceptor. The remaining five variants were located in the protein-coding genes *cox3*, *nad2*, and *nad5* (Table 1). The entire *cox1* gene, in addition to the traditional 5′ barcoding fragment investigated by Heim et al. (2007b), was therefore found to be invariant between the two species. Four of the nucleotide changes in protein-coding genes represented silent substitutions, while two resulted in changes at the amino acid level in *nad2*. Two other potential variants (positions 9537 and 11443) were identified but have conflicting chromatogram traces. An additional nine differences (at nucleotide positions 2221, 11979, 13208, 13317, 13450, 14054, 15860, 18046, and 18608) correspond to ambiguous bases (N's) in the *A. fulva* genome sequence (EU237476.1) and thus cannot be evaluated at present.

Table 1 Summary of nucleotide variation between *Aplysina cauliformis* and *A. fulva*

Position	Locus[a]	Nucleotide: *cauliformis/fulva*[b]	Silent/ substitution[c]
Confirmed			
12147	*trnK*	A/G	
13996	*cox3*	T/A	Silent
14932	*nad2*	A/T	L/I
14941	*nad2*	C/T	F/L
16133	*nad5*	C/T	Silent
17432	*nad5*	C/A	Silent
Unresolved *fulva* base N			
2221	*rnL*	G/N	
11979	*cox2*	T/N	
13208	*trnR*	T/N	
13317	*cox3*	T/N	
13450	*cox3*	A/N	
14054	*cox3*	T/N	
15860	*nad2*	T/N	
18046	*trnMe*	T/N	
18608	*rnS*	A/N	
Conflict			
9537	IG	A or G/A	
11443	*cox2*	A or G/A	K/E

[a] IG refers to intergenic sequence

[b] For each instance of nucleotide variation, the *A. cauliformis* base is listed first, followed by the *A. fulva* base. For the "read conflicts," i.e., the cases in which the *A. cauliformis* chromatograms are inconsistent, both possible bases are listed

[c] When nucleotide variation leads to changes in amino acid sequence, the *A. cauliformis* amino acid is listed first, followed by the corresponding residue in the *A. fulva* gene product

Discussion and conclusions

Comparison of the complete *A. cauliformis* and *A. fulva* mitochondrial genomes, the first congeneric mitochondrial genomes sequenced within the Demospongiae sensu stricto, demonstrates that the rate of molecular divergence is exceedingly low across the entire mtDNA. Only six confirmed nucleotide differences, encoding two predicted amino acid changes, and no structural variation, were identified between these molecules. These complete mitochondrial genomes are so similar that the possibility should remain open that these two forms are in fact the same species. Nevertheless, we follow in this study previous researchers who have found small but consistent morphological (Erwin & Thacker, 2007) and molecular (Erwin & Thacker, 2007; Lamarao et al., 2010) differences between the Caribbean aplysinids and suggested that these be retained as separate species (see also discussion in Schmitt et al., 2005). Further, as only one individual from each species was compared in this study, the comparisons must be viewed with some degree of caution. Specifically, it cannot be determined whether the six observed nucleotide

differences are synapomorphies shared by all members of a given species, or whether they are autapomorphic changes characterizing a specific population. Future studies comparing the complete mitochondrial genomes from multiple individuals in different species will be needed to see if these characters truly delineate the two species.

The entire *cox1* gene, including the 5′ and 3′ flanking sequences, is monomorphic and uninformative between the two species. Our results are in accordance with the minimal *cox1* variation observed between sponge genera (and among other nonbilaterian animals in general; Shearer et al., 2002; Schroder et al., 2003; Heim et al., 2007b; Huang et al., 2008; Pöppe et al., 2010). This overall low level of sequence variation poses a general problem for species discrimination and mitochondrial DNA barcoding within the demosponges.

One solution to the paucity of informative sites in the *cox1* sequence traditionally employed both in barcoding studies and in species- and genus-level phylogenies is to compare other genes. Barcoding studies (and species-level phylogenies in general) have often focused on the use of mitochondrial genes because of the advantages listed above, such as high copy number and uniparental inheritance, but they need not be limited to these genes. The 28S-ITS2 fragment used in the study of Erwin & Thacker (2007), for instance, is not invariant and shows three nucleotide substitutions between the *A. cauliformis* sequenced here and the available *A. fulva* sequences. Thus, while resolution remains low, this fragment does appear to better discriminate between aplysinid species (see the results of that study and Supplemental Figure 1). The future use of these ribosomal genes, introns in protein-coding genes and microsatellite studies may all prove beneficial for demosponge species discrimination and in species-level phylogenies. A comparative review of these genes, however, is outside the scope of this article, and therefore we restrict the discussion to the lessons that might be gained from the variation present in the congeneric mitochondrial genome comparison conducted here, with the caveat that comparison of multiple individuals from each species would give a fuller picture of variation.

The variation observed between *Aplysina* species was confined to single nucleotide variation detected in *trnK*, *nad2*, *nad5*, and *cox3*. In particular, both SNPs in *nad2* cause amino acid substitutions, suggesting balancing selection may contribute to variation in this gene. This hypothesis would predict more widespread variation within populations and between sponge species might exist. Studies of selection acting on the mtDNA of bilaterian species, such as *Drosophila melanogaster*, infer that purifying selection eliminates most novel mutation in these molecules (Haag-Liautard et al., 2008). Our hypothesis of a potential signature of balancing selection in *nad2* does not preclude the possibility that purifying selection eliminates most variation in the rest of the mtDNA.

The mitogenomic location of observable variation in *Aplysina* is congruent with the survey of Wang & Lavrov (2008) who calculated the rate of molecular evolution for protein-coding genes across all published demosponge mitochondrial genomes. They found that *atp8* and *nad6*, both relatively small genes on the order of 77 and 190 predicted amino acids for *Aplysina*, respectively, evolved considerably faster than all other genes. Of the larger genes, the two fastest-evolving genes were *nad2* and *nad5*, both of which show variation in our comparison of *Aplysina* species. In contrast, *cox1* showed the lowest rate of molecular evolution across all genes in the mitochondrial genome, and no variation between the two *Aplysina* species.

The results of this congeneric study, combined with the more global survey of Wang & Lavrov (2008) and other studies of DNA barcoding in demosponges (e.g., Pöppe et al., 2010), suggest that while *cox1* will be useful in resolving relatively ancient species-level divergences, its utility is greatly diminished for recognizing recently diverged sponge species, even when the more variable 3′ region is included. The fastest-evolving genes in demosponge mitochondrial genomes (Wang & Lavrov, 2008), *atp8* and *nad6*, are relatively small, and alignment across demosponges does not reveal any conserved regions within these genes that could be used to build universal degenerate primers. Thus, while primer regions that will work across all sponges are unlikely to be found for *atp6* and *atp8*, with the sequencing of more complete mitochondrial genomes, the possibility of clade-specific targeted primers remains open (e.g., Xavier et al., 2010). Alignments of *nad2* and *nad5*, on the other hand, show several variable regions separated by conserved regions where primers could be placed. Given their relatively higher rates of molecular evolution, these two genes, perhaps in conjunction with ribosomal genes or microsatellite studies, warrant

exploration as a potential complement to the traditional *cox1* gene for mitochondrial DNA barcoding and for species-level phylogenetics in closely related demosponge species.

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References

Bell, J. J., 2008. The functional roles of marine sponges. Estuarine, Coastal and Shelf Sciences 79: 341–353.

Blanquer, A. & M.-J. Uriz, 2007. Cryptic speciation in marine sponges evidenced by mitochondrial and nuclear genes: a phylogenetic approach. Molecular Phylogenetics and Evolution 45: 392–397.

Boore, J. L. & W. M. Brown, 1998. Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. Current Opinions in Genetics and Development 8: 668–674.

Borchiellini, C., C. Chombard, M. Manuel, E. Alivon, J. Vacelet & N. Boury-Esnault, 2004. Molecular phylogeny of Demospongiae: implications for classifications and scenarios of character evolution. Molecular Phylogenetics and Evolution 32: 823–837.

Chen, J. & S. L. Dellaporta, 1994. Urea-based plant DNA miniprep. In Freeling, M. & V. Walbot (eds), The maize handbook. Springer, New York: 526–527.

Drabkin, H. J., M. Estrella & U. L. Rajbhandary, 1998. Initiator-elongator discrimination in vertebrate tRNAs for protein synthesis. Molecular and Cellular Biology 18: 1459–1466.

Duran, S., M. Pascual & X. Turon, 2004. Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poecilosclerida). Marine Biology 144: 31–35.

Erpenbeck, D., J. N. A. Hooper & G. Wörheide, 2005. CO1 phylogenies in diploblasts and the 'Barcoding of Life'—are we sequencing a suboptimal partition? Molecular Ecology Notes 6: 550–553.

Erwin, P. M. & R. W. Thacker, 2007. Phylogenetic analysis of marine sponges within the order Verongida: a comparison of morphological and molecular data. Invertebrate Biology 126: 220–234.

Ewing, B. & P. Green, 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Research 8: 186–194.

Folmer, O., M. Black, W. Hoen, R. Lutz & R. Vrijenhoek, 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Galtier, N., B. Nabholz, S. Glemin & G. D. D. Hurst, 2009. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Molecular Ecology 18: 4541–4550.

Gordon, D., C. Abajian & P. Green, 1998. Consed: a graphical tool for sequence finishing. Genome Research 8: 195–202.

Haag-Liautard, C., N. Coffey, D. Houle, M. Lynch, B. Charlesworth & P. D. Keightley, 2008. Direct estimation of the mitochondrial DNA mutation rate in *Drosophila melanogaster*. PLoS Biology 6(8): e204.

Hebert, P. D. N., A. Cywinska, S. L. Ball & J. R. Dewaard, 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society London B 270: 313–321.

Heim, I., M. Nickel & F. Brummer, 2007a. Phylogeny of the genus *Tethya* (Tethyidae: Hadromerida: Porifera): molecular and morphological aspects. Journal of the Marine Biological Association of the United Kingdom 87: 1615–1627.

Heim, I., N. Nickel & F. Brummer, 2007b. Molecular markers for species discrimination in poriferans: a case study on species of the genus *Aplysina*. Porifera Research 1: 361–371.

Huang, D., R. Meier, P. A. Todd & L. M. Chou, 2008. Slow mitochondrial COI sequence evolution at the base of the metazoan tree and its implications for DNA barcoding. Journal of Molecular Evolution 66: 167–174.

Klautau, M., C. A. M. Russo, C. Lazoski, N. Boury-Esnault, J. P. Thorpe & A. M. Sole-Cava, 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. Evolution 53:1414–1422.

Kloppell, A., A. Putz, M. Pfannkuchen, G. Fritz, A. Jaklin, P. Proksch & F. Brummer, 2009. Depth profile of *Aplysina* ssp.: morphological, histological and biochemical aspects and their role in species distinction. Marine Biodiversity 39: 121–129.

Krzywinski, M., J. Schein, I. Birol, J. Connors, R. Gascoyne, D. Horsman, S. J. Jones & M. A. Marra, 2009. Circos: an information aesthetic for comparative genomics. Genome Research 19: 1639–1645.

Lamarao, F. R. M., E. C. Reis, T. A. Simao, R. M. Albano & G. Lobo-Hadju, 2010. *Aplysina* (Porifera: Demospongiae) species identification through SSCP-ITS patterns. Journal of the Marine Biological Association of the United Kingdom 90: 845–850.

Lavrov, D. V., 2007. Key transitions in animal evolution: a mitochondrial DNA perspective. Integrative and Comparative Biology 47: 734–743.

Lavrov, D. V., W. M. Brown & J. L. Boore, 2000. A novel type of RNA editing occurs in the mitochondrial tRNAs of the centipede *Lithobius forficatus*. Proceedings of the National Academy of Sciences of the USA 97: 13738–13742.

Lavrov, D. V., L. Forget, M. Kelly & B. F. Lang, 2005. Mitochondrial genomes of two demosponges provide insights into an early stage of animal evolution. Molecular Biology and Evolution 22: 1231–1239.

Lavrov, D. V., X. Wang & M. Kelly, 2008. Reconstructing ordinal relationships in the Demospongiae using mitochondrial

genomic data. Molecular Phylogenetics and Evolution 49: 111–124.

Lis, J. T. & R. Schleif, 1975. Size fractionation of double-stranded DNA by precipitation with polyethylene glycol. Nucleic Acids Research 2: 383–389.

Lowe, T. M. & S. R. Eddy, 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Research 25: 955–964.

Maldonado, M., M. C. Carmona, M. J. Uriz & A. Cruzado, 1999. Decline in Mesozoic reef-building sponges explained by silicon limitation. Nature 401: 785–788.

Miller, S. E., 2007. DNA barcoding and the renaissance of taxonomy. Proceedings of the National Academy of Sciences of the USA 12: 4775–4776.

Muramatsu, T., K. Nishikawa, F. Nemoto, Y. Kuchino, S. Nishimura, T. Miyazawa & S. Yokoyama, 1998. Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. Nature 336: 179–181.

Nichols, S. A., 2005. An evaluation of support for order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. Molecular Phylogenetics and Evolution 34: 81–96.

Pöppe, J., P. Sutcliffe, J. N. A. Hooper, G. Wörheide & D. Erpenbeck, 2010. COI barcoding reveals new clades and radiation patterns of Indo-Pacific sponges of the family Irciniidae (Demospongiae: Dictyoceratida). PloS One 5: e9950.

Rosengarten, R. D., E. A. Sperling, M. A. Moreno, S. P. Leys & S. L. Dellaporta, 2008. The mitochondrial genome of the hexactinellid sponge *Aphrocallistes vastus*: evidence for programmed translational frameshifting. BMC Genomics 9: 33.

Schander, C. & E. Willassen, 2005. What can biological barcoding do for marine biology? Marine Biology Research 1: 79–83.

Schmitt, S., U. Hentschel, S. Zea, T. Dandekar & M. Wolf, 2005. ITS-2 and 18S rRNA gene phylogeny of Aplysinidae (Verongida, Demospongiae). Journal of Molecular Evolution 60: 327–336.

Schroder, H. C., S. M. Efremova, V. B. Itskovich, S. Belikov, Y. Masuda, A. Krasko, I. M. Muller & W. E. G. Muller, 2003. Molecular phylogeny of the freshwater sponges in Lake Baikal. Journal of Zoological Systematics and Evolutionary Research 41: 80–86.

Shearer, T. L., M. J. H. Van Oppen, S. L. Romano & G. Wörheide, 2002. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Molecular Ecology 11: 2475–2487.

Sipkema, D., M. C. R. Franssen, R. Osinga, J. Tramper & R. H. Wijffels, 2005. Marine sponges as pharmacy. Marine Biotechnology 7: 142–162.

Sperling, E. A., K. J. Peterson & D. Pisani, 2007. Poriferan paraphyly and its implications for Precambrian palaeobiology. Geological Society, London, Special Publications 286: 355–368.

Sperling, E. A., K. J. Peterson & D. Pisani, 2009. Phylogenetic-signal dissection of nuclear housekeeping genes supports the paraphyly of sponges and the monophyly of Eumetazoa. Molecular Biology and Evolution 26: 2261–2274.

Stortchevoi, A., U. Varshney & U. L. Rajbhandary, 2003. Common location of determinants in initiator transfer RNAs for initiator-elongator discrimination in bacteria and in eukaryotes. Journal of Biological Chemistry 278: 17672–17679.

Tatusova, T. A. & T. L. Madden, 1999. BLAST 2 sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiology Letters 174: 247–250.

Thompson, J. D., D. G. Higgins & T. J. Gibson, 1994. Clustal-W—improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Wang, X. & D. V. Lavrov, 2008. Seventeen new complete mtDNA sequences reveal extensive mitochondrial genome evolution within the Demospongiae. PloS One 3: e2723.

Weber, F., A. Dietrich, J. H. Weil & L. Marechal-Drouard, 1990. A potato mitochondrial isoleucine tRNA is coded for by a mitochondrial gene possessing a methionine anticodon. Nucleic Acids Research 18: 5027–5030.

Wörheide, G., 2006. Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge *Astrosclera willeyana* across the Indo-Pacific. Marine Biology 148: 907–912.

Wörheide, G. & D. Erpenbeck, 2007. DNA taxonomy of sponges—progress and perspectives. Journal of the Marine Biological Association of the United Kingdom 87: 1629–1633.

Wyman, S. K., R. K. Jansen & J. L. Boore, 2004. Automatic annotation of organellar genomes with DOGMA. Bioinformatics 20: 3252–3255.

Xavier, J. R., P. G. Rachello-Dolmen, F. Parra-VElandia, C. H. L. Schonberg, J. A. J. Breeuwer & R. W. M. van Soest, 2010. Molecular evidence of cryptic speciation in the "cosmopolitan" excavating sponge *Cliona celata* (Porifera, Clionaidae). Molecular Phylogenetics and Evolution 56: 13–20.

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SPONGE RESEARCH DEVELOPMENTS

Genetic structure and differentiation at a short-time scale of the introduced calcarean sponge *Paraleucilla magna* to the western Mediterranean

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Abstract The allochthonous calcarean sponge *Paraleucilla magna* has proliferated in the western Mediterranean during the last decade, where it currently shows a highly patchy distribution with dense populations in the neighboring of sea farms and slightly eutrophised marinas, and more sparse populations in well-preserved habitats. To gain knowledge about the species invasive capacity, we studied spatial genetic differentiation and structure, clonality, and temporal differentiation, in three close populations of *P. magna* at the NE of the Iberian Peninsula, in three successive years. The study hypothesis was that the species is able to proliferate under favorable conditions in newly colonized habitats but populations can easily disappear where perturbations occur with some frequency. Samples were genotyped for nine polymorphic microsatellites. Spatial genetic structure was found in the three populations of 2006. One population disappeared in 2007, and the other two remained slightly differentiated, while the three populations were in place again in 2008, and showed very low (but significant) F_{ST} values, and non-significant D values. Low but statistically significant differentiation also occurred for the three populations between years. Results showed high-allele diversity, but heterozygote deficit and changes in allele frequencies in the populations over the 3 years, which are consistent with some genetic drift. The whole population descriptors pointed to the species as a good opportunistic colonizer as it has been hypothesized, but highly sensitive to stochastic events affecting recruitment. This suggests a high impact of the species in favorable habitats (sea culture and sheltered zones) and a low-medium influence in native communities.

Keywords Calcarean sponges · Microsatellites · Population genetics · Introduced species · D versus F_{ST} estimators

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Introduction

The introduction rate of allochthonous species in marine ecosystems has increased extraordinarily during the last few years (http://conserveonline.org/workspaces/global.invasive.assessment). Although biological introductions have been occasionally considered an increase in biodiversity instead of a threat (Briggs, 2007), they represent indeed the second cause of biodiversity losses, and affect to the abundance and

genetic integrity of the autochthonous species (Kaiser & Gallagher, 1997; Thomsen et al., 2011). Marine introductions are especially outstanding in areas such as the Mediterranean Sea, which has an extensive fishery and dense ship traffic, as well as connection with the Indian Ocean through the Suez Canal (Galil, 2006; Steftaris & Zenetos, 2006).

Introduced species may interact in several ways and directions with the autochthonous assemblages, with unpredictable outcomes. Thus, knowledge on the capacity of proliferation and resilience of the newly established populations, acquired from population genetics studies, is a first step for the assessment of their invasive capacities.

Sponges were thought to be poor invaders since they are sessile as adults and have short-life, poor dispersal larvae (Uriz et al., 2008), but the number of introduced sponge species has increased in the last years (DeFelice et al., 2001; Coles et al., 2002; Perez et al., 2006). In spite of their increasing proliferation, aspects such as the genetic variation and connectivity among the newly established populations of introduced sponges have never been addressed although they are key aspects to determine their long-term invasive capacity.

In the last decade, the calcarean sponge *Paraleucilla magna*, which was first described from Rio de Janeiro-Brazil (Klautau et al., 2004), has proliferated in the western Mediterranean (Longo et al., 2007; Zammit et al., 2009, authors, pers. obs). The known current distribution of this species extends to the Atlantic Ocean (Madeira, Açores, Portugal, and Brazil) and Mediterranean: coasts of Italy (Longo et al., 2007), Malta (Zammit et al., 2009), and Spain (Frotscher & Uriz, 2008). Its reproduction period ranged from April to July at the study site. Remarkably, representatives of the genus *Paraleucilla* had only been recorded from the Indo-Pacific (Klautau et al., 2004). *P. magna* presents an annual cycle with total disappearance of the adults after larval release (Frotscher & Uriz, 2008), so that its populations rely exclusively on yearly recruits, and a population consists of a unique cohort class.

In the newly colonized area, the species mainly dwells on habitats with a certain amount of suspended organic material (e.g., close to sea farms, small marinas, and river mouths) (Longo et al., 2007; Zammit et al., 2009, present study). It settles on shallow rocky walls mainly covered by *Halopteris* spp., and *Corallina* spp. algae, but also grows on *Mytilus* sp. and, occasionally, directly on the rock.

Gathering information on the genetic diversity and population differentiation of introduced species allows gaining insight on their capacity for survival and spread in the new habitats, which are among the indicators of their invasive potential. Studies on sponge population genetics are particularly problematic because the molecular markers that are suitable for similar studies in other invertebrates (e.g., mitochondrial genes), show low or no intra-species variation in sponges (Duran et al., 2004b) and, where used, they proved to differentiate cryptic species rather than populations of the target species (Xavier, 2010). Thus, within species polymorphic markers such as species-specific microsatellites need to be developed for studies on sponge population genetics. Only three demosponges (Duran et al., 2004a; Blanquer et al., 2009; Dailianis et al., 2011) and no calcarean sponge have been studied by using microsatellites up to now. Demosponges show contrasting ecological and reproductive strategies compared with most calcarean sponges (e.g., long-lived vs. annual species) and thus population genetic parameters could be predicted to differ in chief traits between representatives of the two sponge Classes. On the other hand, no introduced sponges had been considered for population genetic studies previously.

Here, we address the study of the genetic features of three close populations of *P. magna* established at the Blanes littoral (NW Mediterranean) ca. 10 years ago, during three consecutive years. The study of hypothesis was that the species has an opportunistic behavior with contrasting success in altered or native assemblages. To test our hypothesis, we studied the genetic stability, dispersal, and resilience of the species populations once established in a given area. This is the first time that temporal genetic variation in populations of an introduced sponge is addressed. Moreover this is also the first study on population genetics of a calcarean sponge. The knowledge generated can be decisive for assessing the long-term invasive menace of this introduced species.

Methods

Sampling

Three populations of *Paraleucilla magna* (SA1, SA2, and SA3) from the NE littoral of the Iberian Peninsula (Blanes, NW Mediterranean, 41° 40.12′N, 2° 47.10′E)

were sampled during three consecutive years (2006, 2007, 2008). The populations were spread on rocky walls running from Northeast to Southwest, from 4 to 10 m in depth. The populations SA1 and SA2 were 10 m apart from each other, and 50 m apart from SA3 (Fig. 1). They were a priori considered to be differentiated despite the short distance among them on the basis of a previous study on population genetics of another sponge species (*Scopalina lophyropoda*), which proved to be genetically structured at this very small spatial scale (Blanquer et al., 2009).

Sampling was exhaustive (all individuals were sampled) and was conducted in February (first study year), March (second study year), and April (third study year). The population sizes were 33 individuals (SA1), 20 individuals (SA2), 34 individuals (SA3) in 2006; 17 individuals (SA1), 12 individuals (SA3) in 2007, and 23 individuals (SA1), 24 individuals (SA2), and 24 individuals (SA3) in 2008. A 0.5 cm^3 fragment was removed from the apical part of every individual, to ensure not to take embryo harboring tissue, since the zone close to the oscules uses to be oocyte free (Frotscher & Uriz, 2008). A single multilocus genotype (MLG) was obtained from each sample, confirming that we succeeded at obtaining only parental tissue and can confirm that sampling likely did not affect the annual production of larvae (i.e., the potential number of philopatric recruits). The samples were preserved in absolute ethanol and stored at −20°C until DNA extraction.

DNA extraction, amplification, and microsatellite genotyping

DNA was extracted using the protocol described in Pascual et al. (1997). The samples were amplified using nine polymorphic microsatellite loci (Table 1) previously designed for the species (Agell et al., in press). Forward primers were labeled with a fluorescent dye from Applied Biosystems: PET red (cal_a, cal_h), NED yellow (cal_b, cal_g), VIC green (cal_c, cal_e, cal_j), and FAM blue (cal_d, cal_f). The length of PCR products was estimated relative to the internal size standard GeneScan 500LIZ (Applied Biosystems) and determined using GeneMapper® and PeakScanner® software from Applied Biosystems. Three independent readers checked the results to avoid scoring errors.

Data analysis

Departure from Hardy–Weinberg equilibrium, expected and observed heterozygosities, linkage disequilibrium, allele frequencies, number of alleles per loci, number of private alleles, and the inbreeding coefficient (F_{IS}, Weir & Cockerman, 1984) for each locus individually as well as for all loci combined, were calculated with GENEPOP, web version 4.0 (Raymond & Rousset, 1995; Rousset, 2008). The presence of null alleles was analyzed with Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004). The extent of clonal reproduction was

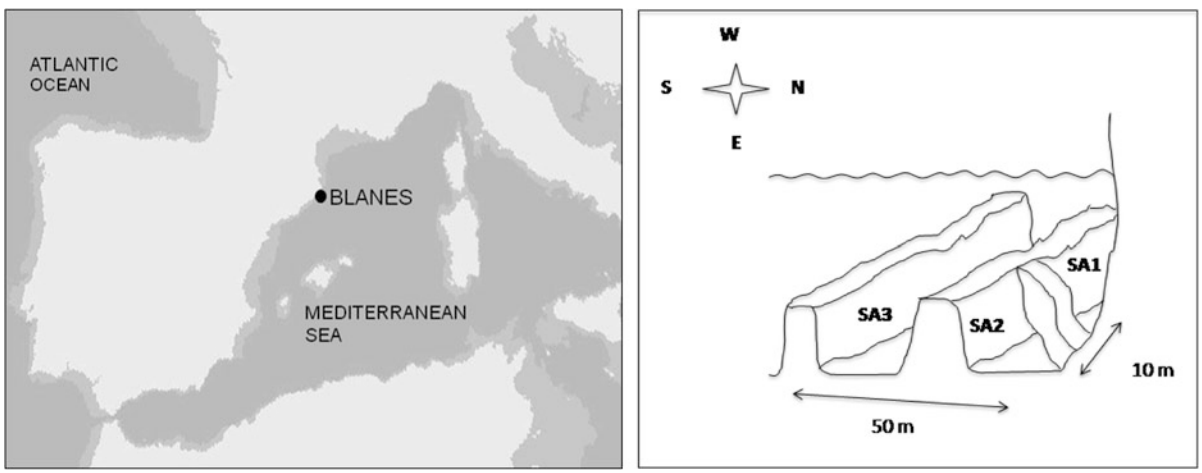

Fig. 1 Sample site

Table 1 Microsatellite loci isolated from the sponge *Paraleucilla magna*

Locus (Dye) (GenBank Accession No.)	Primer sequence (5′-3′) F and R (5′-3′)	Repeat motif	T^a (°C)	Size range
cal_a (PET) (JN628841)	F: TGCAGAAGCTACATGCATGC	$(GT)_3TT(GT)_{11}$	58	171–198
	R: GGTGCATTCGCAAAGCTTCA			
cal_b (NED) (JN628842)	F: GGAGCCAAGGTGTACTTG	$(AC)_7$	60	247–289
	R:GCTACCTGACATGAATGCAAC			
cal_c (VIC) (JN628843)	F: TGGACTGTAGAGCTTCCT	$(GT)_9$	66	145–164
	R: CGAATTGATCAGGCTGTGAG			
cal_d (6FAM) (JN628844)	F: ACTGCTGGTACTCTAGAG	$(CA)_{17}AA(CA)_6$	60	180–207
	R: AACACGACACACTTCGCTTG			
cal_e (VIC) (JN628845)	F: GTGATCAGAGTTGTGAACGC	$(GT)_9T(GT)_2AT(GT)_3$	60	165–179
	R: CGCACCACTTCTGGAACATA			
cal_f (6FAM) (JN628846)	F: CTTCTAGCTATCACAATA	$(TG)_3CG(TG)_4AT(GT)_3$	58	135–169
	R: CACTACTTACCTCTAGCTTC			
cal_g (NED) (JN628847)	F: CTGCATAGCATTGGTACTG	$(GA)_9$	58	178–238
	R: AGGTCACGCGGACTACCACT			
cal_h (PET) (JN628848)	F: CTCAACTCACGACTCATA	$(GT)_{19}$	62	245–352
	R:CTACCGTAACACTGAGCAAGC			
cal_j (VIC) (JN628849)	F: CTTGCTGTTCCCACACTTCAC	$(AC)_8$	64	130–168
	R: TGATTGCCACAACGTTCAGG			

T^a annealing temperature

estimated by analyzing the probability that two distinct sexual reproduction events could produce individuals sharing identical multilocus genotypes (MLGs), using MLGsim v 1.0 (10000 simulations; Stenberg & Lundmark, 2002).

Genetic differentiation and structure, and molecular variance

The genetic differentiation among populations was assessed by calculating the statistic F_{ST} (Weir & Cockerman, 1984) with ARLEQUIN v.3.11 (1000 permutations, Excoffier et al., 2005) and D estimator (Jost, 2008). D, which ranges from 0 to 1 (0 = no differentiation; 1 = absolute differentiation), is independent of within population heterozygosis and thus gives true values of differentiation for any type of population. D was first calculated with SMOGD (Crawford, 2010). The 95% confidence intervals and significance values (P) of pair-wise D comparisons were calculated with DEMEtics v.0.8-2 (Gerlach et al., 2010). This software, which is implemented within the statistical package R v2.12.1 (R Development Core Team 2009), estimates the P values and the confidence intervals according to Manly (1997) with a bootstrap method that distributes the alleles for a specific locus randomly when the populations are in Hardy–Weinberg equilibrium for this locus. If the populations are not in Hardy–Weinberg equilibrium, the genotypes instead of the alleles are distributed randomly among populations (Goudet et al., 1996). The P values indicate how different is the allele distribution in the studied sample from the one randomly obtained. The number of genetically homogenous groups (K) was inferred using a Bayesian algorithm (STRUCTURE v.2.3.3 software, range of $K = 1$–4, MCMC repetitions = 50000, burn-in range = 100000, total replicates = 20, Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009) and the ad hoc statistic ΔK (Evanno et al., 2005). Genetic differentiation was assessed in two steps. First, we analyzed the spatial differentiation of the populations in each of the study years, and then differentiated populations were compared across years. As the species is annual and all the adults of the Blanes populations disappear after larval release (Frotscher & Uriz, 2008), the yearly populations corresponded indeed to population cohorts. A hierarchical analysis of molecular variance (AMOVA) was performed with ARLEQUIN (Excoffier et al., 2005).

Results

Population genetic estimators

A total of 205 individuals of *Paraleucilla magna* were genotyped for the nine microsatellite loci previously designed. All loci were polymorphic, showing from 2 to 14 alleles (Table 2). Several loci showed linkage disequilibrium in a given year but this was not consistent over years. Moreover, the results did not change in any significant way after removing these loci. All the populations showed some "private" alleles in one of the three study years (see Table 3) but most of the alleles that were private for a population in a given year, were also present in any of the other two populations in one of the other two years. Only two private alleles were maintained in the same population across years (Table 3).

The inbreeding coefficient values were positive and significant ($P < 0.001$) for each population at each study year indicating heterozygote deficit and inbreeding in all populations. The exact tests for Hardy–Weinberg equilibrium confirmed these results showing significant deviations for each population (Table 2), although analysis with Micro-Checker indicated the presence of null alleles in the four nine loci that showed FIS, and suggests possible Hardy–Weinberg equilibrium for the populations (Table 4).

The percentage of failed amplifications is reported in Table 4. No evidence of scoring errors due to stuttering or large allele drop out resulted from the Micro-Checker analysis.

Only three cases (1.46% of the individuals analyzed) with identical MLGs were found (two individuals on SA3 2006, 2 on SA1 2007, and 2 on SA2 2007). The probability for the MLGs to be originated by chance from sexual reproduction was significantly low ($P < 0.001$) in the three cases (Table 5).

Population structure

Spatial scale

The genetic differentiation values (D and F_{ST}) were low in all cases. D values among the three populations (SA1, SA2, and SA3) established in 2006 ranged from 0.023 and 0.142 and were statistically significant (Table 6). D but not F_{ST} was statistically significant between the two populations present in 2007 (SA1 and SA2) and, conversely, only F_{ST} was significant between SA1 and SA2 populations in 2008. Bayesian clustering analyses detected the highest likelihood for the model with three genetically homogeneous groups of individuals (K) for year 2006 ($\Delta K = 7.34$) and one homogeneous group for 2008. These analyses did not provide consistent results for populations in 2007 and 2008, since they did not detect K likelihood increments for $K = 2$ or $K = 3$, and ΔK is not able to find the best number of homogeneous groups if $K = 1$. The hierarchical AMOVA revealed significant genetic structuring among the populations in 2006 (3.31%, $P < 0.001$), but the values were not statistically significant for populations in 2007 and 2008 (1.51%, $P = 0.69$ and 0.61%, $P = 0.96$).

Temporal scale

The temporal study was carried out considering the three initial populations separately since they showed spatial genetic structuring. The yearly cohorts of the three populations were also genetically differentiated. SA1 cohorts were genetically differentiated for 2008 and the other two years (D and F_{ST} values between 0.008 and 0.162, $P < 0.05$) (Table 7). The 2006 cohort of SA2 was differentiated from that of 2007 (D and F_{ST}, $P < 0.05$) and that of 2008 (significant D, $P < 0.001$). The cohorts of SA3 in 2006 and 2008 (the two only years where is SA3 was present) were also differentiated (both D and F_{ST}, $P < 0.001$). Bayesian clustering analyses detected the highest likelihood for the model with three genetically homogeneous groups of individuals (K) for SA1 cohorts, and two homogeneous groups for SA2 and SA3 cohorts. The hierarchical AMOVA, however, revealed significant genetic structuring for SA3 among years (2.61%, $P < 0.05$), but not for SA1 and SA2 (1.73%, $P = 0.12$, and 2.33%, $P = 0.20$, respectively).

Discussion

Microsatellites have proved to be informative markers to establish the genetic structure of very close populations of *Paraleucilla magna* across a short temporal scale. Only two sponge species had been studied using microsatellites at very short-spatial scales up to now (*Crambe crambe*, Calderón et al., 2007 and *Scopalina lophyropoda,* Blanquer et al.,

Table 2 Summary of genetic variation at 9 microsatellite loci for each population and year

Locus	Population										
	2006			Mean Na/ locus	2007		Mean Na/ locus	2008			Mean Na/ locus
	SA1	SA2	SA3		SA1	SA2		SA1	SA2	SA3	
cal_a											
Na	7	6	6	6.3	5	4	4.5	5	5	6	5.3
H_E	0.579	0.664	0.630		0.642	0.569		0.535	0.467	0.560	
H_O	0.608	0.600	0.647		0.765	0.750		0.565	0.542	0.667	
F_{IS}	−0.058	0.099	−0.028		−0.199	−0.338		−0.059	−0.165	−0.196	
cal_b											
Na	7	6	7	6.7	6	5	5.5	5	8	6	6.3
H_E	0.712	0.618	0.601		0.638	0.659		0.667	0.613	0.727	
H_O	0.686	0.550	0.529		0.647	0.667		0.609	0.542	0.833	
F_{IS}	0.037	0.113	0.121		−0.014	−0.013		0.089	0.119	−0.150	
cal_c											
Na	2	3	3	2.7	2	2	2.0	3	4	3	3.3
H_E	0.423	0.465	0.442		0.332	0.431		0.420	0.488	0.458	
H_O	0.471	0.350	0.441		0.412	0.417		0.304	0.583	0.417	
F_{IS}	−0.115	0.253	0.002		−0.250	0.035		0.279	−0.202	0.093	
cal_d											
Na	9	5	6	6.7	5	5	5.0	8	9	7	8.0
H_E	0.592	0.673	0.572		0.693	0.680		0.482	0.754	0.713	
H_O	0.137	0.050	0.059		0.059	0.083		0.217	0.500	0.458	
F_{IS}	0.770***	0.928***	0.899***		0.918***	0.883***		0.556***	0.342***	0.362***	
cal_e											
Na	7	7	6	6.7	7	6	6.5	5	7	7	6.3
H_E	0.760	0.781	0.630		0.761	0.601		0.656	0.722	0.778	
H_O	0.843	0.850	0.706		0.824	0.500		0.696	0.833	0.917	
F_{IS}	−0.111	−0.091	−0.123		−0.085	0.175		−0.063	−0.159	−0.183	
cal_f											
Na	5	5	5	5.0	4	4	4.0	8	6	5	6.3
H_E	0.719	0.759	0.739		0.700	0.679		0.801	0.629	0.692	
H_O	0.353	0.500	0.324		0.353	0.500		0.652	0.625	0.583	
F_{IS}	0.512***	0.347**	0.566***		0.504***	0.273		0.189***	0.006	0.160**	
cal_g											
Na	13	9	11	11.0	9	10	9.5	11	13	10	11.3
H_E	0.882	0.774	0.772		0.799	0.866		0.867	0.842	0.822	
H_O	0.510	0.550	0.588		0.765	0.583		0.739	0.625	0.625	
F_{IS}	0.424***	0.295**	0.241**		0.044	0.336**		0.150*	0.262***	0.244**	
cal_h											
Na	12	9	12	11.0	7	7	7.0	14	11	12	12.3
H_E	0.839	0.607	0.733		0.630	0.877		0.813	0.777	0.822	
H_O	0.843	0.550	0.441		0.588	0.583		0.565	0.375	0.458	
F_{IS}	−0.005	0.098	0.402***		0.068	0.345**		0.310**	0.523***	0.448***	

Table 2 continued

Locus	Population										
	2006			Mean Na/ locus	2007		Mean Na/ locus	2008			Mean Na/ locus
	SA1	SA2	SA3		SA1	SA2		SA1	SA2	SA3	
cal_j											
Na	7	9	7	7.7	4	6	5.0	8	9	6	7.7
H_E	0.769	0.714	0.665		0.639	0.714		0.836	0.663	0.708	
H_O	0.863	0.700	0.588		0.529	0.833		0.783	0.833	0.708	
F_{IS}	−0.124	0.020	0.117		0.177	−0.177		0.065	−0.265	0.000	
Mean Na/ population	7.7	6.6	7.0		5.4	5.4		7.4	8.0	6.9	
Mean H_E	0.697	0.673	0.643		0.648	0.609		0.675	0.662	0.698	
Mean H_O	0.590	0.522	0.480		0.549	0.546		0.570	0.606	0.630	
F_{IS}	0.154***	0.229***	0.256***		0.157***	0.199***		0.159***	0.085***	0.100***	
HWE	***	***	***		***	***		***	***	***	

Na number of alleles, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} inbreeding coefficient (estimated as a function of the heterozygosis deficiency), *HWE* probability of departure from Hardy–Weinberg equilibrium. Significant values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

2009), and none of them was a calcarean sponge. Moreover, the target sponge has been introduced recently in the study area, and has an annual life cycle (Frotscher & Uriz, 2008), which allowed us to expect contrasting genetic characteristics compared with the long-lived demosponges previously studied. However, no distinct patterns, which could be ascribed either to demosponges or calcarean sponges can be confirmed by our results.

The mean number of alleles per loci (Na = 8.9) was higher in *P. magna* populations than in the two (*S.lophyropoda* and *C.crambe)* out of the three previously studied species using microsatellites (Na = 3.95, and Na = 5.9, respectively) but lower than in a third species (*Spongia officinalis*, Na = 15.1, Dailianis & Tsigenopoulos, 2010). The populations of *S. lophyropoda* presented an unexplained outcrossing, given its patchy distribution and philopatric larvae (Uriz et al., 1998; Blanquer & Uriz, 2010). Conversely, heterozygote deficit and thus inbreeding were shown by the populations of *C. crambe*, *S. officinalis*, and *P. magna,* despite these species showed contrasting biological and ecological strategies. Inbreeding, which is common in marine modular invertebrates (Addison & Hart, 2005) has mainly been attributed to several causes such as restricted dispersal (due to philopatric larvae, self-fertilization, or/and mating among relatives), and population structure (Grosberg, 1987; Carlon, 1999). However, the presence of null alleles and a founder effect (Duran et al., 2004a, Astanei et al., 2005) has also to be considered.

The inbreeding and the heterozygote deficiency observed in *P. magna* is most probably the combined result of null alleles, larval philopatry, and a founder effect, since the populations have been recently established in the study area, their number of individuals is small and they likely have originated from one or a few colonization events from a nearby larger population.

Clonality played a non-relevant role in structuring *P. magna* populations (1.46% of clones) and thus did not contribute to the excess of homozygotes observed. Indeed asexual reproduction by buds, propagula, or fission events has not been recorded in the studied populations (Frotscher & Uriz, 2008) in contrast to that reported for other sponge species such as *Crambe crambe* (Duran et al., 2004a).

Genetic differentiation estimates

The pros and cons of commonly used estimators of population structure and differentiation have been revised recently (Meirmans & Hedrick, 2011). In this and previous studies, it appears clear that the

Table 3 Frequencies of all alleles

	SA1_06	SA2_06	SA3_06	SA1_07	SA2_07	SA1_08	SA2_08	SA3_08
a								
171	0.063	0.025	0.088	0.088	–	0.053	–	0.046
176	–	–	–	–	–	–	0.050	0.023
178	0.354	0.375	0.485	0.382	0.583	0.474	0.600	0.500
180	0.510	0.450	0.368	0.471	0.333	0.368	0.300	0.386
182	0.010	0.075	0.015	0.029	0.042	0.079	–	–
183	**–**	**0.050**	–	–	–	–	–	–
188	0.010	–	0.015	–	0.042	–	0.025	–
189	–	–	–	**0.029**	–	–	–	–
192	0.021	–	0.029	–	–	–	0.025	0.023
194	0.031	0.025	–	–	–	0.026	–	0.023
b								
247	**0.021**	–	–	–	–	–	–	–
250	0.052	0.075	0.059	0.029	0.091	0.065	0.114	0.146
254	0.073	0.125	0.074	0.059	0.136	0.044	0.091	0.104
257	0.385	0.100	0.177	0.353	0.136	0.109	0.159	0.083
258	0.188	0.075	0.059	0.029	0.136	0.283	0.023	0.125
262	0.042	0.025	0.015	0.029	–	–	0.023	0.063
269	–	–	**0.015**	–	–	–	–	–
270	0.240	0.600	0.603	0.500	0.500	0.500	0.546	0.479
279	–	–	–	–	–	–	**0.023**	–
289	–	–	–	–	–	–	**0.023**	–
c								
145	–	–	–	–	–	0.023	0.024	0.022
147	0.667	0.675	0.691	0.781	0.708	0.705	0.500	0.652
149	0.333	0.300	0.294	0.219	0.292	0.273	0.452	0.326
155	–	–	**0.015**	–	–	–	–	–
163	–	–	–	–	–	–	**0.024**	–
164	–	**0.025**	–	–	–	–	–	–
d								
177	–	–	–	–	–	–	**0.021**	–
180	0.026	–	–	0.063	–	0.053	0.104	0.065
181	0.013	–	–	–	–	0.026	0.021	–
183	0.154	0.167	0.321	0.188	0.100	0.026	0,021	0.130
184	0.410	0.417	0.036	0.406	0.250	0.632	0.458	0.457
185	0.026	0.056	–	–	–	–	–	–
190	–	–	–	–	–	**0.053**	–	–
191	0.128	0.222	0.446	0.281	0.300	0.026	0.083	0.152
199	0.026	–	0.036	0.063	–	0.026	0.063	0.044
201	–	–	–	–	–	–	–	**0.022**
203	0.077	0.139	0.089	–	0.200	0.158	0.146	0.130
205	0.141	–	0.071	–	0.150	–	0.083	–
e								
165	0.060	0.025	0.015	0.031	–	–	0.068	0.068
167	0.190	0.250	0.091	0.156	0.042	0.300	0.091	0.136

Table 3 continued

	SA1_06	SA2_06	SA3_06	SA1_07	SA2_07	SA1_08	SA2_08	SA3_08
169	0.370	0.350	0.561	0.344	0.625	0.350	0.386	0.250
171	0.020	0.025	–	0.031	0.083	–	0.023	0.114
173	0.090	0.200	0.091	0.125	0.125	0.050	0.205	0.205
175	0.060	0.050	0.136	0.094	0.083	0.100	0.114	0.068
179	0.210	0.100	0.106	0.219	0.042	0.200	0.114	0.159
f								
135	0.235	0.300	0.258	0.281	0.182	0.273	0.409	0.435
138	–	–	–	–	–	**0.114**	–	–
145	0.255	0.350	0.333	0.156	0.273	0.205	0.386	0.239
147	–	–	–	–	–	–	**0.023**	–
149	0.049	0.050	0.076	–	–	0.068	0.046	0.044
151	–	–	–	–	–	**0.046**	–	–
159	0.059	0.150	0.091	0.188	0.136	0.046	0.046	0.087
161	0.402	0.150	0.242	0.375	0.409	0.205	0.091	0.196
163	–	–	–	–	–	**0.046**	–	–
g								
184	–	–	–	–	–	–	**0.022**	–
185	–	–	–	–	–	**0.022**	–	–
186	0.060	0.050	0.016	0.031	–	–	0.044	0.087
188	0.030	–	0.016	–	0.167	0.130	0.065	0.109
190	0.180	0.050	0.188	0.156	0.250	0.261	0.196	0.239
192	0.120	0.050	0.125	0.125	0.042	0.109	0.152	0.196
194	0.060	–	0.016	0.063	–	0.044	0.022	0.022
196	**–**	***0.025***	**–**	**–**	***0.042***	**–**	***0.022***	**–**
198	–	–	–	–	–	–	**0.022**	–
200	–	–	–	–	–	–	–	**0.022**
202	0.150	0.350	0.328	0.313	0.250	0.196	0.239	0.196
204	–	–	–	–	–	–	0.022	0.022
210	0.140	0.025	0.078	–	0.042	0.065	0.065	0.044
212	0.070	0.075	0.047	0.094	0.042	0.044	0.044	–
214	0.050	0.325	0.156	0.156	0.083	0.087	0.087	0.065
216	–	–	**0.016**	–	–	–	–	–
220	–	–	–	–	**0.042**	–	–	–
230	**0.020**	–	–	–	–	–	–	–
232	***0.050***	**–**	**–**	***0.031***	**–**	***0.022***	**–**	**–**
234	**0.040**	–	–	–	–	–	–	–
236	0.030	0.050	0.016	0.031	0.042	0.022	–	–
h								
245	–	–	–	**0.036**	–	–	–	–
258	0.110	0.071	0.076	0.071	0.208	0.191	0.296	0.375
261	–	–	–	–	–	–	–	**0.021**
263	0.070	0.107	0.121	–	0.208	0.214	0.227	0.125
264	–	–	–	–	–	0.024	0.046	–
265	–	–	–	–	–	0.024	–	–
266	0.160	0.107	0.046	0.036	0.125	0.024	0.046	0.083

Table 3 continued

	SA1_06	SA2_06	SA3_06	SA1_07	SA2_07	SA1_08	SA2_08	SA3_08
267	–	–	–	–	–	0.048	–	–
269	0.060	0.071	0.076	–	–	0.024	0.046	–
270	–	–	–	–	–	–	–	**0.021**
271	0.020	–	0.015	–	–	0.071	0.023	0.021
272	0.050	0.107	–	–	0.125	–	–	0.021
273	–	0.071	–	–	–	0.048	–	0.021
279	0.050	–	0.015	0.143	–	–	0.046	–
291	0.040	0.071	0.030	0.071	0.167	–	–	–
293	0.010	–	–	–	–	–	–	0.042
297	–	–	0.046	–	–	–	0.023	–
300	–	–	–	–	–	**0.048**	–	–
301	0.090	0.071	0.030	–	–	–	0.114	0.042
303	0.040	–	0.046	0.214	0.083	0.071	0.091	0.104
304	–	–	–	–	–	**0.024**	–	–
319	–	–	**0.030**	–	–	–	–	–
341	0.300	0.321	0.470	0.429	0.083	0.167	0.046	0.125
343	–	–	–	–	–	**0.024**	–	–
j								
130	0.275	0.265	0.161	0.300	0.182	0.261	0.046	0.125
144	0.137	–	0.089	–	0.136	0.152	0.068	0.063
148	0.226	0.147	0.250	0.267	0.409	0.152	0.227	0.417
150	0.304	0.235	0.196	0.367	0.182	0.239	0.477	0.333
152	0.010	0.029	–	–	–	0.022	0.023	0.021
154	0.020	0.029	0.018	0.067	0.046	0.044	0.046	–
160	–	0.059	0.018	–	0.046	0.065	0.046	0.042
164	–	–	–	–	–	–	**0.023**	–
166	0.029	0.177	0.268	–	–	0.065	0.046	–
170	–	**0.029**	–	–	–	–	–	–
172	–	**0.029**	–	–	–	–	–	–

In *bold* the private alleles and, in *bold italic*, the only two private alleles maintained in the same population across years

D estimator (Jost, 2008) is very suitable for genetic differentiation assessment, while F_{ST} should be better considered as a fixation measure. Thus, we have based our interpretation mainly on genetic differentiation on *D* values but have also calculated F_{ST} to compare genetic structure in *P. magna* populations with that of the demosponge *Scopalina lophyropoda,* previously studied in the same zone, at the same spatial scale (Blanquer et al., 2009).

In our populations, F_{ST} and *D* estimators provided in general congruent results. Only between SA1 and SA2 populations in 2008 or between the 2006 and 2008 cohorts of SA2, both statistics gave contrasting significance values. This result does not seem to be influenced by the size of the populations compared (23 and 24 individuals and 20 and 24, respectively). In all these cases, genetic differentiation was low and the Bayesian clustering and the AMOVA indicated no significant genetic structure.

Spatial and temporal genetic differentiation

The two estimators used (*D*, F_{ST}) as well as the AMOVA and Bayesian clustering showed genetic differentiation among the three populations of *P. magna* established in 2006 in the study site despite their proximity (from 10 to 50 m apart) and the short time after their establishment (ca. 6 years). F_{ST}'s values

Table 4 a The presence and frequencies of null alleles across nine microsatellite loci for the populations of *Paraleucilla magna* for each study year; b Percentage of failed amplifications across loci and populations for each year

	2006			2007		2008		
	SA1	SA2	SA3	SA1	SA2	SA1	SA2	SA3
a								
cal_a								
cal_b								
cal_c								
cal_d	0.356	0.045	0.407	0.413	0.405	0.261	0.261	0.162
cal_e								
cal_f	0.242		0.278	0.201				
cal_g	0.207		0.123		0.121			0.122
cal_h			0.169		0.170	0.157	0.267	0.204
cal_j								
b								
cal_a	5.88					17.39	16.67	8.30
cal_b	5.88				5.33		8.33	4.17
cal_c	5.88			5.88		4.35	12.50	4.17
cal_d	23.53	10.00	17.65	5.88	16.67	17.39		8.30
cal_e	1.96		2.94	5.88		13.04	8.33	4.17
cal_f			2.94	5.88	8.33	4.35	8.33	4.17
cal_g	1.96		5.88	5.88			4.17	
cal_h	1.96	30.00	2.94	17.65		8.69	8.30	
cal_j		15.00	17.65	11.76	8.33		8.30	

Table 5 Identical multilocus genotypes (MLGs) detected in *Paraleucilla magna*

	Genotype									N	MLGsim
	cal_a	cal_b	cal_c	cal_d	cal_e	cal_f	cal_g	cal_h	cal_j		
MLG1	171/178	270/270	147/147	000/000	169/175	145/145	192/202	263/341	130/166	2	***
MLG2	178/180	254/258	147/147	000/000	169/169	161/161	188/188	291/341	130/148	2	***
MLG3	178/180	257/270	147/147	191/191	169/175	161/161	202/202	258/279	148/148	2	***

N number of individuals sharing MLG. Significant values: *** $P < 0.001$

Table 6 Pairwise genetic differentiation comparisons between populations for each study year

2006	SA1	SA2	SA3	2007	SA1	SA2	2008	SA1	SA2	SA3
SA1		0.094**	0.142**	SA1		0.097*	SA1		0.044	0.031
SA2	0.038***		0.063**	SA2	0.024		SA2	0.018**		0.000
SA3	0.042***	0.023*					SA3	0.011	0.003	

D values above the diagonal and F_{ST} values below the diagonal. Significant values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

for *P. magna* populations were similar to those reported for *S. lophyropoda* populations in the same zones (e.g., $F_{ST} = 0.023$ between SA1 and SA3), although the later species is native to the Mediterranean and had been detected in the study zone ca. 30 years before the genetic study (Uriz, 1982). This suggests a stronger philopatry for *P. magna* larvae, which is in agreement with the extremely poor swimming ability and the short

Table 7 Pairwise genetic differentiation comparisons between years for each population

SA1	2006	2007	2008	SA2	2006	2007	2008	SA3	2006	2008
2006		0.036	0.075**	2006		0.090**	0.113***	2006		0.162***
2007	0.013		0.090**	2007	0.054*		0.064	2008	0.029***	
2008	0.026***	0.021*		2008	0.022	0.008				

D values above the diagonal and F_{ST} values below the diagonal. Significant values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

life span reported for calcarean larvae in general (Ereskovsky, 2010). The cohorts of the three populations also showed genetic differentiation along the three study years, which could be attributed to genetic drift, given their small size. Moreover, the genetic differentiation among populations diminished with time likely due to some gene flow among the three populations. This is confirmed by the relocation of some private alleles from one population to another in subsequent years. The Bayesian clustering was consistent with the presence of some gene flow, while the AMOVA only revealed significant genetic structuring for SA3 cohorts, probably due to the absence of the population in 2007 and its re-establishment in 2008. All the cohorts showed some private alleles, but only two were constantly present in a given population over years (Table 3).

The disappearance of one out of the three studied populations in 2007 suggests a potential vulnerability of *P. magna* to adverse environmental conditions, which can produce massive mortalities or complete failing of a recruitment episode (Authors pers. obs.). In 2008, the previously missing population was recovered, but no differentiation occurred this year among the three populations, indicating that the reinstalled population was mainly formed by recruits resulting from larvae from any of the two other populations.

Moreover, the studied populations were small so that the influence of stochastic variation in demographic parameters due to failed recruitment and/or mortality may have affected them during the study years. Stochastic variation in demographic rates causes small populations to fluctuate randomly in size, which may have lead to extinction of the SA3 population in 2007.

Species invasive potential

Populations of annual species such as those of *P. magna* rely exclusively on the yearly recruitment success and, consequently, are vulnerable to unfavourable conditions at settlement. This strong dependence on recruitment makes populations of *P. magna* demographically variable, as a function of some stochastic environmental events such as storms, which may determine either recruitment success or failure. Moreover, the small populations of *P. magna* at the study site and other Atlantic and Mediterranean areas (Frotscher & Uriz, 2008) may be prone to disappear due to environmentally generated fluctuations in size and the loss of genetic diversity by genetic drift. However, where the environmental conditions are stable, populations may remain over time and differentiate genetically relatively fast thanks to their philopatric larvae. These characteristics would point to a relatively high invasive potential for *P. magna* with fast proliferation in sheltered habitats but with low to moderate impact in exposed, well-established, native assemblages. Thus, *P. magna* behaves as an opportunistic species in its area of introduction. An opportunistic behavior, with proliferation only close to sea cultures or harbors, may be the underlying cause of its late detection in coastal Mediterranean habitats despite the species had been abundantly recorded in the South of Naples ca. 30 years ago (Longo et al., 2007). A phylogeographic approach (in progress) and ecological studies of *P. magna* interactions with the native species would complement the picture resulting from our genetic study.

Paraleucilla magna is a good target for experimental studies on population genetics, at very small spatial and temporal scales. The knowledge gained from these studies may be extended to other annual introduced invertebrates with limited dispersal, and patchy distributions, which are increasing in number in Mediterranean benthic ecosystems.

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References

Addison, J. A. & M. W. Hart, 2005. Spawning, copulation and inbreeding coefficients in marine invertebrates. Biology Letters 1: 450–453.

Agell, G., J. Frotscher, M. Guardiola, M. Pascual & M. J. Uriz, in press. Characterization of nine microsatellite loci for the calcarean sponge *Paraleucilla magna* Klautau et al. 2004 introduced to the Mediterranean Sea. Conservation Genetics. doi:10.1007/s12686-011-9560-Y.

Astanei, I., E. Gosling, J. Wilson & E. Powell, 2005. Genetic variability and phylogeography of the invasive zebra mussel, *Dreissena polymorpha* (Pallas). Molecular Ecology 14: 1655–1666.

Briggs, J. C., 2007. Marine biogeography and ecology: invasions and introductions. J. Biogeo. 34: 193–198.

Blanquer, A. & M. J. Uriz, 2010. Population genetics at three spatial scales of rare sponge living in fragmented habitats. BMC Evolutionary Biology 10: 13.

Blanquer, A., M. J. Uriz & J. Caujapé-Castells, 2009. Small-scale spatial genetic structure in *Scopalina lophyropoda*, an encrusting sponge with philopatric larval dispersal and frequent fission and fussion events. Marine Ecology Progress Series 380: 95–102.

Calderón, I., N. Ortega, S. Duran, M. Becerro, M. Pascual & X. Turon, 2007. Finding the relevant scale: clonality and genetic structure in a marine invertebrate (Crambe crambe, Porifera). Molecular Ecology 16: 1799–1810.

Carlon, D. B., 1999. The evolution of mating systems in tropical reef corals. Trends in Ecology & Evolution 14: 491–495.

Coles, S. L., R. C. DeFelice, & L. G. Eldredge, 2002. Non indigenous marine species in Kane'Ohe Bay, O'Ahu, Hawaii. Bishop Museum Hawaii. Biological Survey Technical Report, Honolulu, 24: 353 pp.

Crawford, N. G., 2010. SMOGD: software for the measurement of genetic diversity. Molecular Ecology Resources 10: 556–557.

DeFelice, R. C., L. G. Eldredge & J. T. Carlton, 2001. A guidebook of introduced marine species in Hawaii. Non indigenous marine invertebrates. In Eldredge, L. G. & C. M. Smith (eds), Bishop Museum Technical Report, Honolulu, 21: 70 pp.

Dailianis, T. & C. S. Tsigenopoulos, 2010. Characterization of polymorphic microsatellite markersfor the endangered Mediterranean bath sponge *Spongia officinalis* L. Conservation Genetics 11: 1155–1158.

Dailianis T., C. S. Tsigenopoulos, C. Dounas & E. Voultsiadou, 2011. Genetic diversity of the imperiled bath sponge *Spongia officinalis* Linnaeus, 1759 across the Mediterranean sea: patterns of population differentiation and implications for taxonomy and conservation. Molecular Ecology (in press).

Duran, S. M. Pascual, A. Estoup, & X. Turon, 2004a. Strong population structure in the marine sponge Crambe crambe (Poecilosclerida) as revealed by microsatellite markers. Molecular Ecology, 13: 511–522.

Duran, S., M. Pascual & X. Turon, 2004b. Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge Crambe crambe (Poecilosclerida). Marine Biology 144: 31–35.

Evanno, G. S. Regnaut & J. Goudet, 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology 14(8): 2611–2620.

Ereskovsky, A. V., 2010. Development of Sponges from the Class Calcarea Bowerbank, 1864 In The Comparative Embryology of Sponges. Springer, Dordrecht, Chapt. 1: 3–36.

Excoffier, L., G. Laval & S. Schneider, 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1: 47–50.

Falush, D., M. Stephens & J. K. Pritchard, 2003. Inference of population structure: extensions to linked loci and correlated allele frequencies. Genetics 164: 1567–1587.

Falush, D., M. Stephens & J. K. Pritchard, 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes 7: 574–578.

Frotscher, P. J. & M. J., Uriz, 2008. Reproduction and life cycle of the calcarean sponge Paraleucilla magna in the Mediterranean Sea. XV Simposio Ibérico de Estudios de Bentos Marino, Blanes, Book of Abstracts.

Galil, B. S., 2006. The Suez Canal. The marine caravan—the Suez Canal and the erythrean invasion. Monographiae Biologicae 83: 207–300.

Gerlach, G., A. Jueterbock, P. Kraemer, J. Deppermann & P. Harmand, 2010. Calculations of population differentiation based on GST and *D*: forget GST but not all of statistics! Molecular Ecology 19: 3845–3852.

Goudet, J., M. Raymond, T. de-Meeus & F. Rousset, 1996. Testing differentiation in diploid populations. Genetics 144(4): 1933–1940.

Grosberg, R. K., 1987. Limited dispersal and proximity-dependent mating success in the colonial ascidian *Botryllus schlosseri*. Evolution 41: 372–384.

Hubisz, M., D. Falush, M. Stephens & J. Pritchard, 2009. Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources 9: 1322–1332.

Jost, L., 2008. GST and its relatives do not measure differentiation. Molecular Ecology 17(18): 40.

Kaiser, J. & R. Gallagher, 1997. Does diversity lure invaders? Science 277: 1204–1205.

Klautau, M., L. C. Monteiro & R. Borojevic, 2004. First occurrence of the genus Paraleucilla (Calcarea, Porifera) in the Atlantic Ocean: *P. magna* sp. nv. Zootaxa 710:1–8.

Longo, C., F. Mastrototaro & G. Corriero, 2007. Occurrence of *Paraleucilla magna* (Porifera:Calcarea) in the Mediterranean Sea. Journal of the Marine Biological Association of the United Kingdom 87: 1749–1755.

Manly, B. F. J., 1997. Randomization, bootstrap and Monte Carlo methods in biology. Chapman& Hall Computational Statistics 24(2): 371–372.

Meirmans, P. & P. W. Hedrick, 2011. Assessing population structure: F_{ST} and related measures. Molecular Ecology Resources 11(1): 5–18.

Pascual, M., J. Balanyà, A. Latorre & L. Serra, 1997. Analysis of the variability of *Drosophila azteca* and *Drosophila athabasca* populations revealed by random amplified polymorphic DNA. Journal of Zoological Systematics and Evolutionary Research 35: 159–164.

Perez, T., B. Perrin, S. Carteron, J. Vacelet & N. Boury-Esnault, 2006. Celtodoryx girardae gen nov.sp.nov., a new sponge species (Poecilosclerida: Demospongiae) invading the Gulf of Morbihan (North East Atlantic, France). Cahiers de Biologie Marine 47: 205–214.

Pritchard, J. K., M. Stephens & P. Donnelly, 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Raymond, M. & F. Rousset, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248–249.

Rousset, F., 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular Ecology Resources 8: 103–106.

Stenberg, P. & M. Lundmark, 2002. MLGsim: a program for detecting clones using a simulation approach. Umea University, Umea.

Streftaris, N. & A. Zenetos, 2006. Alien marine species in the Mediterranean—the 100 'worst invasives' and their impact. Mediterranean Marine Science 7: 87–118.

Thomsen, M. S., T. Wernberg, J. D. Olden, J. N. Griffin & B. R. Silliman, 2011. A framework to study the context-dependent impacts of marine invasions. Journal of Experimental Marine Biology and Ecology 400: 322–327.

Uriz, M. J., 1982. Morfología y comportamiento, de la larva parenquímula de Scopalina lophyropoda Schmidt 1862 (demospongia, Halichondria) y la formación del rhagon. Investigación Pesquera 42:213–322.

Uriz, M. J., M. Maldonado, X. Turon & R. Martí, 1998. How reproductive output, larval behaviour, and recruitment contribute to adult spatial patterns in Mediterranean encrusting sponges? Marine Ecology Progress Series 167: 137–148.

Uriz, M. J., X. Turon & S. Mariani, 2008. Ultrastructure and dispersal potential of sponge larvae: tufted versus evenly ciliated parenchymellae. Marine Ecology 29(2): 280–297.

Van Oosterhout, C., W. F. Hutchinson, D. P. Wills & P. Shipley, 2004. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4(3): 535–538.

Xavier J., 2010. Biodiversity and phylogeography of Northeast Atlantic and Mediterranean sponges. PhD dissertation. University of Amsterdam, Amsterdam: 149 pp.

Weir, B. S. & C. C. Cockerman, 1984. Estimating F-statistics for the analysis of population structure. Evolution 38: 1358–1370.

Zammit, P. P., C. Longo & P. J. Schembri, 2009. Occurrence of *Paraleucilla magna* Klautau et al., 2004 (porífera: Calcarea) in Malta. Mediterranean Marine Science 10(2): 135–138.

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SPONGE RESEARCH DEVELOPMENTS

Relationship between genetic, chemical, and bacterial diversity in the Atlanto-Mediterranean bath sponge *Spongia lamella*

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Abstract Does diversity beget diversity? Diversity includes a diversity of concepts because it is linked to variability in and of life and can be applied to multiple levels. The connections between multiple levels of diversity are poorly understood. Here, we investigated the relationships between genetic, bacterial, and chemical diversity of the endangered Atlanto-Mediterranean sponge *Spongia lamella*. These levels of diversity are intrinsically related to sponge evolution and could have strong conservation implications. We used microsatellite markers, denaturing gel gradient electrophoresis and quantitative polymerase chain reaction, and high performance liquid chromatography to quantify genetic, bacterial, and chemical diversity of nine sponge populations. We then used correlations to test whether these diversity levels covaried. We found that sponge populations differed significantly in genetic, bacterial, and chemical diversity. We also found a strong geographic pattern of increasing genetic, bacterial, and chemical dissimilarity with increasing geographic distance between populations. However, we failed to detect significant correlations between the three levels of diversity investigated in our study. Our results suggest that diversity fails to beget diversity within a single species and indicates that a diversity of factors regulates a diversity of diversities, which highlights the complex nature of the mechanisms behind diversity.

Keywords Biodiversity · Correlations · Heterozygosity–fitness correlations · Bacterial diversity · Chemical diversity · Porifera · *Spongia agaricina* · *Spongia lamella*

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Introduction

Tremendous efforts are underway to assess the world's biodiversity. The definition of "biodiversity" (or biological diversity) is a challenge in itself partly because of the complex, dynamic, and multifaceted concept of diversity (Gaston & Spicer, 2004). Biodiversity can thus go beyond the widespread species diversity notion to include the habitats, communities, and ecosystems where species live (so called ecosystem diversity), the genes and molecules found within a single species (genetic diversity), and the relationships between them (Feral, 2002). Whether this diversity of diversities is correlated and diversity begets diversity

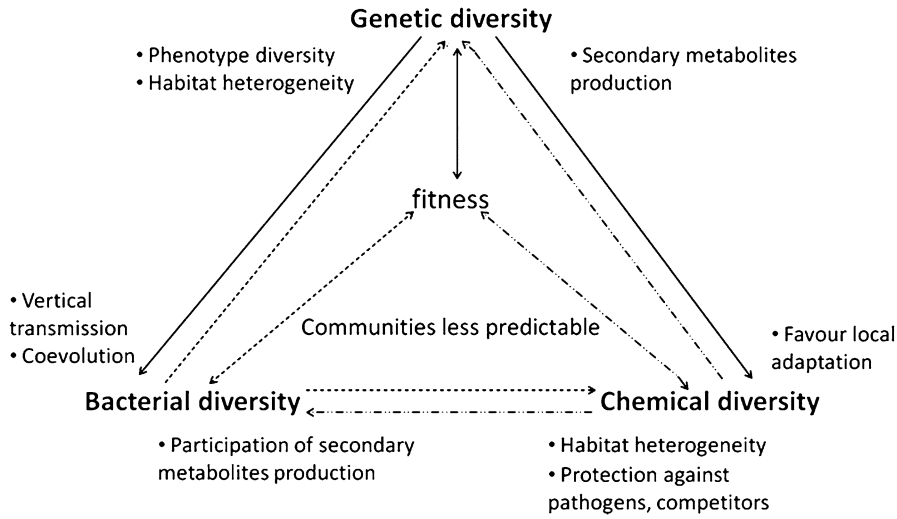

Fig. 1 Potential connections between genetic, bacterial, and chemical diversity

is an important challenge that could shed light on the mechanisms that generate and maintain biodiversity (Whittaker, 1972). Exploring how species diversity affects ecosystem functions has received certain attention from the scientific community (Cardinale et al., 2002; Lamosova et al., 2010). Other studies have aimed at the relationship between species and community diversity, or between species and genetic diversity (Vellend & Geber, 2005; Odat et al., 2010). However, linking diversities is a complex topic that remains controversial and observation-scale dependent (Yue et al., 2005).

According to Whittaker (1972), two main hypotheses explain how diversity in communities could influence diversity in other levels (Whittaker, 1972; Palmer & Maurer, 1997). The first is "diversity begets diversity" and three mechanisms support this suggestion (Palmer & Maurer, 1997). The first mechanism refers to the varying capacity of species to influence the environment, which generates a small-scale heterogeneity that could in turn create opportunities for new species to settle down (Palmer & Maurer, 1997). A second mechanism suggests that diverse communities are less predictable, making competitive exclusion less likely (Palmer & Maurer, 1997). Finally, "diversity breeding diversity" occurs for dependent communities (symbionts, commensals, pathogens…) that are likely to be diverse if their host communities also are diverse (Palmer & Maurer, 1997).

Counteracting the "diversity begets diversity" hypothesis, the niche saturation or "niche limitation" hypothesis assumes biological limits to species number in a community. As niches are limited, the likelihood of successful establishment for a species decreases as the number of species increases (Palmer & Maurer, 1997). Understanding the processes that drive diversity appears to be a challenging and problematic topic with many implications.

In our study, we aimed to unravel whether diversity begets diversity at a species level. We used genetic, bacterial, and chemical diversities that are intrinsically related to sponge evolution to investigate whether or not the data supports the diversity begets diversity hypothesis (Fig. 1). Sponges offer unique opportunities to further understand the multiple levels of diversity and their relationships, yet they have contributed minimally to this field (Becerro, 2008). Sponges are important components of marine sessile communities in a wide range of aquatic ecosystems (Diaz & Rutzler, 2001; Bell, 2008). Sponges host a large diversity of symbiotic microorganisms such as algae, archaea, and bacteria (Lee et al., 2001; Hentschel et al., 2006; Taylor et al., 2007). Sponges are also a major source of natural products, which can present biotechnological interests (Sipkema et al., 2005). Beyond losing species diversity, the loss of sponge species could result in the loss of bacterial and chemical diversities, whose biotechnological applications we are steadily increasing (Arrieta et al., 2010). Thus, these levels of diversity appeared intrinsically related to sponge evolution and could have strong implications for conservation. For this reason, we

tested whether levels of genetic, bacterial, or chemical diversity were associated with or remained independent of each other.

To investigate the relationship between genetic, bacterial, and chemical diversity we sampled nine populations of the endangered Mediterranean bath sponge *Spongia lamella*. *Spongia lamella* may be better known as the Mediterranean bath sponge *Spongia agaricina*. However, *S. agaricina* first description was performed on a now missing specimen from the Indian Sea (Pronzato & Manconi, 2008). Sharing a similar morphology and shape with the Mediterranean sponge (a massive gray color with inner white tissue sponge with a vase or dish-like shape), the name *S. agaricina* was generalized and used for the Mediterranean specimens. Recent evidence showed significant differences between the Philippine and Mediterranean specimens (Castritsi-Catharios et al., 2007; Pronzato & Manconi, 2008), which should be referred to as *S. agaricina* Pallas 1766 and *S. lamella* Schultze 1879, respectively (Pronzato & Manconi, 2008). *Spongia lamella* has a Mediterranean distribution, although occasionally reported in the Atlantic coastal waters of the Iberian Peninsula (Lopes & Boury-Esnault, 1981, J. Xavier, pers. comm.). Natural populations of commercial sponges including *S. lamella* decreased drastically in the Mediterranean as a consequence of overfishing, habitat degradation, and spread of diseases (Gaino & Pronzato, 1989, 1992; Pronzato, 1999) and are now registered in the Annex III of the Berne and Barcelona Conventions (Templado et al., 2004). Genetic studies have shown strong genetic differentiation between populations of this species probably linked to the life cycle (brooding sponge with low dispersal capabilities, slow growing animal) and overexploitation (Noyer et al., 2009; Noyer, 2010). The tissues of *S. lamella* contain abundant bacteria (Bertrand & Vacelet, 1971) dominated by *Chloroflexi* and *Acidobacteria* communities (Noyer, 2010, Noyer et al., 2010) and a great array of structurally diverse secondary metabolites with terpenes as mayor compounds (Cimino et al., 1975; Aiello et al., 1988; Fontana et al., 1996; Rueda et al., 1998; Noyer et al., 2011). Understanding the genetic, chemical, and bacterial diversity of this species can therefore be critical for the conservation and survival of this species. We investigated three of the four components of diversity: (i) the number of entities (richness), (ii) their abundances (evenness), and (iii) their dissimilarities (Magurran, 2004, Sala & Knowlton, 2006). The fourth component of diversity refers to the functional roles of the entities (Magurran, 2004; Sala & Knowlton, 2006) and was left out of this preliminary study because it requires experimental manipulations that will benefit from our observational approach. To quantify the three components of diversity, we used multi-locus heterozygosity (MLH), mean d^2, expected heterozygosity, and gene diversity for genetic data and the Shannon index on bacterial and chemical data, which were used to explore the potential connections between genetic, bacterial, and chemical diversity.

Materials and methods

Sample collection

Sampling was conducting by scuba diving at nine Atlanto-Mediterranean locations representing five regions: South France, Catalonia, Baleares, Gibraltar, and Portugal (Fig. 2). We collected sponges at three locations around Marseille in December 2006 at Plane (Pla), Pharillon (Far), and La Ciotat (Cio). In Catalonia, we sampled *S. lamella* at Cap de Creus (Cre) in summer 2006, Els Bullents (Bul) in summer 2008, and Arenys (Are) in winter 2006. Populations of Cabrera (Cab) in the Baleares Island and Ceuta (Ceu) in Gibraltar were sampled in winter 2005. Population of Portugal was sampled in the Berlengas archipelago in summer 2005 and 2007. For each specimen, a fraction on one edge of the sponge was cut underwater to minimize damage, placed into plastic bag containing sea water, and stored in cool box until further processing (usually 1–2 h after sampling). Sponge tissues were then manually cleaned of foreign tissues, soaked in a series of absolute ethanol baths to prevent ethanol dilution and degradation/contamination of sponge tissues, and conserved in absolute ethanol at −20°C until processed. Samples for population genetic analysis were extracted as in Noyer et al. (2009). For the quantitative analysis of bacterial communities we used DNAeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions, except for a 5 min incubation time before elution in a total volume of 75 ll supplied buffer (Noyer et al., 2010). In parallel, some of the specimens from Marseille (3 populations), Catalonia (3 populations) were also collected for

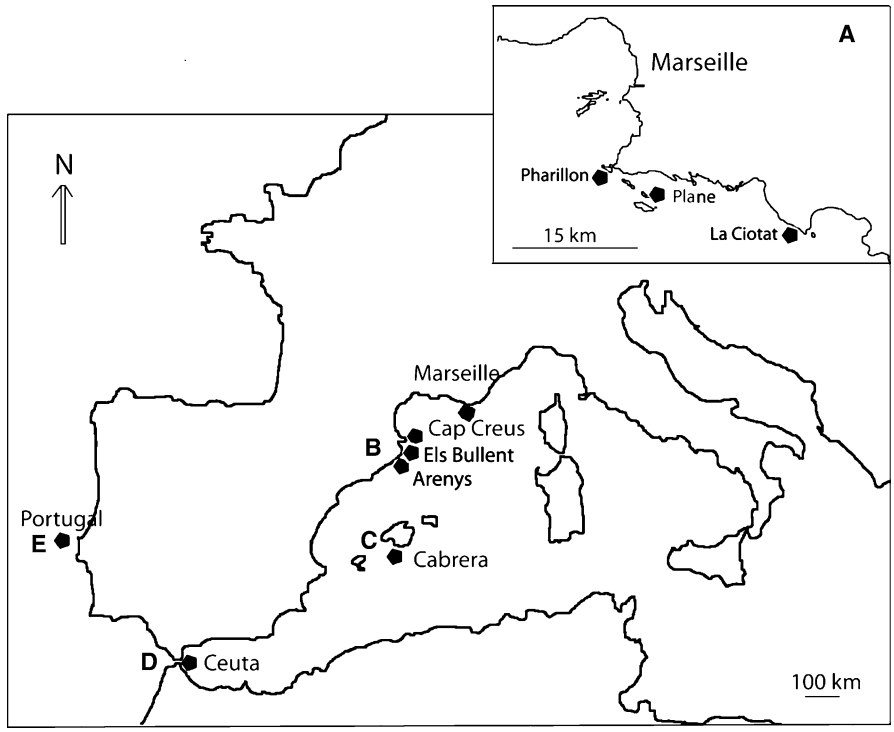

Fig. 2 Sampling sites for *Spongia lamella* in the western Mediterranean and Atlantic Iberian coast

chemical analyses, and were placed in a freezer at −20°C. An additional sample was also obtained from Ceuta. All of those specimens were then freeze-dried and stored in a −20°C freezer until chemical extraction (Noyer, 2010; Noyer et al., 2011).

Data analysis

We used three approaches to test multiple correlations: at individual level, at population level, and at inter-population level (i.e., population dissimilarities). Individuals were typed at seven microsatellite loci, genotyping protocols are described in Noyer et al. (2009, 2010). For each individual, we measured (1) MLH calculated as the proportion of loci that were heterozygous corrected for non-scored loci (Lesbarreres et al., 2007; Chapman et al., 2009; Pujolar et al., 2009) and (2) mean d^2 measured by $LD = \log$ (mean $d^2 + 1$) according to Da Silva et al. (2009). Mean d^2 provides a measure of the genetic distance between parental gamete genomes. Individuals with alleles at a given locus that differ the most in the number of repeat units are presumed to have higher levels of outbreeding and the parental alleles have more ancestral coalescence times (Da Silva et al., 2009; Hansson, 2010). At the population level, we used (1) expected heterozygosities (H_e) as a value for genetic diversity and (2) gene diversity (Noyer, 2010). At the interpopulation level, we used F_{ST} between pairwise populations as an index of genetic dissimilarities between sponge populations (Noyer, 2010).

Shannon index was used to assess bacterial and chemical diversity since (i) it is one of the most frequently used and (ii) it encompasses both richness (number) and evenness (equitability). This index is a measure of information entropy, it measures uncertainty (Sarkar, 2006), and is maximal when components are evenly distributed. Bacterial diversity was estimated by denaturing gel gradient electrophoresis (DGGE) and quantitative PCR (qPCR) analyses. We used the universal bacterial primers BAC358F (5′-CCT ACG GGA GGC AGC AG-3′) and BAC907RM (5′-CCG TCA ATT CMT TTG AGT TT-3′) to amplify fragments approximately 560 bp. We used a 40–75% vertical denaturant gradient (100% denaturant agent is 7 M urea and 40% deionized formamide) on 6% polyacrylamide gel in 1× TAE to separate bacterial bands, i.e., sequences (Muyzer & Smalla, 1998). We analyzed images of the gels using the Gels plot lanes tool of ImageJ software 1.38X (Wayne Rasband, National Institutes of Health, USA) according to Noyer (2010) and references therein. To perform

quantitative analyses using qPCR, we used five specific primer pairs designed in Noyer et al. (2010), to amplify and quantify specifically bacterial clades using a Stratagene Mx3005P QPCR system and 2× Brilliant SYBR® Green QPCR Master Mix (Stratagene) (Noyer, 2010). For each individual screened, we used Shannon diversity index from the DIVERSE procedure available in PRIMER 6 (Clarke & Warwick, 2001). Bacterial diversity was assessed by bacterial richness as the number of bands on the DGGE gels in an individual sample, and evenness through the relative intensity (quantity) of the bands on the gels (Casamayor et al., 2000). Individual Shannon index was also used to estimate diversity of the bacterial clades quantified by qPCR (*Chloroflexi* clade 1 and clade 2; *Acidobacteria* clade 1 and clade 2, and *Actinobacteria*). For each population, we averaged the individual indices within their original populations and we obtained the average index of bacterial diversity per sponge population. For the interpopulation comparisons, we used pairwise dissimilarities obtained from the SIMPER procedure available in PRIMER v6, both for the DGGE banding patterns and for the quantitative proportions of bacterial clades amplified.

Chemical diversity was estimated at the same individual, population and interpopulation levels. Secondary metabolites were extracted by dichloromethane/methanol (1:1) solution and extracts were analyzed by high performance liquid chromatography (HPLC) to characterize chemical profiles (Noyer, 2010; Noyer et al., 2011). In agreement with previous studies on *S. agaricina* secondary metabolites, chemical profiles showed two major compounds, the nitenin being the major compound, and a variety of minor metabolites belonging to the product class "terpenes" (Aiello et al., 1988; Rueda et al., 1998). For each individual, we used Shannon indices obtained from the DIVERSE procedure (available in PRIMER v6) from the chemical SPE–ELSD–HPLC profiles (Noyer et al., 2011). Thus, chemical diversity was assessed by chemical richness as the number of compounds in an individual sample, and chemical evenness through the abundance or quantity of this compound in the sample. For each population, we averaged individual Shannon indices within their original populations. For the interpopulation comparisons, we used pairwise dissimilarities from the SIMPER procedure (available in PRIMER v6). Pairwise dissimilarities were performed on 10 out of the 22 compounds that presented significant variations between sponge populations (Noyer, 2010; Noyer et al., 2011).

Multiples correlations were globally performed first between individual measures. However, only subsamples of each sponge populations used for the genetic analyses were used for bacterial and chemical analyses. Thus, only individuals that have triple genetic, bacterial, and chemical data were used in the individual-level analysis. As 10 individuals per populations were used for the qPCR analysis, we also investigated more closely the relationship between genetic and bacterial diversity at the individual-level per population. At the population level, multiple correlations were carried between the different measures of diversity averaged per population. At the interpopulation level, we visualized population dissimilarities with hitmaps using JColorGrid software (Joachimiak et al., 2006). We performed correlations between three matrixes to investigate whether there are relationships between genetic, bacterial, and chemical dissimilarities. The analyses of correlations were run separately for bacterial diversity using DGGE data or qPCR data to test one measure of genetic, bacterial, and chemical dissimilarities.

Results

Individual-level correlations

Multiple correlations of individuals that were typed for genetic, bacterial, and chemical analyses are summarized on Fig. 3. We observed negative correlations for both genetic measures, MLH and mean d^2 with chemical diversity, however, those correlations were not significant ($P = 0.325$ for MLH, and $P = 0.175$ for mean d^2 after Bonferroni corrections). We did not detect any correlations between chemical diversity and bacterial diversity using both DGGE and qPCR Shannon indices, or between genetic diversity (MLH and mean d^2) and bacterial diversity (DGGE and qPCR). The only significant correlation was obtained between the two measures of genetic diversity ($P = 0.015$, after Bonferroni corrections, Fig. 3).

As more individuals were scored for qPCR, we also investigated more deeply relationships between bacterial and genetic diversity at the individual-level per population (only individuals with paired genetic and

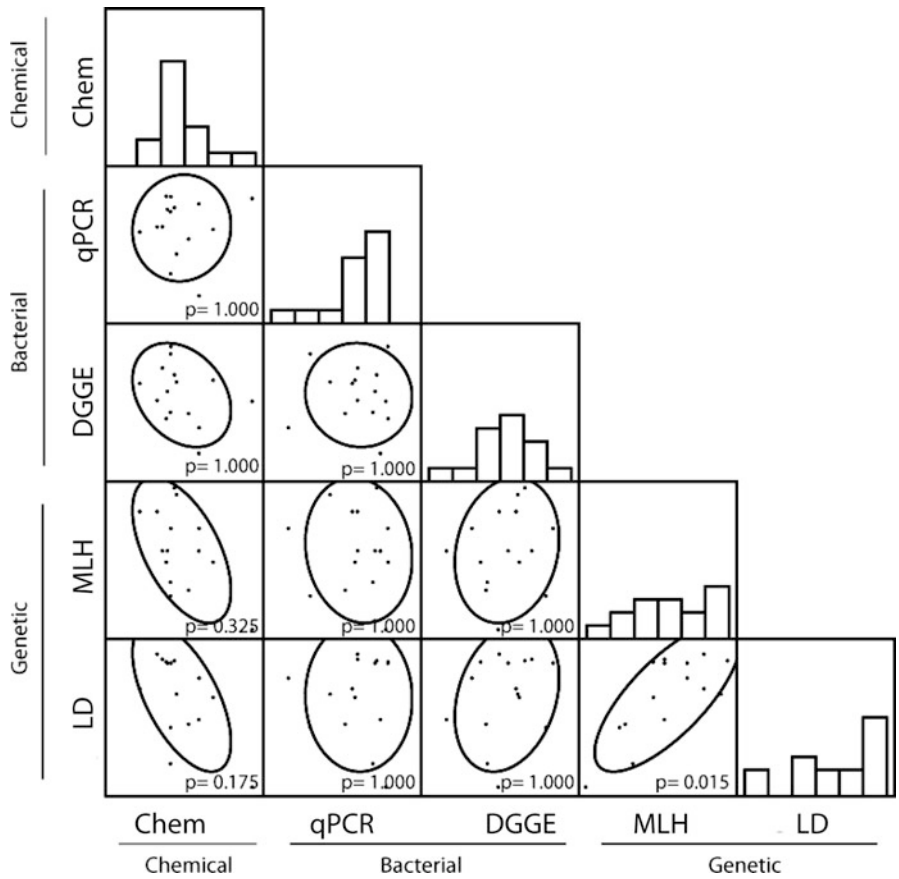

Fig. 3 Multiple individual-level correlations between chemical, bacterial, and genetic diversity. Chemical diversity was estimated by the Shannon index on the SPE–ELSD–HPLC profiles, qPCR diversity was estimated by Shannon index on proportions of bacterial clades amplified, DGGE diversity was estimated by Shannon index on the relative intensity of DGGE banding patterns, and genetic diversity was estimated on MLH (multi-locus heterozygosity) and *LD* [log (mean $d^2 + 1$)]. *P* values are Bonferroni corrected

qPCR data were included, Fig. 4). We did not find significant relationships between bacterial and genetic (both using MLH and mean d^2) diversities in any population. Thus, for genetic and qPCR bacterial diversities, we obtained the same consistent results when individual analyses were performed both globally (Fig. 3) or by populations (Fig. 4).

Population-level correlations

At the population level, when all indices were averaged by sponge population, we obtained similar results to those at the individual level (Figs. 3, 5). We did not detect significant correlation between chemical and bacterial (DGGE and qPCR), chemical and genetic (H_e and gene diversity), or bacterial (DGGE and qPCR) and genetic diversity (H_e and gene diversity). As at the individual level, we found significant correlations between He and gene diversity ($P = 0.012$, after Bonferroni corrections). However, we also detected a negative and significant correlation between the two methods used to assess bacterial diversity, mean qPCR and mean DGGE Shannon indices ($P = 0.018$, after Bonferroni corrections).

Population dissimilarities

Genetic, bacterial, and chemical dissimilarities showed the same pattern of higher dissimilarities for more distant sponge populations (Noyer, 2010; Noyer et al., 2011). Pairwise populations including Ceuta and Portugal presented the highest dissimilarities (Fig. 6). When we looked at correlations between population pairwise dissimilarities for the three levels, we obtained a positive and significant correlation between sponge genetic and chemical dissimilarities (Table 1). However, increasing genetic differences between sponge populations were not linked to increasing bacterial dissimilarities ($P = 1.000$ for both DGGE and qPCR data after Bonferroni corrections, Table 1). Increasing chemical dissimilarities was not linked to an increase of DGGE differences ($P = 1.000$). However, we observed a negative correlation between chemical dissimilarities and qPCR dissimilarities

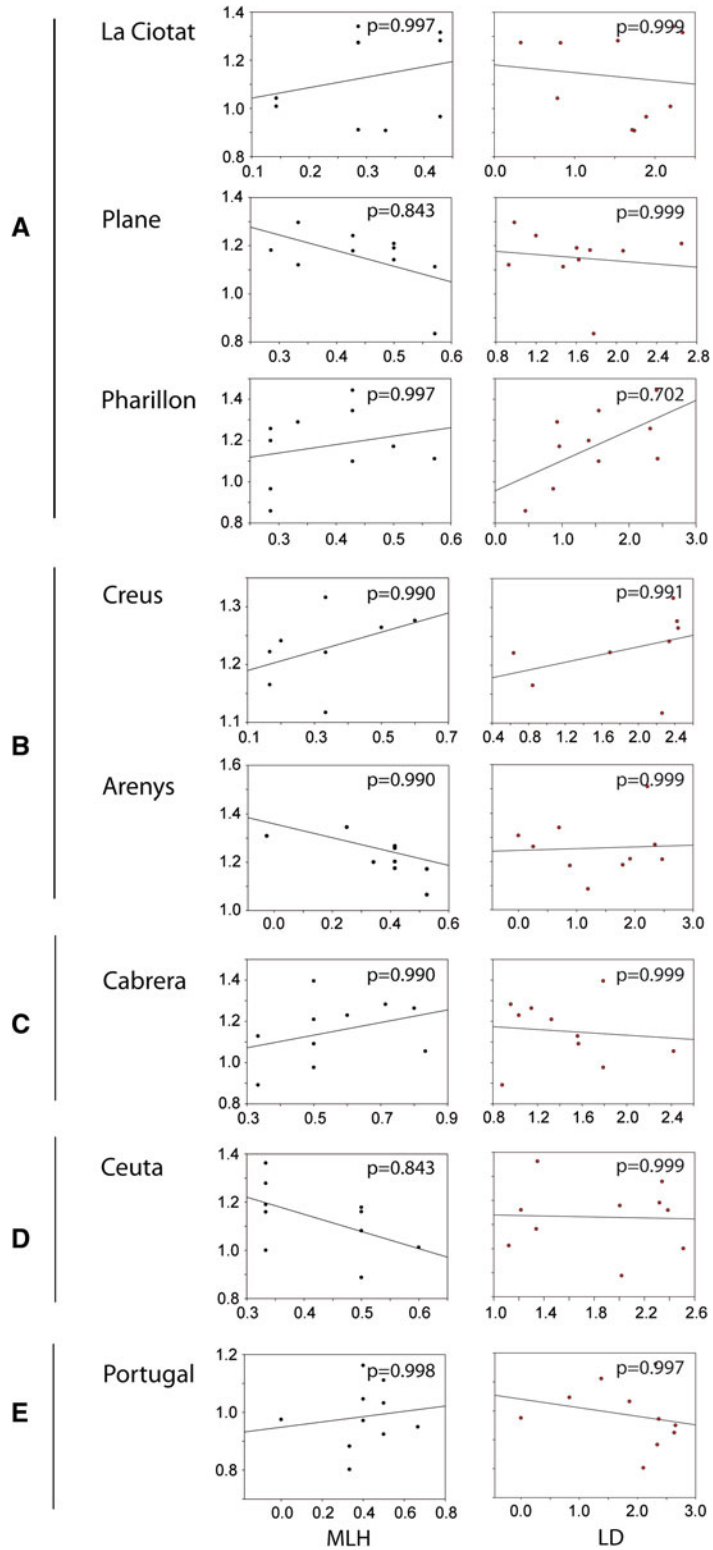

Fig. 4 Relationships between genetic diversity: MLH (*left*), *LD* (*right*), and diversity of bacterial clades amplified by qPCR, per sponge population. *P* values are Bonferroni corrected

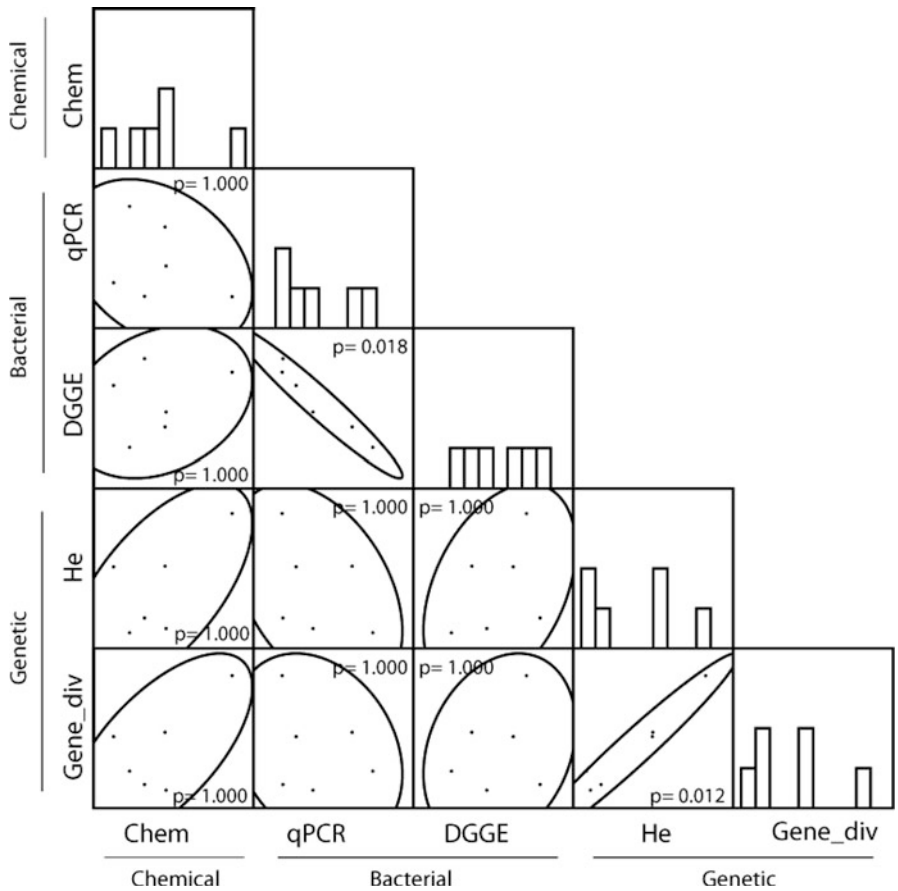

Fig. 5 Multiple population-level correlations between chemical, bacterial, and genetic diversity. Chemical and bacterial diversity were estimated as for Fig. 3, averaged per sponge population (except for Portugal and Cabrera populations, which did not contained chemical data and for Els Bullents, which did not contain bacterial data). Genetic diversity was estimated by expected heterozygosity under Hardy–Weinberg equilibrium and gene diversity among individuals. *P* values are Bonferroni corrected

($R = -0.587$, uncorrected $P = 0.021$). Increasing differences in bacterial clades amplified by qPCR were linked to a decrease of chemical dissimilarities between sponge populations, although this relationship turned not significant after Bonferroni corrections ($P = 0.064$).

Discussion

Does diversity beget diversity in *Spongia lamella*?

We used genetic, bacterial, and chemical diversities to investigate whether diversity begets diversity within the endangered sponge *S. lamella* (Fig. 1). We failed to detect multiple correlations between the three levels of diversity using two distinct methods to assess bacterial diversity and two measures of genetic diversity. Results were consistent both at the individual and population levels. The only significant and positive correlations were between the two measures of genetic diversity at individual level: heterozygosity (MLH) which is a measure of inbreeding, and mean d^2; and at population level: expected heterozygosity (H_e) and gene diversity. Paired measures of genetic diversity were highly correlated, which is in agreement with Chapman et al. (2009). At the population level, we also obtained a negative and significant correlation between the two methods of bacterial diversity. As Shannon index considers both richness and evenness, and as the same number of clades (richness) was amplified by qPCR for all sponge individuals, diversity of bacterial clades relied only on the evenness. However, each DGGE band refers theoretically to a bacterial sequence (Muyzer & Smalla, 1998). Thus, a bacterial clade could be represented by various sequences on the DGGE gels. Many sequences of the same clades at different positions on the DGGE gels increase number of DGGE bands and DGGE bacterial diversity. However, increasing proportions of a single clade would decrease bacterial clade evenness, and also decrease qPCR diversity. The two

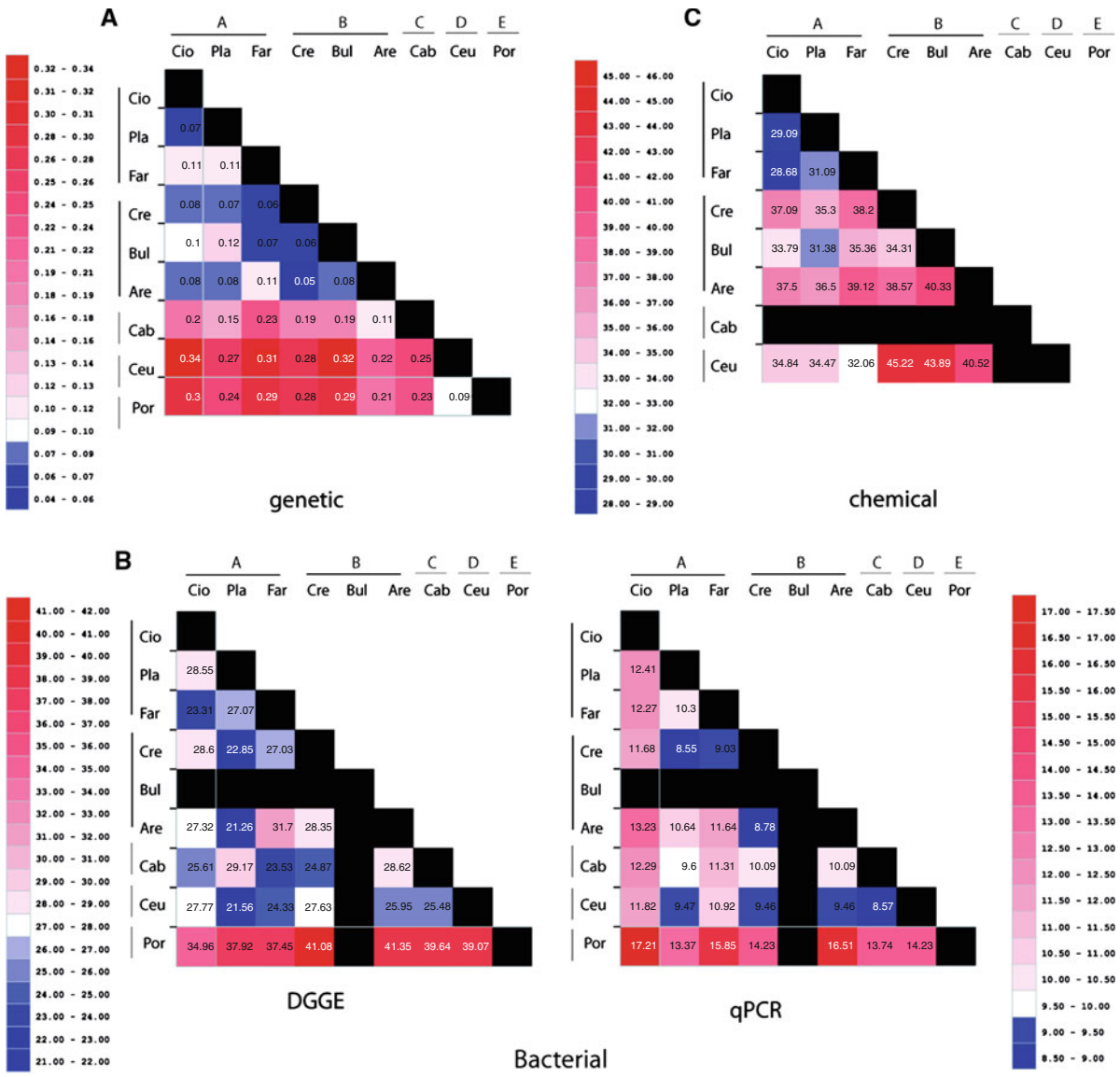

Fig. 6 Hit map of sponge population dissimilarities for **A** genetic differences; **B** bacterial (DGGE and qPCR) dissimilarities; and **C** chemical dissimilarities

measures of bacterial diversity are complementary rather than redundant as they focus on different phylogenetic levels, sequences, and clades.

In previous studies, we observed positive and significant relationships between geographical and genetic, bacterial, and chemical dissimilarities (Noyer, 2010). Among the multiple correlations between population dissimilarities we tested for those three levels, only chemical and genetic dissimilarities were significantly correlated. Increasing geographical distances between pairwise populations increased genetic differences by isolation by distance and, increasing genetic differences enhanced chemical dissimilarities. Though genetic and chemical diversity were not correlated per se, a certain connection seemed to exist. Production of secondary metabolites may have a genetic basis. Thus, increasing population genetic divergence through isolation and/or local selection might increase chemical dissimilarities between populations that are likely to be locally

Table 1 Correlations between genetic, bacterial, and chemical dissimilarities

	Genetic	Bacteria		Chemistry
	$F_{ST}/(1 - F_{ST})$	DGGE	qPCR	Chem.
Genetic				
$F_{ST}/(1 - F_{ST})$	–	−0.154	−0.059	**0.634**
Bacteria				
DGGE	$P = 1.000$	–		−0.013
qPCR	$P = 1.000$		–	−0.587
Chemistry				
Chem.	$P = 0.033$	$P = 1.000$	$P = 0.064$	–

Bonferroni corrected P values are given below the diagonal; Pearson correlation coefficients above the diagonal

adapted. Alternatively under specific ecological factors, some secondary metabolites only could present an advantage and increase sponge fitness. Then, differential production of secondary metabolites could also indirectly promote and strengthen genetic dissimilarities between populations through the selection of these genotypes. Finally, increasing genetic differences did not have any influence on bacterial dissimilarities as calculated either with DGGE and qPCR data. DGGE pairwise dissimilarities did not have any influence on chemical dissimilarities. Results suggested that all these factors may vary geographically, and highlight the complex nature of the mechanisms behind the variability in life.

The lack of significant correlations in this study could also reflect limitations in the ability of our data or analyses to detect them. Measures of genetic diversity, and its relevance to explain correlations (especially with fitness), remains a controversial and complex topic (Coltman & Slate, 2003; Balloux et al., 2004; Chapman et al., 2009; Da Silva et al., 2009). In this study, we used MLH and mean d^2 at the individual level. Various measures are generally used in heterozygosities–fitness correlations (HFCs). MLH has been the index most frequently used and has generally displayed strong size effect (Chapman et al., 2009; Pujolar et al., 2009).

Microsatellite variability is widely used to infer levels of genetic diversity in natural populations. However, criticisms have challenged the use of these markers to explain genome-wide variability (Vali et al., 2008). Nonetheless, microsatellites provide significantly stronger predictions of genome-wide-heterozygosity than do SNPs (Ljungqvist et al., 2010). In sponges, adequate genetic markers are limited and sequencing could be problematic. The mitochondrial COI gene, widely and extensively used, appears poorly polymorphic in Porifera (Duran et al., 2004a). Alternatively, microsatellites are highly polymorphic and have proven their usefulness in sponge studies (Duran et al., 2004b; Noyer et al., 2009; Blanquer and Uriz, 2010). Thus, microsatellite markers still remain the marker of choice for evaluating genetic diversity and assessing HFCs.

Alternative methods could have been applied to compare bacterial community and study bacterial diversity. Construction of bacterial clone libraries is a method widely used and gives indications of the identities/phylogeny and relative abundances of bacteria present in the sponge populations (Taylor et al., 2007 and reference herein). However, it is generally performed on few specimens of various species. First, it would appear time and money consuming when comparing nine populations from the same sponge species; second, clone libraries should be done at the population level which hinders analyses at the individual level. For these reasons, we choose the DGGE banding patterns to have a rapid and general insight of the bacterial community at the individual level and qPCR to compare quantitatively the main bacterial clades present.

One limitation of the Shannon index is the necessity to be compared across equivalent sampling designs (Clarke & Warwick, 2001). Here, we applied the same experimental design and method on each sample to sample the same "chemical" and "bacterial" diversity within each sponge specimen. Measure of bacterial diversity using qPCR was different. As we used the same set of primers for each sponge specimen, diversity values reflect only evenness (richness being equal to all samples, i.e., five bacterial clades). However, we chose to keep Shannon index to compare the same index between the different levels of chemical and bacterial diversity. Other diversity indices could be used too, but Simpson or Pielou's evenness indices lead to the same conclusions (data not shown).

Finally, to obtain a full picture of *S. lamella* system and get an insight on the functional role that sponge populations play in the associated organisms, it could

have been interesting to assess sponge habitat forming (diversity of sponge dwellers). Sponges also host a variety of other benthic invertebrates, such as scyphozoan, barnacles, decapodes, polychaetes, or amphipods among others (Rützler, 1976; Voultsiadou-Koukoura et al., 1987; Uriz et al., 1992; Ilan et al., 1999; author's unpublished data) and behave as efficient ecological niches.

Putative connections between genetic diversity, bacterial diversity, and chemical diversity

Our data failed to support the hypotheses that could explain potential links between genetic and bacterial diversity in *S. lamella* (Fig. 1). Increasing genetic diversity in a sponge population could increase diversity of host phenotypes for microorganisms to adapt. This would create habitat microheterogeneity in the host population and a greater possibility for various bacteria to use the resources. In their study based on the gastrointestinal (GI) tract, Zoetendal et al. (2001) found a positive and significant correlation between the similarity indices of bacterial communities and the genetic relatedness of their hosts, suggesting that the host genotype has a significant effect on the bacterial composition (Zoetendal et al., 2001). Another explanation relies on HFC and suggests that genetic diversity could be positively correlated with fitness. Populations with higher individual fitness would be less prone to local extinction promoting population survival especially for small inbreeding populations (Brook et al., 2002; Frankham, 2005). Survival of host populations would lead to the survival and evolution (coevolution) of associated symbionts. Increasing host genetic diversity through an increase of host fitness could thus promote bacterial diversity. An alternative relation could also occur if different bacteria would impart different functions that contribute to sponge metabolism (Lee et al., 2001; Taylor et al., 2007). Then, bacteria diversity would be linked to higher sponge fitness and potentially favor sponge populations. However, if only a few strains of microorganisms serve the sponge, increase diversity of microorganisms would be a burden because of higher competition between microorganism strains. Finally, relationship between genetic and bacterial dissimilarities could exist, since vertical transmission (Usher et al., 2001; Ereskovsky et al., 2005; de Caralt et al., 2007; Schmitt et al., 2007, 2008; Sharp et al., 2007; Noyer, 2010) and coevolution (Erpenbeck et al., 2002) occur. Thus, genetically different sponge populations could have differed more in their associated symbionts.

Whether genetic diversity controls or is controlled by chemical diversity is still an unresolved issue. Spatial and temporal variations in secondary metabolites could affect trophic and competitive interactions, increasing the chemical patchiness of the environment and promoting biodiversity at both genetic and species levels (Hay & Fenical, 1996). Alternatively, sponge genetic diversity could promote chemical diversity if secondary metabolites are produced by the sponge and the production has a heritable basis. Secondary metabolites present multiple ecological roles such as anti-predation, anti-fouling, mediation of spatial competition, facilitation for reproduction or protection against UV (Becerro et al., 1997; Mcclintock & Baker, 2001) and can be critical for species survival. Production of a cocktail of chemical compounds could confer advantages to a sponge individual that faces various threats, e.g., predators, competitors, pathogens, or environmental factors. If so, chemical diversity would increase sponge fitness. However, if only a limited number of chemical compounds are advantageous, increasing diversity of chemical compounds with ecologically irrelevant metabolites may dilute this benefit by allocating less energy to the production of the specific relevant compound. Thus, sponge populations with a low diversity of ecologically relevant compounds would be at an advantage.

If sponge-associated microorganisms produce even partially some of the secondary metabolites used by the host, the relationship between bacterial and chemical diversity could present direct effects. The production of some bioactive compounds has been ascribed to bacterial symbionts (Unson & Faulkner, 1993; Oclarit et al., 1994; Unson et al., 1994; Bewley et al., 1996). Also, symbiotic microorganisms could provide precursors or enzymes, being just one of the multiple cell compartments involved in the production of natural products (Kreuter et al., 1992; Ebel et al., 1997; Siegl & Hentschel, 2010; Sacristan-Soriano et al., 2011). We could therefore foresee a positive relationship between bacterial and chemical diversities. Alternatively, if a limited number of advantageous chemical compounds are produced by few strains of bacteria, increasing bacterial diversity may

increase competition between these different strains inhabiting sponge tissue, and could limit this advantage. Considering that bacteria do not influence production of secondary metabolites; secondary metabolites could potentially protect sponges from pathogens by preventing them to overrun the sponge tissues. This could have positive effect on both sponge fitness and symbiotic bacterial communities. Nevertheless, many factors may operate in these relationships, and the explanations represent basic interpretations that are not exhaustive. Hence, caution is required when attempting to understand mechanisms that could link those levels of diversity.

Measuring sponge fitness may have been interesting data to collect in order to unravel underlying ecological processes. Sponge fitness, defined as the capability to survive and reproduce, could be impacted by these three components (cf. above). Sponge populations with greater fitness are more likely to survive and adapt to environmental changes. Survival of various populations at different locations would promote maintenance of genetic diversity, of bacterial communities associated to the sponges and chemical diversity. Thus, besides the question "does diversity begets diversity," another fundamental question arises: does diversity at different levels favor the evolutionary potential of species?

Conclusion

Previous work performed on this species revealed that genetic, bacterial, and chemical diversity varied between sponge populations and presented significant geographical patterns (Noyer, 2010). However, we failed to show that those levels of diversity are related in *S. lamella* despite the various methods and measures used in this study. Measuring and understanding biological diversity is an important task with direct and indirect implications for conservation issues. However, assessing biodiversity remain very complex as there is no single all-embracing measure (Gaston & Spicer, 2004) and under different scales of observation, different indices of diversity might lead to different conclusions (Yue et al., 2003, 2005). Nevertheless, these three levels are intrinsically related to the sponge evolution and connections may still occur between the three components. However, in this model (target species, sampling design, technical, and statistical analyses), our data failed to support the diversity begets diversity hypothesis. This suggests that multiple factors can regulate the genetic, bacterial, and chemical diversity in *S. lamella*. In our study, all factors varied with geography as we found a clear pattern of changes in genetic, chemical, and bacterial diversity associated with geographical distance. These factors could represent multiple biotic and abiotic differences between populations, highlighting the complex nature of the mechanisms behind biodiversity.

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References

Aiello, A., P. Ciminiello, E. Fattorusso & S. Magno, 1988. 3-Beta, 5-alpha-dihydroxy-6-beta-methoxycholest-7-enes from the marine sponge *Spongia agaricina*. Journal of Natural Products 51: 999–1002.

Arrieta, J. M., S. Arnaud-Haond & C. M. Duarte, 2010. What lies underneath: conserving the oceans' genetic resources. Proceedings of the Natural Academy of Sciences 107: 18318–18324.

Balloux, F., W. Amos & T. Coulson, 2004. Does heterozygosity estimate inbreeding in real populations? Molecular Ecology 13: 3021–3031.

Becerro, M. A., 2008. Quantitative trends in sponge ecology research. Marine Ecology 29: 167–177.

Becerro, M. A., X. Turon & M. J. Uriz, 1997. Multiple functions for secondary metabolites in encrusting marine invertebrates. Journal of Chemical Ecology 23: 1527–1547.

Bell, J. J., 2008. The functional roles of marine sponges. Estuarine, Coastal and Shelf Science 79: 341–353.

Bertrand, J. C. & J. Vacelet, 1971. Association between Corneal and Bacterial Sponges. Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D 273: 638–641.

Bewley, C. A., N. D. Holland & J. D. Faulkner, 1996. Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. Experientia 52: 716–722.

Blanquer, A. & M. J. Uriz, 2010. Population genetics at three spatial scales of a rare sponge living in fragmenteed habitats. BMC Evolutionary Biology 10: 13.

Brook, B. W., D. W. Tonkyn, J. J. Q'Grady, & R. Frankham, 2002. Contribution of inbreeding to extinction risk in threatened species. Conservation Ecology 6: 16.

Cardinale, B. J., M. A. Palmer & S. L. Collins, 2002. Species diversity enhances ecosystem functioning through interspecific facilitation. Nature 415: 426–429.

Casamayor, E. O., H. Schafer, L. Baneras, C. Pedros-Alio & G. Muyzer, 2000. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 66: 499–508.

Castritsi-Catharios, J., M. Magli & J. Vacelet, 2007. Evaluation of the quality of two commercial sponges by tensile strength measurement. Journal of the Marine Biological Association of the United Kingdom 87: 1765–1771.

Chapman, J. R., S. Nakagawa, D. W. Coltman, J. Slate & B. C. Sheldon, 2009. A quantitative review of heterozygosity–fitness correlations in animal populations. Molecular Ecology 18: 2746–2765.

Cimino, G., S. de Stefano, L. Minale & G. Sodano, 1975. Chemical patterns and classification of the Demospongiae. Comparative Biochemistry and Physiology 50B: 279–285.

Clarke, K. R. & R. M. Warwick, 2001. Change in Marine Communities: An Approach to Statistical Analysis and Interpretation. 5-Primer-E Ltd, Plymouth Marine Laboratory, UK.

Coltman, D. W. & J. Slate, 2003. Microsatellite measures of inbreeding: a meta-analysis. Evolution 57: 971–983.

Da Silva, A., et al., 2009. Heterozygosity–fitness correlations revealed by neutral and candidate gene markers in roe deer from a long-term study. Evolution 63: 403–417.

De Caralt, S., M. J. Uriz, A. V. Ereskovsky & R. H. Wijffels, 2007. Embryo development of Corticium candelabrum (Demospongiae: Homosclerophorida). Invertebrate Biology 126: 211–219.

Diaz, M. C. & K. Rutzler, 2001. Sponges: an essential component of Caribbean coral reefs. Bulletin of Marine Science 69: 535–546.

Duran, S., M. Pascual, A. Estoup & X. Turon, 2004a. Strong population structure in the marine sponge Crambe crambe (Poecilosclerida) as revealed by microsatellite markers. Molecular Ecology 13: 511–522.

Duran, S., M. Pascual & X. Turon, 2004b. Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge Crambe crambe (Poecilosclerida). Marine Biology 144: 31–35.

Ebel, R., M. Brenzinger, A. Kunze, H. J. Gross & P. Proksch, 1997. Wound activation of protoxins in marine sponge *Aplysina aerophoba*. Journal of Chemical Ecology 23: 1451–1462.

Ereskovsky, A. V., E. Gonobobleva & A. Vishnyakov, 2005. Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge Halisarca dujardini Johnston (Porifera, Demospongiae, Halisarcida). Marine Biology 146: 869–875.

Erpenbeck, D., J. A. J. Breeuwer, H. C. Van Der Velde & R. W. M. Van Soest, 2002. Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae, Porifera) using a mitochondrial marker. Marine Biology 141: 377–386.

Feral, J. P., 2002. How useful are the genetic markers in attempts to understand and manage marine biodiversity? Journal of Experimental Marine Biology and Ecology 268: 121–145.

Fontana, A., L. Albarella, G. Scognamiglio, M. J. Uriz & G. Cimino, 1996. Structural and stereochemical studies of C-21 terpenoids from Mediterranean Spongiidae sponges. Journal of Natural Products 59: 869–872.

Frankham, R., 2005. Genetics and extinction. Biological Conservation 126: 131–140.

Gaino, E. & R. Pronzato, 1989. Ultrastructural evidence of bacterial damage to *Spongia officinalis* fibers (Porifera, Demospongiae). Diseases of Aquatic Organisms 6: 67–74.

Gaino, E. & R. Pronzato, 1992. Disease and overfishing drive commercial sponges to the brink of extinction in the Mediterranean basin. Bollettino del Museo Istituto di Biologia Università di Genova 56-57: 209–224.

Gaston, K. J. & J. I. Spicer, 2004. Biodiversity: An introduction. Blackwell Science, Oxford. 191 p.

Hansson, B., 2010. The use (or misuse) of microsatellite allelic distances in the context of inbreeding and conservation genetics. Molecular Ecology 19: 1082–1090.

Hay, M. E. & W. Fenical, 1996. Chemical ecology and marine biodiversity: insights and products from the Sea. Oceanography 9: 10–20.

Hentschel, U., K. M. Usher & M. W. Taylor, 2006. Marine sponges as microbial fermenters. FEMS Microbiology Ecology 55: 167–177.

Ilan, M., Y. Loya, G. A. Kolbasov & I. Brickner, 1999. Sponge-inhabiting barnacles on Red Sea coral reefs. Marine Biology 133: 709–716.

Joachimiak, M. P., J. L. Weisman & B. C. H. May, 2006. JColorGrid: Software for the visualization of biological measurements. BMC Bioinformatics 7: 225.

Kreuter, M. H., A. Robitzki, S. Chang, R. Steffen, M. Michaelis, Z. Kljajic, M. Bachmann, H. C. Schröder & W. E. G. Müller, 1992. Production of the cytostatic agent aeroplysinin by the sponge *Verongia aerophoba* in in vitro culture. Comparative Biochemistry and Physiology Part C-Pharmacology, Toxicology and Endocrinology 101: 183–187.

Lamosova, T., J. Dolezal, V. Lanta & J. Leps, 2010. Spatial pattern affects diversity–productivity relationships in experimental meadow communities. Acta Oecologica-International Journal of Ecology 36: 325–332.

Lee, Y. K., J. H. Lee & H. K. Lee, 2001. Microbial symbiosis in marine sponges. Journal of Microbiology 39: 254–264.

Lesbarreres, D., D. S. Schmeller, C. R. Primmer & J. Merila, 2007. Genetic variability predicts common frog (Rana temporaria) size at metamorphosis in the wild. Heredity 99: 41–46.

Ljungqvist, M., M. Akesson & B. Hansson, 2010. Do microsatellites reflect genome-wide genetic diversity in natural populations? A comment on Vali et al. (2008). Molecular Ecology 19: 851–855.

Lopes, M. T. & N. Boury-Esnault, 1981. Contribution à la connaissance des éponges cornées de la côte de l'Arrábida et de l'Algarve. Arquivos do Museu Bocage, A, I 6: 95–110.

Magurran, A. E., 2004. Measuring Biological Diversity. Blackwell Science Ltd., Oxford.
Mcclintock, J. B. & B. J. Baker, 2001. Marine Chemical Ecology. CRC Press, Boca Raton.
Muyzer, G. & K. Smalla, 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 73: 127–141.
Noyer, C., 2010. Relationship between genetic, bacterial and chemical diversity of the Mediterranean sponge *Spongia agaricina* (*S. lamella* Schultze). PhD thesis, University of Barcelona: 278 pp.
Noyer, C., G. Agell, M. Pascual & M. A. Becerro, 2009. Isolation and characterization of microsatellite loci from the endangered Mediterranean sponge Spongia agaricina (Demospongiae: Dictyoceratida). Conservation Genetics 10: 1895–1898.
Noyer, C., A. Hamilton, O. Sacristán-Soriano & M. A. Becerro, 2010. Quantitative comparison of bacterial communities in two Mediterranean sponges. Symbiosis 51: 239–243.
Noyer, C., O. P. Thomas & M. A. Becerro, 2011. Patterns of chemical diversity in the mediterranean sponge Spongia lamella. PLoS ONE 6(6): e20844. doi:10.1371/journal.pone.0020844.
Oclarit, J. M., H. Okada, S. Ohta, K. Kaminura, Y. Yamaoka, T. Iisuka, S. Miyashiro & S. Ikegami, 1994. Anti-bacillus substance in the marine sponge, *Hyatella* species, produced by an associated *Vibrio* species bacterium. Microbios 78: 7–16.
Odat, N., F. H. Hellwig, G. Jetschke & M. Fischer, 2010. On the relationship between plant species diversity and genetic diversity of Plantago lanceolata (Plantaginaceae) within and between grassland communities. Journal of Plant Ecology-UK 3: 41–48.
Palmer, M. W. & T. A. Maurer, 1997. Does diversity beget diversity? A case study of crops and weeds. Journal of Vegetation Science 8: 235–240.
Pronzato, R., 1999. Sponge-fishing, disease and farming in the Mediterranean Sea. Aquatic Conservation-Marine and Freshwater Ecosystems 9: 485–493.
Pronzato, R. & R. Manconi, 2008. Mediterranean commercial sponges: over 5000 years of natural history and cultural heritage. Marine Ecology-An Evolutionary Perspective 29: 146–166.
Pujolar, J. M., D. Bevacqua, F. Capoccioni, E. Ciccotti, G. A. De Leo & L. Zane, 2009. Genetic variability is unrelated to growth and parasite infestation in natural populations of the European eel (Anguilla anguilla). Molecular Ecology 18: 4604–4616.
Rueda, A., E. Zubia, M. J. Ortega, J. L. Carballo & J. Salva, 1998. New metabolites from the sponge *Spongia agaricina*. Journal of Natural Products 61: 258–261.
Rützler, K., 1976. Ecology of Tunisian commercial sponges. Tethys 7: 249–264.
Sacristan-Soriano, O., B. Banaigs, E. O. Casamayor & M. A. Becerro, 2011. Exploring the links between natural products and bacterial assemblages in the sponge *Aplysina aerophoba*. Applied and Environmental Microbiology 77: 862–870.
Sala, E. & N. Knowlton, 2006. Global marine biodiversity trends. Annual Review of Environment and Resources 31: 93–122.
Sarkar, S., 2006. Ecological diversity and biodiversity as concepts for conservation planning: comments on Ricotta. Acta Biotheoretica 54: 133–140.
Schmitt, S., J. B. Weisz, N. Lindquist & U. Hentschel, 2007. Vertical transmission of phylogenetically complex microbial consortium in the vivipare sponge *Ircinia felix*. Applied and Environmental Microbiology 73: 2067–2078.
Schmitt, S., H. Angermeier, R. Schiller, N. Lindquist & U. Hentschel, 2008. Molecular microbial diversity survey of sponge reproductive stages and mechanistic insights into vertical transmission of microbial symbionts. Applied and Environmental Microbiology 74: 7694–7708.
Sharp, K. H., B. Eam, D. J. Faulkner & M. G. Haygood, 2007. Vertical transmission of diverse microbes in the tropical sponge Corticium sp. Applied and Environmental Microbiology 73: 622–629.
Siegl, A. & U. Hentschel, 2010. PKS and NRPS gene clusters from microbial symbiont cells of marine sponges by whole genome amplification. Environmental Microbiology Reports 2: 507–513.
Sipkema, D., M. C. R. Franssen, R. Osinga, J. Tramper & R. H. Wijffels, 2005. Marine sponges as pharmacy. Marine Biotechnology 7: 142–162.
Taylor, M. W., R. Radax, D. Steger & M. Wagner, 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiology and Molecular Biology reviews 71: 295–347.
Templado, J., M. Calvo, A. Garcia, A. A. Luque, M. Maldonado & L. Moro, 2004. Guía de Invertebrados y Peces Marinos protegidos por la legislación nacional e internacional. In N.y.P.N.s. (ed) Guía de Invertebrados y Peces Marinos protegidos por la legislación nacional e internacional. M.M.A. & C.S.I.C.: 214.
Unson, M. D. & D. J. Faulkner, 1993. Cyanobacterial symbiont biosynthesis of chlorinated metabolites from *Dysidea herbacea* (Porifera). Experientia 49: 349–353.
Unson, M. D., N. D. Holland & D. J. Faulkner, 1994. A brominated metabolite by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. Marine Biology 119: 1–11.
Uriz, M. J., D. Rosell & M. Maldonado, 1992. Parasitism, commensalism or mutualism—the case of Scyphozoa (Coronatae) and Horny sponges. Marine Ecology-Progress Series 81: 247–255.
Usher, K. M., J. Kuo, J. Fromont & D. C. Sutton, 2001. Vertical transmission of cyanobacterial symbionts in the marine sponge Chondrilla australiensis (Demospongiae). Hydrobiologia 461: 15–23.
Vali, U., A. Einarsson, L. Waits & H. Ellegren, 2008. To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? Molecular Ecology 17: 3808–3817.
Vellend, M. & M. A. Geber, 2005. Connections between species diversity and genetic diversity. Ecology Letters 8: 767–781.
Voultsiadou-Koukoura, H. E., A. Koukouras & A. Eleftheriou, 1987. Macrofauna associated with the sponge Verongia

aerophoba in the north Aegean Sea. Estuarine, Coastal and Shelf Science 24: 265–278.

Whittaker, R. H., 1972. Evolution and measurement of species diversity. Taxon 21: 231–251.

Yue, M., L. J. Zhang, F. X. Gu, X. L. Pan & G. F. Zhao, 2003. The relationships between plant community species diversity and soil factors. Ecosystems Dynamics, Ecosystem-Society Interactions, and Remote Sensing Applications for Semi-Arid and Arid Land, Pts 1 and 2 4890: 534–540.

Yue, T. X., et al., 2005. Considerable effects of diversity indices and spatial scales on conclusions relating to ecological diversity. Ecological Modelling 188: 418–431.

Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermnas-Van Vliet, J. A. G. M. De Visser & W. M. De Vos, 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microbial Ecology in Health and Disease 13: 129–134.

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Short Research Note

First evaluation of mitochondrial DNA as a marker for phylogeographic studies of Calcarea: a case study from *Leucetta chagosensis*

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Abstract In most animals mitochondrial DNA (mtDNA) evolves much faster than nuclear DNA. Therefore, and because of its shorter coalescent time, mitochondrial (mt) markers provide better resolution to trace more recent evolutionary events compared to nuclear DNA. But in contrast to most other Metazoa, previous studies suggested that in sponges mitochondrial sequence evolution is much slower, making mtDNA less suitable for studies at the intraspecific level. However, these observations were made in the class Demospongiae and so far no data exist for calcareous sponges (Class Calcarea). We here provide the first study that evaluates intraspecific mt sequence variation in Calcarea. We focus on arguably the best-studied species *Leucetta chagosensis*, for which three nuclear DNA marker datasets existed previously. We here sequenced the partial mitochondrial *cytochrome oxidase subunit III gene* (*cox3*). Our analyses reveal an unexpected variability of up to 8.5% in this mitochondrial marker. In contrast to other sponges where this marker evolves considerable slower than the nuclear *internal transcribed spacer region* (*ITS*), we found that *cox3* in *L. chagosensis* evolves about five times as fast as *ITS*. The variability is similar to that of nuclear intron data of the species. The phylogeny inferred with *cox3* is congruent with other markers, but separates earlier reported genetic groups much more distinctively than nuclear DNA. This provides further evidence for cryptic speciation in *L. chagosensis*. All these features make calcarean mtDNA exceptional among sponges and show its suitability for phylogeographic studies and potential as a species-specific (DNA barcoding) marker to distinguish morphologically identical cryptic species.

Keywords *Leucetta chagosensis* · Calcarea · Mitochondrial DNA · Cytochrome oxidase subunit III · Phylogeography · DNA barcoding

Mitochondrial DNA (mtDNA) sequences are frequently applied DNA markers in studies of phylogeny, phylogeography, and, more recently, are used in DNA barcoding approaches, e.g., in the sponge barcoding project (Wörheide et al., 2007). Several features of mtDNA make its application in these

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instances advantageous over nuclear markers. Because it is solely maternal inherited, most metazoans carry only one haploid version of mitochondrial genotype, the so-called haplotype. Therefore, mtDNA lacks the problems in sequencing occasionally occurring with nuclear DNA (ncDNA) due to allelic copies. In phylogenetic analyses, haplotypes represent individuals, which simplifies the interpretation of, for example, haplotype networks. As a consequence of their maternal inheritance, effective population size of mt haplotypes is about fourfold decreased compared to nuclear genes (Zink & Barrowclough, 2008). Consequently, lineage sorting proceeds much faster in these genes (Birky et al., 1983), hence more recent isolation events can be uncovered than is possible with nuclear markers (Avise et al., 1987; Zink & Barrowclough, 2008).

Despite these advantages, the use of mtDNA for phylogeographic studies of sponges is hampered by their slow evolution in contrast to other animals, where mitochondrial genes evolve faster than nuclear genes (Shearer et al., 2002, Huang et al., 2008). Especially on the intraspecific level sponge mtDNA genes show only limited variability (e.g., Duran et al., 2004; Lavrov et al., 2005; Wörheide, 2006, but see Rua et al., 2011, who showed higher variability in mt intergenic regions). However, all this data refer to sequences from the class Demospongiae. In Calcarea, such data has not been available. We here present the first intraspecific analysis of calcarean mitochondrial sequences from *Leucetta chagosensis*. The phylogeography of this species across the Indo-Pacific has been intensively studied with nuclear markers, i.e., with the internal transcribed spacer region of the ribosomal RNA cistron (*ITS*, including *ITS1*, *5.8S* ribosomal rRNA gene and *ITS2*), partial 28S ribosomal RNA gene (*28S*) and the second intron of the ATP synthase beta gene (*ATPSb-iII*) (Wörheide et al., 2002, 2008). This system allows us to compare the performance of a mtDNA marker to previously employed nuclear markers for phylogeographic studies of Calcarea.

We found that PCR amplification of mt genes with universal mt primers (e.g., Folmer et al., 1994) did usually result in sequences from commensal organisms instead of calcarean sequences, as revealed by BLAST searches (Altschul et al., 1997) and phylogenetic analysis (data not shown). To overcome this problem, we applied a primer independent approach. By screening an EST library of *L. chagosensis* (Philippe et al., 2009) for mt sequences we identified the mt *cytochrome oxidase subunit III gene* (*cox3*) sequence.

We designed specific primers (Leuc_cha_cox3_fw: CTA TTT ATC TTA TCT GAA GT; Leuc_cha_cox3_rv: AAA TAA TCA TAC AAC ATC AAC) and amplified 460 bp (without primer sequences) of the *cox3* gene from a subset of 36 *L. chagosensis* samples (Supplementary Table 1). From previous studies, it was already known that *L. chagosensis* comprises several deeply diverged clades, of which two have an overlapping distribution on the Great Barrier Reef (Wörheide et al., 2002, 2008). For our case study we focused on samples from these two groups and also included one sequence per gene from a sample from the Red Sea, a genetically isolated geographical region (Wörheide et al., 2008).

PCR products were purified by ammonium acetate precipitation and used in cycle sequencing with the BigDye Terminator Kit v. 3.1 chemistry (ABI) following the manufacturer's instructions. DNA sequencing was performed by the LMU sequencing service (http://www.gi.bio.lmu.de/sequencing). Sequences were assembled with CodonCode Aligner (http://www.codoncode.com/aligner) and the resulting contigs were aligned in SeaView (Gouy et al., 2010). Sequences of the *cox3* fragment were submitted to GenBank (accession numbers JN031968-JN032003, see Supplementary Table 1). *ITS*, *28S* and *ATPSb-iII* sequences for our analyses were obtained from the previous studies (Wörheide et al., 2002, 2008).

Unrooted phylogenetic trees were reconstructed with PHYML (Guindon & Gascuel, 2003), each analysis included a 1,000 replicate bootstrap analysis. For the Maximum likelihood (ML) analyses we determined the evolutionary model proposed by the AIC criterion in jModelTest (Posada, 2008) by comparison of 88 models (chosen models: *cox3*: TVM+I; *ITS+28S*: TIM1+I; *ATPSb-iII*: HKY+G). Phylogenies were reconstructed for *cox3*, combined *ITS* and *28S* and *ATPSb-iII*. *ITS* and *28S* sequences were concatenated, because *28S* alone (306 base pairs) provided too little information to reconstruct a resolved phylogeny and sequence variation was similar in both genes (data not shown). Also, both genes are part of the nuclear rRNA cistron. Bayesian analyses were performed in MrBayes v3.2.1

(Ronquist & Huelsenbeck, 2003).The number of substitutions and rate parameters were set according to the proposed ML models. Two runs with four chains were run for 5 million generations. Parameter stabilization and convergence of runs were verified with Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) and AWTY (Nylander et al., 2008). The first 2 million generations were excluded as burn-in phase. Uncorrected pairwise *p* distances for all genes were calculated in PAUP* 4.0b10 (Swofford, 2003). Boxplots were generated in R (R Development Core Team, 2001).

A search in GenBank revealed only one published study that provided intraspecific sequences for *cox3* in Porifera: Park et al. (2007) compared *ITS* and *cox3* sequences of four species representing the genera *Hymeniacidon* and *Halichondria* in family Halichondriidae (class Demospongiae) from Korea. Although the sampling scheme and evolutionary history of these species and *L. chagosensis* prohibit direct comparison of genetic distances of each gene, a comparison of *cox3* and *ITS* differences within each group can reveal relative mutation rates between these mitochondrial and nuclear sequences. For this reason the sequences from GenBank were used to calculate mean pairwise distances between the species of each genus. Additionally, we also compared genetic distances of *cox3* and *ITS* sequences of the two relatively closely related freshwater sponges *Lubomirskia baikalensis* and *Ephydatia muelleri*, because both markers were available for these species. A list of taxa and accession numbers of included sequences from GenBank is given in the Supplementary Table 2.

Results from Maximum Likelihood analyses and Bayesian inference yielded largely congruent topologies for each gene (see Fig. 1 for ML tree including Bayesian support values). All phylogenetic trees confirmed the presence of two defined groups in *L. chagosensis* with partial geographic overlap (group 1 and 2), from which the Red Sea sample was genetically distinct (Fig. 1), as expected from previous studies (Wörheide et al., 2002, 2008). For the samples included in this study, this subdivision is clearest displayed in the *cox3* tree, where branches between the groups were considerably longer than within groups. The definition of groups (1, 2, Red Sea, Fig. 1) was used to calculate 'within group' and 'between group' genetic distances.

In *cox3*, we observed considerable variation in *L. chagosensis* with a maximum sequence difference of 8.5%. This distance was comparable to the nuclear intron data (*ATPSb-iII:* 7.2%) but much higher than in *ITS* or *28S* (1.7 and 2.2%, respectively). Concordant to the findings of slow evolution of mt genes in demosponges (Duran et al., 2004;, Lavrov et al., 2005; Wörheide, 2006; Huang et al., 2008), in our analyses the mitochondrial *cox3* of the halichondrid sponges from Korea and the freshwater sponges evolved slower than the nuclear encoded *ITS* (Fig. 2). In the Halichondriidae, *cox3* evolves on average about half as fast as *ITS* (overall: 5.5% vs. 12.5%). In the freshwater sponges, *Lubomirskia baikalensis* and *Ephydatia muelleri*, differences between *cox3* and *ITS* evolutionary rates are even more prominent. While in *cox3*, almost no variation occurs between both species (0.12%), the variation in *ITS* is very high (*5.8S rRNA+ITS2*: 13.5%, STDV: 0.0056).

In contrast to the demosponges, in *L. chagosensis*, the ratio of mitochondrial to nuclear evolutionary rates is reversed. Here, *cox3* evolves on average more than five times faster than *ITS*. In general, high genetic distance can be caused by either a long independent evolution or in elevated rates of sequence evolution. At least for other nuclear markers like *18S* rRNA and another fragment of *28S*, calcareous sponges do not have very long branches compared to other taxa (e.g., Dohrmann et al., 2006). Therefore, it seems more plausible that elevated evolutionary rates in the mitochondrial genome of Calcarea are responsible for the high genetic distances in *cox3*.

When comparing distances of nuclear markers 'within' and 'between groups' in *Leucetta*, we also observe another special feature of the *cox3* marker. While the maximum distance 'between groups' values in *cox3* are similar to the ones observed in nuclear *ATPSb-iII*, *cox3* variation 'within groups' are much smaller and closer to the range observed in *ITS* (Fig. 3). Most strikingly, when comparing 'within' and 'between groups' genetic distances, the distance of both categories overlap in the nuclear markers, while in cox3 'within group' distances are very low and with a maximum of 2.17% still much smaller than the lowest 'between groups' distance (5%).

It has been suggested that *L. chagosensis* populations possibly had been isolated in refugia during glaciation sea-level lowstands and were already

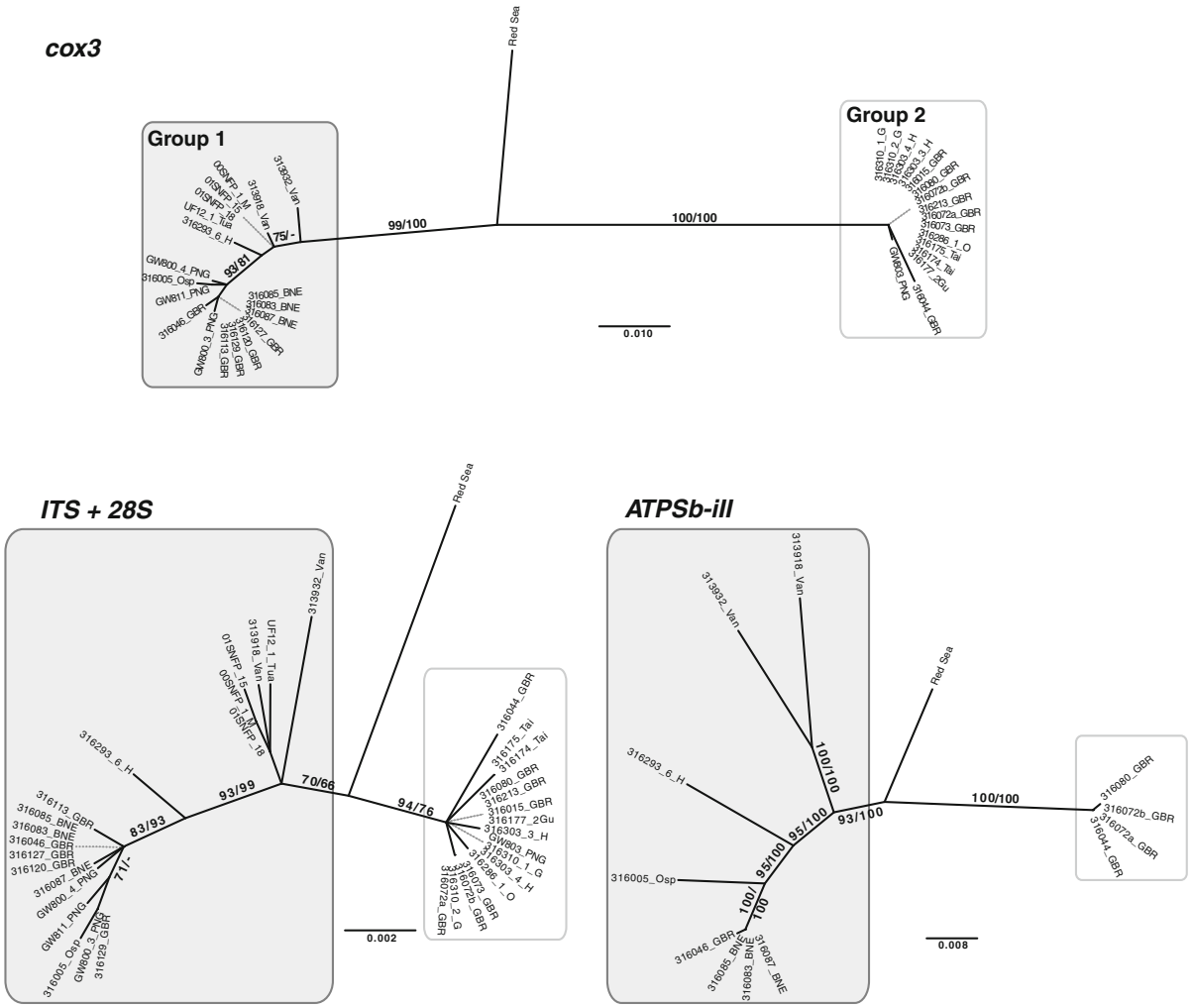

Fig. 1 Unrooted phylogenetic trees (ML) calculated from the three datasets of *L. chagosensis*. The two groups with overlapping distributions are boxed (group 1, group 2). Sample numbers refer to Queensland Museum specimens (beginning with numbers) or present in our sample collection (all others). Bootstrap (ML)/posterior probability (Bayesian inference) values are given at the branches if greater than 69 and on branches with visible length. *BNE* Sunshine Coast & Brisbane, *GBR* Great Barrier Reef, *Gu* Guam, *H* Holmes Reef (Coral Sea), *Osp* Osprey Reef (Coral Sea), *PNG* Papua New Guinea, *Tai* Taiwan, *Van* Vanuatu

reproductively isolated when later recolonizing the shallower areas of their overlapping current distribution (Wörheide et al., 2002, 2008). Whatever the cause, the patterns of these isolation events are recovered much more clearly in the mitochondrial data than from the nuclear data and corroborate the hypothesis, that *L. chagosensis* is indeed a complex of several cryptic species (Wörheide et al., 2002, Wörheide et al., 2008). With the clear distinction of 'within' and 'between group' sequence divergence, i.e., the presence of a "barcoding gap" (Meyer & Paulay, 2005), mtDNA might proof a useful diagnostic tool to define and identify indistinguishable species based on gross morphology in the *L. chagosensis* species complex. However, a more thorough examination of mitochondrial data with specimens from the entire distributional range will have to be conducted. Possibly such study should be extended to the cytochrome oxidase subunit I gene (*cox1*), the standard marker for DNA barcoding. In demosponges, *cox3* and *cox1*, have been shown to have similar rates of evolution on the amino acid level (Wang & Lavrov, 2008), which remains to be shown for Calcarea. Nonetheless, our findings show

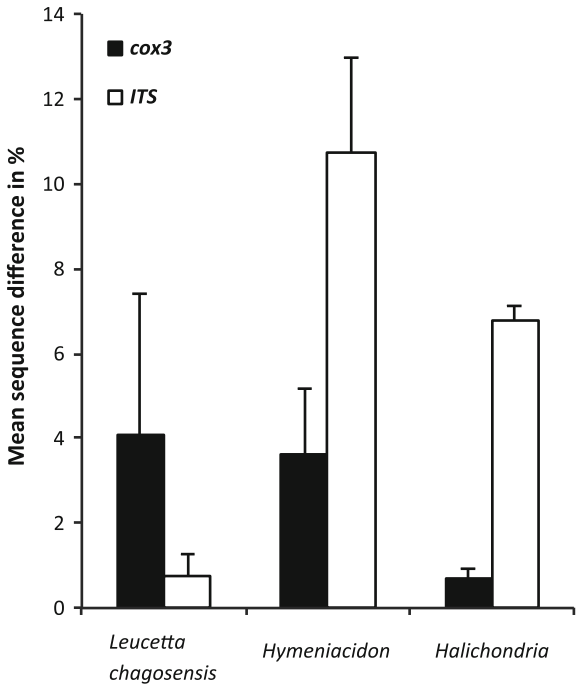

Fig. 2 Comparisons of the relation of *cox3* and *ITS* variation (uncorrected *p* distances) in *L. chagosensis* and in halichondrid demosponges from *Hymeniacidon* and *Halichondria*. For the latter interspecific congeneric comparisons are shown. *Error bars* display the standard deviation. Note that contrary to the demosponges,in *L. chagosensis cox3* is much more variable than *ITS*

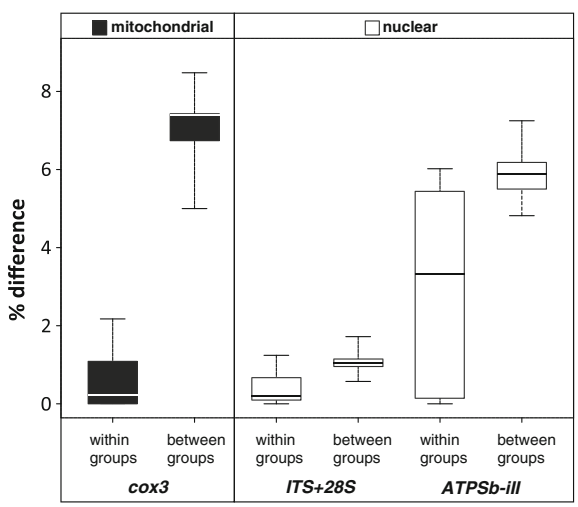

Fig. 3 Boxplots showing the percentage sequence differences (uncorrected *p* distance) in three datasets within and between the groups of *L. chagosensis*. Whiskers in plots give the extreme values in the data

that mitochondrial *cox3* in Calcarea evolves much faster than in other sponges. This suggests a fast evolution of all mitochondrial genes and would yet be a unique feature of this class in Porifera. From our results, mtDNA will be a valuable marker in phylogeographic studies of Calcarea. For example, a previous study on the phylogeography of *Pericharax heteroraphis* (a calcareous sponge closely related to *Leucetta chagosensis*) using *ITS* and *ATPSb-iII* data showed only little population structure (Bentlage & Wörheide, 2007). Further and more detailed structure is likely to be recovered by analyzing mtDNA markers in this and other species.

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References

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller & D. J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389–3402.

Avise, J. C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb & N. C. Saunders, 1987. Intraspecific phylogeography—the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst 18: 489–522.

Bentlage, B. & G. Wörheide, 2007. Low genetic structuring among *Pericharax heteroraphis* (Porifera: Calcarea) populations from the Great Barrier Reef (Australia), revealed by analysis of nrDNA and nuclear intron sequences. Coral Reefs 26: 807–816.

Birky, C. Jr., T. Maruyama & P. Fuerst, 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. Genetics 103: 513–527.

Dohrmann, M., O. Voigt, D. Erpenbeck & G. Wörheide, 2006. Non-monophyly of most supraspecific taxa of calcareous sponges (Porifera, Calcarea) revealed by increased taxon sampling and partitioned Bayesian analysis of ribosomal DNA. Molecular Phylogenetics and Evolution 40: 830–843.

Duran, S., M. Pascual & X. Turon, 2004. Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poecilosclerida). Marine Biology 144: 31–35.

Folmer, O., M. Black, W. Hoeh, R. Lutz & R. Vrijenhoek, 1994. DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan

invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.
Gouy, M., S. Guindon & O. Gascuel, 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27: 221–224.
Guindon, S. & O. Gascuel, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704.
Huang, D., R. Meier, P. A. Todd & L. M. Chou, 2008. Slow mitochondrial COI sequence evolution at the base of the metazoan tree and its implications for DNA barcoding. Journal of Molecular Evolution 66: 167–174.
Lavrov, D. V., L. Forget, M. Kelly & B. F. Lang, 2005. Mitochondrial genomes of two demosponges provide insights into an early stage of animal evolution. Molecular Biology and Evolution 22: 1231–1239.
Meyer, C. P. & G. Paulay, 2005. DNA barcoding: error rates based on comprehensive sampling. PLoS Biology 3: 2229–2238.
Nylander, J. A., J. C. Wilgenbusch, D. L. Warren & D. L. Swofford, 2008. AWTY (Are We There Yet?): a system for graphical exploration of MCMC convergence in Bayesian phylogenetics. Bioinformatics 24: 581–583.
Park, M. H., C. J. Sim, J. Baek & G. S. Min, 2007. Identification of genes suitable for DNA barcoding of morphologically indistinguishable Korean Halichondriidae sponges. Molecules and Cells 23: 220–227.
Philippe, H., R. Derelle, P. Lopez, K. Pick, C. Borchiellini, N. Boury-Esnault, J. Vacelet, E. Renard, E. Houliston, E. Quéinnec, C. Da Silva, P. Wincker, H. Le Guyade, S. Leys, D. J. Jackson, F. Schreiber, D. Erpenbeck, B. Morgenstern, G. Wörheide & M. Manuel, 2009. Phylogenomics revives traditional views on deep animal relationships. Current Biology 19: 706–712.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25: 1253–1256.
R Development Core Team, 2001. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria [available on internet at http://www.R-project.org].
Ronquist, F. & J. P. Huelsenbeck, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.
Rua, C., C. Zilberberg & A. Solé-Cava., 2011. New polymorphic mitochondrial markers for sponge phylogeography. Journal of the Marine Biological Association of the UK. doi:10.1017/S0025315410002122.
Shearer, T. L., M. J. H. Van Oppen, S. L. Romano & G. Wörheide, 2002. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Molecular Ecology 11: 2475–2487.
Swofford, D. L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (and Other Methods). Version 4. Sinauer Associates, Sunderland.
Wang, X. & D. V. Lavrov, 2008. Seventeen new complete mtDNA sequences reveal extensive mitochondrial genome evolution within the Demospongiae. PLoS ONE 3: e2723.
Wörheide, G., 2006. Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge *Astrosclera willeyana* across the Indo-Pacific. Marine Biology 148: 907–912.
Wörheide, G., J. N. A. Hooper & B. M. Degnan, 2002. Phylogeography of western Pacific *Leucetta 'chagosensis'* (Porifera : Calcarea) from ribosomal DNA sequences: implications for population history and conservation of the Great Barrier Reef World Heritage Area (Australia). Molecular Ecology 11: 1753–1768.
Wörheide, G., D. Erpenbeck & C. Menke, 2007. The Sponge Barcoding Project: aiding in the identification and description of poriferan taxa. In Custódio, M. R. & E. Hajdu (eds), Porifera Research: Biodiversity, Innovation & Sustainability. Museu Nacional Serie Livros, Rio de Janerio: 123–128.
Wörheide, G., L. S. Epp & L. Macis, 2008. Deep genetic divergences among Indo-Pacific populations of the coral reef sponge *Leucetta chagosensis* (Leucettidae): founder effects, vicariance, or both? BMC Evolutionary Biology 8: 24.
Zink, R. M. & G. F. Barrowclough, 2008. Mitochondrial DNA under siege in avian phylogeography. Molecular Ecology 17: 2107–2121.

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SPONGE RESEARCH DEVELOPMENTS

Diversity patterns and zoogeography of the Northeast Atlantic and Mediterranean shallow-water sponge fauna

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Abstract Recognizing and understanding present-day biodiversity and biogeographical patterns and how these relate to contemporary and past climate is pivotal to predict the effect of future climate on marine biodiversity and promote adequate conservation policies. Sponges constitute an important and dominant component of the marine benthos and are therefore an excellent model group for such investigations. In this study, we assessed the diversity patterns and the zoogeographical affinities of the Northeast Atlantic and Mediterranean shallow-water demosponge assemblages. Data on the distribution of 745 species throughout 28 areas was compiled from the literature and used to build a presence/absence matrix. Diversity patterns were assessed from estimates of species richness (S) and taxonomic distinctness (AvTD). The Mediterranean Sea proved to be more diverse both in terms of species richness and taxonomic distinctness ($S = 539$, AvTD $= 94.74$) than the Northeast Atlantic ($S = 480$, AvTD $= 92.42$) and the two regions together were found to constitute a diversity hotspot harbouring approximately 11% of the global demosponge diversity. We found an Atlantic N–S and a Mediterranean NW–SE gradient of increasing taxonomic distinctness that is strongly correlated to both contemporary ($R^2 = 0.5667$; $P < 0.01$) and historical values ($R^2 = 0.7287$; $P < 0.01$) of sea surface temperature (SST) at the Last Glacial Maximum (LGM). The zoogeographical affinities examined through classification (cluster analysis) and ordination (non-metric multidimensional scaling, nMDS) based on the Bray–Curtis similarity index, revealed the presence of three groups approximately corresponding to the Northern European Seas, Lusitanian and Mediterranean provinces outlined in the 'Marine Ecoregions of the World' (MEOW) classification system. Geographical distance and oceanographic circulation were shown to constitute important factors in shaping the zoogeographical affinities among areas. The vast majority of the species occurring in the Northeast

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Atlantic and the Mediterranean (67 and 57%, respectively) was shown to have extremely restricted geographical ranges, as single-area or narrow-range (2–3 areas) endemics, which raises some concerns regarding their conservation.

Keywords Porifera · Demospongiae · Biogeography · Marine biodiversity · Paleoclimate · Taxonomic distinctness

Introduction

The study of the patterns and the underlying processes of origin and maintenance of diversity from populations to ecosystems and how these relate to contemporary and historical factors are the focus of disciplines such as ecology, biogeography and phylogeography. The shallow waters of the Atlanto-Mediterranean region encompass an important area covering a wide climatic range of subtropical, temperate and subarctic conditions. Since its formation, the Northeast Atlantic and Mediterranean have experienced an intricate geological and climatological history, with major events such as the Messinian Salinity Crisis (5.96–5.33 Myr BP) in the late Miocene and the formation of permanent ice sheets ($\approx$3 Myr BP) at high northern latitudes at the onset of the Quaternary oscillations between cold glacial and warm interglacial periods (the Pleistocene glaciations). The last glacial cycle, from 130 kyr BP to the present, culminated in the Last Glacial Maximum (LGM, 30–19 kyr BP) during which the North Atlantic and Mediterranean shores endured glacial conditions with the advance of the major ice sheets and concomitant drop in sea surface temperature (SST), eustatic sea-level fluctuations, and changes in surface circulation patterns (Lambeck et al., 2002; Pflaumamm et al., 2003; Hayes et al., 2005). This event produced great changes in species distributions: some went extinct over large parts of their range or dispersed to new areas, while others survived in refugia, leaving a traceable genetic imprint in its populations. While the effects at the intraspecific level are relatively well documented (Hewitt, 1996, 1999, 2000, 2004), the effects on the diversity patterns of regional assemblages are far less known (e.g. Jansson, 2003; Araújo et al., 2008). Within the marine realm, the most compelling evidence of such effects emerge from phylogeographic studies that have identified structured populations and signatures of refugia, as well as range-expansion for several Atlanto-Mediterranean species (see reviews in Patarnello et al., 2007; Maggs et al., 2008).

Sponges are one of the dominant invertebrate groups of hard-bottom benthic communities, both in terms of biomass and species richness, and they play important roles in ecosystem functioning throughout temperate, tropical and polar habitats (Sarà & Vacelet, 1973; Bell, 2008). Yet, studies on the diversity and distribution patterns of sponge faunas at alpha, beta or gamma scales remain few (e.g. Hooper & Kennedy, 2002; Samaai, 2006; Van Soest et al., 2007a), and an even smaller number of studies have attempted to relate the observed patterns to ecological variables (e.g. de Voogd et al., 2006; de Voogd & Cleary, 2007; Van Soest et al., 2007a).

Sponges are sessile in their adult stage and exhibit low dispersal capabilities during their larval stage, due to the production of lecithotrophic larvae with a short life span and philopatric behaviour (Mariani et al., 2005, 2006; Maldonado, 2006; Uriz et al., 2008). As in many other marine organisms, the reproductive timing in sponges is highly correlated with water temperature (see Riesgo & Maldonado, 2008 and references therein). Taking these characteristics into account, we hypothesise that when examined over a large spatial scale, the demosponge fauna should exhibit clear zoogeographical and diversity patterns with a signature of the geological and paleoclimatic events of the Mio-Pleistocene.

Some previous studies examined the zoogeographical affinities of the sponge fauna in the Northeast Atlantic and Mediterranean, but these mostly focused on particular areas within this region such as Cape Verde and Mauritania (Van Soest, 1993a, b), Strait of Gibraltar (Carballo et al., 1997), Alboran Sea (Maldonado & Uriz, 1995) or the Aegean Sea (Voultsiadou, 2005). Pansini & Longo (2003), followed by Voultsiadou (2009), were the first to provide a comprehensive account of the diversity and biogeography of the Mediterranean sponges. Both studies recognized the contribution of the Atlantic Ocean to the diversity patterns seen in the Mediterranean, but the lack of an analysis combining data from both areas has obscured our understanding of the relationship between them. The present work aims to uncover the diversity

patterns and zoogeographical affinities of the shallow-water demosponge fauna of the entire Northeast Atlantic and Mediterranean region, and determine to which extent these patterns are related to the climatic and geological events of the late Pleistocene.

Methods

Study area

Our study encompasses the area located approximately between latitudes 15–70°N and longitudes 32°W–35°E (Fig. 1). According to the Marine Ecoregions of the World (MEOW) classification system (Spalding et al., 2007), this area comprises four provinces (Northern European Seas, Lusitanian, Mediterranean Sea, and West African Transition) and 17 out of the 19 marine ecoregions (excl. Iceland and the Baltic Sea) nested within these provinces.

Species distribution data

Among the three extant classes of the Porifera, the Demospongiae is the most speciose group harbouring approximately 85% of all sponge species (Van Soest et al., 2009). The two remaining classes, Calcarea and Hexactinellida account for a much smaller proportion of species. As the former are much in need of taxonomic revision and the latter are mostly restricted to the deep-sea environment (Hooper & Van Soest, 2002), these lesser groups have been excluded from the analyses. Given that shallow and deep-water sponge assemblages differ in species composition, in

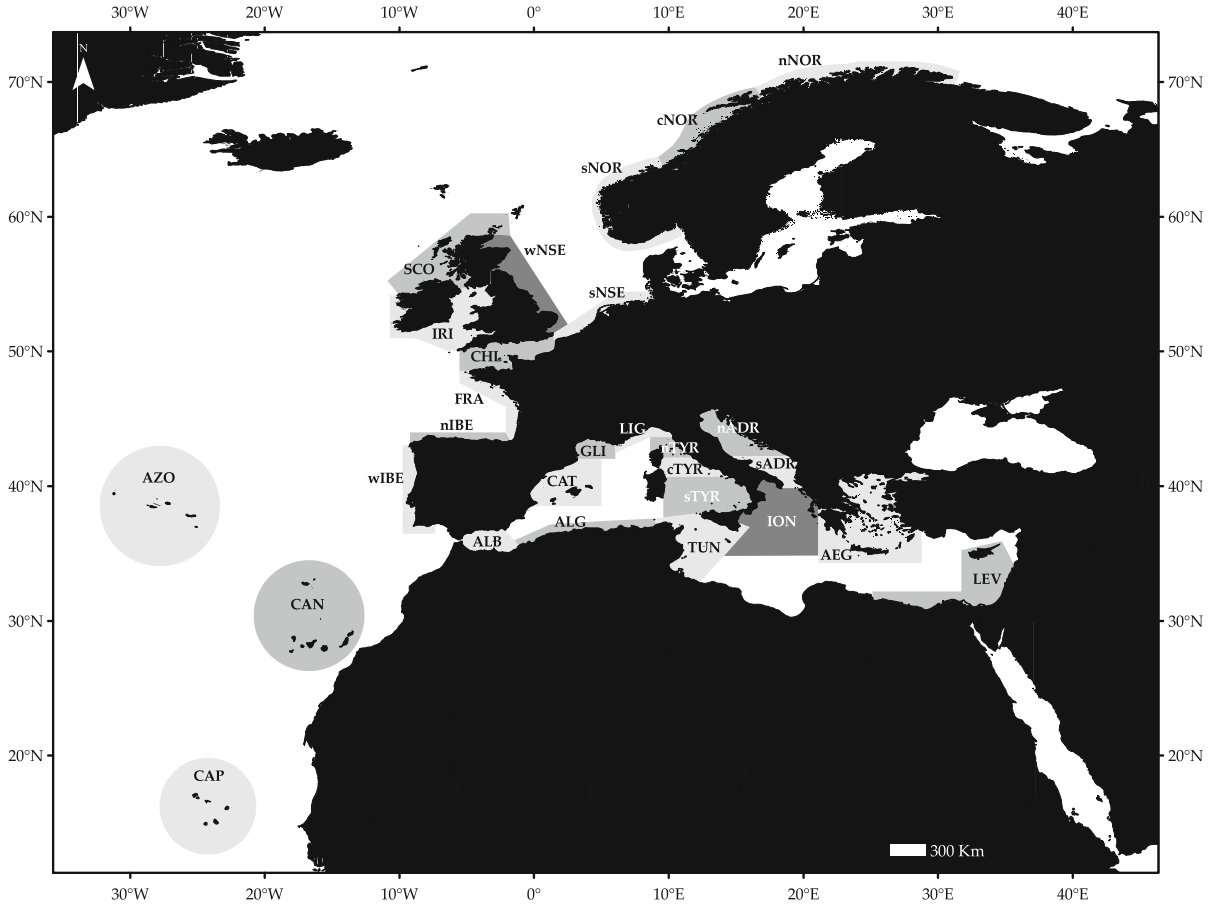

Fig. 1 The Northeast Atlantic and Mediterranean Sea, highlighting the areas considered in this study (Mediterranean areas were redrawn from Pansini & Longo, 2003). For area codes see "Methods" section

this comparison we have only taken into account species inhabiting the sublittoral and circalittoral zones to an approximate depth of 120 m.

Species distributions (presence/absence) were compiled from the literature, which covers an approximately 115 year—period of publications concerning the Northeast Atlantic and Mediterranean sponge fauna (e.g. Topsent, 1892; Vacelet et al., 2007). We have collected data covering a total of 14 Northeast Atlantic and 14 Mediterranean areas (Fig. 1). These areas were delimited on the basis of either the geographical orientation of a coastal segment (e.g. West and North coasts of the Iberian Peninsula) or natural isolation (e.g. Canary or Azores islands), and for which a relatively well-studied sponge fauna was available.

The Northeast Atlantic was divided into (main data sources in parentheses): AZO—Azores archipelago (Topsent, 1892, 1904; Boury-Esnault & Lopes, 1985; De Weerdt & Van Soest, 1986; Moss, 1992; Xavier, 2003; J. Xavier, unpublished data); CAN—Canary islands (Cruz, 1980, 1984, 2002; Cruz & Bacallado, 1982, 1983, 1984, 1985a, b) also including Madeira island (Johnson, 1899; Topsent, 1928; Lopes, 1995; Pestana, 2002; J. Xavier, unpublished data); CAP—Cape Verde also including Mauritania (Van Soest, 1993a, b); wIBE—West coast of the Iberian Peninsula (Portugal and Spain) (Hanitsch, 1895; Lévi & Vacelet, 1958; Lopes & Boury-Esnault, 1981; Lopes, 1989; Naveiro, 2002; Pires, 2007; J. Xavier, unpublished data) also including the Gorringe Bank (Xavier & Van Soest, 2007); nIBE—North coast of Spain (Ferrer-Hernández, 1914, 1918, 1922; Solórzano, 1991; Cristobo, 1997; Preciado, 2002); FRA—West coast of France (Descatoire, 1966, 1969); CHL—English Channel (Burton, 1930, 1957; Borley, 1931; Lévi, 1950; Borojevic et al., 1968; Cabioch, 1968, 1973; Cabioch & Glaçon, 1975; Ackers et al., 1992); IRI—Irish and Celtic Seas (Burton, 1963; Van Soest & Weinberg, 1980; Van Soest et al., 1983; Hiscock et al., 1984; Ackers et al., 1992; Bell & Barnes, 2000); SCO—North coast of Scotland and Ireland (Ackers et al., 1992; Picton & Goodwin, 2007); sNSE, wNSE—South and West coasts of the North Sea (Topsent, 1899; Ackers et al., 1992; Van Soest et al., 2007b; M. de Kluijver, unpublished data); sNOR, wNOR and nNOR—South, West and North coasts of Norway (Tendal et al., 2001; Hans Tore Rapp, pers. comm.).

For the Mediterranean areas, we followed the matrix in Pansini & Longo (2003), removed the deep-sea species and complemented the data of the shallow-water species for some areas with later studies (e.g. Kefalas et al., 2003; Voultsiadou, 2005; Kefalas & Castritsi-Catharios, 2007 for the Aegean Sea; Vacelet et al., 2007 for the Lebanon coast; Mustapha et al., 2003 for Tunisia). The areas considered in the Mediterranean were: ALB—Alboran Sea; CAT—Catalunya and Balearic islands; GLI—Golfe du Lion; ALG—Algerian Basin; LIG—Ligurian Sea; n, c, sTYR—North, Central and South Tyrrhenian Sea; TUN—Tunisian coast, Malta and southwest Sicily; n, sADR—North and South Adriatic; ION—Ionian Sea; AEG—Aegean Sea; and LEV—Levantine Basin including the coast of Egypt. Some of the studies we consulted have a larger geographical coverage (e.g. Topsent, 1892, 1928; Van Soest et al., 2000).

Species lists were extracted from each reference, compiled for each area and then aggregated, eliminating redundant records. These lists were crosschecked with the World Porifera Database (WPD, Van Soest et al., 2009 available at: http://www.marinespecies.org/porifera/) and only valid names were considered. These lists were then compiled into a distribution matrix which has approximately 21,000 records (745 spp. × 28 areas).

Diversity: species richness (*S*) and taxonomic distinctness (AvTD)

Species richness (*S*) is a diversity measure commonly used when no quantitative information is available. However, making comparisons and interpretations based on this measure is difficult as it is highly dependent on sampling effort (Clarke & Warwick, 2001a). Given that we have assembled our data from an extremely heterogeneous array of sources, in terms of sampling techniques and intensity, this measure will only be used as indicative.

Average taxonomic distinctness (AvTD or Δ^+), developed by Warwick & Clarke (1995), is a diversity-measure that takes into account the relationships between species following a Linnean classification. For presence/absence data, the AvTD of an assemblage at a particular area (a species list) is defined as the average taxonomic distance (path length) between all its species pairs, measured along the classification tree. This approach is therefore an indicative measure of the taxonomic 'breadth' of an assemblage and the relatedness of its constituent species (Clarke &

Warwick, 1998). The advantages of the AvTD over simple species-richness estimates are that AvTD captures 'phylogenetic' diversity and is robust to variation in sampling effort (Clarke & Warwick, 1998; Warwick & Clarke, 1998). These statistical properties make this method particularly suitable for analyses of historic data, i.e. data which does not follow a standardised sampling design or effort (Clarke & Warwick, 1998). Another advantage of this method is that, contrary to the species-richness measure, it allows a test for departure from expectation, i.e. it compares the taxonomic distinctness of a location with the one which would be expected if that assemblage had been randomly drawn from the total species pool present in the wider area (master list). As an example, considering the Azores list of size $S = 95$, the method will randomly extract sublists of the same size from the complete species list of the Northeast Atlantic and compute the AvTD values for each of these lists. From the AvTD-simulated values, it will construct a histogram of the expected range of AvTD values for sublists of that size. If the observed AvTD falls outside the 95% confidence interval of the simulated Δ^+, it is considered to have departed significantly from expectation, thus reflecting enhanced or reduced diversity of the Azorean assemblage. Because the 95% expectation interval was calculated from the master list from which the species have been drawn at random, and because we found a large proportion of species that are endemic to either the Northeast Atlantic or the Mediterranean (see 'Results' section), we further calculated this interval using only Mediterranean or Atlantic locations. Note that the AvTD value for each area remains the same and that only the 95% funnel boundaries and mean AvTD are re-adjusted, due to the change of the master list. Thus, this analysis provides a more accurate estimate of the distinctness of these areas' assemblages, relative to expectation, within their geographical contexts.

Another index associated with the AvTD is the variation in taxonomic distinctness (varTD or Λ^+), which measures the variance of the pairwise path lengths and reflects the unevenness of the taxonomic tree, i.e. the assemblage (Clarke & Warwick, 2001b). A presence/absence data matrix and an aggregation file containing the taxonomic classification of the species in the data matrix are the basis for these analyses. Because the assemblages studied here are restricted to the Class Demospongiae, we have considered four phylogenetic levels: species, genus, family and order (e.g. *Oscarella lobularis*; *Oscarella*; Plakinidae; Homosclerophorida) and constant step lengths between levels (Clarke & Warwick, 1999). The analyses were performed through the TAXDTEST routine in Primer v. 6.1.11 (Clarke & Gorley, 2006).

Correlation between taxonomic distinctness (AvTD) and climate (SST)

Using taxonomic distinctness as a measure of assemblage diversity, we tested whether the observed pattern was more correlated with contemporary or historical values of SST, as this factor is known to be a critical one for species distribution (Sarà & Vacelet, 1973). The SST data used was that resulting from the project 'Climate: long range investigation, mapping and prediction' (CLIMAP, 1976, 1981, 1984). In order to make use of this data, we averaged the values of mean contemporary SST and mean SST anomaly (LGM—contemporary) for each area and performed a linear regression analyses between these and the values of AvTD.

Zoogeographical affinities: cluster analysis

From the presence/absence data matrix we built a pairwise similarity matrix employing the Bray–Curtis coefficient. Hierarchical agglomerative clustering and non-metric multidimensional scaling (nMDS) were performed to assess the faunistic similarities between areas. In order to assure convergence to the global minimum of stress, which is a goodness-of-fit measure of the representation in the 2D space, we performed several MDS runs with 50 random starts (stress <0.1 corresponds to a good ordination with no prospect for misleading interpretations). These analyses were carried out for both species and genera.

Using a similarity percentage routine (SIMPER), we assessed the extent of similarity within the Northeast Atlantic and Mediterranean regions, as well as the dissimilarity between these two areas. A matrix was built with geographical marine distances between each pair of locations, and then analysed along with a Bray–Curtis similarity matrix through non-parametric correlation (Spearman's rank correlation) in order to ascertain the role of geographical distance in the zoogeographical affinities among areas. This was performed using Primer's RELATE routine.

Species affinities and geographical range

The zoogeographical affinities of the species were assessed through classification of each species into Atlanto-Mediterranean (AM), Atlantic endemic (AE) and Mediterranean endemic (ME). Distribution ranges were classified into: single-area occurrence (sa) if a species is reported in the literature for only one of the considered areas; a narrow-ranged (nr) occurrence if a species is found in two or three out of the 14 Northeast Atlantic or Mediterranean areas; and a wide-ranged (wr) occurence if a species is found in four or more areas. As an example, a species which occurs in only one Atlantic area would be classified as a single-area Atlantic endemic (or saAE), while one occurring in six Mediterranean regions would be classified as a wide-ranged Mediterranean endemic (wrME). Note that the term endemic here stands for species that occur either in the NEA or MED, and that we do not take into account whether the species occurs in other regions outside our study area.

Results

Diversity: species richness and taxonomic distinctness

The sponge literature yielded data on the distribution of 745 shallow-water demosponge species occurring in the study area. This value encompasses 187 genera in 64 families and 14 orders and represents approximately 11% of the global demosponge diversity (Table 1). The distribution of the diversity at the higher taxonomic levels (Orders) found for the NEA/MED is similar to that found at the global scale (Fig. 2a, b). Overall, the Mediterranean is more species-rich than the Northeast Atlantic, harbouring 539 versus 480 species, respectively. The most speciose Mediterranean areas are the South coasts of Spain, France and Italy (CAT, GLI, LIG, cTYR), each harbouring over 230 shallow-water demosponge species. In the Northeast Atlantic the highest species-richness values were found in the North coast of Iberia (nIBE), the English Channel (CHL), and the Macaronesian archipelagos of the Canaries, Madeira (CAN) and Cape Verde (CAP), with over 160 species reported for each of these locations (Table 1).

Taxonomic distinctness among areas varied considerably (Table 1). The values of taxonomic distinctness of the Mediterranean assemblages were higher than the values seen in the Atlantic, with the exception of those located in the Lusitanian region (CAN, wIBE and CAP), which exhibited similar values to the Mediterranean. All Mediterranean locations except the Alboran Sea had AvTD above the mean and many were above the 95% upper limit of expectation (Fig. 3a). All of the Atlantic areas, except for those previously mentioned, exhibited AvTD values below the mean, and many localities (n, sNOR; SCO; IRI; CHL; FRA and nIBE) were found to be even below the lower limit of expectation (Fig. 3a).

When the Atlantic and Mediterranean areas were considered separately, we found that in the Atlantic only the west coast of Iberia and the Macaronesian archipelagos of the Canaries and Cape Verde exhibited a taxonomic distinctness above the 95% expectation limit. All other Northeast Atlantic areas revealed an AvTD below the mean, and several were close to and even below the lower expectation limit (Fig. 3b). The Mediterranean areas were more centred on the mean AvTD, and only the easternmost localities approached or rose above the upper limit. The Alboran Sea is the only Mediterranean locality whose assemblages fall at the lower limit of expectation (Fig. 3c).

The values of taxonomic distinctness of each area were found to be correlated with both modern ($R^2 = 0.5667$; $P < 0.01$) and historical values of SST ($R^2 = 0.7287$; $P < 0.01$), but more so with the latter (Fig. 4a, b). Note that for this analysis some areas could not be taken into account, either because the SST anomaly was so extreme that the sea had been covered in ice (e.g. off the coasts of Scotland and Norway), or because during times of lowered sea level the areas were totally emerged (e.g. the northern Adriatic and North Seas).

Zoogeographical affinities

The Bray–Curtis classification analysis revealed three main groups with further sub-structuring. Group I comprises the assemblages found in the north and easternmost section of the study area, with one subgroup with the localities of the Norwegian coasts (Ia) and another with the localities of the North Sea (Ib), each exhibiting a mere 20% average similarity with the remaining NEA and MED. Group II includes

Table 1 Diversity estimates for each of 14 Northeast Atlantic (NEA) and 14 Mediterranean (MED) areas

Area	Species	Genera	Families	Orders	AvTD (Δ^+)	VarTD (Λ^+)
Northeast Atlantic (NEA)						
AZO	95	51	35	11	92.42	266.4
CAP	181	106	49	11	94.65	172.4
CAN	163	89	50	13	94.85	190.5
wIBE	135	71	39	10	94.70	182.9
nIBE	171	83	44	10	92.66	216.4
FRA	83	45	29	10	91.54	267.4
CHL	167	77	38	12	91.11	278.3
SCO	100	51	31	11	89.52	297.6
IRI	107	55	31	11	91.86	270.0
wNSE	34	25	20	9	93.23	254.9
sNSE	40	25	17	7	91.99	312.4
sNOR	119	60	32	10	90.97	257.6
wNOR	92	51	30	10	92.40	236.7
nNOR	81	47	28	10	92.04	244.8
Mediterranean Sea (MED)						
ALB	159	85	45	9	93.57	206.8
CAT	236	114	52	13	94.41	217.9
ALG	94	64	37	12	95.45	157.1
GLI	226	114	56	13	94.58	199.6
LIG	250	113	52	14	94.24	197.0
nTHY	100	62	36	11	94.94	194.0
cTHY	270	116	54	14	94.18	205.8
sTHY	92	61	38	11	95.12	181.5
TUN	120	63	40	13	94.32	212.4
nADR	147	77	41	11	94.70	212.8
sADR	154	85	46	13	95.12	190.5
ION	166	92	52	13	94.98	180.3
AEG	183	93	51	14	95.14	179.5
LEV	91	59	35	13	95.58	167.3
Total NEA	480	147	58	13	92.42	246.31
Total MED	539	167	62	14	94.74	193.04
Total NEA/MED	745	187	64	14	93.58	219.67

For abbreviations see "Methods" section

the Lusitanian areas and it is further subdivided into the NW coast of Europe (IIa) and the Iberian coasts (IIb). Group III comprises all of the Mediterranean locations, as well as the Canaries and Cape Verde archipelagoes. This latter group is further divided in subgroups that represent the central, western and eastern Mediterranean (IIIa, IIIb and IIIc, respectively). The Azorean assemblage assumes an outlying position in relation to the Lusitanian region, with which it exhibits a 40% similarity, as do the assemblages of Alboran, Algeria, Canaries and Cape Verde in relation to the Mediterranean subgroups (Fig. 5). Similar structure and groupings were obtained from the nMDS for both species and genera presence/absence (Fig. 6a, b).

The analysis of similarity percentages (SIMPER) showed that the MED areas exhibit an average pairwise similarity (49.11%) higher than the NEA areas

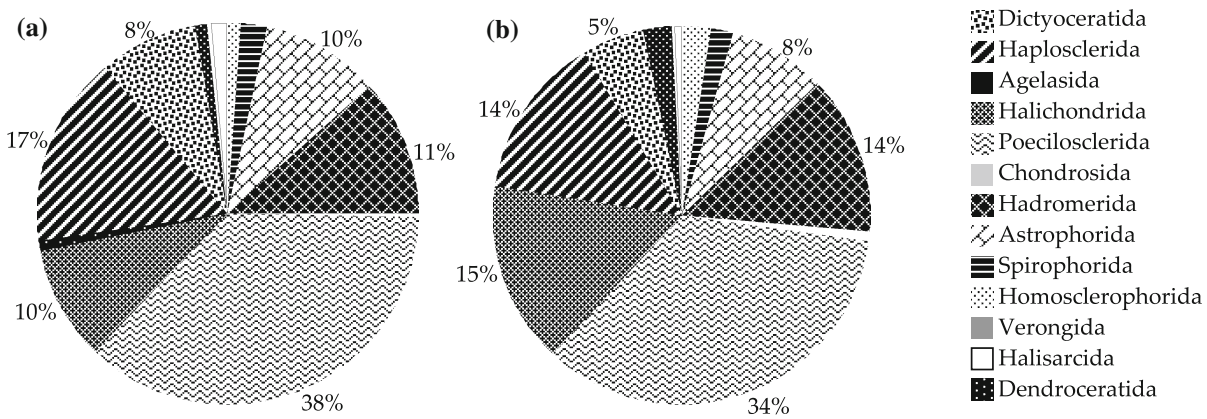

Fig. 2 Higher level (Order) taxonomic composition of the demosponge fauna at **a** global scale ($S = 6{,}777$; WPD, Van Soest et al., 2009); **b** Northeast Atlantic and Mediterranean ($S = 745$; this study)

(36.96%), while NEA and MED are on average 72% dissimilar. A significant rank correlation was found between the biotic affinities among areas (Bray–Curtis similarities) and the geographical distances separating these areas (Fig. 7; Spearman's $r_S = 0.767$; $P < 0.01$).

Range and endemism

Out of the total number of 745 species, 37% were found to have an Atlanto-Mediterranean distribution, while 206 (28%) and 265 (36%) were found to be endemic to the Northeast Atlantic and to the Mediterranean, respectively. Overall, the Northeast Atlantic is composed of 206 Atlantic endemic (44%) and 274 Atlanto-Mediterranean (56%) species. Similar proportions were found for the species inhabiting the Mediterranean: 265 (49%) are endemic to this enclosed Sea while 274 (51%) also occur in the Atlantic. Regarding the geographical range of the Northeast Atlantic and the Mediterranean species, we found that single-area occurrences or narrow-ranged species account for 67% of the Northeast Atlantic fauna and 57% of the Mediterranean fauna, whereas a wide distribution range accounts for only a very small proportion of the fauna endemic to each of the basins (6 and 8%, respectively) (Fig. 8a, b).

Discussion

There is growing scientific evidence that present-day biodiversity and biogeographical patterns are a result of both contemporary and historical climatic conditions. Uncovering and understanding these patterns is pivotal to predict both proximate and emergent ecological responses (e.g. changes in physiology, reproductive cycles, distributional shifts, changes in species composition, diversity and community structure, population dynamics and evolution) to the ongoing anthropogenically induced climate change (see Harley et al., 2006 for a review).

Diversity patterns of the Northeast Atlantic and Mediterranean sponge fauna

Harbouring over 700 species, the Northeast Atlantic and the Mediterranean appear to be a diversity hotspot for shallow-water demosponges, containing approximately 11% of the currently known demosponge species at a global scale (Van Soest et al., 2009). Although this value may result from a long-standing sponge taxonomic tradition in Europe, and because the true diversity of this group is estimated to harbour twice the current number of described species (approx. 7,000 demosponges, WPD, Van Soest et al., 2009), it still represents a remarkably rich sponge fauna, comparable only with regions such as the Caribbean, Australian Seas, or the Indo-West Pacific (see Van Soest, 1994). This species richness is even more remarkable if we take into account the fact that at least another 300 demosponge species are known to occur in this area but at greater depths (J.R. Xavier and R. Van Soest, unpublished data).

To understand the diversity patterns of the shallow-water sponge fauna found in the Northeast Atlantic

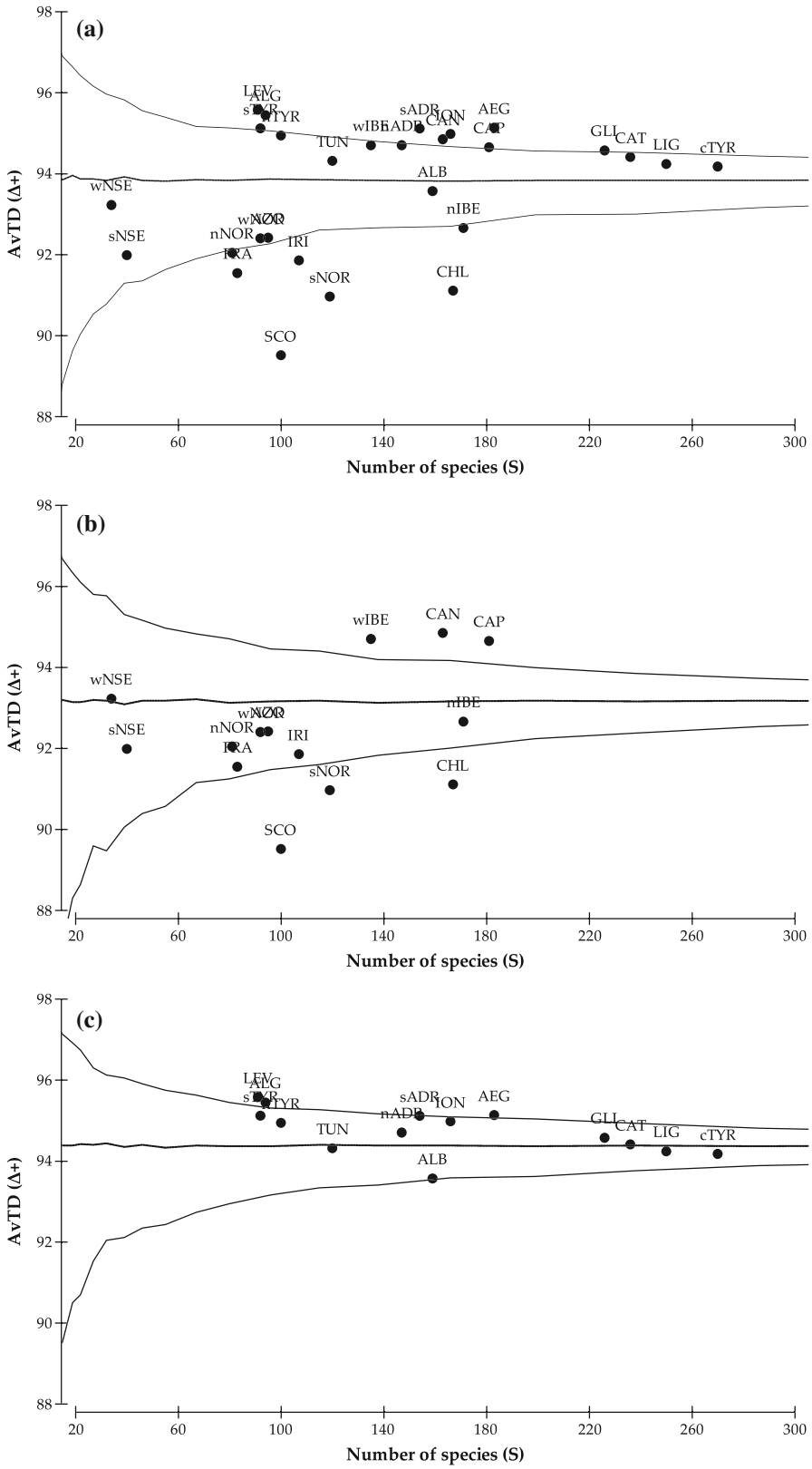

Fig. 3 Taxonomic distinctness (AvTD or Δ^+): **a** Northeast Atlantic and Mediterranean, **b** only Northeast Atlantic, and **c** only Mediterranean areas. The central line indicates mean Δ^+ (AvTD of the master list) while funnel lines constitute the 95% limits of the simulated Δ^+ values. The *points* represent the AvTD values for each area plotted against the number of species

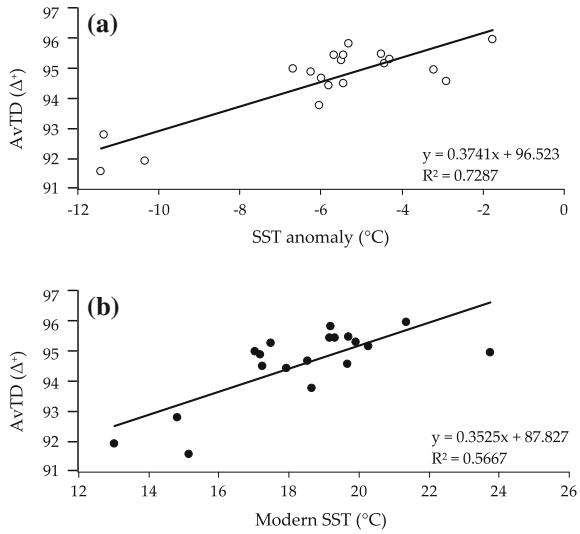

Fig. 4 Linear regression between taxonomic distinctness (AvTD or Δ^+) and sea surface temperature (SST): **a** historical SST (Last Glacial Maximum—modern-time), and **b** modern-time SST

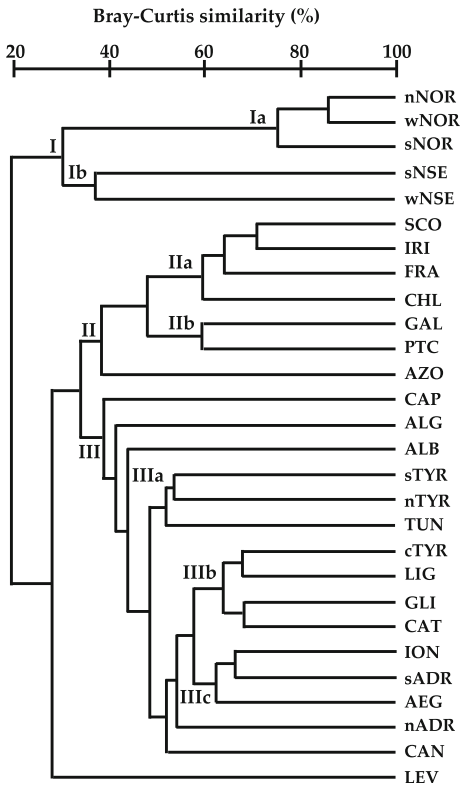

Fig. 5 Dendogram of hierarchical agglomerative clustering based on the Bray–Curtis similarity index. For area codes see "Methods" section

and Mediterranean region, one must consider the paleo-history of these areas in the Mio-, Plio- and Pleistocene. The two most prominent events of this period were (i) the Messinian salinity crisis of the Mediterranean in the late Miocene (MSC, 5.96–5.33 Myr BP), and (ii) the Quaternary glacial–interglacial cycles that culminated in the LGM (30–19 kyr BP).

The MSC was a major desiccation of the Mediterranean basin caused by a tectonic uplift of the marine gateways between the Atlantic and Mediterranean (Hsü et al., 1973; Krijgsman et al., 1999; Rouchy & Caruso, 2006). This event, considered one of the most dramatic episodes of the Cenozoic, resulted in a massive extinction of the Mediterranean marine fauna, except for some organisms that may have survived under brackish or hypersaline conditions. Once reopened, the connection to the Atlantic enabled a massive replenishment of the basin with Atlantic water and its fauna. As such, the present-day Mediterranean sponge fauna is of post-MSC assembly and composed of Atlantic (and a few Red Sea) immigrants, species that have evolved within the Mediterranean basin, along with a few Tethyan relics as suggested by previous authors (Maldonado & Uriz, 1995; Pansini & Longo, 2003).

The glaciations of the Pleistocene, and particularly the LGM, are known to have constituted the most influential events in shaping present-day distributions of terrestrial and aquatic biota, as a consequence of extinction, displacement and range-contraction, followed by range-expansion and re-colonization (Hewitt, 1996, 1999, 2000, 2004). During the LGM, the British and the Irish as well as the Scandinavian ice sheets advanced and covered large areas of northern Europe (Bowen et al., 2002; Clark & Mix, 2002). The concurrent eustatic sea-level fluctuations that reached a nadir of 130 m below current level (Yokoyama et al., 2000; Lambeck & Chappell, 2001; Lambeck et al., 2002) dramatically changed the contour of the European shores, exposing most of the North Sea Basin, part of the Bay of Biscay shelf and the northern Adriatic, along with some seamounts between the southwest coast of Portugal and Madeira island. In addition, there has been a strong decrease in SST (anomaly), which has differentially affected the various areas of the Atlanto-Mediterranean. Based on several paleoclimatic reconstructions, the maximum Atlantic SST anomaly during the LGM ($\approx 12°C$ lower than today) was located at approximately 45°N,

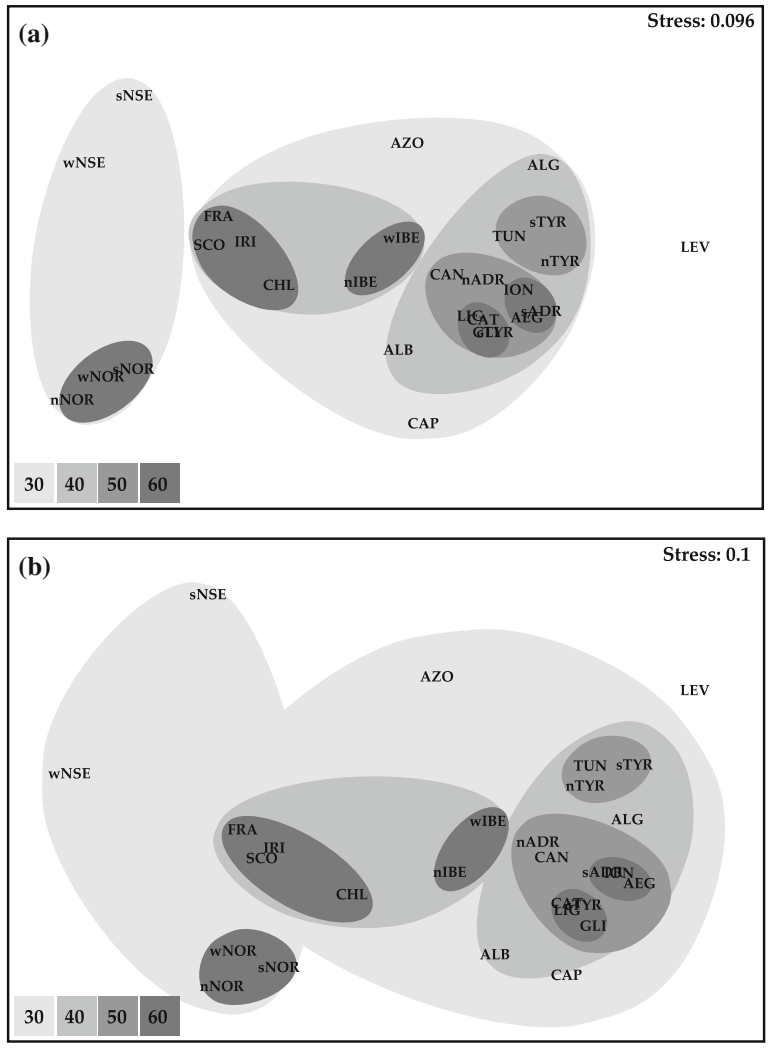

Fig. 6 Non-metric MDS plots of the Northeast Atlantic and Mediterranean shallow-water demosponge assemblages based on **a** species and **b** genera presence/absence. The increasingly *dark shades* join areas of increasing % similarity (see *scale bar*)

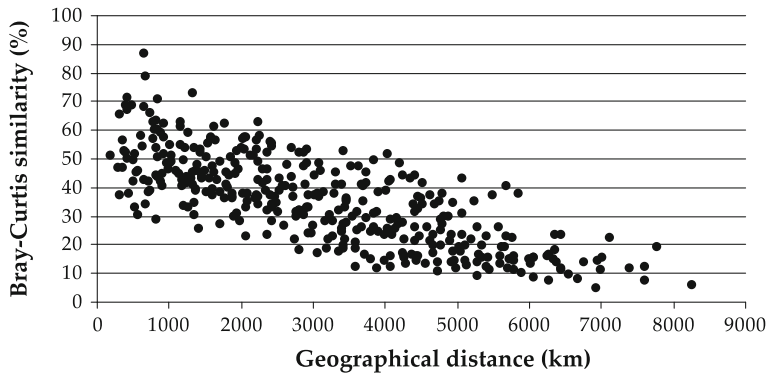

Fig. 7 Scatter plot of Bray–Curtis similarities and pairwise geographical distances (km) between the studied areas ($N = 279$ pairwise comparisons). Spearman's $r_S = 0.767$; $P < 0.01$

steeply decreasing along the Portuguese coast down to the West African coast (including the Madeira and the Canary and Cape Verde islands), where SST's were slightly lower than the current ones (see Pflaumamm et al., 2003). In the Mediterranean, reconstructions of the SST at the LGM suggest an east–west temperature gradient of 9 and 6°C during glacial winter and glacial summer, respectively, with maximum SST anomalies

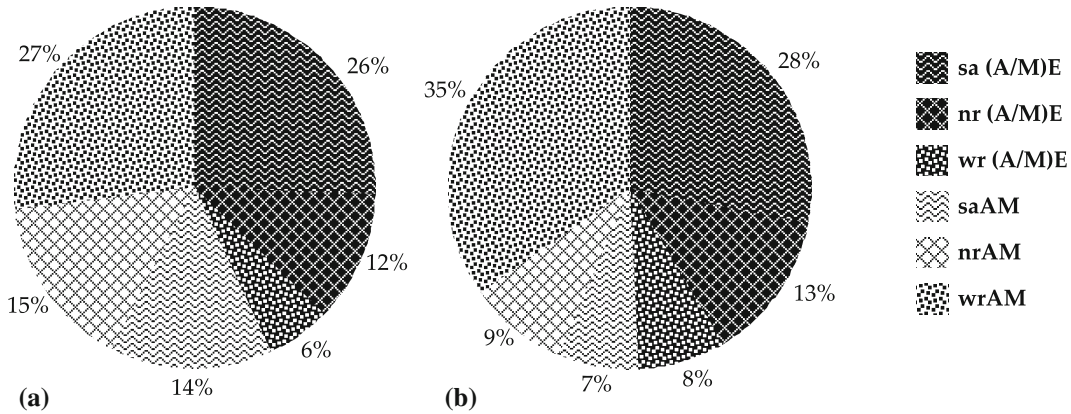

Fig. 8 Distribution range of the species in the **a** Northeast Atlantic ($S = 480$); **b** Mediterranean Sea ($S = 539$). *sa* single-area occurrence, *nr* narrow-range, *wr* wide-range, *(A/M)E* Atlantic or Mediterranean endemic, *AM* Atlanto-Mediterranean

(11°C lower than today) occurring along the northwestern coasts (Hayes et al., 2005) (Fig. 9).

Despite the massive extinction of its fauna during the MSC, the Mediterranean experienced more stable climatic conditions during the Pleistocenic glaciations than the Northeast Atlantic, which repeatedly lost a significant proportion of suitable marine habitat over relatively short periods of time. This relative stability for the Mediterranean over the past 5 Myr along with an on-going input of Atlantic shallow-water fauna, explains the higher present-day species richness (by about 12%), as well as the taxonomic distinctness of this basin.

The N–S Atlantic and the NW–SE Mediterranean gradient of increasing taxonomic distinctness that we observed in our study seems to strongly correlate with the SST anomaly gradient of the LGM. For example, the largest shift in taxonomic distinctness, from above to below 95% expectation limits, occurs in the Northeast Atlantic between the North and West coasts of Iberia ($AvTD_{wIBE} = 94.70$, $AvTD_{nIBE} = 92.66$), coinciding with the area where a shift from strong (−11.4°C) to moderate (−6.5°C) SST anomaly occurred (Fig. 9). Similarly, the eastern and southernmost areas of the Mediterranean Sea, which exhibit the highest AvTD values, coincide with areas where smaller SST anomalies occurred. Furthermore, due to the on- to offshore gradient of decreasing SST anomaly, the Macaronesian archipelagos displayed more stable environmental conditions throughout the glaciations (Pflaumamm et al., 2003), therefore serving as glacial refugia for populations and species. This is reflected in the high taxonomic-distinctness values of the Cape Verde and Canary islands ($AvTD_{CAP} = 94.65$, $AvTD_{CAN} = 94.85$), both above the 95% expectation. The apparent counter-pattern found for the Azores, which despite having suffered a smaller SST anomaly than the wIBE has a relatively low AvTD value, may be related to other factors such as geographical isolation.

Interglacial conditions began to become restored approximately 19 kyr BP, with the retreat of the ice sheets, a rise in sea-level, increasingly warmer SST's, and the re-establishment of the sea surface circulation patterns. During this period, species are known to have expanded their ranges into previously glaciated areas at a pace compatible with their dispersal abilities (Hewitt, 1999). Thus, the northern latitude assemblages should be composed of re-colonizers expanding from southern refugia and potential cold-adapted species that could have sought refugia in deep-waters. The particularly high species richness observed along the coast of Norway, especially in the south, is probably an example of the latter phenomenon, where some groups (thus explaining the relatively low AvTD) could have found refugia in the deeper Norwegian trench (e.g. the Skagerrak with a maximum depth of 700 m) or off the continental shelf. These species would have subsequently expanded their ranges to shallower water at the end of the LGM.

While some areas (e.g. nIBE, CHL) seem to have recovered their species richness, their assemblages remain taxonomically unbalanced (which is shown by their low AvTD and high varTD). Other more highly-impacted and isolated areas such as the North Sea, have yet to recover their presumably richer pre-LGM

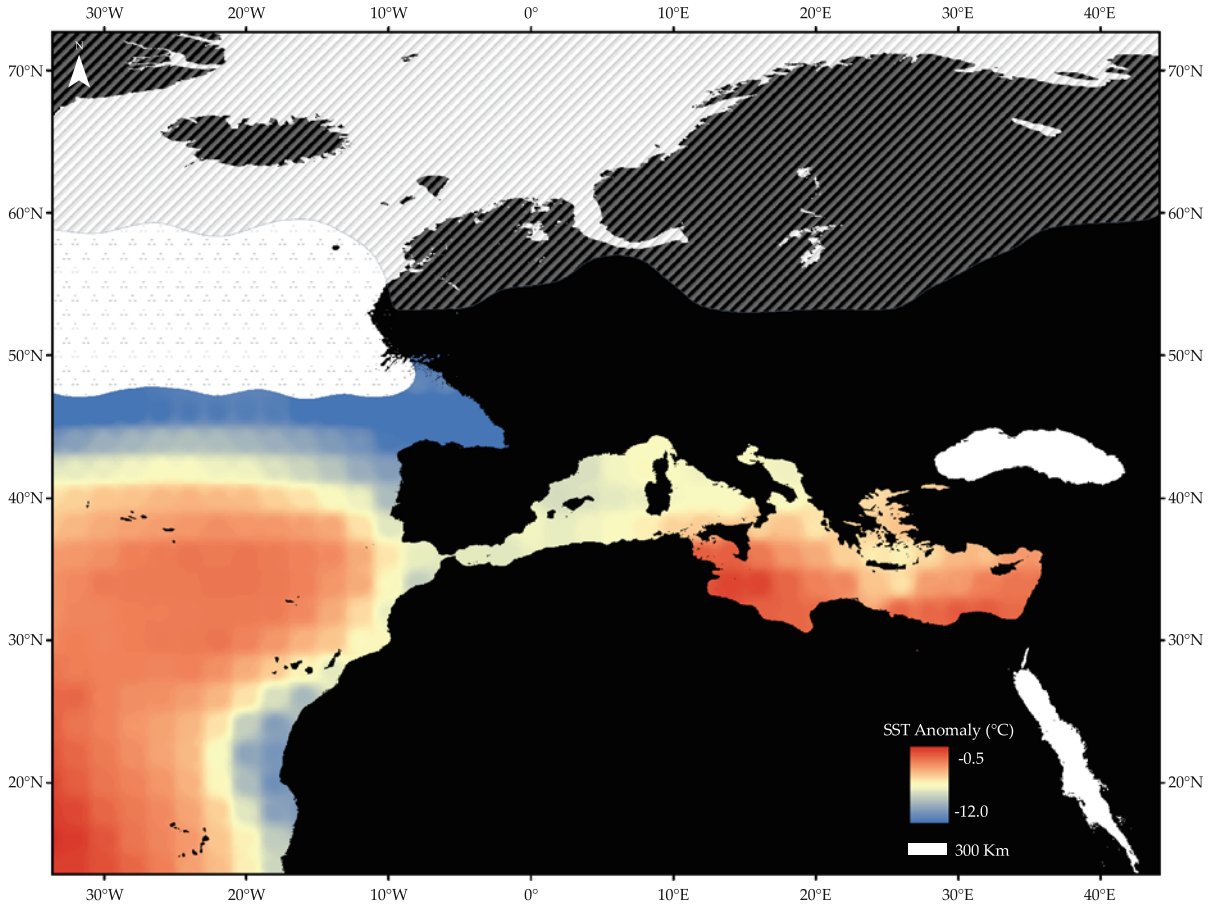

Fig. 9 Approximate representation of the Northeast Atlantic and Mediterranean at the Last Glacial Maximum (LGM; 21 kya). Coastlines extended to a −130 m sea-level lowstand (bathymetric data from GEBCO 1 min grid). *Shaded striped areas* represent permanent land and sea ice cover and *dotted areas represent* seasonal sea ice. Palaeoclimatic data from CLIMAP (1981)

sponge fauna, and their current assemblages appear to be the result of chance dispersals and human-mediated introductions (see Van Soest et al., 2007b).

The strong correlation between AvTD and SST values, although not necessarily implying causation, at least provides a plausible explanation for the influence of present and past climate on the regional sponge assemblages. Due to the intricate links between the different environmental factors (temperature, salinity, sea-level, ocean circulation), SST was used as a proxy in the present study. Yet, these and other factors, such as the area of rocky substrate at present and at LGM, summer maxima and winter minima in SST, distance to nearest potential refugia in both shallow and deeper water, cannot be ignored and should be further explored to better our understanding of sponge diversity patterns in this area. Furthermore, understanding the relationships between biological traits of individual species or groups and their ecological requirements, in addition to their responses to past climate, will prove essential in predicting future climate effects on marine biodiversity (see Bianchi, 2007).

The patterns found in our study at a meta-community scale are concordant with phylogeographic patterns observed for several marine taxa in the Atlanto-Mediterranean region (see review in Maggs et al., 2008). In these studies, gradients of genetic diversity and endemism were used to infer refugial areas for diverse marine organisms. While most studies have supported the southern refugia hypothesis for the Mediterranean, Iberian Peninsula and Macaronesian islands (e.g. Chevolot et al., 2006; Domingues et al., 2006, 2007a, b, 2008), others have shown that some populations may have persisted in northern periglacial

refugia (e.g. Provan et al., 2005; Hoarau et al., 2007). In Porifera, the southern island refugium pattern has been observed for the poecilosclerid *Phorbas fictitius* (Xavier et al., 2010a).

Zoogeographical affinities

The zoogeographical groups found in our study are concordant with the overall geologic and oceanographic setting of the Atlanto-Mediterranean region, and are mostly consistent with the main provinces and eco-regions outlined in the MEOW classification system proposed by Spalding et al. (2007). The only exceptions that we found were the English Channel, Irish, and Celtic Seas that cluster in the Lusitanian Province, and the Canaries that group with the Mediterranean Sea.

The Northern European Seas province in the Atlantic, constituted by the North and Norwegian Seas (group I), is characterized by an impoverished cold-water fauna, which along with its geographic and oceanographic isolation, explains its low similarity (approximately 30%) to the remaining NEA areas. The Lusitanian province (group II) comprises all of the western European coastal areas running from the coast of Portugal northwards to the coast of Scotland. Within this group, the northernmost areas (subgroup IIa) fall under the influence of the North Atlantic Current, whereas the Iberian Peninsula (subgroup IIb) is additionally influenced by the Portugal coastal current, which flows southward in the summer and polewards in the winter (Ambar & Fiúza, 1994; Reverdin et al., 2003).

In contrast to the complex surface circulation of the NEA, the more enclosed Mediterranean circulation explains the highest mean similarities that are found among its areas in comparison to the Atlantic. Nevertheless, three subgroups (IIIa–c) separating the western, central and eastern Mediterranean assemblages have been found. The low similarity and outlying position of the Alboran and Algerian assemblages to the remaining western Mediterranean is consistent with the well-known hydrographical barrier of the Almeria-Oran front, which is associated with the circulation in two anticyclonic gyres of the inflowing Atlantic water (Tintore et al., 1988).

We cannot, however, explain the closer similarity between westernmost (subgroup IIIb) and easternmost (subgroup IIIc) Mediterranean areas, whose faunas would have to have been connected through the Siculo-Tunisian Strait, but exhibit a surprisingly lower similarity to this area. The low similarity found between the Levantine basin and the remaining areas is most likely due to a combination of our poor knowledge of the fauna in this area along with an influence of species from the Red Sea entering the Mediterranean through the Suez Canal.

Our observations confirmed the strong affinity of the Mauritanian (Canaries) and Senegalian faunas (Cape Verde) with those of the westernmost Mediterranean (Alboran, Catalunya, Algeria) reported by previous authors (e.g. Van Soest, 1993b; Maldonado & Uriz, 1995; Carballo et al., 1997). This affinity has been suggested to be the result of a past inflow of Senegalian–Mauritanian elements into the Mediterranean (Maldonado & Uriz, 1995; Pansini & Longo, 2003) and may reflect a pattern of post-glacial range-expansion of species from southern island refugia. Alternatively this similarity could have resulted from the parallel dispersal of species from the Iberian Peninsula both into the Canary/Cape Verde and western Mediterranean through the Portugal current system.

Both geographical distance and oceanographic currents seem to constitute important factors shaping the zoogeographical affinities of the sponge fauna among the different areas, as is to be expected since this taxonomic group relies on currents for the transport of its poorly dispersing larvae.

Range, endemism and implications for conservation

Despite their ecological and biotechnological relevance, sponges have thus far received little if any attention from a conservation viewpoint, in contrast to other lower metazoan groups such as corals. This is likely due to a yet unacknowledged importance that sponges play on ecosystem functioning and the false perception that sponge species are widely distributed, which in turn is a result of overconservative systematic traditions and morphological stasis of some groups. However, since the advent of molecular techniques this later view has begun slowly but steadily to change as a growing number of allegedly cosmopolitan species are being shown to constitute

complexes of cryptic species (Klautau et al., 1999; Xavier et al., 2010b). In an assessment of global distribution patterns of demosponges, Van Soest (1994) found that over 70% of all species were confined to one of the 35 areas in which he divided the globe. These restricted distributions have been corroborated in the present study as we provided further evidence for the small distribution range of most sponge species. In fact, more than half of the total Northeast Atlantic and Mediterranean species occur in only 1–3 out of the 28 considered areas. Even more striking is that this level of endemism seems to hold true over much smaller spatial scales. Examining the diversity patterns of the sponge fauna of several reefs within a 50 km radius in eastern Australia, Hooper & Kennedy (2002) found that 60% of all species occurred on a single reef. These observations suggest that populations are mostly sustained by local recruitment of their larvae.

Due to this high level of endemism and the important roles played by this taxonomic group in ecosystem functioning, such as in bentho-pelagic coupling, substrate dynamics or its biotic interactions (see review in Bell, 2008), sponges should in the future be consistently represented in monitoring programmes and conservation strategies. Furthermore, given the growing commercial interest in this taxonomic group due to its biotechnological potential in pharmaceutics (Munro et al., 1999), further advancement in the aquaculture of sponges, and total synthesis of discovered compounds, should be promoted to avoid depletion of the existing populations.

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References

Ackers, R. G., D. Moss & B. E. Picton, 1992. Sponges of the British Isles (Sponge V): A Colour Guide and Working Document. Marine Conservation Society: 175 pp.

Ambar, I. & A. Fiúza, 1994. Some features of the Portugal Current System: a poleward slope undercurrent, an upwelling-related summer southward flow and an autumn–winter poleward coastal surface current. In Katsaros, K., A. Fiúza & I. Ambar (eds), Proceedings of the Second International Conference on Air–Sea Interaction and on Meteorology and Oceanography of the Coastal Zone. American Meteorological Society, Lisbon: 286–287.

Araújo, M. B., D. Nogués-Bravo, J. A. F. Diniz-Filho, A. M. Haywood, P. J. Valdes & C. Rahbek, 2008. Quaternary climate changes explain diversity among reptiles and amphibians. Ecography 31: 8–15.

Bell, J. J., 2008. The functional roles of marine sponges. Estuarine, Coastal and Shelf Science 79: 341–353.

Bell, J. J. & D. K. A. Barnes, 2000. A sponge diversity centre within a marine island. Hydrobiologia 440: 55–64.

Bianchi, C. N., 2007. Biodiversity issues for the forthcoming tropical Mediterranean Sea. Hydrobiologia 580: 7–21.

Borley, H. J. H., 1931. Some additions to the sponge fauna of Plymouth. Journal of the Marine Biological Association of the United Kingdom 17: 839–846.

Borojevic, R., L. Cabioch & C. Lévi, 1968. Inventaire de la faune marine de Roscoff: Spongiaires. Cahiers de Biologie Marine 9: 1–44.

Boury-Esnault, N. & M. T. Lopes, 1985. Les Démosponges littorales de l'archipel des Açores. Annales de l'Institute Océanographique 61: 149–225.

Bowen, D. Q., F. M. Phillips, A. M. McCabe, P. C. Knutz & G. A. Sykes, 2002. New data for the Last Glacial Maximum in Great Britain and Ireland. Quaternary Science Reviews 21: 89–101.

Burton, M., 1930. Additions to the sponge fauna at Plymouth. Journal of the Marine Biological Association of the United Kingdom 16: 489–507.

Burton, M., 1957. Phylum Porifera. In Wilson, D. P. (ed.), Plymouth Marine Fauna, 3rd ed. Marine Biological Association of the United Kingdom, Plymouth: 26–36.

Burton, M., 1963. Porifera. In Bruce, J. R., J. S. Colman & N. S. Joes (eds), Marine Fauna of the Isle of Man and its Surrounding Seas. Memoir No. 36. Liverpool University Press, Liverpool: 42–47.

Cabioch, L., 1968. Contribution à la conaissance de la faune des spongiaires de la manche occidentale. Démosponges de la région de Roscoff. Cahiers de Biologie Marine 9: 211–246.

Cabioch, L., 1973. Additions a l'inventaire de la faune marine de roscoff: Spongiaires. Travaux Station Biologique Roscoff 31: 5–6.

Cabioch, L. & R. Glaçon, 1975. Distribution des peuplements benthiques en Manche orientale, de la baie de Somme au Pas de Calais. Compte Rendu de l'Académie des Sciences de Paris série D 280: 491–494.

Carballo, J. L., S. Naranjo & J. C. Garcia-Gomez, 1997. Where does the Mediterranean Sea begin? Zoogeographical affinities of the littoral sponges of the Straits of Gibraltar. Journal of Biogeography 24: 223–232.

Chevolot, M., G. Hoarau, A. Rijnsdorp, W. Stam & J. Olsen, 2006. Phylogeography and population structure of thornback rays (*Raja clavata* L., Rajidae). Molecular Ecology 15: 3693–3705.

Clark, P. U. & A. C. Mix, 2002. Ice sheets and sea level of the Last Glacial Maximum. Quaternary Science Reviews 21: 1–7.

Clarke, K. R. & R. N. Gorley, 2006. PRIMER v6: User Manual/ Tutorial. PRIMER-E, Plymouth.

Clarke, K. R. & R. M. Warwick, 1998. A taxonomic distinctness index and its statistical properties. Journal of Applied Ecology 35: 523–531.

Clarke, K. R. & R. M. Warwick, 1999. The taxonomic distinctness measure of biodiversity: weighting of step lengths between hierarchical levels. Marine Ecology Progress Series 184: 21–29.

Clarke, K. R. & R. M. Warwick, 2001a. Change in Marine Communities: An Approach to Statistical Analysis and Interpretation, 2nd ed. PRIMER-E, Plymouth.

Clarke, K. R. & R. M. Warwick, 2001b. A further biodiversity index applicable to species lists: variation in taxonomic distinctness. Marine Ecology Progress Series 216: 265–278.

CLIMAP Project Members, 1976. The surface of the ice-age earth. Science 191: 1131–1144.

CLIMAP Project Members, 1981. Seasonal Reconstruction of the Earths Surface at the Last Glacial Maximum. Geological Society of America, Map and Chart Series, 36.

CLIMAP Project Members, 1984. The last interglacial ocean. Quaternary Research 21: 123–224.

Cristobo, F. J., 1997. Esponjas del Orden Poecilosclerida (Porifera: Demospongiae) de la Ría de Ferrol (NW de España). PhD thesis, Universidad de Santiago de Compostela.

Cruz, T., 1980. Contribucion al estudio de los espongiarios de las Islas Canarias. Demosponjas (Homosclerophorida, Astrophorida y Hadromerida) del litoral de Tenerife. Memoria para aspirar al grado de licenciado, Universidad de La Laguna.

Cruz, T., 1984. Espongiarios. In Estudio del Bentos Marino del Archipielago Canario. Catalogo preliminar de los invertebrados marinos bentonicos de Canarias.

Cruz, T., 2002. Esponjas Marinas de Canarias. Banco de datos de Biodiversidad de Canarias. Gobierno de Canarias: 258 pp.

Cruz, T. & J. J. Bacallado, 1982. Contribucion al conocimiento de los espongiarios de las Islas Canarias. I – Demosponjas Homosclerophorida y Astrophorida del Litoral de Tenerife. Boletin del Instituto Espanol de Oceanografia 6: 75–87.

Cruz, T. & J. J. Bacallado, 1983. Esponjas perforantes (Porifera, Clionidae) de Tenerife, Islas Canarias. Vieraea 12: 37–48.

Cruz, T. & J. J. Bacallado, 1984a. Contribucion al conocimiento de los espongiarios de las Islas Canarias. II – Demosponjas Hadromerida del Litoral de Tenerife. Servicio Publicaciones Universidad La Laguna: 63–73.

Cruz, T. & J. J. Bacallado, 1985a. Contribucion al conocimiento de los espongiarios de las Islas Canarias: demosponjas de los fondos de *Dendrophyllia ramea* en Tenerife. Anales Facultad de Ciencias 10: 71–98.

Cruz, T. & J. J. Bacallado, 1985b. Introduccion a los poblamientos de espongiarios de las Islas Canarias. Proceedings of the IV Simposio Iberico Bentos Marino: 141–150.

de Voogd, N. J. & D. F. R. Clearly, 2007. Relating species traits to environmental variables in Indonesian coral reef sponge assemblages. Marine & Freshwater Research 58: 240–249.

de Voogd, N. J., D. F. R. Cleay, B. W. Hoeksema, A. Noor & R. W. M. Van Soest, 2006. Sponge beta diversity in the Spermonde Archipelago, SW Sulawesi, Indonesia. Marine Ecology Progress Series 309: 131–142.

De Weerdt, W. & R. W. M. Van Soest, 1986. Marine shallow-water Haplosclerida (Porifera) from the South-Eastern part of the North Atlantic Ocean. Zoologische Verhandelingen 225: 1–49.

Descatoire, A., 1966. Sur quelques Démosponges de l'Archipel de Glénan. Cahiers de Biologie Marine 7: 231–246.

Descatoire, A., 1969. Les peuplements sessiles de l'Archipel de Glénan de l'infralittoral rocheux. II. Notes systématiques a propos de l'inventaire des Spongiaires. Vie et Millieu 20: 9–30.

Domingues, V. S., R. S. Santos, A. Brito & V. C. Almada, 2006. Historical population dynamics and demography of the eastern Atlantic pomacentrid *Chromis limbata* (Valenciennes, 1833). Molecular Phylogenetics and Evolution 40: 139–147.

Domingues, V. S., R. S. Santos, A. Brito, M. Alexandrou & V. C. Almada, 2007a. Mitochondrial and nuclear markers reveal isolation by distance and effects of Pleistocene glaciations in the Northeastern Atlantic and Mediterranean populations of the white seabream (*Diplodus sargus,* L.). Journal of Experimental Marine Biology and Ecology 346: 102–113.

Domingues, V. S., C. Faria, S. Stefanni, R. S. Santos, A. Brito & V. C. Almada, 2007b. Genetic divergence in the Atlantic-Mediterranean Montagu's blenny, *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and morphological characters. Molecular Ecology 16: 3592–3605.

Domingues, V. S., S. Stefanni, A. Brito, R. S. Santos & V. C. Almada, 2008. Phylogeography and demography of the Blenniid *Parablennius parvicornis* and its sister species *P. sanguinolentus* from the Northeastern Atlantic Ocean and the western Mediterranean Sea. Molecular Phylogenetics and Evolution 46: 397–402.

Ferrer-Hernández, F., 1914. Esponjas del Cantabrico. Parte 2. III. Myxospongida. IV. Tetraxonida. V. Triaxonida. Trabajos del Museo Nacional de Ciencias Naturales 17: 1–46.

Ferrer-Hernández, F., 1918. Esponjas del litoral de Asturias. Trabajos del Museo Nacional de Ciencias Naturales 36: 1–39.

Ferrer-Hernández, F., 1922. Más datos para el conocimiento de las esponjas de las costas españolas. Boletin de Pescas 7: 247–272.

Hanitsch, R., 1895. Notes on a collection of sponges from the west coast of Portugal. Transactions Liverpool Biological Society 9: 205–219.

Harley, C. D. G., A. R. Hughes, K. M. Hultgreen, B. G. Miner, C. J. B. Sorte, C. S. Thornber, L. F. Rodriguez, L. Tomanek & S. L. Williams, 2006. The impacts of climate change in coastal marine systems. Ecology Letters 9: 228–241.

Hayes, A., M. Kucera, N. Kallel, L. Sbaffi & E. J. Rohling, 2005. Glacial Mediterranean Sea surface temperatures based on planktonic foraminiferal assemblages. Quaternary Science Reviews 24: 999–1016.

Hewitt, G. M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society 58: 247–276.
Hewitt, G. M., 1999. Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society 68: 87–112.
Hewitt, G. M., 2000. The genetic legacy of the Quaternary Ice Ages. Nature 405: 907–913.
Hewitt, G. M., 2004. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions Royal Society B 359: 183–195.
Hiscock, K., S. Stone & J. D. George, 1984. The marine fauna of Lundy: Porifera (Sponges). Report of the Lundy Field Society 34: 16–35.
Hoarau, G., J. A. Coyer, J. H. Veldsink, W. T. Stam & J. L. Olsen, 2007. Glacial refugia and recolonization pathways in the brown seaweed *Fucus serratus*. Molecular Ecology 16: 3606–3616.
Hooper, J. N. A. & J. A. Kennedy, 2002. Small-scale patterns of sponge biodiversity (Porifera) on Sunshine Coast reefs, eastern Australia. Invertebrate Systematics 16: 637–653.
Hooper, J. N. A. & R. W. M. Van Soest, 2002. Systema Porifera: A Guide to the Classification of Sponges. Kluwer Academic/Plenum Publishers, New York.
Hsü, K. J., W. B. F. Ryan & M. B. Cita, 1973. Late Miocene desiccation. Nature 242: 240–244.
Jansson, R., 2003. Global patterns in endemism explained by past climatic change. Proceedings of the Royal Society London B 270: 583–590.
Johnson, J. Y., 1899. Notes on some sponges belonging to the Clionidae obtained at Madeira. Journal of the Royal Microscopical Society 9: 461–463.
Kefalas, E. & J. Castritsi-Catharios, 2007. Taxonomy of some sponges (Porifera: Demospongiae) collected from the Aegean Sea and description of a new species. Journal of the Marine Biological Association of the United Kingdom 87: 1527–1538.
Kefalas, E., G. Tsirtsis & J. Castritsi-Catharios, 2003. Distribution and ecology of Demospongiae from the circalittoral of the islands of the Aegean Sea (Eastern Mediterranean). Hydrobiologia 499: 125–134.
Klautau, M., C. A. M. Russo, C. Lazoski, N. Boury-Esnault, J. P. Thorpe & A. M. Solé-Cava, 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. Evolution 53: 1414–1422.
Krijgsman, W., F. J. Hilgen, I. Raffi, F. J. Sierro & D. S. Wilson, 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400: 652–655.
Lambeck, K. & J. Chappell, 2001. Sea level change through the last glacial cycle. Nature 292: 679–686.
Lambeck, K., T. M. Esat & E. K. Potter, 2002. Links between climate and sea levels for the past three million years. Nature 419: 199–206.
Lévi, C., 1950. Remarques sur la faune des Spongiaires de Roscoff. Archives de Zoologie expérimentale et générale 87: 10–21.
Lévi, C. & J. Vacelet, 1958. Éponges récoltées dans l'Atlantique Oriental par le "Président Théodore Tissier" (1955–1956). Recueil des Travaux de l'Institut des Pêches maritimes 22: 225–246.
Lopes, M. T., 1989. Demosponjas intertidais da Costa Portuguesa. PhD thesis, Universidade de Lisboa.
Lopes, M. T., 1995. Littoral sponges from Selvagens Islands. Boletim do Museu Municipal do Funchal 4: 387–394.
Lopes, M. T. & N. Boury-Esnault, 1981. Contribution a la connaissance des eponges cornees de la cote de l'Arrabida et de l'Algarve. Arquivos do Museu Bocage 1: 95–109.
Maggs, C. A., R. Castilho, D. Foltz, C. Henzler, M. T. Jolly, J. Kelly, J. Olsen, K. E. Perez, W. Stam, R. Vainola, F. Viard & J. Wares, 2008. Evaluating signatures of glacial refugia for North Alantic benthic marine taxa. Ecology 89: 108–122.
Maldonado, M., 2006. The ecology of sponge larvae. Canadian Journal of Zoology 84: 175–194.
Maldonado, M. & M. J. Uriz, 1995. Biotic affinities in a transitional zone between the Atlantic and the Mediterranean: a biogeographical approach based on sponges. Journal of Biogeography 22: 89–110.
Mariani, S., M. J. Uriz & X. Turon, 2005. The dynamics of sponge larvae assemblages from northwestern Mediterranean nearshore bottoms. Journal of Plankton Research 27: 249–262.
Mariani, S., M. J. Uriz, X. Turon & T. Alcoverro, 2006. Dispersal strategies in sponge larvae: integrating the life history of larvae and the hydrologic component. Oecologia 149: 174–184.
Moss, D. L., 1992. A summary of the Porifera collected during "Expedition Azores 1989". Arquipélago, Life and Earth Sciences 10: 45–53.
Munro, M. H. G., J. W. Blunt, E. J. Dumdei, S. J. H. Hickford, R. E. Lill, S. Li, C. N. Battershill & A. R. Duckworth, 1999. The discovery and development of marine compounds with pharmaceutical potential. Journal of Biotechnology 70: 15–25.
Mustapha, K. B., S. Zarrouk, A. Souissi & A. El Abed, 2003. Diversite des demosponges Tunisiennes. Bulletin Institut National Sciences et Technologie de la Mer de Salammbô 30: 55–78.
Naveiro, A., 2002. Poriferos de la costa da Arrábida (Portugal): Classe Demospongiae. Memoria para optar al grado de licenciado, Universidad de Santiago de Compostela.
Pansini, M. & C. Longo, 2003. A review of the Mediterranean Sea sponge biogeography with, in appendix, a list of the demosponges hitherto recorded from this sea. Biogeographia 24: 59–90.
Patarnello, T., F. M. A. Volckaert & R. Castilho, 2007. Pillars of Hercules: is the Atlantic-Mediterranean transition a phylogeographic break? Molecular Ecology 16: 4426–4444.
Pestana, R., 2002. Sistemática e ecologia de esponjas de substratos rochosos marinhos verticais e inclinados da costa Sul da Madeira. Tese de licenciatura, Universidade da Madeira.
Pflaumamm, U., M. Sarnthein, M. Chapman, L. d'Abreu, B. Funnell, M. Huels, T. Kiefer, M. Maslin, H. Schulz, J. Swallow, S. van Kreveld, M. Vautravers, E. Vogelsang & M. Weinelt, 2003. Glacial North Atlantic: sea surface conditions reconstructed by GLAMAP 2000. Paleoceanography 18: 10–28.
Picton, B. E. & C. Goodwin, 2007. Sponge biodiversity of Rathlin Island, Northern Ireland. Journal of the Marine Biological Association of the United Kingdom 87: 1441–1458.

Pires, F., 2007. Padrões de distribuição e taxonomia para os Porifera da região central do Algarve. Tese de mestrado, Universidade do Algarve.

Preciado, I., 2002. Desmosponjas litorales del entorno de la Isla de Mouro (Santander, Mar Cantábrico): Taxonomia y Ecologia. PhD thesis, Facultad de Ciencias de la Universidad Autónoma de Madrid, Spain.

Provan, J., R. Wattier & C. Maggs, 2005. Phylogeographic analysis of the red seaweed *Palmaria palmata* reveals a Pleistocene marine glacial refugium in the English Channel. Molecular Ecology 14: 793–803.

Reverdin, G., P. P. Niiler & H. Valdimarsson, 2003. North Atlantic Ocean surface currents. Journal of Geophysical Research 108(C1): 3002.

Riesgo, A. & M. Maldonado, 2008. Differences in reproductive timing among sponges sharing habitat and thermal regime. Invertebrate Biology 127: 357–367.

Rouchy, J. M. & A. Caruso, 2006. The Messinian salinity crisis in the Mediterranean basin: a reassessment of the data and an integrated scenario. Sedimentary Geology 188: 35–67.

Samaai, T., 2006. Biodiversity "hotspots", patterns of richness and endemism, and distribution of marine sponges in South Africa based on actual and interpolation data: a comparative approach. Zootaxa 1358: 1–37.

Sarà, M. & J. Vacelet, 1973. Écologie des Démosponges. In Grassé, P. (ed.), Traité de Zoologie, Spongiaires. Masson et Cie, Paris: 462–576.

Solórzano, M. R., 1991. Inventario dos Poríferos do Litoral Galego (Porifera). Caderno da Área de Ciencias Biolóxicas 7: 1–54.

Spalding, M. D., H. E. Fox, G. R. Aleen, N. Davidson, Z. A. Ferdaña, M. Finlayson, B. Halpern, M. A. Jorge, A. Lombana, S. A. Lourie, K. D. Martin, E. McManus, J. Molnar, C. A. Recchia & J. Robertson, 2007. Marine ecoregions of the world: a bioregionalization of coastal and shelf areas. BioScience 57: 573–583.

Tendal, O. S., T. Brattegard & H. T. Rapp, 2001. Phylum Porifera. In Brattegard, T. & T. Holthe (eds), Distribution of Marine, Benthic Macro-organisms in Norway. A Tabulated Catalogue. Research Report for DN 2001–3. Directorate for Nature Management: 36–51.

Tintore, J., P. E. La Violette, I. Blade & A. Cruzado, 1988. A study of an intense density front in the Eastern Alboran Sea: the Almeria-Oran front. Journal of Physical Oceanography 18: 1384–1397.

Topsent, E., 1892. Contribution á l'étude des Spongiaires de l'Atlantique Nord (Golfe de Gascogne, Terre-Neuve, Açores). Résultats des Campagnes Scientifiques accomplies par le Prince Albert I Monaco 2: 1–165.

Topsent, E., 1899. Documents sur la faune des Spongiaires des côtes de Belgique. Archives de Biologie 16: 105–115.

Topsent, E., 1904. Spongiaires des Açores. Résultats des Campagnes Scientifiques accomplies par le Prince Albert I Monaco 25: 1–280.

Topsent, E., 1928. Spongiaires de l'Atlantique et de la Méditerranée provenant des croisières de Prince Albert 1er de Monaco. Résultats des Campagnes Scientifiques accomplies par le Prince Albert I Monaco 74: 1–376.

Uriz, M. J., X. Turon & S. Mariani, 2008. Ultrastructure and dispersal potential of sponge larvae: tufted versus evenly ciliated parenchymellae. Marine Ecology 29: 280–297.

Vacelet, J., G. Bitas, S. Carteron, H. Zibrowius & T. Perez, 2007. Five new sponge species (Porifera: Demospongiae) of subtropical or tropical affinities from the coast of Lebanon (eastern Mediterranean). Journal of the Marine Biological Association of the United Kingdom 87: 1539–1552.

Van Soest, R. W. M., 1993a. Distribution of sponges on the Mauritanian continental shelf. Hydrobiologia 258: 95–106.

Van Soest, R. W. M., 1993b. Affinities of the marine demosponge fauna of the Cape Verde Islands and Tropical West Africa. Courier Forschungsinstitute Senckenberg 159: 205–219.

Van Soest, R. W. M., 1994. Demosponge distribution patterns. In Van Soest, R. W. M., Th. M. G. Van Kempen & J. C. Braekman (eds), Sponges in Time and Space. Balkema, Rotterdam: 213–223.

Van Soest, R. W. M. & S. Weinberg, 1980. A note on the sponges and octocorals from Sherkin Island and Lough Hyne, Co. Cork. Irish Naturalists Journal 20: 1–14.

Van Soest, R. W. M., J. D. Guiterman & M. Sayer, 1983. Sponges from Roaringwater Bay and Lough Hyne. Journal of Sherkin Island 1: 35–49.

Van Soest, R. W. M., B. E. Picton & C. Morrow, 2000. Sponges of the North East Atlantic. World Biodiversity Database CD-ROM Series, ETI. University of Amsterdam, The Netherlands.

Van Soest, R. W. M., D. F. R. Cleary, M. J. de Kluijver, M. S. S. Lavaleye, C. Maier & F. C. van Duyl, 2007a. Sponge diversity and community composition in Irish bathyal coral reefs. Contributions to Zoology 76: 121–142.

Van Soest, R. W. M., M. J. Kluijver, P. H. Van Bragt, M. Faasse, R. Nijland, E. J. Beglinger, W. H. De Weerdt & N. J. de Voogd, 2007b. Sponge invaders in Dutch coastal waters. Journal of the Marine Biological Association of the United Kingdom 87: 1733–1748.

Van Soest, R. W. M, N. Boury-Esnault, J. N. A. Hooper, K. Rützler, N. J. de Voogd, B. Alvarez, E. Hajdu, A. B. Pisera, J. Vacelet, R. Manconi, C. Schoenberg, D. Janussen, K. R. Tabachnick & M. Klautau, 2009. World Porifera Database [available online at http://www.marinespecies.org/porifera]. Accessed 15 Jan 2009.

Voultsiadou, E., 2005. Demosponge distribution in the Eastern Mediterranean: a NW-SE gradient. Helgoland Marine Research 59: 237–251.

Voultsiadou, E., 2009. Reevaluating sponge diversity and distribution in the Mediterranean Sea. Hydrobiologia 629: 1–12.

Warwick, R. M. & K. R. Clarke, 1995. New biodiversity measures reveal a decrease in taxonomic distinctness with increasing stress. Marine Ecology Progress Series 129: 301–305.

Warwick, R. M. & K. R. Clarke, 1998. Taxonomic distinctness and environmental assessment. Journal of Applied Ecology 35: 532–543.

Xavier, J. R., 2003. *Esponjas marinhas* (Porifera: Demospongiae e Calcarea) do litoral de São Miguel – Açores: abordagem taxonómica. Tese de licenciatura, Universidade dos Açores.

Xavier, J. R. & R. W. M. Van Soest, 2007. Demosponge fauna of Ormonde and Gettysburg Seamounts (Gorringe Bank, Northeast Atlantic): diversity and zoogeographical

affinities. Journal of the Marine Biological Association of the United Kingdom 87: 1643–1653.

Xavier, J. R., R. W. M. Van Soest, J. A. J. Breeuwer, A. M. F. Martins & S. B. J. Menken, 2010a. Phylogeography of the poecilosclerid sponge *Phorbas fictitius*: genetic structure and refugia at oceanic islands. Contributions to Zoology 79: 119–129.

Xavier, J. R., P. G. Rachello-Dolmen, F. Parra-Velandia, C. H. L. Schönberg, J. A. J. Breeuwer & R. W. M. Van Soest, 2010b. Molecular evidence of cryptic speciation in the "cosmopolitan" excavating sponge *Cliona celata* (Porifera, Clionaidae). Molecular Phylogenetics and Evolution 56: 13–20.

Yokoyama, Y., K. Lambeck, P. Deckker, P. Johnston & L. K. Fifield, 2000. Timing of the Last Glacial Maximum from observed sea-level minima. Nature 406: 713–716.

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SPONGE RESEARCH DEVELOPMENTS

Living on the edge: the sponge fauna of Australia's southwestern and northwestern deep continental margin

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Abstract This first assessment of sponges on Australia's deep western continental margin (100–1,100 m) found that highly species-rich sponge assemblages dominate the megabenthic invertebrate biomass in both southwestern (86%) and northwestern (35%) areas. The demosponge orders Poecilosclerida, Dictyoceratida, Haplosclerida, and Astrophorida are dominant, while the presence of the order Agelasida, lithistid sponges, and the Verongida are noteworthy in providing contrasts to other studies from the deep temperate Australian margin. Most sponge species appeared to be rare as two-thirds were present in only one or two samples—a finding consistent with studies of the shallow Australian sponge fauna. The Demospongiae and Calcarea had similar distribution and abundance patterns being found in the greatest numbers in the south on the outer shelf and shelf edge in hard substrates. In contrast, the Hexactinellida were more abundant at deeper depths and in soft substrates, and were more common in the north. Although the environmental factors that influence sponge distributions on the western margin cannot be completely understood from the physical covariates analyzed in this study, the data suggest depth-related factors, substrate type, and current regimes are the most influential. Incompletely documented historic demersal trawling may partly account for the lower sponge biomass found in the north. The potentially high importance of sponges to benthic ecosystems, as well as the potential for high impacts on sponges by bottom trawling, indicates that maintaining healthy sponge assemblages should be an important consideration for marine conservation planners. Successful management will need to be underpinned by additional research that better identifies the ecological roles of sponges, and their distributions over local and broad environmental scales.

Keywords Continental margin · Porifera · Diversity · Western Australia

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Introduction

Continental margins host some of the highest species diversity in the oceans (Grassle & Maciolek, 1992).

Deep benthic diversity is often enhanced by the presence of habitat-forming biota (Buhl-Mortensen et al., 2010), with deep sea sponge assemblages recognized as forming complex structural living space for large numbers of species from many taxa (Buhl-Mortensen et al., 2010; Howell et al., 2010). The potentially high ecological importance of deep sea sponges is indicated by their roles in coral reef ecosystems; these are suggested by Rützler (2004) to include habitat provision, predation, space competition, chemical defense, primary production, nutrient cycling, nitrification, food chains, bioerosion, mineralization, and cementation of substrates.

Western Australia encompasses one-third of Australia's coastline and has a topographically and oceanographically diverse continental margin. Emergent paleo-coastlines are a feature of the deep continental shelf in northern areas, and the south below 31°S; large rocky banks occur in the central west around the Houtman Abrolhos, while coarse sediments are found in the south around Point Hillier and Bald Island. The deeper margin includes small seamounts, rocky banks and plateaus, and many submarine canyons including large features off Kalbarri, Two Rocks, and Perth Williams et al., 2010). The deep margin is influenced by complex oceanography (Ridgway & Condie, 2004; Waite et al., 2007): the Leeuwin Current, a warm, oxygen poor, low salinity, tropically originating current flows southward from Ningaloo along the shelf and shelf edge to a depth of ~250 m; at greater depths, the upper continental slope from 250 to 400 m is influenced by the northward flowing cool, oxygen-rich, Leeuwin Undercurrent, and the deep upper slope greater than 400-m depth by Antarctic Intermediate waters.

Despite the massive area and environmental complexity of Western Australia's deep margin, there was little information available of its benthic biodiversity, and there were no systematic surveys of its sponge fauna deeper than diving depths (~30 m) before 2005. This situation changed after two wide ranging surveys of benthic epifauna in 2005 and 2007 that collected sponges from depths between 100 and 1,100 m between latitudes 12°S to 36°S. Results from the first survey provided a regional scale account of habitat heterogeneity and megabenthos biodiversity based on distribution patterns of sponges, crustaceans, molluscs, echinoderms, ascidians, and corals (Williams et al. (2010). A species checklist of those taxa was presented by McEnnulty et al. (2011) and photographic information collected during the 2005 survey has been used to evaluate the potential of geomorphic features to act as surrogates for benthic biodiversity distributions (Althaus et al., 2011). The faunal data from the 2007 survey had not previously been used for analyses of biodiversity distributions.

This article presents the first integration of data from the two surveys by examining the sponge fauna of the outer continental shelf, shelf edge and continental slope adjacent to the southwestern and northwestern coasts of Australia (36°S to ~12°S, 112°E to 124°E). The article has two primary aims: first, to document the diversity and abundance of sponges on the continental margin, and second, to examine environmental influences on sponge distribution patterns. Our findings are also discussed in the context of biodiversity conservation planning on Australia's western continental margin.

Methods

Field collections

Sponges were collected along with other benthic marine invertebrates on two surveys on *RV Southern Surveyor* in 2005 and 2007 (SS200510 and SS200705). The 2005 survey sampled Australia's southwestern margin (~22°S to 36°S); the 2007 survey continued the sampling program along the northwestern margin (12°S to 22°S). The field design detailed below was chosen to sample at four nested hierarchical spatial scales: biogeographic provinces, bathomes (ecologically meaningful depth zones), geomorphic features, and substrates (Williams et al., 2010; Althaus et al., 2011).

Samples were collected systematically at sites located at one degree longitudinal or latitudinal intervals off Australia's western continental margin, following the coastline from Bald Island to Ashmore Reef. The sites ranged in latitude from 12°S to 36°S and longitude from 112°E to 124°E (over 3,400 km of margin). One additional site at the Perth Canyon was included as a geological feature of special interest, resulting in 31 sites being surveyed (Fig. 1). This sampling approach was designed to position sampling

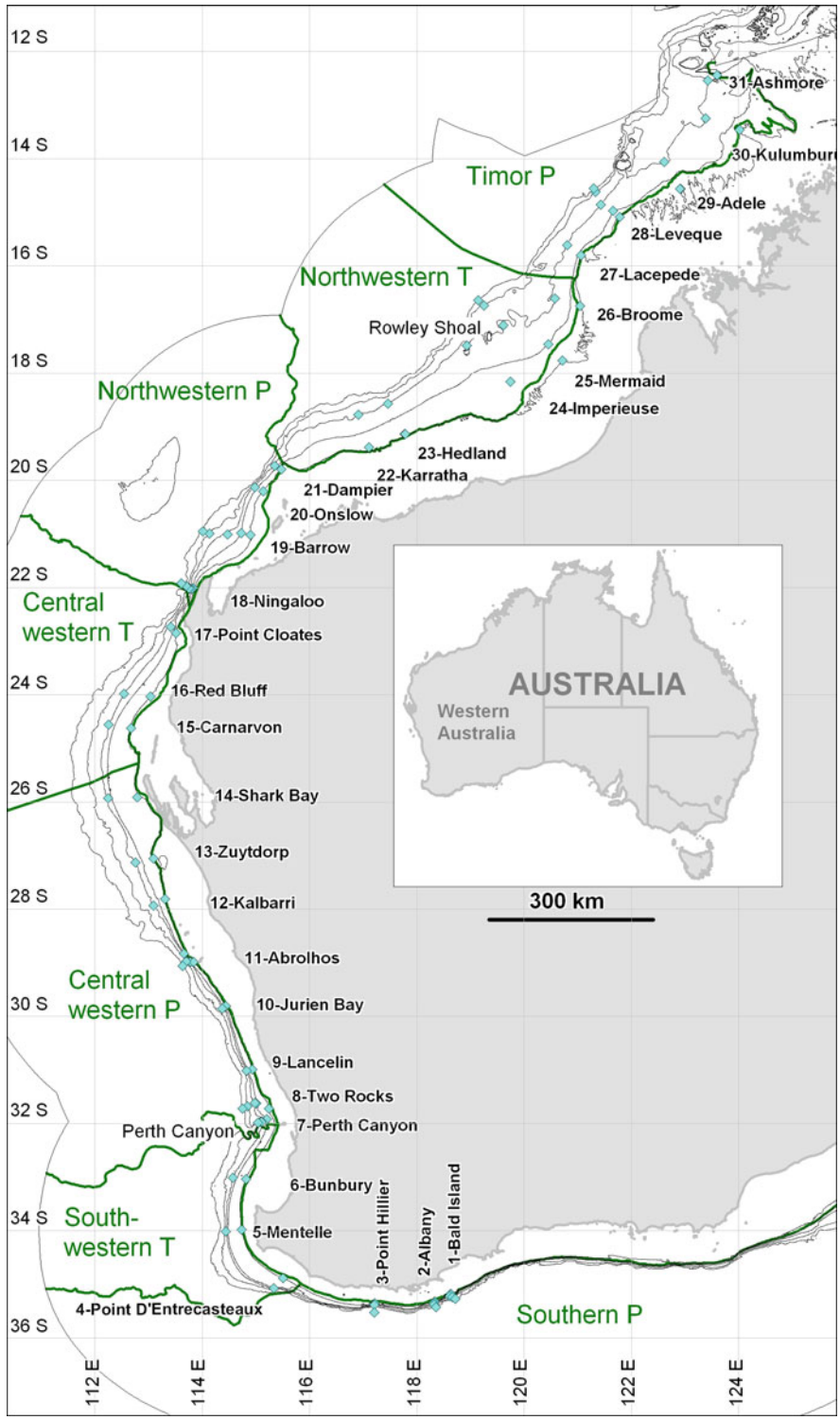

Fig. 1 Map of the study region showing the 31 sites, depth contours (100, 200, 400, 700, and 1000 m), and provincial scale bioregions (Commonwealth of Australia, 2005). *Diamonds* indicate the average location of samples taken by study site and bathome (ecologically meaningful depth zones). The locations of the Perth Canyon and the Rowley Shoals are shown. *T* transition zone and *P* province

locations within each of the four biogeographic provinces and the three transition zones adjacent to Australia's western coastline that have been designated in the national marine bioregionalization scheme (Commonwealth of Australia, 2005) (boundaries are shown in Fig. 1).

Table 1 Number of samples taken over two surveys by gear type: (a) for each bathome (ecologically meaningful depth zones after Last et al., 2010); and (b) for each substrate type

Total number of samples:	Depth (m)	Sherman sled 72	Beam trawl 117	McKenna trawl 13	Total 202
(a) Bathome					
Outer shelf	~80–150	39	25	4	68
Shelf-break	150–250	6	12	3	21
Upper slope (shallow)	250–500	14	54	3	71
Upper slope (deep)	500–800	3	10	2	15
Mid-slope (shallow)	800–1,100	9	15		24
Mid-slope (deep)	1,100–1,500	1	1	1	3
(b) Substrate					
Hard		44	17	4	65
Mixed		17	19	1	37
Soft		11	81	8	100

At each site, samples were collected from two ecologically meaningful depth zones (bathomes sensu Last et al., 2010): the outer continental shelf (~100 m depth) and shallow upper continental slope (~400 m). At selected sites within each province, the sampling design was extended to include additional samples at the following bathomes: continental shelf break (~200 m), deep upper continental slope (~700 m), and shallow mid-continental slope (~1,000 m). Additional samples were taken at the northwestern offshore atolls comprising the Rowley Shoals (at sites Imperieuse and Mermaid), and three very deep samples were taken on the mid-continental slope at 1,200 m at Ningaloo and Leveque, and at 1,500 m in the Perth Canyon (Table 1). The substrate of each sampling location was classified as hard, soft or mixed based on visual examination of backscatter maps generated by multibeam acoustic surveys of the same areas. Separate soft and hard substrates were sampled within depths if both types were present at a survey site (Table 1). Owing to time constraints, only a few replicate samples were taken.

Sampling equipment included two epibenthic samplers with 25-mm stretched-mesh net cod ends to collect the megabenthos. The Sherman sled (Lewis, 1999, 2009) was used for sampling hard substrates; and a modified version of the French Institute de Recherche pour le Dévelopment (IRD) designed and CSIRO modified light beam trawl (Forest, 1981; Lewis, 2010) was used for sampling mainly soft substrates (Table 1). At one site (Lacepede), a demersal fish trawl (McKenna trawl) was also used. Geolocation of samples was based on the ship's GIS. The tow track and distance were derived from dynamic GIS mapping and the duration of bottom contact. Tow direction and duration were dependent on the roughness and topography of the substrate. In general, tows were along depth contours and of about 20-min duration (but were less for samples when the sled was full of catch within 5–10 min). Images and basic descriptions of gear types can be found at http://www.cmar.csiro.au/research/seamounts/epibenthic.htm.

Shipboard and laboratory processing

The total sponge catch was weighed for each sample and sponges were separated into morphospecies and weighed to the nearest gram to give a species weight for each sample. Voucher specimens were labeled with a field number and name and photographed with a scale bar and label to record live color. Details were recorded on field datasheets. Voucher specimens were preserved in 75% ethanol, and some large specimens were frozen. Specimens were identified by examining classical taxonomic characters including morphological, skeletal, and spicule features. Spicule preparations and skeletal slides were made for each specimen as per the methods outlined in Fromont et al., (2008). Specimens of the class Demospongiae except for the lithistids were identified to a species level operational taxonomic unit (OTU), and given a species code. Specimens of the classes Calcarea and Hexactinellida were confirmed in the laboratory and

recorded at class level. The Calcarea and lithistids are being identified in separate studies. All the specimens have been lodged in the collections of the Western Australian Museum. Specimens smaller than 5 g (less than the 25 mm mesh size of the epibenthic samplers) were not identified but have been retained at the WA Museum pending future study.

Data preparation

Data were analyzed at the class level: Demospongiae, Calcarea, Hexactinellida, and the dominant class Demospongiae (excluding the lithistids) were analyzed at species level. The biomass (wet catch weight) was standardized to g m^{-2} based on the area sampled (mouth width * tow distance) for each sample. The McKenna trawl samples from a single site were not comparable in sampling effort, and were excluded from the statistical analyses.

Analyses

Analyses at class level were based on sponge biomass, averaged across all the samples by site and bathome. Univariate contrasts in the distribution over the three substrate types (hard, mixed, and soft) were made with the non-parametric Kruskal–Wallis test. Species accumulation curves, based on the non-lithistid demosponge species, were produced for each province in Primer-e v6 (Clarke & Gorley, 2006), using 999 permutations. To make the curves comparable, they were scaled to the total area sampled in each province.

For multivariate analyses, the standardized biomass data at class and species-level were square-root transformed to reduce the effect of dominant taxa. The Bray–Curtis dissimilarity between samples was visualized in a non-metric multi-dimensional scaling (nMDS) ordination (Clarke, 1993), and the differences between a priori groups of provinces and bathomes was tested using analysis of similarities (ANOSIM—Clarke, 1993). The relationship of the multivariate community structure, at class and at species level, with the environmental covariates (described below) was examined using distanced-based linear models (DISTLM). This method models the relationship between the multivariate data cloud (Bray–Curtis dissimilarity of biological data) and multiple predictor variables (environmental covariates) based on a multivariate regression (Anderson et al., 2008). In case a relationship between the covariates and the biological data was found, distance-based redundancy analysis (dbRDA) was used for graphical representation of these results (Legendre & Anderson, 1999). The environmental covariates were superimposed onto the dbRDA plot as vectors whose direction and length are related to their partial correlation with the dbRDA axes (i.e. the role they played in generating them), creating a biplot (Clark et al., 2010).

Environmental covariate data for each sample's location were extracted from three databases: (1) Geoscience Australia—MARS modeled sediment data (http://www.ga.gov.au/oracle/mars/index.jsp); (2) CSIRO Atlas of Regional Seas—an interpolated oceanographic dataset (CARS 2006—http://www.marine.csiro.au/~dunn/cars2006/; Dunn & Ridgway, 2002; Ridgway et al., 2002); and (3) SeaWiFS—satellite seasurface data (http://oceancolor.gsfc.nasa.gov/SeaWiFS). Twenty-eight covariates were tested: depth, latitude, longitude (from survey data), aspect, slope, carbonate content, percentage of gravel, sand and mud (MARS), mean and variability (intra-annual standard deviation—SD) of oxygen (O_2), salinity, temperature, nitrates (NO_3), phosphates (PO_4) and silicates (Si) at sampling depth (CARS), and mean and variability (intra-annual standard deviation—SD) of SeaWiFS surface measures of productivity (Chlorophyll *a*), turbidity (K490), irradiation, and sea surface temperature (SST).

Results

Overview of sponge collection

In total, 202 samples were taken over six depth zones (bathomes); 72 with the Sherman sled, 117 with the beam trawl and 13 with the McKenna trawl (Table 1). Seventy-five samples did not contain sponges, and these were predominantly deep bathomes and soft substrates. Sherman sleds were predominantly used on hard substrata (61%) and beam trawl and McKenna trawl on soft substrata (69% and 62% respectively, Table 1).

Porifera dominated the entire invertebrate catch by biomass; in the southwestern survey (SS200510), sponges accounted for 86% of the total catch weight

(see McEnnulty et al., 2011), and in the northwestern survey (SS200705), they accounted for 35%—followed by echinoderms (30%) and cnidarians (20%).

The largest Demospongiae specimen collected was an individual of the genus *Biemna* that weighed 37 kg and was collected at 200-m depth off Point Hillier in the southern province. The largest single catch of Calcarea was more than 1,700 individuals (total weight 850 g) taken from 400-m depth at Two Rocks in the central western province. In total, 941 kg of sponges and more than 4,600 individuals were collected from the 127 samples containing sponges (Table 2). The Sherman sled collected the majority of the sponge samples (758 kg, ~78%), followed by the beam trawl (177 kg, ~18%), and the McKenna trawl (42 kg, ~4% of the total catch, Table 2). The sled collected the majority of the Demospongiae (78%) and Calcarea (63%) and the McKenna trawl collected the least (4 and 0%, respectively), whereas the beam trawl collected most of the Hexactinellida (63%) (Table 2). The McKenna trawl samples were not comparable to the beam trawl and sled samples in sampling effort, and thus were excluded from the statistical analyses.

Distribution of sponge classes

Sponges were collected in all biogeographic provinces and transition zones sampled but predominantly from the outer shelf and shelf break bathomes; glass sponges (Hexactinellida) were sampled to 1,000-m depth on the mid slope (Fig. 2; Table 3). Most of the sponge biomass (averaged by sampling effort) was collected in the southern province and southwestern transition zone on the outer shelf and shelf break (197 g m^{-2}, Fig. 2). High biomass of sponges was also seen in the central western (50 g m^{-2}) and north western provinces (15 g m^{-2}). Lower biomass occurred in the other three biogeographic regions (~9 g m^{-2}; Fig. 2; Table 3).

The Demospongiae (including lithistids) comprised the majority (98.31%) of the sponge biomass collected (Table 2) and were found principally in the outer shelf and shelf break bathomes (Table 3) in the southernmost temperate provinces (Fig. 2a). Only very low abundances were detected in the central western transition zone. In general, demosponge biomass decreased with decreasing latitude, with the exception of the northwestern province, in particular at Ningaloo and Onslow (sites 18 and 20), but also at Imperieuse (site 24) in the northwestern transition zone, and at Adele (site 29) in the Timor province (Fig. 2a). Within the total 90 samples that contained demosponges, their biomass was significantly higher in hard (mean = 22.25 g m^{-2}) than on soft (mean = 0.220 g m^{-2}) or mixed (mean = 6.182 g m^{-2}) biotopes (Kruskal–Wallis test: 67.91, $P < 0.0001$).

The lithistid demosponges were only collected in the northwestern and central western provinces and transition zones; they were not found south of Perth Canyon (site 7) at 32°S latitude (Fig. 2a). The largest biomass of lithistids was collected from site 20 at Onslow in the north western province (Fig. 2a). In total, 26 samples collected lithistids.

The Calcarea comprised a very low proportion (0.36%) of the sponge biomass collected in the study (Table 2). Most of the biomass of this class was

Table 2 Number of individuals and catch weights of sponges sampled by Class; also shown is the catch weight by gear type

	No. of stations	No. of individuals	Total catch weight (kg)	% of total weight	Sherman sled (kg)	Beam trawl (kg)	McKenna trawl (kg)
Porifera total	127	4,657	976.687		758.124	176.532	42.042
Demospongiae total	119	2,521	960.174	98.31	753.425	166.930	39.820
Lithistids	27	122	35.702	3.66	31.907	3.757	0.038
Other	92	2,399	924.472	94.65	721.518	163.173	39.782
Calcarea	17	1,883	3.557	0.36	2.192	1.365	0.000
Hexactinellida	47	131	12.829	1.31	2.470	8.147	2.222
Unidentified	10	122	0.127	0.01	0.037	0.090	0.000

The Demospongiae are separated into lithistids and other (all other Orders); the latter were identified to species for all samples

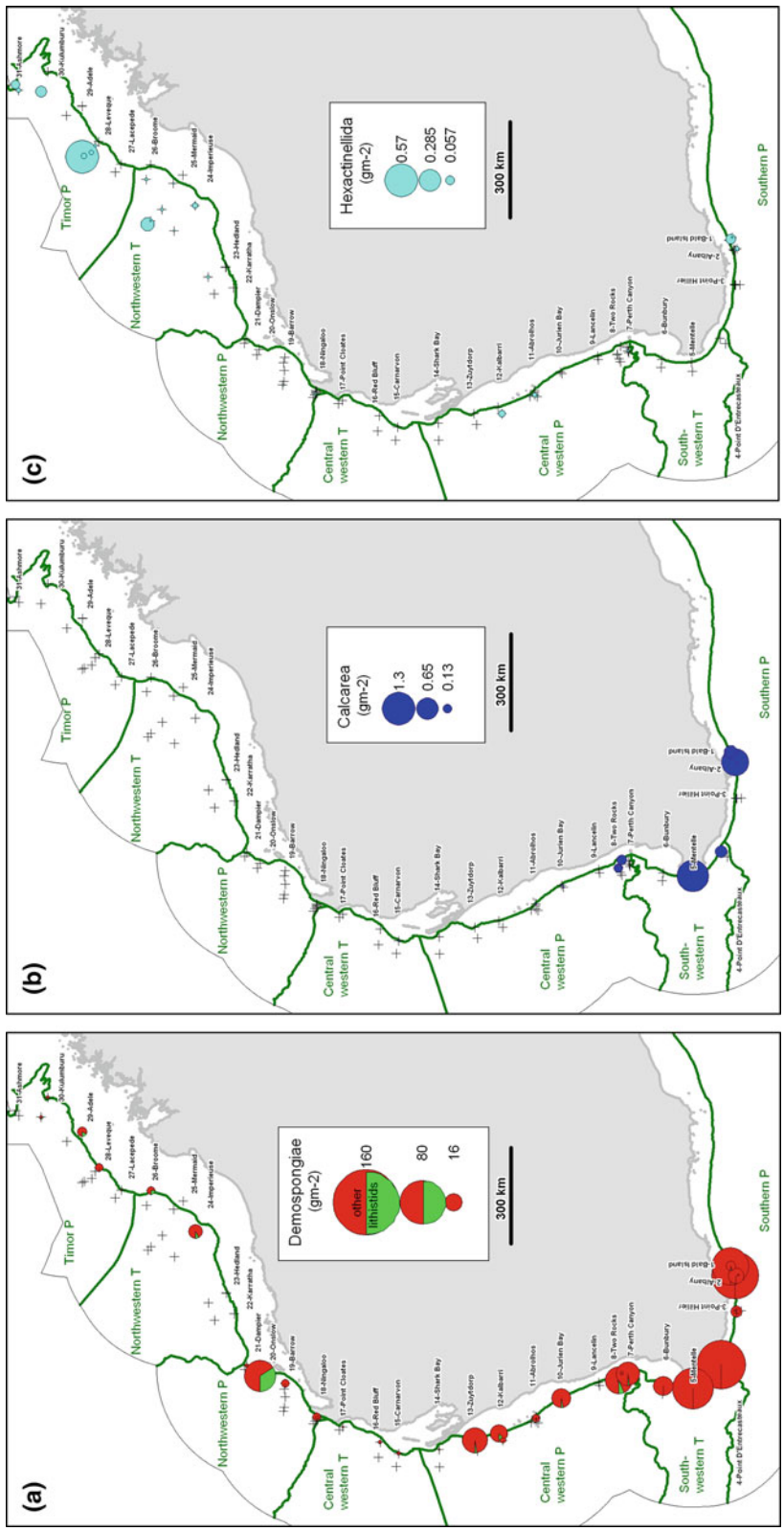

Fig. 2 Map of Australia's western coast showing the 31 sites and the provincial scale bioregions (Commonwealth of Australia, 2005). *Graded circles* show the averaged biomass of **a** Demospongiae (subdivided to show the proportion of lithistids), **b** Calcarea, **c** Hexactinellida collected at each sampled site-bathome combination; the *plus-symbol* indicates site-bathome combinations where no biomass of the respective classes was recorded

Table 3 Averaged standardized biomass (g m^{-2}) for the three sponge classes by province and bathome (including an indicative depth)

Province (S to N)	No. of sites	Bathome	Indicative depth (m)	No. of samples	Demospongiae (g m^{-2})	Calcarea (g m^{-2})	Hexactinellida (g m^{-2})
SP	3	Outer shelf	100	7	83.84	0.31	
		Shelf-break	200	5	13.72	0.12	
		Upper slope shallow	400	6	1.30		
		Upper slope deep	700	4	0.02		0.02
		Mid-slope shallow	1,000	11	0.13		0.01
SWT	3	Outer shelf	100	3	99.26	0.49	
		Upper slope shallow	400	6	0.01		
CWP	8	Outer shelf	100	14	15.74	0.01	
		Shelf-break	200	3	34.38	0.02	
		Upper slope shallow	400	15	0.63	0.02	0.01
		Upper slope deep	700	3			0.01
		Mid-slope shallow	1,000	5			0.00
		Mid-slope deep	1,200	1			
CWT	3	Outer shelf	100	7	1.65	0.00	
		Upper slope shallow	400	6	0.00		
NWP	4	Outer shelf	100	14	14.18		
		Shelf-break	200	4	0.97		
		Upper slope shallow	400	10	0.01		
		Upper slope deep	700	4			0.00
		Mid-slope shallow	1,000	4			0.00
		Mid-slope deep	1,200	1			
NWT	5	Outer shelf	100	13	4.81		0.01
		Shelf-break	200	6	0.02		0.00
		Upper slope shallow	400	18	0.01		0.01
		Upper slope deep	700	3			0.00
		Mid-slope shallow	1,000	2	0.00		0.12
		Mid-slope deep	1,200	1			
Timor P	5	Outer shelf	100	10	2.44		0.01
		Shelf-break	200	3	0.00		0.01
		Upper slope shallow	400	10	0.35		0.03
		Upper slope deep	700	1			0.02
		Mid-slope shallow	1,000	2	0.00		0.57

Number of sites and samples collected also provided

SP Southern province, *SWT* southwestern transition, *CWP* central western province, *CWT* central western transition, *NWP* northwestern province, *NWT* northwestern transition, *Timor P* Timor province

collected in the southern province and southwestern transition zone on the outer shelf and shelf break (Fig. 2b) on hard (10) or mixed (4) substrates. Two upper slope (soft substrate) samples contained Calcarea. This class was not collected north of 24.5°S latitude at Carnarvon (site 15) in the central western transition zone. In total, 17 samples collected Calcarea which were significantly more commonly sampled in hard (mean = 0.0667 g m^{-2}) and mixed (mean = 0.0124) than on soft (mean = 0.0.003 g m^{-2}) substrates (Kruskal–Wallis test: 8.164, $P = 0.017$).

The Hexactinellida represented only 1.31% of the sponge biomass collected (Table 2). This class was collected in the southern and central western provinces with none being collected in the southwestern or central western transition zones (Fig. 2c). A low biomass of glass sponges was also collected from site 18 at Ningaloo in the northwestern province. North of this province, glass sponge biomass increased with decreasing latitude with most glass sponge biomass being collected in the tropical Timor province, in particular at Leveque (site 28). In total, 42 samples collected Hexactinellida with the majority being collected from soft substrates in the shallow upper shelf and deeper slope (27 samples), with fewer from hard substrates in the outer shelf to mid-slope bathomes (9 samples), and from mixed substrate in the shelf break to mid-slope bathomes (6 samples). Glass sponges were found at shallower depths on hard substrates in lower latitudes but the general trend was for them to be collected from soft substrates on the slope bathomes (Fig. 2c). Statistically, there were no significant differences in the distribution of the Hexactinellida over the three substrates—$mean_{hard} = 0.0086$ g m^{-2}; $mean_{mixed} = 0.0304$ g m^{-2}; $mean_{soft} = 0.0086$ g m^{-2} (Kruskal–Wallis test: 4.334, $P = 0.115$).

Analysis of similarity of the distributions of the sponge classes detected a significant difference between the bathomes across provinces $R = 0.442$, $P = 0.001$, in particular the outer shelf and shelf-break differed strongly from the deeper bathomes. There were no significant differences between provinces across bathomes detected in the multivariate distribution of the sponge classes.

Seven of the 28 environmental variables considered in the DISTLM model account for 37% of the variance in the distribution of the three classes over the samples. Depth was the most influential explaining 18% of the variation, the sequential addition of six other covariates accounted for the remaining 19%—nitrate (+4% of variance explained), phosphate (+4%), chlorophyll *a* (+2%), and variability (SD) in silicate (+2%), chlorophyll *a* (+2%), and irradiation (+1%). The marginal tests showed that in regard to single covariates, depth accounted for most of the variance (18%), followed by chlorophyll *a* (12%), silicate (11%), and nitrate (10%); the remaining covariates made less than 10% individual contributions. Figure 3a gives a visual impression of the distribution of the samples, coded by bathome, in the covariate space, with the first two dbRDA axes explaining 33% of the variance in the fitted data. The biplot also shows that depth is negatively correlated to the variability in silicates (SD) and to the irradiance (their vectors point in the opposite direction to the depth vector). Thus, the effect of each of these variables cannot be fully separated from the others. Depth showed little correlation to nitrate and variability in chlorophyll *a* (SD) (their vectors are near perpendicular to the depth vector).

Among classes, Fig. 3b shows that the Demospongiae distribution was clearly associated with decreasing depth and/or increasing variability in silicates, as measured by the standard deviation (SD), with the highest biomass in the shallowest depths where the silicates were the most variable and irradiance was the highest. A slight positive association with chlorophyll *a* can be seen in Fig. 3b. The distribution of the Calcarea (Fig. 3c) was very similar to the Demospongiae. In contrast, the Hexactinellida distribution (Fig. 3d) was associated with increasing depth with the highest biomass at deeper depths on the shelf break and upper slope, where silicate supplies were more constant—i.e., low silicate variability.

Demospongiae composition and distribution

The Demospongiae (excluding the lithistids) in the southwestern (SS200510) survey were identified to be 451 species in 141 genera, 51 families, and 11 orders (see McEnnulty et al., 2011). Of the 154 species collected and identified in the northwestern survey (SS200705), 72 had been collected in the south but have an extended range along the Western Australian coast as a consequence of this study, and 50 have extended depth ranges as a result of this

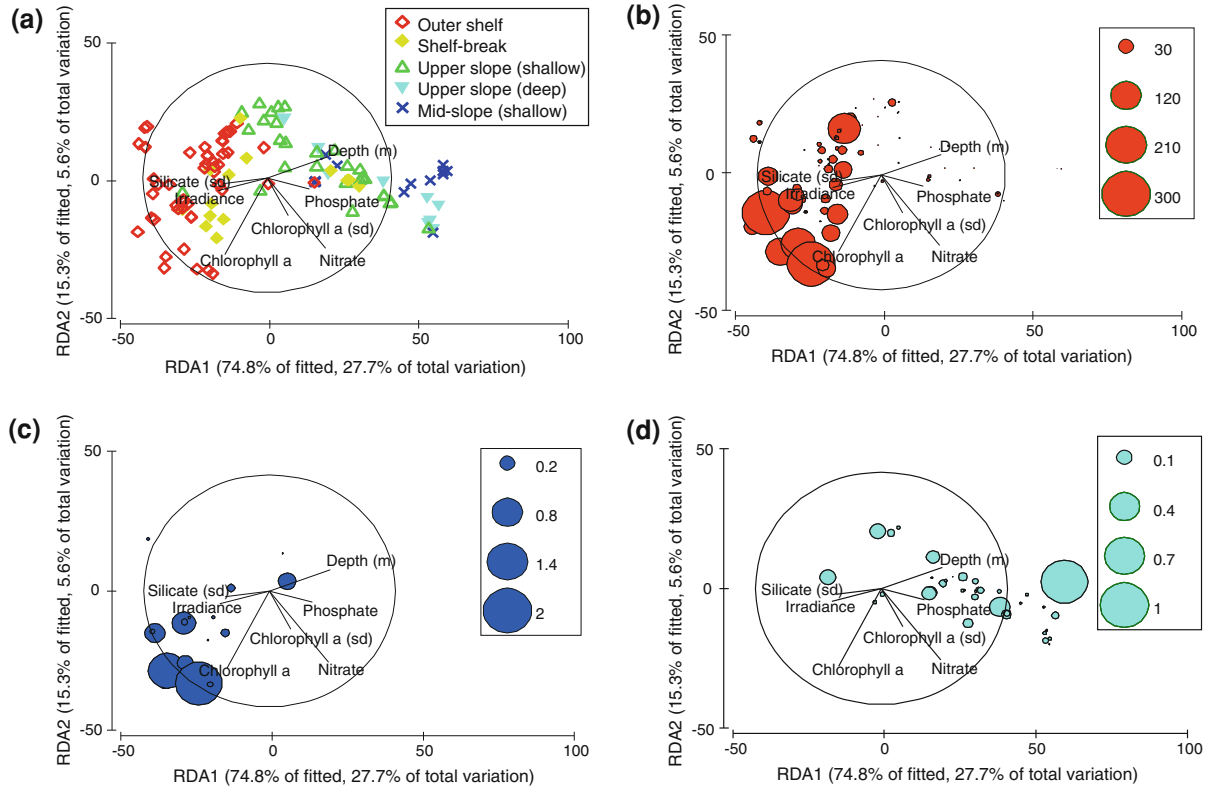

Fig. 3 Sample distribution in relation to the first two dbRDA axes explaining 90.1% of the variance in the data. Samples are coded by **a** symbols for bathomes, and **b**–**d** bubbles graduated by the abundance of **b** Demospongiae, **c** Calcarea, **d** Hexactinellida; the *lines* indicate the direction and influence of the relative covariates in the dbRDA space; the *circle radius* is equal to the unity vector

publication. The remaining 82 species were only found in the northern survey (see species list in supplementary material).

The order Poecilosclerida was the most speciose (110 species) followed by the Dictyoceratida (81), Haplosclerida (75), and Astrophorida (70 species). Of the demosponge species collected 205 (45.5%) were present in only a single sample (singletons), and 105 species (23.3%) were in two samples (doubletons). *Sarcotragus* Ng1 was the most widely distributed species, collected in 14 samples, from Bald Island to Dampier (sites 1–21). At the genus level, 25.5% of genera were only collected from a single sample and 14.9% were collected from two samples. The genus *Sarcotragus* was the most widely distributed (31 samples). The majority of species were relatively restricted in their distributions, however this result may in part be due to the lack of replication in the sample design, and the distance of 1° of latitude between sites.

Analyses of demosponge species composition were restricted to the sub-set of outer shelf samples in which they were highly abundant. Species accumulation curves by province (scaled to area sampled) showed that the demosponge species richness on the outer shelf generally decreased from south to north with the exception of the northwestern province that had higher richness than the central western transition zone (Fig. 4).

The nMDS ordination of the outer shelf sponge biomass data, after removal of two outlier samples, showed no clear separation into groups (e.g., provinces), although there is a slight latitudinal trend with the northernmost samples to the right of the graph (Fig. 5). This is confirmed by the significant, although low R-value of the ANOSIM comparing provinces $R = 0.21$, $P = 0.001$ (999 permutations). The two outlier samples excluded from both the nMDS and the ANOSIM each contained only one singleton species. DISTLM analysis of demosponge

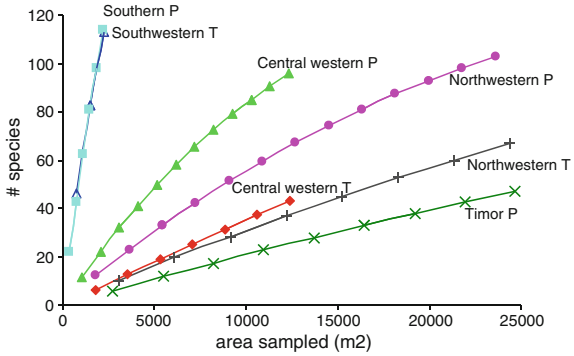

Fig. 4 Species accumulation curves for demosponge species collected on the outer shelf, by province (*P*)/transition zones (*T*) based on 999 permutations, scaled to the total area sampled

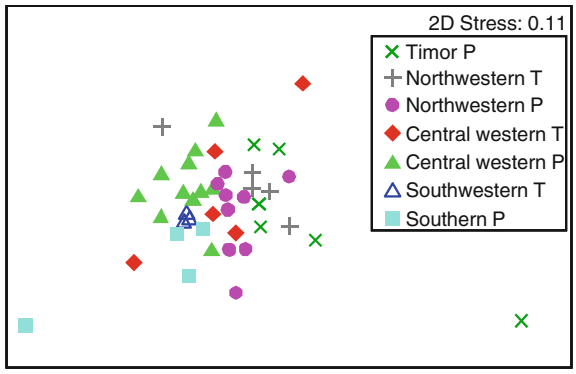

Fig. 5 nMDS ordination of the Bray–Curtis dissimilarities of the square-root transformed demosponge species biomass data from the outer shelf off Australia's west coast; symbols indicating bioregional provinces (*P*) and transition zones (*T*) (Commonwealth of Australia, 2005). Two outlier samples were excluded (SS20705-141 & SS200705-171)

species showed that single covariates explained less than 4.5% of the variation in the data (the top three were SST 4.47%, latitude 4.43, and salinity 4.41%). Combining multiple covariates did not improve the model. Thus, the variation in the species-level demosponge data could not be satisfactorily explained by the 28 covariates that were available for statistical analyses. The exclusion of singletons and doubletons did not fundamentally change the distribution patterns of the demosponges, and thus did not improve the explanatory power of our environmental covariates.

Discussion

Composition and diversity of demosponges

The most abundant and most speciose groups of sponges on Australia's western continental margin were the demosponge orders Poecilosclerida, Dictyoceratida, Haplosclerida, and Astrophorida. These orders are known to be highly speciose in other locations including the Great Australian Bight (GAB) continental shelf (Sorokin et al., 2007), shelf edge canyons off Tasmania (Schlacher-Hoenlinger, unpublished sponge mudmaps from survey SS200404), and off the Bahamas between 30- and 922-m depths (Reed & Pomponi, 1997). Notable differences between the western margin and those from the GAB and Tasmania are the presence of the orders Agelasida, and lithistid sponges, and about 10 species of the Verongida—a group apparently absent in the GAB and represented by only one species off Tasmania (Table 4). Off the Bahamas, Reed & Pomponi (1997) found a high number of lithistids, Agelasida and Verongida, and fewer Dictyoceratida. In our study, the lithistids only occurred as latitudes decreased when fewer Dictyoceratida were also found, suggesting that these changes may be related to tropical environments which we sampled, but which were not present in the GAB and Tasmania which are temperate.

Mounting evidence supports the finding that sponge communities in shallow Australian waters consist of many rarely occurring species and few commonly occurring ones (Hooper & Kennedy,

Table 4 Demosponge orders, number of species present, and number of stations where they occurred

Order	No. of species	No. of stations recorded (summed for all species)
Agelasida	3	15
Astrophorida	70	182
Dendroceratida	2	2
Dictyoceratida	81	230
Hadromerida	31	79
Halichondrida	54	128
Haplosclerida	75	192
Homosclerophorida	4	11
Poecilosclerida	110	181
Spirophorida	9	22
Verongida	10	16

2002; Fromont et al., 2006; McQuillan, 2006, Fromont & Vanderklift, 2009). The study by Sorokin et al. (2007) and Schlacher et al. (2007) are the first reports of this phenomenon in Australian deepwater habitats. Sorokin et al. (2007) reported that 77% of the sponge species had limited distributions, with only 22% widely distributed; Schlacher et al. (2007) found on average only 10.3% of species were common to all five canyons, while 76% were spot endemics (= singletons). More than 68% of the species in this study were only found in one or two samples, thus further supporting the finding that sponges in deeper environments also have very limited distributions. This finding may be partly explained by low sampling replication within each site and depth zone, but replicated sampling in shallow diving depths did not decrease the high proportion of rare species (Fromont & Vanderklift, 2009).

Several theories exist to explain why sponge assemblages have numerous rare species and small-scale patchiness in species distributions. These include limited dispersal ability, specific microhabitat requirements, and exposure to episodic disturbance (see Fromont et al., 2006 and references therein).

High apparent rarity of species, in particular in poorly sampled environments, is not uncommon, but the replicate sampling necessary to distinguish rarity from patchiness or sampling efficiency is often lacking (Williams et al., 2010). Examples are known for most marine and terrestrial communities (Gray et al., 2005), indicating that the causal factors can be expected to be complex and variable between taxa.

Factors influencing sponge distributions

Sponge assemblages in the study area were characterized by high biomass and high species richness. The Demospongiae were most abundant on the outer shelf and shelf edge ($\sim$100 and $\sim$200 m depth) where the majority of substrate types sampled were hard (Althaus et al., 2011). Most species of Demospongiae and Calcarea attach to hard substrates, and the availability of hard seabed as a factor influencing sponge distribution was confirmed to be significant in analyses. Relatively high sponge biomass usually occurred where the continental shelf was relatively narrow with a steep edge, for example, at Albany, Ningaloo, and Onslow (see Fig. 1).

Depth was the most influential of the 28 environmental covariates in models of the distribution of the three sponge classes. There was also a latitudinal trend (although not statistically significant) with higher biomass of Demospongiae in the southwestern regions compared to higher biomass of Hexactinellida in the tropical northwestern regions. More than 60% of the variation in the distribution of these classes was unexplained by the covariates we used in the DISTLM model. This is partly explained by the lack of replicate sampling. However, factors such as current dynamics that are not accounted for in the model, are assumed to also influence sponge distributions along this margin.

The Leeuwin Current and a deeper undercurrent dominate the oceanography of the shelf and shelf edge. The Leeuwin Current flows most strongly and continuously for 6 months of the year to $\sim$200-m depth along the shelf and shelf break in the southwest where sponge biomass was high, and is weaker in the central western region above Shark Bay (Feng et al., 2010) where sponge biomass was lower. The average current speed is 0.4 m s^{-1} (Ridgway & Condie, 2004) with a maximum flow of 0.64–0.68 m s^{-1} southward from below Shark Bay (Hanson et al., 2007). This fast flow may increase particle encounter for suspension feeding organisms. Although the Leeuwin Current is oligotrophic, sponges are efficient filter feeders reported to filter between 60 and 900 times their volume per hour (Jimenez & Ribes, 2007 and references therein). They have high retention efficiencies of picoplankton and can take up dissolved organic matter, including carbon, nanoplankton and microplankton (Jimenez & Ribes, 2007 and references therein), as well as free-living bacteria and other particles to $\sim$0.1-μm diameter, with a near 100% retention of particles (Riisgard & Larsen, 2010).

The transport of nutrient poor water via the Leeuwin Current, and consequent low associated pelagic primary productivity appears inconsistent with the high abundance of seagrass beds and macroalgal communities reported in shallow coastal waters (Lourey et al., 2006 and references therein), and the high sponge biomass we found on the outer shelf and shelf edge in the southernmost regions of the study area. Low dissolved nitrate concentrations and a low N-to-P ratio suggests that primary producers in this region may be primarily nitrogen limited

(Lourey et al., 2006). Recent studies suggest that nitrification associated with sponges appears to be a general feature in oligotrophic zones (Jimenez & Ribes, 2007) and future studies examining the excretion products of the high sponge biomass in this region may provide some explanation for the observed nutrient budget imbalance.

Silicate concentrations in the Leeuwin Current are highest at its source with a gradual decrease as it travels south. This could be attributed to biological uptake, although silicate kinetics have not been studied to date (Lourey et al., 2006). Although the role of diatoms would be important in any uptake study, the high biomass of sponges we reported here may be an additional sink for silicates. Maldonado et al. (2010) suggest a diatom-driven loop may appropriately represent silicate cycling in the open ocean, but may not realistically reflect the situation on some continental shelves with substrates densely populated by siliceous consuming sponges. Hexactinellid sponges lay down more silica per unit biomass than many demosponges (Hogg et al., 2010), and this might in part account for the higher incidence of glass sponges in shallow depths in the north—at the source of the Leeuwin current.

The distribution of the Calcarea mirrored the distribution of the Demospongiae—being the highest in biomass in the southwest on the outer shelf and shelf break (100–200-m depth). However, their distribution did not extend northward, and no specimens were collected above Carnarvon at 24.5°S. A similar pattern has been found in shallow coastal sites along the Western Australian coast with higher abundance of Calcarea in the south than in northern tropical waters (Fromont unpublished data). No details are currently available on calcium carbonate budgets in northwest Australia but calcareous sponges may not compete well with reef-building scleractinian corals which dominate in shallow tropical seas.

Demosponge biomass was low in the central western and northwestern transition zones and the Timor province. Although our analyses did not find any strong correlation between sponge distribution and environmental variables aside from depth and substrate hardness, other factors are believed to have contributed to this pattern. In general, regions where demosponge biomass was low have a wider shelf compared to areas where biomass was high—although the central western province had a high sponge biomass and a wide shelf area. The central western transition zone is influenced by high salinity outflows from Shark Bay, and is where the Leeuwin Current decelerates and flows shallower at $\sim$50-m depth (Woo et al., 2006) compared to the central western province where sponge biomass and current flow are higher and deeper respectively.

Hexactinellida were found to have the opposite distribution pattern to the Demospongiae and Calcarea as they mostly occurred at depths greater than 300 m on the upper slope, and increased in biomass in the tropical provinces, where they were also collected at shallower depths ($\sim$100 m). The Leeuwin undercurrent flows northward below the Leeuwin current at 250–600 m depth with an average velocity of 0.1 m s^{-1} (Meuleners et al., 2007). In general, the glass sponges in the southwest were found in the path of the Leeuwin undercurrent in deeper waters.

Conservation of sponge biodiversity

Although recently trawled areas were avoided by our sampling, the removal of sponges by historical demersal trawling, which was not accurately mapped, cannot be ruled out as a contributor to the low sponge biomass observed in the northern part of our study area. The outer shelf was trawled up to the 1990s, intensively in the north western transition zone and, to a lesser extent, in the Timor province. It can be inferred from demersal trawl bycatch records that sponge biomass must have been substantially reduced over large areas, although the exact distribution of trawl fishing was not accurately recorded. The catch rate of epibenthic fauna (primarily sponges, alcyonarians and gorgonians) was $\sim$500 kg h^{-1} up to $\sim$2,600 kg h^{-1} in 1963, but reduced to less than 300 to $\sim$0 kg h^{-1} by 1979 (Sainsbury, 1987). Subsequent modeling (NWSJEMS, 2007) suggested that depletion rates of epibenthic fauna are rapid and recovery times can be slow (potentially more than 20 years). We found high biomass of demosponges at Onslow, where the steep shelf break is inaccessible to trawling activities and creates a natural refuge area. Sponge richness is also known to be high in shallow waters inshore of these areas, for instance, in the Dampier Archipelago (Fromont et al., 2006) and off Port Hedland (Fromont, unpublished data) where demersal trawling has not occurred. The importance of structural benthic fauna to creating habitats for fishes is

demonstrated here by the associated change in fish community structure. Large commercial fishes (species of *Lethrinus* and *Lutjanas*) that are associated with structured habitats, accounted for 40–60% of the catch in the early fishing years when sponge bycatch was high (Sainsbury, 1987). A significantly reduced bycatch of structural benthic fauna by the mid-1980s was accompanied by a shift to dominance by small fishes associated with open unstructured habitats, *Nemipterus* and *Saurida*, on the same fishing grounds. Trawling effects vary with gear type, so that lighter ground-gear has been reported to remove fewer sponges. Wassenberg et al. (2002) found that the less flexible and larger sponges are more likely to be dislodged, with a removal rate of 13.8% per tow using a McKenna demersal trawl, a significantly smaller proportion than reported by Sainsbury (1987) of 89% removal of sponges by demersal pair trawlers in the northwest of Australia. Wassenberg et al. (2002) suggested the biggest gap in our current understanding of these impacts is knowledge on the rate of recovery of sponges. More recently large, habitat-forming deep-water sponges have been found to be particularly slow-growing (Hogg et al., 2010), but more studies are required to fully understand the turnover of these important assemblages.

The high proportions of apparently rare species within broadly distributed taxa—including sponges—will require careful management outside any designated marine protected areas, as well as inside them (Williams et al., 2010). It will be important to compare species richness across major taxonomic groups to determine where individual marine areas will efficiently conserve benthic invertebrate biodiversity across taxa. Data for sponges illustrates the importance of adequately representing outer shelf and shelf break habitats in marine reserves on the western Australian margin because demosponges and Calcarea have few representatives in slope depths. It is also important to determine whether hard outer shelf areas support high diversity of other taxa in addition to sponges. This has been examined for higher taxa in the southwestern margin by Williams et al. (2010) who noted that mollusc, echinoderm, and sponge species richness increased with increasing substrate hardness. Hard substrates are largely confined to the shelf (61% of observations), whereas soft substrates were recorded in 78% of observations in depths greater than 200 m (Althaus et al., 2011).

Apart from substrate type, depth, and historic fishing, we found few factors that explained the distribution of sponges. Recent analyses by Huang et al. (2011) found depth to be the most important contributing factor in some models, with bottom-water temperature and variables that describe bottom-water nutrient status, such as nitrates and chlorophyll, being important in some cases. They conclude that these factors could be useful surrogates for the distribution of sponge assemblages at the regional scale. Maldonado & Uriz (1999) noted that fragmented habitats, characteristic of the deep sea, often appear to produce discrete, spatially separated populations. We found fine scale patchiness of the sponge communities examined here which appear to be largely related to substrate type and depth. Broad regional scale analyses are not sensitive to fine scale changes in species composition in these sponge assemblages. The lack of understanding of the function and role of deep sponge assemblages in the ecological processes of the marine environment off Western Australia suggest that more study is required to underpin conservation planning.

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References

Althaus, F., A. William, R. J. Kloser, J. Seiler & N. J. Bax, 2011. Evaluating geomorphic features as surrogates for benthic biodiversity on Australia's western continental margin. In Harris, P. (ed.), Seafloor Geomorphology as

Benthic Habitat: GeoHab Atlas of Seafloor Geomorphic Features and Benthic Habitats. http://geohab.org/atlas.html. Accessed on 9 Aug 2011.

Anderson, M. J., R. N. Gorley & K. R. Clarke, 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. PRIMER-E, Plymouth, UK: 214 pp.

Buhl-Mortensen, L., A. Vanreusel, A. J. Gooday, L. A. Levin, I. G. Priede, P. Buhl-Mortensen, H. Gheerardyn, N. J. King & M. Raes, 2010. Biological structures as a source of habitat heterogeneity and biodiversity on the deep ocean margins. Marine Ecology – An Evolutionary Perspective 31: 21–50.

Clark, M. R., F. Althaus, A. Williams, E. Niklitschek, G. M. Menezes, N.-R. Hareide, P. Sutton & C. O'Donnell, 2010. Are deep-sea demersal fish assemblages globally homogenous? – insights from seamounts. Marine Ecology – An Evolutionary Perspective 31(Suppl. 1): 39–51.

Clarke, K. R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18(1): 117–143.

Clarke, K. R. & R. N. Gorley, 2006. PRIMER-e v6: User Manual/Tutorial. Primer-E, Plymouth.

Commonwealth of Australia, 2005. National Marine Bioregionalisation of Australia. Department of the Environment and Heritage, Geoscience Australia, CSIRO Marine Research. National Oceans Office, Canberra, ISBN 1-877043-61-3.

Dunn, J. R. & K. R. Ridgway, 2002. Mapping ocean properties in regions of complex topography. Deep-Sea Research Part I-Oceanographic Research Papers 49: 591–604.

Feng, M., D. Slawinski, L. E. Beckley & J. K. Keesing, 2010. Retention and dispersal of shelf waters influenced by interactions of ocean boundary current and coastal geography. Marine and Freshwater Research 61: 1259–1267.

Forest, J., 1981. Compte rendu et remarques générales (texte bilingue)/Report and general comments (bilingual text). Résultats des campagnes MUSORSTOM, 1, Mémoires ORSTOM (Office de la Recherche Scientifique et Technique Outre-Mer): 91 pp.

Fromont, J. & M. A. Vanderklift, 2009. Porifera (sponges) of Mermaid Reef (Rowley Shoals), Scott and Seringapatam Reefs, Western Australia. Records of the Western Australian Museum. Supplement 77: 89–104.

Fromont, J., M. A. Vanderklift & G. A. Kendrick, 2006. Marine sponges of the Dampier Archipelago, Western Australia: patterns of species distributions, abundance and diversity. Biodiversity and Conservation 15: 3731–3750.

Fromont, J., K. L. Usher, D. C. Sutton, S. Toze & J. Kuo, 2008. Species of the sponge genus *Chondrilla* (Demospongiae: Chondrosida: Chondrillidae) in Australia. Records of the Western Australian Museum 24(4): 469–486.

Grassle, J. F. & N. J. Maciolek, 1992. Deep-sea species richness – regional and local diversity estimates from quantitative bottom samples. American Naturalist 139: 313–341.

Gray, J. S., A. Bjoergesaeter & K. I. Ugland, 2005. The impact of rare species on natural assemblages. Journal of Animal Ecology 74: 1131–1139.

Hanson, C. E., S. Pesant, A. M. Waite & C. B. Pattiaratchi, 2007. Assessing the magnitude and significance of deep chlorophyll maxima of the coastal eastern Indian Ocean. Deep-Sea Research Part II-Topical Studies in Oceanography 54: 884–901.

Hogg, M. M., O. S. Tendal, K. W. Conway, S. A. Pomponi, R. W. M. van Soest, J. Gutt, M. Krautter & J. M. Roberts, 2010. Deep-Sea Sponge Grounds: Reservoirs of Biodiversity, UNEP-WCMC Biodiversity Series No. 32. UNEP-WCMC, Cambridge, UK.

Hooper, J. N. A. & J. A. Kennedy, 2002. Small-scale patterns of sponge biodiversity (Porifera) on Sunshine Coast reefs, eastern Australia. Invertebrate Systematics 16: 637–653.

Howell, K. L., J. S. Davies & B. E. Narayanaswamy, 2010. Identifying deep-sea megafaunal epibenthic assemblages for use in habitat mapping and marine protected area network design. Journal of the Marine Biological Association of the United Kingdom 90: 33–68.

Huang, Z., B. Brooke & J. Li, 2011. Performance of predictive models in marine benthic environments based on predictions of sponge distribution on the Australian continental shelf. Ecological Informatics 6: 205–216.

Jimenez, E. & M. Ribes, 2007. Sponges as a source of dissolved inorganic nitrogen: nitrification mediated by temperate sponges. Limnology and Oceanography 52: 948–958.

Last, P. R., V. D. Lyne, A. Williams, C. R. Davies, A. J. Butler & G. K. Yearsley, 2010. A hierarchical framework for classifying seabed biodiversity with application to planning and managing Australia's marine biological resources. Biological Conservation 143: 1675–1686.

Legendre, P. & M. J. Anderson, 1999. Distance-based redundancy analysis: testing multi-species responses in multifactorial ecological experiments. Ecological Monograph 69: 1–24.

Lewis, M., 1999. CSIRO-SEBS (Seamount, Epibenthic Sampler), a new epibenthic sled for sampling seamounts and other rough terrain. Deep-Sea Research I 46: 1101–1107.

Lewis, M., 2009. Sherman the epibenthic sled for rough terrain (CSIRO Marine and Atmospheric Research paper; 029). CSIRO Marine and Atmospheric Research, Canberra, ACT: 15 pp.

Lewis, M., 2010. The CSIRO 4m Beam Trawl (CSIRO Marine and Atmospheric Research Paper 033). CSIRO Marine and Atmospheric Research, Hobart, Tas: 17 pp.

Lourey, M. J., J. R. Dunn & J. Waring, 2006. A mixed-layer nutrient climatology of Leeuwin Current and western Australian shelf waters: seasonal nutrient dynamics and biomass. Journal of Marine Systems 59: 25–51.

Maldonado, M. & M. J. Uriz, 1999. Sexual propagation by sponge fragments. Nature 398: 476.

Maldonado, M., A. Riesgo, A. Bucci & K. Rutzler, 2010. Revisiting silicon budgets at a tropical continental shelf: silica standing stocks in sponges surpass those in diatoms. Limnology and Oceanography 55: 2001–2010.

McEnnulty, F. R., K. L. Gowlett-Holmes, A. Williams, F. Althaus, J. Fromont, G. C. B. Poore, T. D. O'Hara, L. Marsh, P. Kott, S. Slack-Smith, P. Alderslade, & M. V. Kitahara, 2011. The deepwater megabenthic invertebrates on the western continental margin of Australia (100–1100 m depths): composition, distribution and novelty. Records of Western Australian Museum Supplement 80: 1–189.

McQuillan, L., 2006. Species richness, density and cover of sponges on temperate reefs in Western Australia. Unpublished MSc thesis, Edith Cowan University.

Meuleners, M. J., C. B. Pattiaratchi & G. N. Ivey, 2007. Numerical modelling of the mean flow characteristics of the Leeuwin Current System. Deep-Sea Research Part II-Topical Studies in Oceanography 54: 837–858.

North West Shelf Joint Environmental Management Study, 2007. CSIRO and Department of the Environment of Western Australia: 40 pp.

Reed, J. K. & S. A. Pomponi, 1997. Biodiversity and distribution of deep and shallow water sponges in the Bahamas. In Proceedings of the Eighth International Coral Reef Symposium, Panama, June 24–29, 1996: 1387–1392.

Ridgway, K. R. & S. A. Condie, 2004. The 5500-km-long boundary flow off western and southern Australia. Journal of Geophysical Research-Oceans 109: 18 pp.

Ridgway, K. R., J. R. Dunn & J. L. Wilkin, 2002. Ocean interpolation by four-dimensional least squares – application to the waters around Australia. Journal of Atmospheric and Oceanic Technology 19: 1357–1375.

Riisgard, H. U. & P. S. Larsen, 2010. Particle capture mechanisms in suspension-feeding invertebrates. Marine Ecology-Progress Series 418: 255–293.

Rützler, K., 2004. Sponges on coral reefs: a community shaped by competitive cooperation. In Pansini, M., R. Pronzato, G. Bavestrello & R. Manconi (eds), Sponge Science in the New Millennium. Officine Grafiche Canessa, Rapallo, Genova: 85–148.

Sainsbury, K. J., 1987. Assessment and management of the demersal fishery on the continental shelf of northwestern Australia. In Polovina, J. J. & S. Ralston (eds), Tropical snappers and groupers – biology and fisheries management. Westview Press, Boulder, CO: 465–503.

Schlacher, T. A., M. A. Schlacher-Hoenlinger, A. Williams, F. Althaus, J. N. A. Hooper & R. Kloser, 2007. Richness and distribution of sponge megabenthos in continental margin canyons off southeastern Australia. Marine Ecology-Progress Series 340: 73–88.

Sorokin, S., J. Fromont & D. Currie, 2007. Demosponge biodiversity in the benthic protection zone of the Great Australian Bight. Transactions of the Royal Society of South Australia 131: 192–204.

Waite, A. M., P. A. Thompson, S. Pesant, M. Feng, L. E. Beckley, C. M. Domingues, D. Gaughan, C. E. Hanson, C. M. Holl, T. Koslow, M. Meuleners, J. P. Montoya, T. Moore, B. A. Muhling, H. Paterson, S. Rennie, J. Strzelecki & L. Twomey, 2007. The Leeuwin current and its eddies: an introductory overview. Deep-Sea Research Part II-Topical Studies in Oceanography 54: 789–796.

Wassenberg, T. J., G. Dews & S. D. Cook, 2002. The impact of fish trawls on megabenthos (sponges) on the north-west shelf of Australia. Fisheries Research 58: 141–151.

Williams, A., F. Althaus, P. K. Dunstan, G. C. B. Poore, N. J. Bax, R. J. Kloser & F. R. Mcennulty, 2010. Scales of habitat heterogeneity and megabenthos biodiversity on an extensive Australian continental margin (100–1100 m depths). Marine Ecology – An Evolutionary Perspective 31: 222–236.

Woo, M., C. Pattiaratchi & W. Schroeder, 2006. Summer surface circulation along the Gascoyne continental shelf, Western Australia. Continental Shelf Research 26: 132–152.

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SPONGE RESEARCH DEVELOPMENTS

Sponge gardens of Ningaloo Reef (Carnarvon Shelf, Western Australia) are biodiversity hotspots

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Abstract During a multi-agency Australian Government initiative sponges were sampled at three areas from Carnarvon Shelf, NW Australia. Sponges were identified to lowest possible level, largely as morphospecies (84%). A searchable and interactive taxonomic catalogue was created and is publicly accessible through the Ningaloo Atlas collated by the Australian Institute for Marine Science. The sponge gardens on Carnarvon Shelf are patchily distributed but highly diverse and occasionally extremely dense. We examined 754 specimens and distinguished 261 species belonging to 112 genera. Species accumulation curves indicate that this species number does not represent local sponge biodiversity, which is projected to reach up to 840 species with additional sampling. Many observed species appear to be new to science, 81% occurred only at one of the three areas, about 56% were singletons, and 76% had a wet weight of $\leq$500 g. Detailed spatial analyses were difficult due to sampling design, but general trends could be detected. The northern areas, where the Australian continental shelf is narrowest, favour sponges with a higher content of inorganic skeleton and growth forms able to withstand the strong tidal currents and exposure typical for this area. Sponges were most abundant at the shelf edge, where massive forms dominated. While the central and northern areas are protected by zoning regulations, the southern area had the highest species diversity, the largest individuals and the densest distributions, suggesting additional conservation measures may be required. Western Australia is clearly an important, but understudied bioregion for sponges, and future research foci are proposed.

Keywords Western Australia · Porifera · Demospongiae · Species richness · Distribution · Biomass

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Guest editors: M. Maldonado, X. Turon, M. A. Becerro & M. J. Uriz / Ancient animals, new challenges: developments in sponge research

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Introduction

When we consider that the taxon of sponges has about six times as many species as the taxon of hard corals, sponges are under-represented in the literature

compared to corals, and also to other organism groups (Table 1; Becerro, 2008). This is especially true at the community level for studies on sponge gardens that fulfil an ecological role comparable to coral reefs by providing a nutrient source for other organisms as well as a three-dimensional habitat. Sponge gardens can occur intermingled with coral reefs or in the immediate neighbourhood, but usually they start at deeper depths where corals cannot compete (e.g. Australian Broadcasting Corporation, 1998; Heyward et al., 2010). Australian filter feeder communities dominated by sponges have been known to sports divers for many years (e.g. Coleman & Marsh, 2003), yet scientists are not as familiar with this habitat. Nevertheless, these environments increasingly attract the attention of scientists and management agencies. For example, within the last 2 years there has been a surge in Internet-generated information on sponge gardens in various contexts from almost zero in 2009 to many hits in 2010–2011 (Table 1), and a significant proportion of this material is the result of marine management and protection associated activities. Most of these sources agree that sponge gardens are attractive, rich habitats co-occurring with and supporting a multitude of other marine life, but also that they are vulnerable and endangered by changes caused by human activities such as trawling, dredging, land use, sedimentation, pollution, climate change and species shifts due to over-fishing or introduced species (e.g. Australian Broadcasting Corporation, 1998; State of New South Wales, 2005; Reef Watch Victoria, 2006). However, in contrast to ever-popular coral reefs, the vast majority of sponge gardens have not been primary drivers for the establishment of marine protected areas (MPAs).

A traditional perception is that the densest and richest sponge communities in garden or reef form occur in the Caribbean and the Antarctic (e.g. Diaz & Rützler, 2001; McClintock et al., 2005), with more recent data on Canadian glass sponge communities (e.g. Chu & Leys, 2010), while Australia has yet to gain the same level of recognition as major sponge habitat. We know little about Australian sponge gardens, even though they are very common along Australia's coasts and were previously reported from Sydney on the east coast, continuing around the southern half of Australia and to Exmouth Gulf in Australia's northwest (e.g. Australian Broadcasting Corporation, 1998). It was recently discovered that similar sponge gardens occur as far northwest as the Kimberley region (Andrew Heyward, unpubl. data), and northern bioregions of Australia's coasts have been recorded with very high sponge diversities (Hooper, 1988; Hooper et al., 2002; Hooper & Ekins, 2004). Nevertheless, any information beyond descriptive records of existence of Australian sponge gardens rapidly decreases from east to west and from south to north, and confirmed accounts usually concentrate on Victoria and Tasmania (e.g. Australian Broadcasting Corporation, 1998).

The present publication refers to sponges sampled during a survey on the Carnarvon Shelf, Western Australia conducted by the Commonwealth Environmental Research Facilities (CERF) Marine Biodiversity Hub, a multi-agency Australian Government initiative (Brooke et al., 2009; Bax et al., 2011). An earlier study in the area was conducted through the deepwater biodiversity project of the Western Australian Marine Science Institute (WAMSI; see Colquhoun & Heyward, 2008; Heyward et al., 2010). The purpose of the CERF project was 'surrogacy', i.e. evaluation of physical parameters in relation to patterns of benthic biodiversity (see Brooke et al., 2009). The sampling design was based on limited a priori knowledge of the distribution of key habitats and was therefore largely unsuitable for detailed spatial analyses of sponge data across all sampled areas. Consequently, we have focussed on increasing awareness of northwestern Australian sponge gardens by examining the following aspects: 1. What sponge species occur on the Carnarvon Shelf and how diverse is the community? 2. How can we describe basic characters of this community, e.g. what can be said about sponge sizes and growth forms that may relate to functions? 3. Are there any general spatial patterns that can be recognised? 4. Can we provide new information for management agencies who protect unique benthic communities? Ultimately, we would like to invite colleagues to continue work on the present collection and to describe the many new species likely to have been discovered from the Carnarvon Shelf.

Materials and methods

Sampling was conducted from 12th August to 15th September 2008 on the RV *Solander* through the CERF program. The study area was the southern section of the Carnarvon Shelf, Western Australia,

Table 1 Web-based assessment of the sponge world versus the coral world

Item	In the sponge world	In the coral world	Source	Rough factor
Valid species	8,290 (status 22.6.2011)	Approx. 1,300 Scleractinia (7,500 Anthozoa; status Nov. 2007)	van Soest et al. (2011), Daly et al. (2007)	6× for sponges
General internet search (search as of 20.10.2010)	Keyword 'sponge garden': 1,910,000	Keyword 'coral reef': 3,290,000 hits	www.google.com.au	2–3× for corals
	9,140,000	18,300,000 hits	www.bing.com	
	21,000,000	61,200,000 hits	www.au.altavista.com	
(search as of 24.6.2011)	20,500,000	17,400,000 hits	www.google.com.au	1–3× for corals
	13,300,000	25,500,000 hits	www.bing.com	
	24,100,000	67,700,000 hits	www.au.altavista.com	
General literature search	Keyword 'porif*': 409 hits	Keyword 'scleract*': 699 hits	Thomson Reuter's ISI Web of Knowledge (2011)	2×–32× for corals
Search in 'all databases' for 'title' and 'all years'(as of 24.11.2011)	Keyword 'sponge': 3,984 hits adjusted to ca. 2,630 relevant hits	Keyword 'coral': 6,608 hits adjusted to ca. 5,550 relevant hits		
	Keyword 'sponge reef': 66 hits but usually referring to sponges on coral reefs	Keyword 'coral reef': 2,139 hits		
	Keyword 'sponge garden': 0 relevant hits	Keyword 'coral garden': 4 hits		
Literature specifically relating to animals, search 1945 to present (as of 24.6.2011)	Keyword 'Porifera' 12,416 hits	Keyword 'Scleractinia' 7,900 hits	Zoological Record (2011)	2× for sponges
	Keyword 'sponge' 12,995 hits	Keyword 'coral' 18,377 hits		
Email list members (as of October 2010)	433	2,500	Coral-List (2010), Porifera-List (2010)	6× for corals
International conference statistics	8th WSC	11th ICRS	11th International Coral Reef Symposium (2008), 8th World Sponge Conference (2010)	5×–7× for corals
Registered participants	265	ca. 1,400		
Concurrent sessions	1	12		
Accepted abstracts	354	2,500		

Internet hits were not corrected for irrelevant entries, and many items may not relate to the search ('sponge garden' includes gardening advice and information re-environmental architecture and 'coral reef' often refers to travel information). The literature in ISI Web of Knowledge search was coarsely adjusted for relevancy by using the 100 most recent entries as a guideline and subtracting the proportion of irrelevant hits. Comparing e-mail list and conference statistics may also be slightly biased, because the sponge community is small and focussed on the taxon, while large proportions of the coral reef community concentrate on fish or other reef-associated fauna and flora

including Ningaloo Reef Marine Park (Fig. 1). Sampled areas were (from north to south): Muiron Islands north of Exmouth (not included in the present analyses), Mandu (30 sites), Point Cloates (39 sites), an area south of Pt. Cloates (no sponge samples taken), and Gnaraloo (34 sites; background information available in Brooke et al., 2009; sampling details—see Supplementary material). Each area was examined from shallow depths around 30 m to the shelf break at about 100 m. Investigations included physical (waves and currents, multi-beam sonar bathymetric mapping and backscatter, sediment and substrate properties) and biological data (viewing communities with towed underwater video and still photography, plankton samples, in- and epifauna). The present publication focuses on data related to sponge samples, all other data will be published elsewhere (preliminary report available in Brooke et al., 2009). Depending on site, sponges were sampled by one to three 100 m tows with a custom-made epibenthic sled of 1.5 m width (Colquhoun & Heyward, 2008, pp. 110–112), of which only tow one was analysed. Tows followed the paths of previously taken underwater video tracks.

Fieldwork was conducted in two halves, with the first half at Mandu and Pt. Cloates followed by a team change for the second half at Gnaraloo (Fig. 1, Supplementary material). The two teams recorded sponge collection data differently, so that some weights for the first half and some specimen counts for the second half had to be amended with estimates based on photographs and field notes, which introduced some uncertainty into the data. Weights were a mix of wet, ethanol-preserved and frozen, but preservation method did not significantly alter weights, which were adjusted to subtract weight of foreign materials adhering to or embedded in the specimens. Therefore, differences in sampling prevented a full analysis of the sponge data, and we refrained from detailed spatial and similarity analyses. A significant data issue was caused by much of the material from the second half of the cruise (Gnaraloo) being discarded due to lack of freezer space on the vessel. Field notes did not always conclusively cross reference, and discarded material could only be partially identified. As a consequence, only area 1 and 2 (Mandu and P. Cloates) can be reliably compared, while all data from area 3 (Gnaraloo) were regarded with caution. We attempted tentative spatial analyses across all three sites assuming that lost material was not biased for growth forms or taxon groups, and we standardized for sample effort (number of tows per area).

In the WAMSI project very large sponge sample volumes were obtained, and the Western Australian Museum (WAM) restricted identifications to samples of fresh biomass $\geq$1 kg per species and tow (=dominants) to achieve project milestones. In the CERF collection most available material was very small and rare, and 'dominant' species made up only 26%, therefore all specimens were evaluated. Available vouchers of adequate quality comprised 754 specimens which were examined over 1.5 years. They were identified to operational taxonomic units (OTU) as morphospecies or where possible to known species. Resulting descriptive data were summarised in a 'sponge mudmap system' following Hooper & Ekins (2004), but adding the categories 'smell', 'symbionts' and 'associated fauna', and adhering to the format used at WAM (example in Heyward et al., 2010). 'Mudmaps' were generated per specimen rather than per species, which facilitated the initial sorting process and now provides additional information at species level. Final versions of the 'mudmaps' were saved in PDF format and combined in a portfolio to generate a catalogue. This catalogue can be keyword-searched and is available as part of the Ningaloo Atlas (Schönberg et al., 2011). Catalogue pages are linked to all available images, and interactive images of skeletal sections that can be viewed on a virtual microscope on a PC with the freeware ImageScope Viewer (further information about this technique: Internet pages Aperio, 2010; Centre for Microscopy, Characterisation and Analysis at the University of Western Australia—CMCA at UWA, 2010).

Species richness estimates were generated with the freeware program EstimateS (Colwell & Coddington, 1994; Colwell, 2005). The following definitions were used: unique species = species that exclusively occurred at one site, i.e. on one transect, singletons = species with only one specimen in the entire collection, duplicates = species that occur at two sites, i.e. on two transects, doubletons = species with two specimens in the entire collection. Trial calculations of expected total species counts included abundance-based coverage estimation of species richness (ACE), incidence-based coverage estimation of species richness (ICE), Chao1 and Chao2 richness estimations (mean among runs), first- and second-order jackknife

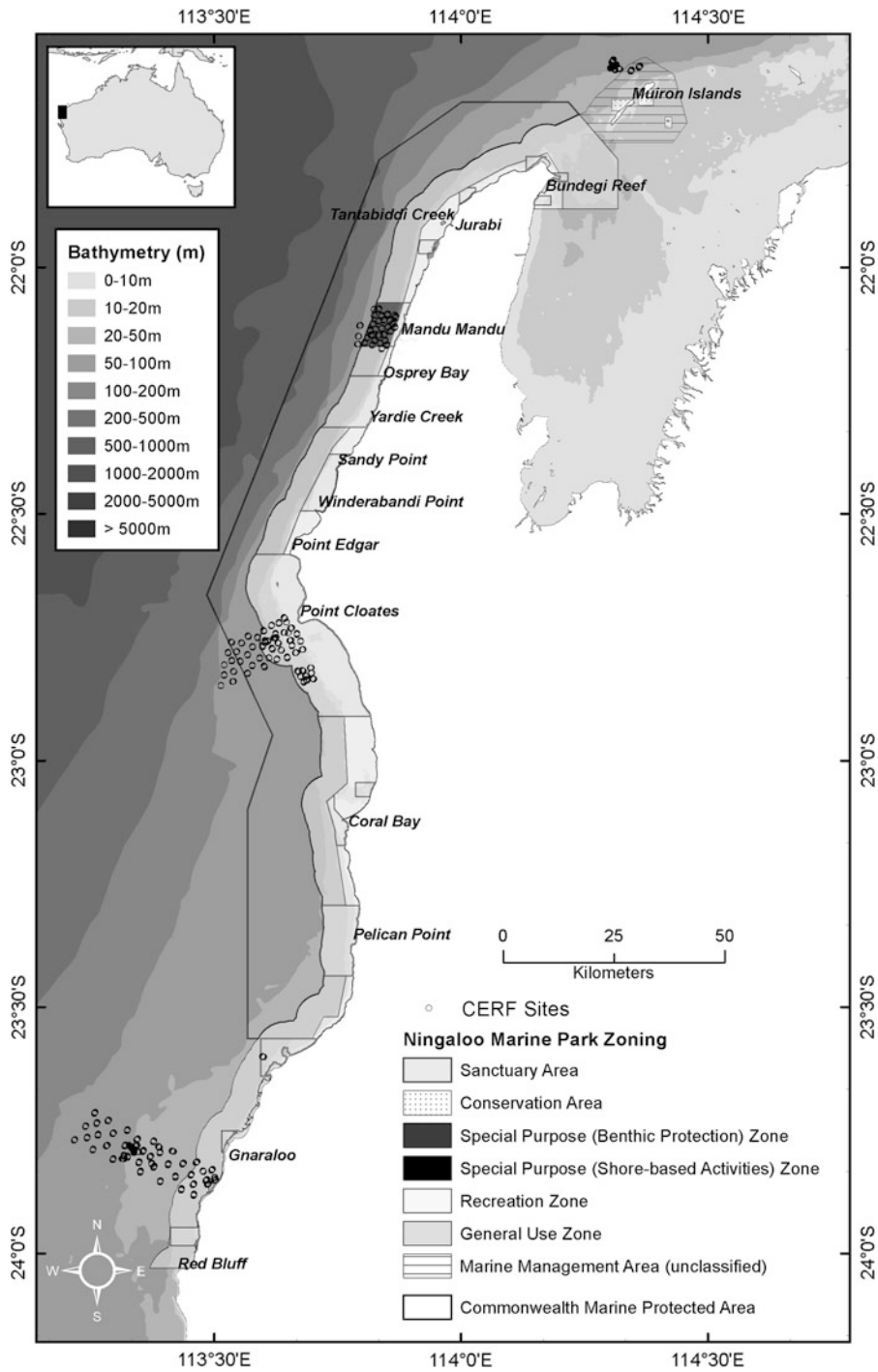

Fig. 1 Map of Carnarvon Shelf showing sample areas and bathymetry at Ningaloo Reef. The Muiron Islands and an area south of Point Cloates were part of the CERF project, but only Mandu, Point Cloates and Gnaraloo were sampled for sponges and used in the present study

estimations (Jack1 and Jack2), bootstrap richness estimations (Boot), and a Michaelis–Menten richness estimation (MM) with estimators averaged over randomizations, because data distribution was extremely heterogeneous (respective references as suggested in Colwell, 2005). Here, we graphically display only the data for the nonparametric Chao1 analysis as it is based on uncommon species in the overall collection, which reflects the results in this study. It also generated accumulation curves very similar to those obtained

with ACE and Jack2, and produced total species estimations that fell between those resulting from the other two methods. Diversity settings for Chao1 used 100 runs and randomization without replacements and were adjusted to 'classic', as the coefficient of variation for Chao's incidence distribution was >0.5, and calculations were based on individuals rather than on sites, which creates estimates based on richness instead of densities (Colwell, 2005). For the estimations, missing samples and samples that were not fully distinguished from or matched with other species were omitted. It should also be noted that for some transects at Gnaraloo no specimen counts were available, and these were conservatively estimated from biomass values and photographs. Therefore, calculated total species estimates for Gnaraloo may be higher if a full data set had been available.

Gathering descriptive information on the sponges was restricted to the laboratory, supplemented by field notes where available. Specimens were evaluated after being frozen or preserved in ethanol for about 1.5 years. Only very small subsamples were taken of frozen specimens for identification to retain them suitable for biodiscovery. Photos of fresh specimens or from segments of specimens were available for the second half of the field trip. All other specimens or specimen fragments were photographed after preservation in ethanol.

Spicule preparations were made for each sponge specimen and viewed by microscopy. Photographs and basic spicule measurements ($N = 5$) were obtained for many preparations. Unstained hand- and about four hundred 90 μm microtome sections were used to assess the arrangement of the skeletons. All microtome sections were digitally scanned as brightfield images at the Aperio Scanscope facility at the CMCA at UWA (magnification 20×). For over 130 samples scanning electron microscopy preparations were made for portions of tissue and for spicules and viewed at the CMCA at UWA. Representative micrographs were taken from over 100 SEM samples for the 'mudmaps'. Details of procedures, 'mudmaps' and additional photographs are available through the CERF Sponge Catalogue as part of the Ningaloo Atlas on the Internet (Schönberg et al., 2011), which contains updated taxonomic decisions as available in July 2011.

All material was tentatively pre-identified to OTU by the first author. In February 2010, a 5-day taxonomy workshop was conducted at the Perth branch of the Australian Institute of Marine Science (AIMS) for final OTU identifications by specialists. About 5% of the sled 1 material remained unidentified due to poor material properties or small sample size and will contain a number of species additional to those recognised. After the workshop all specimens and preparations were relabelled with new names and inconsistencies were eliminated. Species counts and identifications presented in this publication may change with future work on the collection. All specimens, one set of spicule preparations and all microtome sections were lodged with WAM, while AIMS retained a duplicate set of spicule preparations and all handsections as a reference collection. Remaining replicate samples and area 5 specimens are presently in AIMS temperature-controlled storage and are not identified. CERF identifications to OTU are currently being matched with WAMSI Ningaloo specimens to be incorporated into the State collection held at the WAM. Results available mid-September 2010 and March 2011 were summarised in project reports and Newsletters of Geoscience Australia (2010) and presented at the 8th World Sponge Conference in Girona, Spain 2010 and the 2011 Conference of the Australian Marine Sciences Association in Fremantle, Australia.

Results

From the 754 identified and matched sled 1 specimens 112 genera and 261 species were differentiated, either as morphospecies and OTU (84%) or as known species (16%), with 105, 112 and 100 species, respectively, for the areas Mandu, Pt. Cloates and Gnaraloo (Table 2 and Supplementary material). No hexactinellids were found, and the vast majority of specimens were demosponges. Calcareous sponges contributed only 23 specimens (3%), and only occurred at Pt. Cloates and Mandu. About 5% of the vouchers remain unidentified due to poor quality of the material (Table 2). The 11 most diverse genera in the collection with over five species each were *Aka*, *Clathria* and *Oceanapia* (10 spp. each), *Petrosia* (8 spp. or more), *Axinella*, *Cliona* together with *Spheciospongia*, *Crella, Raspailia* and *Xestospongia* (7 spp. each; for the purpose of species richness per genus *Cliona* and *Spheciospongia* are here considered congeneric based on results of Yuji Ise, pers. comm.), and *Erylus* (6 spp.).

Table 2 Summary of sample design and material, yields per area and total species estimates based on identified material

Sample area	1: Mandu ~22°10′S	2: Pt. Cloates ~22°45′S	3: Gnaraloo ~23°50′S	For all areas
Number of sampled sites per area	30	39	34	103
Number of sites yielding sponges	20	29	28	78
Proportion of sites yielding sponges	67%	74%	82%	74%
Assumed total no. of specimens per area	237	325	345	907
Identified vouchers and matched missing specimens	217	297	240	754
Missing specimens with insufficient notes to match them to existing material	3	2	105	110
Proportion of missing specimens (Gnaraloo: reconstructed min. value from field notes, likely more)	1.3% = negligible	0.6% = negligible	30.4%	12.1%
Of available specimens: not yet fully identified to OTU or species, unmatched	7	26	6	39
Of available specimens: proportion of not yet fully identified to OTU or species, unmatched	3.1%	8.0%	2.4%	4.9%
Confirmed species count (value at Gnaraloo too low, relies on available samples)	105	112	100	261
Estimated no. of species per area, based on identified material				
Chao1/Chao2	245/367	221/293	163/273	524/651
ACE/ICE	243/378	205/308	166/308	468/618
Jack1/Jack2	186/253	193/254	177/234	440/577
Boot/MM	137/365	145/608	131/708	334/844
Site average species count, based on field notes	5.0	4.5	5.3	4.9
Site average species count, based on identified material (SD)	4.6 (5.5)	4.1 (5.8)	3.7 (4.2)	4.1 (0.5)
Site average specimen count, based on field notes	7.5	8.1	9.9	8.5
Site average specimen count, based on identified material (SD)	7.2 (9.1)	7.6 (13.5)	7.1 (10.3)	7.3 (0.3)
Site average sponge biomass, based on field notes (kg)	2.5	1.7	9.6	4.6
Site average sponge biomass, based on identified material (kg) (SD)	4.7 (6.8)	1.6 (4.0)	8.2 (14.4)	4.8 (3.3)
Site average specimen biomass, based on identified material (g) (SD)	471.8 (851.9)	125.4 (196.2)	699.1 (709.1)	432.1 (288.9)

Chao1 and Chao2 Chao richness estimations 1 and 2, *ACE* abundance-based coverage estimation of species richness, *ICE* incidence-based coverage estimation of species richness, *Jack1 and Jack2* first and second order jackknife estimations, *Boot* bootstrap richness estimations, *MM* Michaelis–Menten richness estimation, *SD* standard deviation. Site = one 100 m transect

Cumulative species estimations of Carnarvon Shelf sponges revealed that the sampling effort was insufficient to adequately reflect the local sponge biodiversity, and many more species are predicted with increased sampling effort (Table 2). Overall, the linear relationships for all samples and observed cumulative species richness by sample area suggested that a significantly larger sample than 103 tows would have been needed to describe the sponge biodiversity of Carnarvon Shelf (Fig. 2a, b). A large disparity was apparent between observed and expected species, with the curve for expected species at Carnarvon Shelf (Fig. 2a) and Mandu (Fig. 2b) not reaching an asymptote, even when reaching estimates of 500 and 250 species, respectively. Based on the present sample, Chao1 estimations gave expected numbers of total species as 245 for Mandu (Fig. 2b), 221 for Pt. Cloates (Fig. 2b), 163 for Gnaraloo (Fig. 2b) and 524 for Carnarvon Shelf (Fig. 2a). The Chao1 estimate therefore indicates that only about 50% of the putatively existing sponge species were sampled in this study. With other estimation methods this proportion varied considerably, often suggesting higher biodiversity. Moreover, error bars for species

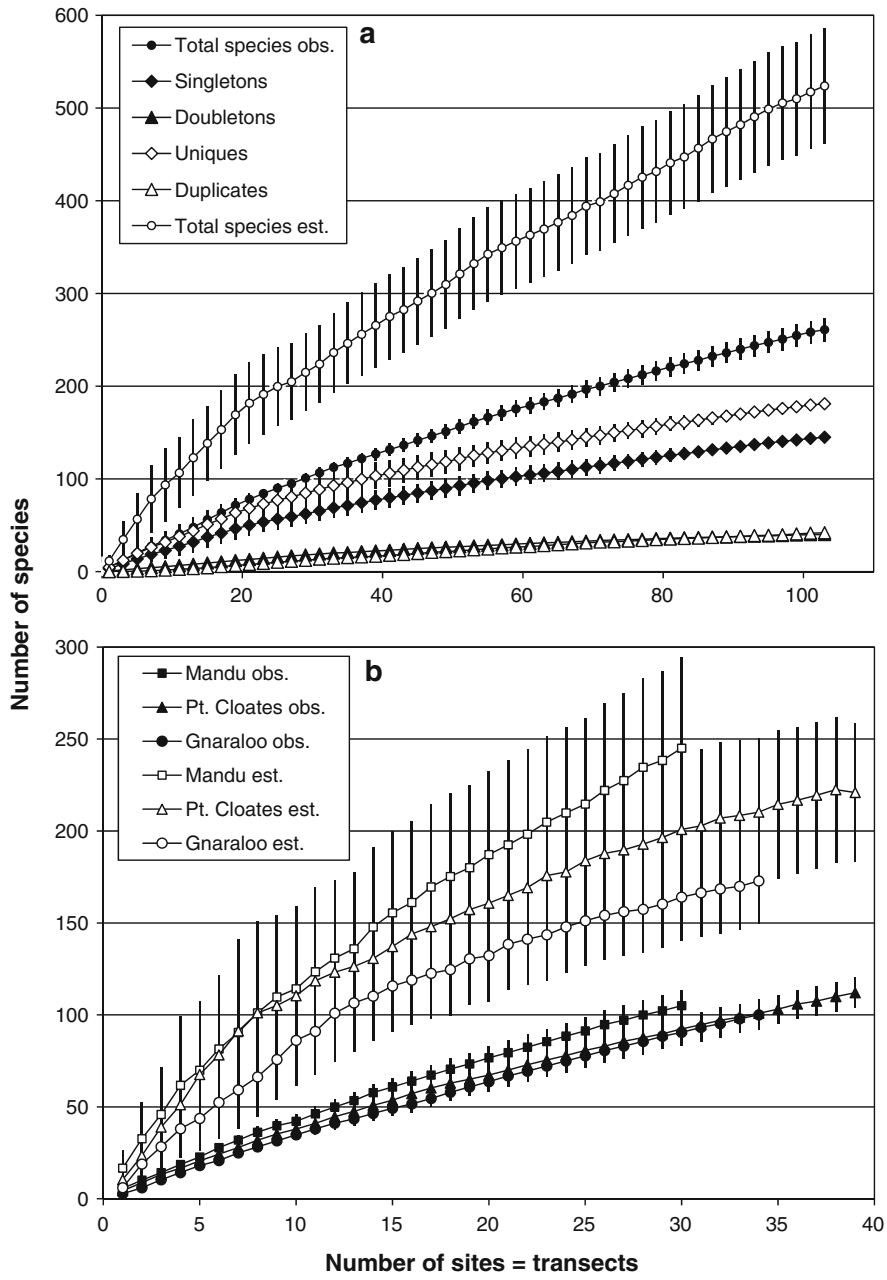

Fig. 2 Observed (obs.) and estimated (est.) sponge species richness on Carnarvon Shelf as generated by EstimateS (Colwell, 2005). Values for estimated species richness are represented by Chao1 (based on rare species in the entire collection), which was almost identical to results generated by ACE (based on abundance) and Jack2 (based on rare species per site). *Error bars* are here given as standard deviations, because the usually displayed 95% confidence intervals were very large, obscuring the figure. **a** Total species richness for Carnarvon Shelf. **b** Species richness by sample area

estimates were very large (Fig. 2a, b), indicating that the results should be regarded with caution. The resulting conservative assumptions we made when amending the data sets may mean that species numbers are higher than the calculated estimations.

CERF and WAMSI sponge collections from Carnarvon Shelf were compared to provide further evidence for high sponge biodiversity and the status of research in this bioregion (Fig. 3a). While morphospecies of the two collections are not yet fully matched, a comparison between the identified genera was used to estimate species level comparisons when the collections are combined. Per collection diversity behaved proportionally at the genus and species levels, with a ratio between genera and species of 1:2.3. The CERF collection furthermore comprised 1.7 times as many genera and species than in the WAMSI dominants collection for the same region. Both collections

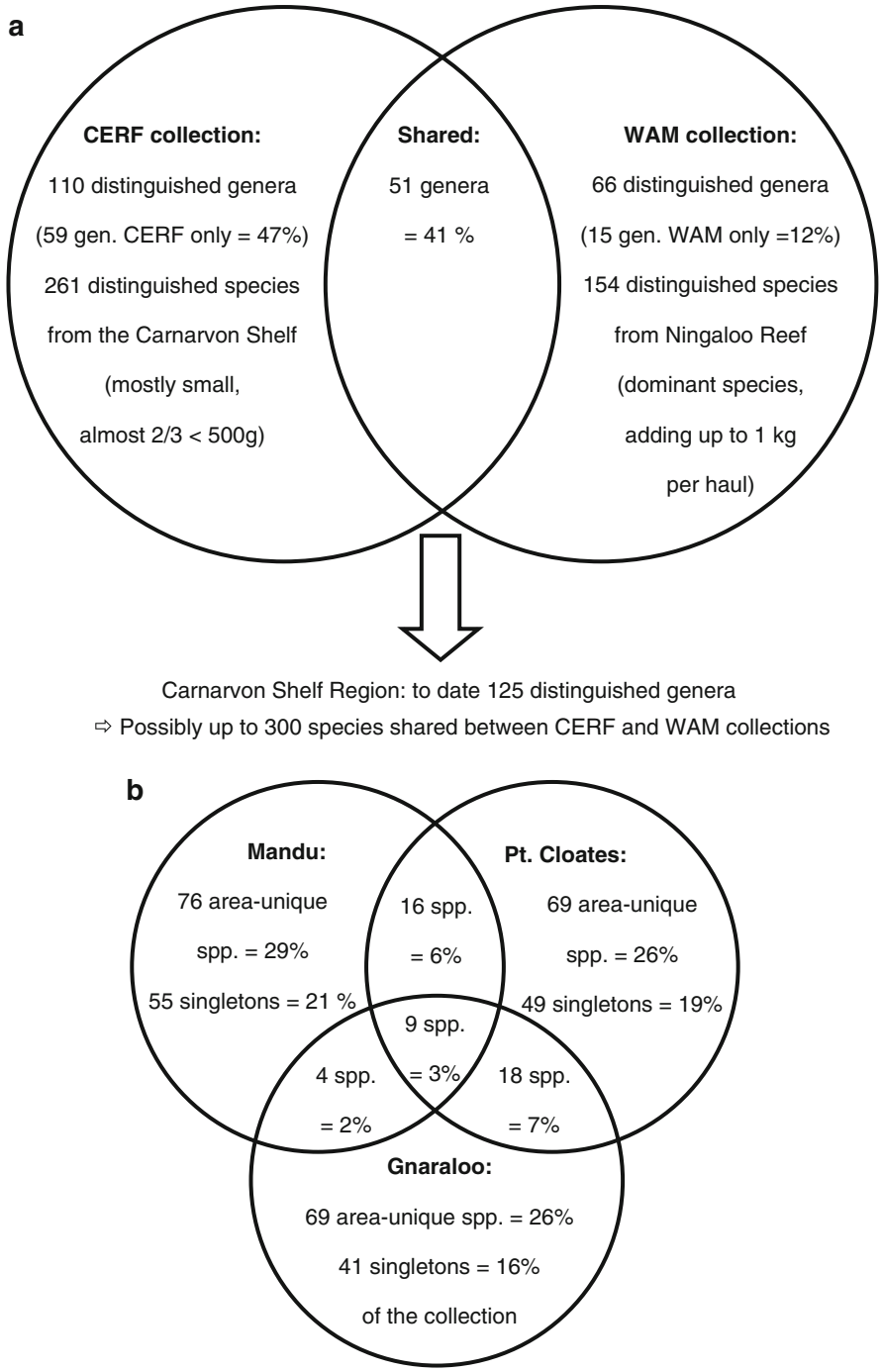

Fig. 3 Overview of proportions of Carnarvon Shelf sponge collections. **a** A comparison between the CERF and WAMSI collections, with a projection of shared and total species available from these two collections. **b** Area proportions of the CERF collection, showing shared components and proportions of perceived endemics

shared 51 sponge genera, with a total of 125 genera identified from Carnarvon Shelf. From this we calculated that the combined collections will hold about 300 sponge species from the bioregion, i.e. 47% represented only in the CERF collection, 12% only in the WAMSI collection and 41% shared between the two collections. Again, when taking the estimated species total from the Chao1 analysis, <60% of the sponge species presumably existing on the Carnarvon Shelf have been found and described.

Sponge species biomass estimates, occurrences and distributions were very patchy, with yield per

sled varying widely from no sponge specimens (Mandu 33%, Pt. Cloates 30% and Gnaraloo 18% of the tows) to a peak harvest of 58 or more specimens at a site at Pt. Cloates, and 84 kg total sponge biomass at a site at Gnaraloo (details in Supplementary material). When using area means calculated from site means, these differences were reduced, but standard deviations larger than the calculated means again revealed heterogeneity of the data (Table 2).

Most species were very rare, and while under-sampled there was a high level of perceived endemism in the present collection, with about 56% singletons, 81% of the species unique to one of the three sample areas, 69% unique to a transect, and about 15% of the species contributed only two specimens in the entire collection (=doubletons; Figs. 3b, 4a). Despite the close proximity between the three sample areas, there was very little overlap between their species complements, with the northern area Mandu and central Pt. Cloates sharing 16 species (6%), central Pt. Cloates and southern Gnaraloo 18 species (7%), and only 13 species (5%) occurring at all three areas or both the northern and southern areas, the latter case assuming occurrence in the central area. The nine species that were sampled at all three areas were *Stelletta* CERF sp. 1, *Caulospongia plicata*, *Diacarnus* CERF sp. 1, *Axinella* CERF sp. 1, *Petrosia* (*Petrosia*) CERF sp. 4, *Hyattella intestinalis*, *Acanthella pulcherrima, Theonella* CERF sp. nov. 1 and *Pararhaphoxya* CERF sp. 1 (further details in Supplementary material). The species that were collected from Mandu and Pt. Cloates and thus probably occurred at all three areas were: *Ecionemia* CERF sp. 1, *Ircinia* CERF sp. 1, *Polymastia* CERF sp. 1 and *Coelosphaera* (*Coelosphaera*) CERF sp. 2 (further details in Supplementary material). Only 12 species (<5%) had more than 10 specimens in the collection (Table 3). Among these, *Stelletta* CERF sp. 1 was by far the most common, with a total of 89 vouchers and up to 28 specimens per haul (Fig. 4a).

The collection is not only remarkable for its high biodiversity, but also because most specimens were small and would have been ignored if only dominants had been identified (=species >1 kg per tow). Only 26% of the species reached ≥1 kg in total (Fig. 4b), only 22% of transects had sponges ≥1 kg weight (the 14 most dominant listed in Table 3), and only 18% of the vouchers had an average wet weight of ≥1 kg (Fig. 4c). In contrast, 76% of the specimens weighed ≤500 g on average, and about half of the species (44%) had a mean specimen weight of ≤50 g (Fig. 4c). Only eight species contributed a total fresh weight above 10 kg, and the highest total biomass contributed by a single species was for *Forcepia* (*Forcepia*) CERF sp. 1 (Table 3). Nine species were represented by specimens over 5 kg average specimen weight, with cf. *Asteropus* CERF sp. 1 having the heaviest specimens (Table 3). Average individual weights and total biomass were significantly correlated (Fig. 4d).

Taking into account limitations to the sampling, under-sampling, lack of 1/3 of the vouchers from Gnaraloo, and partly extrapolated data, tentative spatial analyses still generated useful information. For a comparison between areas, absolute values illustrated the overall importance of the bioregion, but were slightly misleading (Fig. 5a–c), as sample effort (=number of transects, i.e. sites) varied between the areas. Using nested means it was evident that given the large variation, areas did not differ significantly from each other in mean sponge biodiversities and abundances (Fig. 5d, e). Differences in biomass per area were more evident, with Pt. Cloates displaying the smallest biomass per site and the smallest specimens, followed by Mandu, while at Gnaraloo site-related biomass and average specimen weight were approximately double those from Mandu and were roughly six times greater than from Pt. Cloates, depending on the contribution of the missing vouchers (Fig. 5f, g).

Taxon groups were not evenly distributed over the three sample areas. Diversity of the spirophorids, halichondrids and haplosclerids decreased from north to south, while that of hadromerids increased (Fig. 6a). As abundances of the heavily silicified spirophorods, astrophorids, lithistids and haplosclerids declined from north to south, the softer and more resilient poecilosclerid and fibre sponge abundances increased (Fig. 6b). Distribution patterns for biomass per taxon were obscured by the large weights sampled from Gnaraloo, so that biomass contributions for most taxon groups increased from north to south (Fig. 6c). However, standard deviations were again extremely large due to the variability of the data (not displayed).

Based on the patterns of taxon abundances, we considered evidence that might provide additional

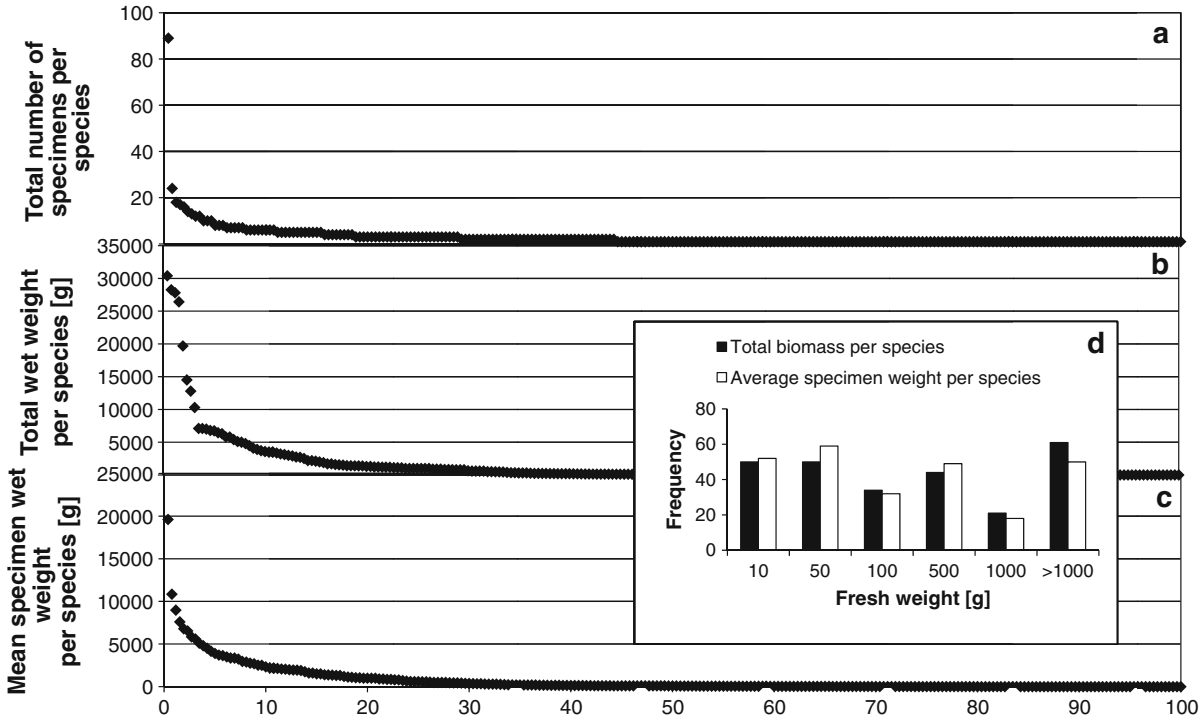

Fig. 4 Collection proportions of specimen replicates per species (**a**), total biomass per species (**b**) and mean specimen biomass per species (**c**). Data used do not include missing or unidentified vouchers. The single most extreme data points in the graphs represent *Stelletta* CERF sp. 1 with 89 specimens (**a**) and *Forcepia* (*Forcepia*) CERF sp. 1 as the species with the highest total biomass of over 30 kg (**b**), and cf. *Asteropus* CERF sp. 1 as the species with the heaviest specimens of 19 kg on average (**c**). *Inset* (**d**) depicts the relationship between total and average specimen biomass

support for differences in distribution from north to south. Six categories of growth forms were chosen to reflect sponge functions in relationship to environmental conditions (Christine Schönberg, unpubl. data). Number of specimens of robust growth forms increased northwards (simple-massive and encrusting forms), while more vulnerable growth forms increased southwards (barrels and cups, laminar, branching and other erect growth forms; Fig. 7).

No clear bathymetric patterns were perceived for taxon groups (Fig. 8a) or for functional growth forms (Fig. 8b). Overall, there were medium–high densities of taxa and growth forms in medium depths at 30–60 m. Abundances decreased between 60 and 100 m, with the exception of massive astrophorids that doubled in abundance compared to shallow depths: at the shelf edge all sponge categories strongly increased in abundance (Fig. 8a, b; data deeper than 100 m are from Mandu only). The spirastrellids, hadromerids and the simple-erect forms appeared to be categories of sponges more common in shallower than in deeper depths and were missing entirely in depths >100 m (Fig 8a, b; but based on $N = 3$ transects).

Discussion

Sponge communities in the Carnarvon Region have been regarded as comparatively species poor compared to other sites in Australia (Hooper et al., 2002; Hooper & Ekins, 2004; Wörheide et al., 2005). However, the region now ranks among sites in the Indo-Pacific with highest reported diversities, and of 34 tropical Australian bioregions only seven other regions presently exceed the number of species found on the Carnarvon Shelf (Table 4; Hooper et al., 2002; Hooper & Ekins, 2004). With 261 (morpho)species reported here it represents another 'hotspot' for sponge diversity, and with expected diversities of >500 species would contribute about 10% of the conservative estimates of the total sponge species

Table 3 Tabulated list of the most common, dominant and heaviest sponge species from Carnarvon Shelf, from the most important to the least important

Species with total specimen nos. >10 (then ranked by total weight)		Species with fresh weight per transect ≥1 kg, occurring on at least two transects (then ranked by total weight)		Species contributing a total weight of >10 kg to the collection		Species with an average specimen weight of >5 kg	
Species	Specimens	Species	No. of transects	Species	Total weight (kg)	Species	Average specimen weight (kg)
Stelletta CERF sp. 1	89	*Petrosia (Petrosia)* CERF sp. 1	5	*Forcepia (Forcepia)* CERF sp. 1	30.4	cf. *Asteropus* CERF sp. 1	19.6
Iotrochota CERF sp. 1	24	*Ecionemia* CERF sp. 1	5	cf. *Asteropus* CERF sp. 1	28.3	*Ecionemia* CERF sp. 1	10.8
Diacarnus CERF sp. 1	18	cf. *Asteropus* CERF sp. 1	4	*Petrosia (Petrosia)* CERF sp. 1	27.8	*Petrosia (Petrosia)* CERF sp. 1	9.0
Ancorina CERF sp. 1	17	*Iotrochota* CERF sp. 1	3	*Ecionemia* CERF sp. 1	26.4	*Forcepia (Forcepia)* CERF sp. 1	7.6
Axinella CERF sp. 1	16	*Sidonops* CERF sp. 1	2	*Iotrochota* CERF sp. 1	19.7	*Zyzzya* CERF sp. 1	6.8
Ecionemia CERF sp. 1	14	*Zyzzya* CERF sp. 1	2	*Sidonops* CERF sp. 1	14.5	*Petrosia (Strongylophora)* CERF sp. 2	6.5
Acanthella pulcherrima	13	cf. *Ancorina* CERF sp. 1	2	*Ecionemia* CERF sp. 2	12.8	*Diacarnus* CERF sp. 1	5.8
Petrosia (Petrosia) CERF sp. 1	12	*Iotrochota* CERF sp. 2	2	*Zyzzya* CERF sp. 1	10.3	*Ecionemia* CERF sp. 2	5.6
Acanthella cavernosa	12	*Theonella* CERF sp. nov. 1	2			*Agelas* CERF sp. 1	5.1
Monanchora clathrata	10	*Psammocinia halmiformis*	2				
Polymastia CERF sp. 1	10	*Spheciospongia* cf. *papillosa*	2				
indet. soleneiscid, calcarean CERF sp. 1	10	*Hyattella intestinalis*	2				
		Monanchora cf. *clathrata*	2				
		Coelosphaera (*Coelosphaera*) CERF sp. 2	2				

More detailed data and a complete species list can be found as Supplementary material

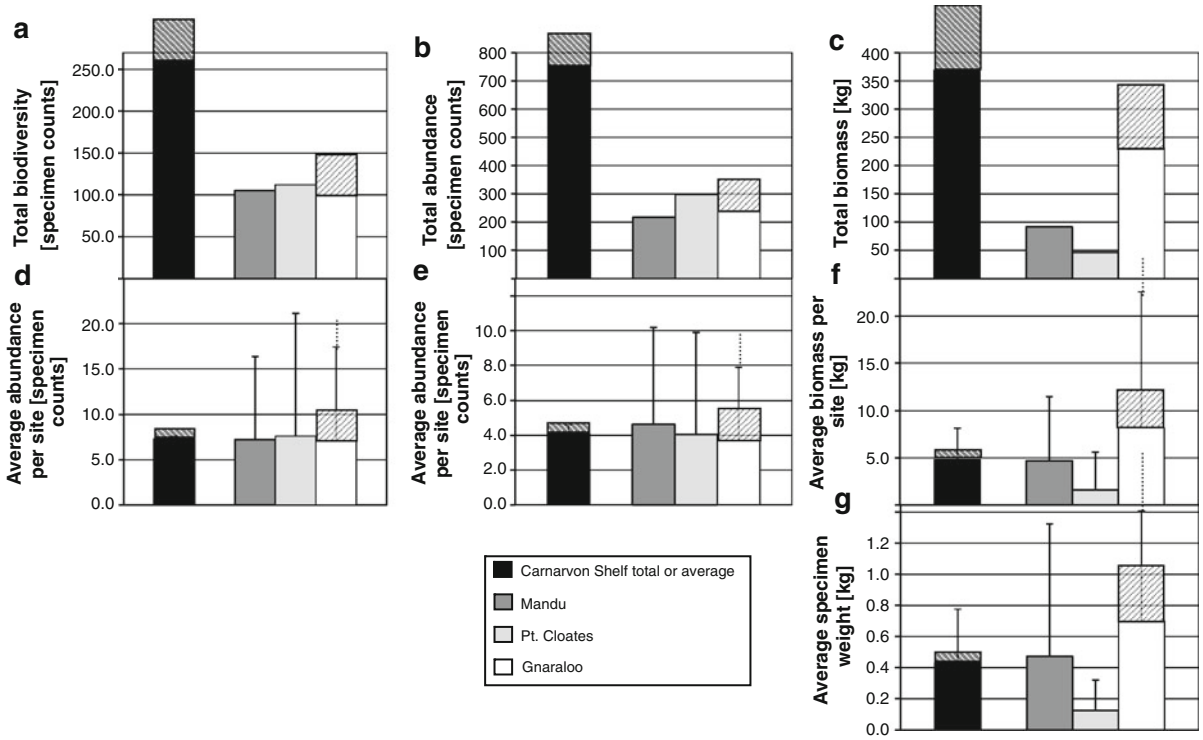

Fig. 5 Area total (**a**–**c**) and average (**d**–**g**) estimates for sponge biodiversities, abundances and biomass at Carnarvon Shelf. *Hashed boxes* for Gnaraloo and Carnarvon Shelf are estimates for missing specimens. Estimates could coarsely be calculated for specimen numbers and biomass, and were assumed to reflect the same proportion for species

record for Australia (Hooper & Ekins, 2004). This again stresses the importance of Australia's coasts as major sponge habitats (see also Heyward et al., 2010).

As in many previous studies in the Indo-Pacific (e.g. de Voogd et al., 2009; Sutcliffe et al., 2010) sponges of the CERF collection were not fully described, due to the scope and funding of the present investigation. While generating total species counts and comparisons between collections are possible (Hooper & Ekins, 2004), new species can remain unrecognised or at least unavailable to the public. At the present stage, we cannot indicate how many new species were sampled from the Carnarvon Shelf, and the material is still to be fully matched to other Australian collections (through OZCAM, see Hooper & Ekins, 2004). However, the proportion of undescribed material is thought to be significant, and our present estimate is that over 30% of the species sampled in these collections may be new to science. Our hope is that our study, including the online Sponge Catalogue for Ningaloo (Schönberg et al., 2011), will attract the interest of other taxonomists and that new species will be described, eventually providing a total number of new species found in the area.

Present results need to be compared to other studies to establish a context, but one has to consider that different sample effort and methods can affect the outcome (Table 4, see also Hooper & Ekins, 2004). Observations on the sponge community composition on the Carnarvon Shelf indicated significant small-scale heterogeneity with a high percentage of rare species and perceived endemism, and low levels of similarity between adjacent sites. This supports previous findings from Australia (e.g. Hooper, 1994; Hooper & Kennedy, 2002; Hooper & Ekins, 2004; Fromont et al., 2006). These results may in part be caused by under-sampling (Fig. 2), which has been discussed in some of these studies (e.g. Hooper & Ekins, 2004), but it may also be an inherent character of tropical sponge communities. As has also been noted during other studies (e.g. Kostylev et al., 2001; Hooper & Ekins, 2004) we found that bioeroding

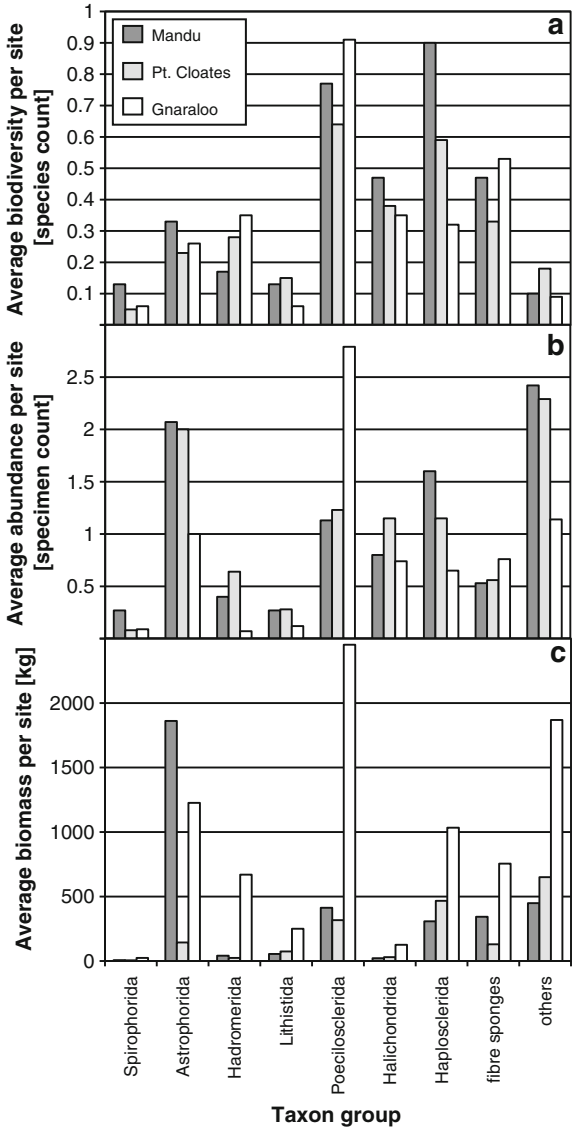

Fig. 6 Distribution of sponge taxon groups at Carnarvon Shelf. **a**—biodiversity, **b**—abundance, **c**—biomass, as average per transect to adjust for differences in sample effort. Large error bars caused by patchy distributions are not presented in order to improve the display of the graph

sponges were very important in the Carnarvon Shelf sponge collections and contributed some of the most diverse genera, often with high specimen counts and average species weights (Table 3 and Supplementary material), hence they can play a significant role in environments not immediately part of coral reefs. Free-living massive bioeroding sponges are usually included in biodiversity surveys (e.g. de Voogd & Cleary, 2008; Sutcliffe et al., 2010), but encrusting or papillate forms are often ignored (see studies conducted in Indonesia summarised in Table 4). Ignoring the papillate and fistulose bioeroding sponges from the Carnarvon Shelf would have meant 14 putative new species were not detected, which represent over 5% of the voucher collection.

Distribution patterns of the Carnarvon sponges were most likely related to substrate properties and hydrodynamic conditions. Underwater video surveys in this study suggested patchiness in sponge distribution on the Carnarvon Shelf can be related to the occurrence and distribution of hard substrate. The observed north–south trend of taxa with inorganic skeletons and more robust growth forms in the north versus more flexible sponges in the south may reflect increasing physical exposure towards the north associated with the narrowing of the shelf (Fig. 1).

The newly defined hotspot for sponge biodiversity on the Carnarvon Shelf may call for additional considerations for future protection. Carnarvon Shelf borders Ningaloo Reef, a well-known MPA recently listed for UNESCO World Heritage status (Australian Government, 2011), and shallower waters of the two northern sample areas are already protected by non-anchoring and benthic protection zones (Mandu) and Ningaloo's largest no-take, sanctuary zone (Pt. Cloates; Fig. 1). The southern area around Gnaraloo and the deeper waters, however, will need to be considered in any future zoning amendments. Gnaraloo had highest overall biodiversity, abundance, biomass and the largest sponges (Fig. 5d–g), but is not within the Ningaloo MPA. Furthermore, dense filter feeding communities extend beyond the state boundaries into deeper water. Yet, all three studied areas are important and exceptional in their species composition, with over 3/4 of the species unique to a given area and only 18% of all species occurring at more than one area (Fig. 3b). Therefore, any one area cannot be protected over the other two, a situation also noted for other regions (Hooper & Ekins, 2004). Forthcoming publications from the WAMSI deepwater biodiversity project will provide information from sample sites outside our study area (see Colquhoun & Heyward, 2008; Heyward et al., 2010), and ultimately allow more detailed assessments and more specific management recommendations.

These results provide useful baseline information, but also strongly suggest the need to work through the remaining unidentified material in the collections. In

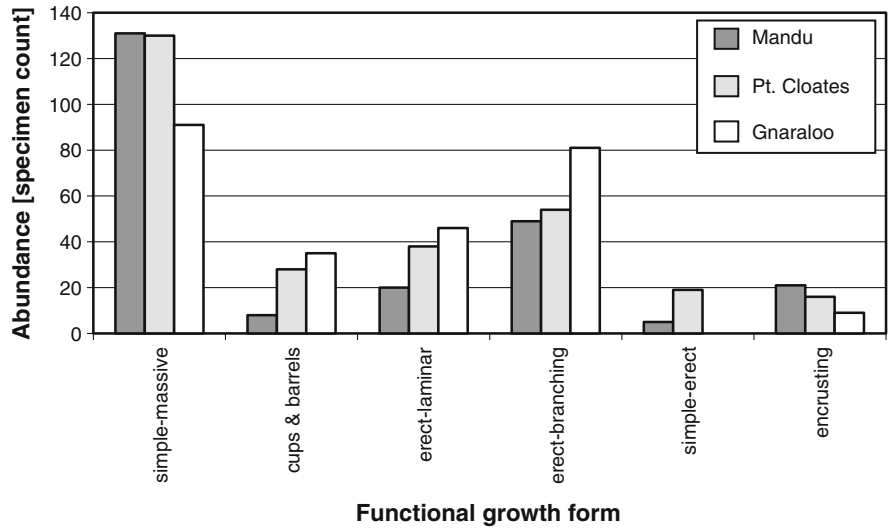

Fig. 7 Distribution of sponge functional growth forms on Carnarvon Shelf, based on total counts

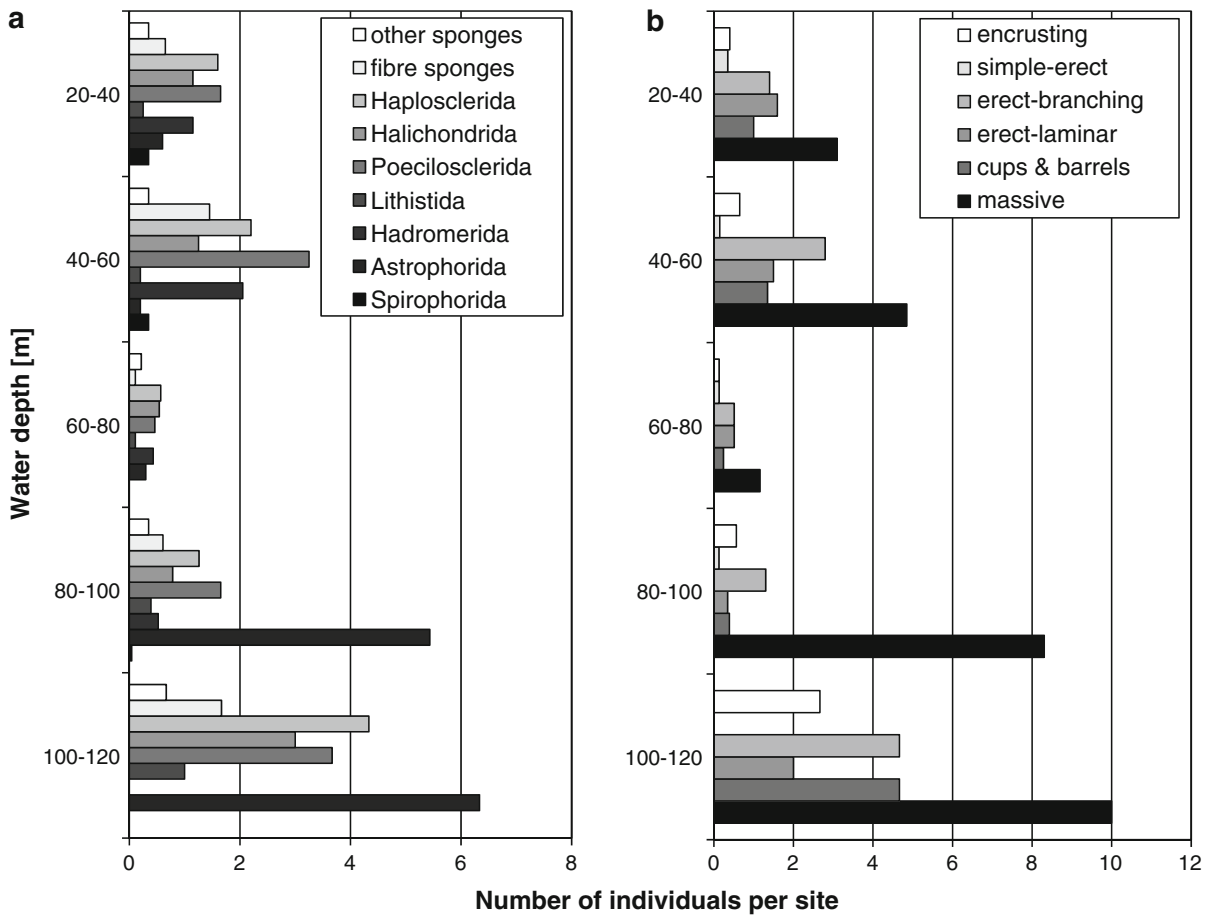

Fig. 8 Bathymetric distribution of sponge taxon groups (**a**) and functional growth forms (**b**) at Carnarvon Shelf. Data are displayed as means per transect, because number of transects per depth range varied (between 3 and 19). No samples were obtained shallower than −20 m. Large error bars caused by patchy distributions are not presented in order to improve the graphic display

Table 4 A selection of publications providing an overview of sponge biodiversities and related study methods in Australasia

Known number of species/ genera	Area	Methods	Source
A	Overview		
2,324/?	Australia, Thailand, Vanuatu and Palau	Review of other works: 'complete' faunal inventories of 1,343 localities over 15 years, various sampling strategies in 0–70 m depth	3
1,500/313 valid but ca. 4,000 morphospecies distinguished, conservative estimate: 5,000 spp. in total	Australia	Review of other works and collation of databases	6, 7
B	Selected studies from around Australia, where spp. richness is ±100:		
153/66	Sydney, Illawarra, Newcastle Regions	Review of other works and collation of databases	3, 6
214/78	Moreton Bay, Stradbroke and Moreton Islands	Review of other works and collation of databases	3, 6
233/97	Sunshine Coast, Queensland	10-year sampling program on 10 reefs, in shallow water on SCUBA	3, 4, 6
110/?	Hervey Bay	Review of other works and collation of databases	6
387/118	Capricorn Bunker Group, S Great Barrier Reef, Queensland, Australia	Review of other works and collation of databases	3, 6
304/93	Swain Reefs	Review of other works and collation of databases	3, 6
162/77	Pompey Group	Review of other works and collation of databases	3, 6
135/69	Whitsunday Group	Review of other works and collation of databases	3, 6
239/77	Townsville Region	Review of other works and collation of databases	3, 6
99/65	Cairns Region	Review of other works and collation of databases	3, 6
131/62	Low Isles	Review of other works and collation of databases	3, 6
464/104	Lizard and Direction Islands, Ribbon, No Name and Yonge Reefs, N Great Barrier Reef, Queensland, Australia	Review of other works and collation of databases	3, 6
106/?	N Queensland Plateau	Review of other works and collation of databases	6
146/71	Far northern GBR reefs	Review of other works and collation of databases	3, 6
>1,200	GBR Seabed, Queensland, Australia	Six cruises in 2 years, 457 trawls (8 fathom prawn otter trawl net towed over 1 km), 1,189 epibenthic sled samples (1.5 m width towed 200 m each) = 1,254 sites	17
135/?	Torres Straight, N Australia	Review of other works and collation of databases	6
315/116	Wessel Islands, N Australia	Review of other works	3
395/111	Darwin and Coburg Peninsula Regions, N Australia	Review of other works and collation of databases	3, 6
124/77	Ashmore, Carteria and Hibernia Reefs, N Sahul Shelf	Review of other works	3, 6
130/42	Houtman Abrolhos, Western Australia	14 stations in shallow water on SCUBA	1, 6
275/106	Dampier Archipelago, Western Australia	Compilation of data from four fieldtrips, collection by wading, on snorkel, SCUBA and by dredging; sponges <2 cm in size were ignored	5
347/129	Dampier and Port Hedland Regions, NW Australian Shelf	Review of other works and collation of databases	3, 6
132/59	North West Atolls, Western Australia	15 day survey on SCUBA in shallow water at 45 stations	15
261/112 ca. 300/125 when including WAMSI collection, but >500 spp. estimated	Carnarvon Shelf at Mandu, Pt. Cloates and Gnaraloo	1 month cruise, 103 sites, 100 m tows with 1.5 m wide epibenthic sledge	Present study
109/61	Great Australian Bight	Study at 65 sites, epibenthic sled tows between 48 and 195 m, 5 min tows at 3.5 knots	11
165/65	Continental margin canyons, SE Australia	2 week cruise, sampling at 14 epibenthic sledge samples in 114–612 m depth (sled 0.72 m wide)	10

Table 4 continued

Known number of species/ genera	Area	Methods	Source
C	Selected studies from the Indian Ocean:		
96/64	iSimangaliso Wetland Park, N KwaZulu-Natal, South Africa	5 year bathymetry survey by wading in the intertidal, SCUBA and trimix diving subtidal to 140 m, submersible and ROV to 500 m	16
262/? full spp. record: 325/?	South Africa	Review of other works	8
>1,500	South China Sea	Review of other works	2
196/73	Thailand (Gulf and Andaman Sea)	Review of other works	3
D	Selected studies from the Pacific Ocean:		
118/64	Thousand Islands and Jakarta Bay Areas, W Java, Indonesia	Almost 3 weeks of surveys on SCUBA in shallow water at 30 sites; thinly encrusting, cryptic and bioeroding species excluded	12
168/62	Derawan Islands, NE Kalimantan, Indonesia	2 weeks of SCUBA diving at shallow depth at 18 different sites; thinly encrusting, cryptic and bioeroding species excluded	13
151/68	Spermonde Archipelago, SW Sulawesi, Indonesia	Almost 1 year of surveys on SCUBA in shallow depths at 34 stations at seven reefs; thinly encrusting, cryptic and bioeroding species excluded	9
212/86	Palau	Review of other works	3
193/91	Vanuatu	Review of other works	3
149/94	New Caledonia	Review of other works	14

Values for sponge biodiversity 'hotspots' in bold, defined by Hooper & Ekins (2004) as 250+ species occurring per bioregion. Sources below are: (1) Fromont (1999), (2) Hooper et al. (2000), (3) Hooper et al. (2002), (4) Hooper & Kennedy (2002), (5) Fromont (2004), (6) Hooper & Ekins (2004), (7) Wörheide et al. (2005), (8) Samaai (2006), (9) de Voogd et al. (2006), (10) Schlacher et al. (2007), (11) Sorokin et al. (2007), (12) de Voogd & Cleary (2008), (13) de Voogd et al. (2009), (14) Hooper & Schlacher-Schoenlinger (2007), (15) Fromont & Vanderklift (2009), (16) Samaai et al. (2010), (17) Sutcliffe et al. (2010). Where 3 and 6 are cited, 6 refers to species count, 3 to genera distinguished

addition, studies with an ecological and physiological focus would be useful, and future research efforts should seek explanations for observed high diversities and densities of sponges in northern Australia (e.g. Hooper et al., 2002; Hooper & Ekins, 2004), how these sponge communities survive, and what resources they provide to other organisms. We need a better understanding of the role (e.g. Chu & Leys, 2010) and vulnerabilities of sponge gardens compared to coral reefs. When we can quantify for example the filtering capacity of such communities and provide predictions on how human impacts may affect their functions, we can better estimate how the system may cope with change and recommend ways to protect these unique environments.

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References

11th International Coral Reef Symposium. Fort Lauderdale, Florida, USA, July 7–11, 2008. Available at http://www.nova.edu/ncri/11icrs/index.html. Accessed 12/10/2010.

8th World Sponge Conference, 2010. Ancient Animals, New Challenges. Available at http://www.spongeconference2010.org/. Accessed 12/10/2010.

Aperio, 2010. SCAN—High-Quality, Ultra-Resolution Digital Scanning. Available at http://www.aperio.com/pathology-services/scanscope-digital-scanners.asp. Accessed 12/10/2010.

Australian Broadcasting Corporation, 1998. Jewels of the Sea. Sponge Gardens. Available at http://www.abc.net.au/oceans/jewel/sponge/default.htm. Accessed 12/10/2010.

Australian Government, 2011. Department of Sustainability, Environment, Water, Population and Communities, Ningaloo Coast. Available at http://www.environment.gov.au/heritage/places/world/ningaloo/index.html. Accessed 27/6/2011.

Bax, N. J., B. Brooke, D. Gledhill, R. Pitcher & C. Wilcox, 2011. CERF Marine Biodiversity Hub Final Report 2007–2010. CERF Marine Biodiversity Hub, Hobart: 76 pp. Available at http://www.marinehub.org/cerf-marine-biodiversity-hub-final-report-2007-2010. Accessed 22/6/2011.

Becerro, M. A., 2008. Quantitative trends in sponge ecology research. Marine Ecology 29: 167–177.

Brooke, B., S. Nichol, M. Hughes, M. McArthur, T. Anderson, R. Przeslawski, J. Siwabessy, A. Heyward, C. Battershill, J. Colquhoun & P. Doherty (eds), 2009. Carnarvon Shelf Survey. Post Survey Report August 12th–September 15th, 2008. Geoscience Australia Record 2009/02: 89 pp.

Centre for Microscopy, Characterisation and Analysis, 2010. Aperio Scanscope Digital Slide Scanner. Available at http://www.cmca.uwa.edu.au/facilities/ocm/aperio-scanscope. Accessed 12/10/2010.

Chu, J. W. F. & S. L. Leys, 2010. High resolution mapping of community structure in three glass sponge reefs (Porifera, Hexactinellida). Marine Ecology Progress Series 417: 97–113.

Coleman, N. & N. Marsh, 2003. Diving Australia. A Guide to the Best Diving Down Under. Tuttle Publishing, Sydney.

Colquhoun, J. & A. Heyward (eds), 2008. Ningaloo Reef Marine Park Deepwater Benthic Biodiversity Survey. Annual Report 2007. Joint Report by the Australian Institute of Marine Science, Curtin University of Technology, The University of Western Australia and the Western Australian Museum. Perth: 195 pp.

Colwell, R. K., 2005. EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples. Version 7.5. User's Guide and Application Published at: http://purl.oclc.org/estimates. Accessed 29/11/2010.

Colwell, R. K. & J. A. Coddington, 1994. Estimating terrestrial biodiversity through extrapolation. Philosophical Transactions of the Royal Society B 345: 101–118.

Coral-List, 2010. NOAA's Coral Health and Monitoring Program (CHAMP) Listserver for Coral Reef Information and News. Available at http://coral.aoml.noaa.gov/mailman/listinfo/coral-list/. Accessed 12/10/2010.

Daly, M., M. R. Brugler, P. Cartwright, A. G. Collins, M. N. Dawson, D. G. Fautin, S. C. France, C. S. McFadden, D. M. Opresko, E. Rodriguez, S. L. Romano & J. L. Stake, 2007. The Phylum Cnidaria: a review of phylogenetic patterns and diversity 300 years after Linnaeus. Zootaxa 1668: 127–182.

de Voogd, N. J. & D. F. R. Cleary, 2008. An analysis of sponge diversity and distribution at three taxonomic levels in the Thousand Islands/Jakarta Bay reef complex, West-Java, Indonesia. Marine Ecology 29: 205–215.

de Voogd, N. J., D. F. R. Cleary, B. W. Hoeksema, A. Noor & R. W. M. van Soest, 2006. Sponge beta diversity at the Spermonde Archipelago, SW Sulawesi, Indonesia. Marine Ecology Progress Series 309: 131–142.

de Voogd, N. J., L. E. Becking & D. F. R. Cleary, 2009. Sponge community composition in the Derawan Islands, NE Kalimantan, Indonesia. Marine Ecology Progress Series 396: 169–180.

Diaz, M. C. & K. Rützler, 2001. Sponges: an essential component of Caribbean coral reefs. Bulletin of Marine Science 69: 535–546.

Fromont, J., 1999. The demosponge fauna of the Houtman Abrolhos Islands. Memoirs of the Queensland Museum 44: 175–183.

Fromont, J., 2004. Porifera (sponges) of the Dampier Archipelago, Western Australia: habitats and distribution. In Jones, D. S. (ed.), Aquatic Fauna of the Waters of the Dampier Archipelago, Western Australia. Report of the Woodside Energy Ltd/Western Australia Museum Partnership to Explore the Marine Biodiversity of the Dampier Archipelago, 1998–2001. Records of the Western Australian Museum Supplement 66: 69–100.

Fromont, J. & M. A. Vanderklift, 2009. Porifera (sponges) of Mermaid reef (Rowley Shoals), Scott and Seringapatam Reefs, Western Australia. Records of the Western Australian Museum Supplement 77: 81–104.

Fromont, J., M. A. Vanderklift & G. A. Kendrick, 2006. Marine sponges of the Dampier Archipelago, Western Australia: patterns of species distributions, abundance and diversity. Biodiversity and Conservation 15: 3731–3750.

Geoscience Australia, 2010. Marine Biodiversity Hub. Available at http://www.marinehub.org/index.php/site/home. Accessed 1/7/2011.

Heyward, A., J. Fromont, C. H. L. Schönberg, J. Colquhoun, B. Radford & O. Gomez, 2010. The sponge gardens of Ningaloo Reef, Western Australia. Open Marine Biology Journal 4: 3–11.

Hooper, J. N. A., 1988. Structural features of the benthic community of East Point Reef Fish Reserve. A comparative study between oceanic, near-shore and inshore reefs of northwest Australia. In Larson, H. K., M. G. Michie & J. R. Hanley (eds), Darwin Harbour. Australian National University North Australian Research Unit Mangrove Monograph 4. North Australian Research Unit, ANU, Darwin: 214–225.

Hooper, J. N. A., 1994. Coral reef sponges of the Sahul Shelf—a case for habitat preservation. Memoirs of the Queensland Museum 36: 93–106.

Hooper, J. N. A. & M. Ekins, 2004 (published online 2009). Collation and Validation of Museum Collection Databases Related to the Distribution of Marine Sponges in Northern Australia. Report to the National Oceans Office Contract C2004/020 and Technical Reports of the Queensland Museum 002: 1-224. Available at http://www.environment.gov.au/coasts/mbp/publications/general/nmb-sponge-report.html and http://www.qm.qld.gov.au/Research/Publications/Publications/Technical+reports/

002+Collation+and+validation+of+museum+collection+databases. Accessed 22/6/2011.

Hooper, J. N. A. & J. A. Kennedy, 2002. Small-scale patterns of sponge biodiversity (Porifera) on Sunshine Coast reefs, eastern Australia. Invertebrate Systematics 16: 637–653.

Hooper, J. N. A., J. A. Kennedy & R. W. M. van Soest, 2000. An annotated checklist of sponges (Porifera) of the South China Sea Region. The Raffles Bulletin of Zoology 8: 125–207.

Hooper, J. N. A. & M. A. Schlacher-Schoenlinger, 2007. Porifera of New Caledonia. Remarks on the check list of shallow water species. In Payri, C. E. & D. Richer De Forges (eds), Compendium of marine species from New Caledonia, Special Volume II(7), 2nd edn. Institut de Recherche pour le Developpement. Documents Scientifiques et Techniques of IRD Centre de Noumea: 113–117.

Hooper, J. N. A., J. A. Kennedy & R. J. Quinn, 2002. Biodiversity 'hotspots', patterns of richness and endemism, and taxonomic affinities of tropical Australian sponges (Porifera). Biodiversity and Conservation 11: 851–885.

Kostylev, V. E., B. J. Todd, G. B. J. Fader, R. C. Courtney, G. D. M. Cameron & R. A. Pickrill, 2001. Benthic habitat mapping on the Scotian Shelf based on multibeam bathymetry, surficial geology and sea floor photographs. Marine Ecology Progress Series 219: 121–137.

McClintock, J. B., C. D. Amsler, B. J. Baker & R. W. M. van Soest, 2005. Ecology of Antarctic marine sponges: an overview. Integrative and Comparative Biology 45: 359–368.

Porifera-List, 2010. Jiscm@il Porifera homepage. Available at https://www.jiscmail.ac.uk/cgi-bin/webadmin?A0=porifera. Accessed 12/10/2010.

Reef Watch Victoria, 2006. Take a Dive that Counts. Sponge Gardens. Available at http://www.reefwatchvic.asn.au/MarineLife/Habitats/3_SpongeHabitats.htm. Accessed 12/10/2010.

Samaai, T., 2006. Biodiversity 'hotspots', patterns of richness and endemism, and distribution of marine sponges in South Africa based on actual and interpolation data: a comparative approach. Zootaxa 1358: 1–37.

Samaai, T., M. J. Gibbons, S. Kerwath, D. Yemane & K. Sink, 2010. Sponge richness along a bathymetric gradient within the iSimangaliso Wetland Park, South Africa. Marine Biodiversity 40: 205–217.

Schlacher, T. A., M. A. Schlacher-Hoenlinger, A. Williams, F. Althaus, J. N. A. Hooper & R. Kloser, 2007. Richness and distribution of sponge megabenthos in continental margin canyons off southeastern Australia. Marine Ecology Progress Series 340: 73–88.

Schönberg, C. H. L., J. Fromont, B. Alvarez, C. Battershill, A. Pisera, P. Sutcliffe, S. Sorokin, L. Goudie, O. Gomez & M. Case, 2011. The Ningaloo sponge catalogue. As part of: B. Radford & T. Ridgway (eds.) The Ningaloo Atlas. Available at http://ningaloo-atlas.org.au/. Accessed 30/8/2011.

Sorokin, S., J. Fromont & D. Currie, 2007. Demosponge biodiversity in the Great Australian Bight's benthic protection zone. Transactions of the Royal Society of South Australia 131: 192–204.

State of New South Wales, 2005. Primary Industries Fisheries and Aquaculture. Marine Habitats. Available at http://www.dpi.nsw.gov.au/fisheries/habitat/aquatic-habitats/marine. Accessed 12/10/2010.

Sutcliffe, P. R., J. N. A. Hooper & C. R. Pitcher, 2010. The most common sponges on the Great Barrier Reef seabed, Australia, include species new to science (Phylum Porifera). Zootaxa 2616: 1–30.

Thomson Reuter's (ISI) Web of Knowledge. Citation and Journal Database for Academic Users. Available at http://apps.isiknowledge.com/UA_GeneralSearch_input.do?product=UA&search_mode=GeneralSearch&SID=X2pFj1294BKAEN82Ph3&preferencesSaved=. Accessed through the Australian Institute of Marine Sciences information services 24/6/2011.

van Soest, R. W. M, N. Boury-Esnault, J. N. A. Hooper, K. Rützler, N. J. de Voogd, B. Alvarez de Glasby, E. Hajdu, A. B. Pisera, R. Manconi, C. H. L. Schönberg, D. Janussen, K. R. Tabachnick, M. Klautau, B. Picton & M. Kelly, 2011. World Porifera Database. Available at http://www.marinespecies.org/porifera. Accessed 22/6/2011.

Wörheide, G., A. M. Solé-Cava & J. N. A. Hooper, 2005. Biodiversity, molecular ecology and phylogeography of marine sponges: patterns, implications and outlooks. Integrative and Comparative Biology 45: 377–385.

Zoological Record, 2011. The World's Oldest Continuing Database of Animal Biology. Available at http://thomsonreuters.com/products_services/science/science_products/a-z/zoological_record/. Accessed through the University of Western Australia information services 24/6/2011.

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SPONGE RESEARCH DEVELOPMENTS

Role of deep sponge grounds in the Mediterranean Sea: a case study in southern Italy

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Abstract The Mediterranean spongofauna is relatively well-known for habitats shallower than 100 m, but, differently from oceanic basins, information upon diversity and functional role of sponge grounds inhabiting deep environments is much more fragmentary. Aims of this article are to characterize through ROV image analysis the population structure of the sponge assemblages found in two deep habitats of the Mediterranean Sea and to test their structuring role, mainly focusing on the demosponges *Pachastrella monilifera* Schmidt, 1868 and *Poecillastra compressa* (Bowerbank, 1866). In both study sites, the two target sponge species constitute a mixed assemblage. In the Amendolara Bank (Ionian Sea), where *P. compressa* is the most abundant species, sponges extend on a peculiar tabular bedrock between 120 and 180 m depth with an average total abundance of 7.3 ± 1.1 specimens m^{-2} (approximately 230 gWW m^{-2} of biomass). In contrast, the deeper assemblage of Bari Canyon (average total abundance 10.0 ± 0.7 specimens m^{-2}, approximately 315 gWW m^{-2} of biomass), located in the southwestern Adriatic Sea between 380 and 500 m depth, is dominated by *P. monilifera* mixed with living colonies of the scleractinian *Madrepora oculata* Linnaeus, 1758, the latter showing a total biomass comparable to that of sponges (386 gWW m^{-2}). Due to their erect growth habit, these sponges contribute to create complex three-dimensional habitats in otherwise homogenous environments exposed to high sedimentation rates and attract numerous species of mobile invertebrates (mainly echinoderms) and fish. Sponges themselves may represent a secondary substrate for a specialized associated fauna, such zoanthids. As demonstrated in oceanic environments sponge beds support also in the Mediterranean Sea locally rich biodiversity levels. Sponges emerge also as important elements of benthic–pelagic coupling in these deep habitats. In fact, while exploiting the suspended organic matter, about 20% of the Bari sponge assemblage is also severely affected by cidarid sea urchin grazing, responsible to cause visible damages to the sponge tissues (an average of 12.1 ± 1.8 gWW of individual biomass removed by grazing). Hence, in deep-sea ecosystems, not only the coral habitats, but also the grounds of massive sponges

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represent important biodiversity reservoirs and contribute to the trophic recycling of organic matter.

Keywords Deep benthos · Porifera · Mediterranean Sea · Biodiversity · Pelagic–benthic coupling

Introduction

The Porifera fauna of the Mediterranean Sea is one of the best documented in the world although this holds particularly true only for habitats shallower than 100 m. In fact, information on sponges inhabiting deeper settings is scant, fragmentary and sparse in the literature (Babic, 1922; Topsent, 1928; Vacelet, 1960, 1961, 1969, 1996; Uriz, 1981, 1983, 1984; Uriz & Bibiloni, 1984; Zibrowius, 1985; Pansini, 1987; Uriz & Rosell, 1990; Pansini & Musso, 1991; Maldonado, 1992; Voultsiadou-Koukouras & Van Soest, 1993, Boury-Esnault et al., 1994; Ilan et al., 1994, 2003; Magnino et al., 1999; Uriz & Maldonado, 2000; Longo et al., 2002). Furthermore, most of these observations are derived from bottom sampling not deliberately targeting sponges in a systematic way. Sampling in the deep sea is often biased towards soft-bottom habitats, which are easier to sample by trawling than the less-accessible hard substrata. The first in situ observations that focused on deep sea sponges are those carried out in the Cassidaigne Canyon, near Marseille, by Vacelet (1969) in a manned submarine (*Soucoupe plongeante Cousteau*: SP 300). In the last few years, the exploration of the deep sea realm, due to the implementation of non-invasive techniques such as Remotely Operated Vehicles (ROV), increased steadily. Using this technique, important new information were obtained on Mediterranean deep sea inhabitants, including sponges (Freiwald et al., 2009; Taviani & Angeletti, 2009; Beuck et al., 2010; Mastrototaro et al., 2010; Vertino et al., 2010; Bo et al., 2011a).

The term sponge ground is commonly accepted as referring to the case of sponges dominating in size and abundance, and also often by the accumulation of skeletal remains, the seabed (Hogg et al., 2010). They commonly occur on patchy hard substrata surrounded by unstable soft bottoms, but some species are specialized to live on sand and mud. Deep-water sponge grounds are also known to occur in distinct areas where local environmental conditions are suitable for their, usually slow, growth (Hogg et al., 2010). Although in the Mediterranean Sea sponge beds have not been recorded so far, grey literature and fishermen bycatch indicate the existence of large sponge assemblages also for this temperate basin.

Seamounts and submarine canyons, intrinsically difficult to survey due to their rough topography and vigorous current regime, were chosen as study sites since they may host structured and diverse suspension feeders communities, in which Porifera often play a leading role (Bourcier & Zibrowius, 1973; Boehlert & Genin, 1987; Tunesi et al., 2001; Samadi et al., 2007; Freiwald et al., 2009; Clark et al., 2010; Hogg et al., 2010). In this context, the Amendolara Bank (Ionian Sea) (Fig. 1) has been extensively investigated in the past on its shallow euphotic top resulting in substantial information on the benthic coralligenous biota (Rossi & Colantoni, 1976; Panetta et al., 1985; Perrone, 1985; Strusi et al., 1985; D'Addabbo Gallo et al., 1987; Cecere & Perrone, 1988; Di Geronimo et al., 1998). On the contrary, no precise documentation was given for the deep mesophotic assemblages below 100 m depth, characterized by sparse rocks surrounded by muddy bottoms, suitable for the settling of deep suspension feeders such as sponges. The Bari Canyon (south-western Adriatic Sea: Fig. 1), instead, is known to be an optimal area where to investigate the complex interplay between topographic setting, hydrologic regime and the biological response of benthic communities (Trincardi et al., 2007; Bianchelli et al., 2008). Indeed, recently, lush deep-water coral communities dominated by the frame-building scleractinians *Madrepora oculata* Linnaeus, 1758 and *Lophelia pertusa* Linnaeus, 1758, often intimately associated with sponges and serpulids, have been documented from the steep flanks and other hard-bottom settings in the canyon system between 350 and 650 m depth (Freiwald et al., 2009). As the nearby deep water coral province of Santa Maria di Leuca (Taviani et al., 2005a, b; Longo et al., 2005; Freiwald et al., 2009; Mastrototaro et al., 2010; Rosso et al., 2010, with references therein), the Bari Canyon represents an important biodiversity hotspot dominated by suspension feeders, mainly corals and sponges.

Fig. 1 Locations of the study areas (*black dots*) in the southern Italian seas: Amendolara Bank (Ionian Sea) and Bari Canyon (southern Adriatic Sea)

The aims of this study are to characterize the population structure and to test the structuring role of the sponge assemblages in deep habitats of the Mediterranean Sea mainly focusing on the two demosponges, *Pachastrella monilifera* Schmidt, 1868 and *Poecillastra compressa* (Bowerbank, 1866).

Materials and methods

Data collection and analyses

Data here presented were recorded during two ROV campaigns performed in August 2009 and July 2010 on board R/V *Astrea* of ISPRA (MoBioMarCal project) and R/V *Urania* of CNR (E.U. HERMIONE project) for the Amendolara Bank and the Bari Canyon, respectively. The ROV *Pollux* was equipped with a digital camera (Nikon D80, 10 megapixels), an underwater strobe (Nikon SB 400), and a high definition video camera (Sony HDR-HC7). In addition, it was equipped with a depth sensor, a compass, and two parallel laser beams providing a 10 cm scale for measuring the size of the sampled bottom areas.

Eleven adult sponges, 6 orange and 5 white, were collected from the Amendolara Bank with the ROV grabbers for taxonomic identification. Samples were fixed on-board in 4% formaldehyde in filtered sea water and then preserved in 70% ethanol. Spicule complement and skeletal architecture were examined under light microscopy following Hooper (2000). For SEM analyses, dissociated spicules were transferred onto stubs and sputter coated with gold then observed under a Philips XL20 SEM. No specimens were collected in the Bari Canyon and the species identification was based on the similarity with the Amendolara samples and on the base of a previous taxonomic work conducted in the sponge-white coral habitat of S. Maria Di Leuca (Longo et al., 2005).

In total, 50 and 135 photographs have been analyzed with areas comprised between 0.5 and 3 m^2, respectively, for the Amendolara Bank (54 m^2 of total explored surface) and the Bari Canyon (128 m^2 of total explored surface). Images were analyzed by ImageJ 1.33 software in order to record both abundance (±SE, standard error) and size (height × width in cm) of the sponges counted in the ROV images (211 measures in the Amendolara Bank and 770 in the Bari Canyon). Data were used to depict the community structure in terms of species identification, average abundance, bathymetric distribution, size-frequency distribution and percentage of sea urchin bites. Observations on the coral component (in the Bari Canyon) and on the associated organisms were also recorded for each analyzed image.

To depict the sponge biomass contribution in the two studied assemblages, the average wet weights (WW, expressed as g cm^{-2} ± SE) for the two studied species (0.33 ± 0.02 and 0.30 ± 0.05 g cm^{-2}, respectively, for *Pachastrella monilifera* and *Poecillastra compressa*) were calculated by weighting the collected specimens. With the same procedure, the biomass of the coral *Madrepora oculata* (0.73 ± 0.01 g cm^{-2}) was calculated for comparison. From the areas of the specimens, measured from ROV images with ImageJ software, the average individual biomass and the total biomass for each species and for each site were obtained. With the same method, the area and biomass grazed.

Study sites

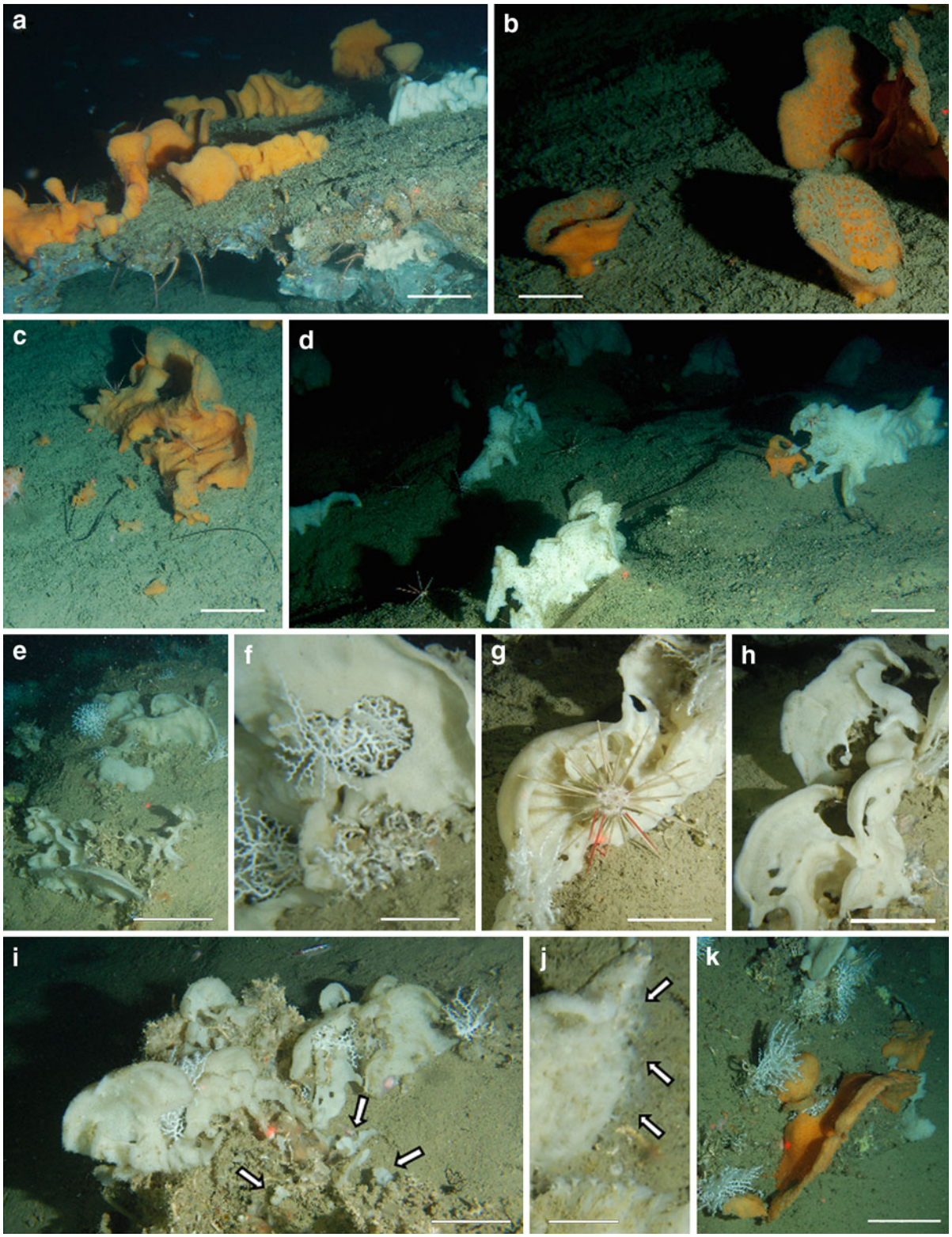
Amendolara Bank is located on the western side of the Gulf of Taranto, in the northern Ionian Sea, about 10 miles offshore Corigliano Calabro (Fig. 1). It is a seamount with an almost ellipsoidal surface extending for 31 km^2 from 200 to 26 m depth (Rossi & Colantoni, 1976; Strusi et al., 1985; Di Geronimo et al., 1998; Romagnoli, 2004). Its top is quite rugged because of peaks, depressions and ridges (Rossi & Colantoni, 1976; Cecere & Perrone, 1988; Ceramicola et al., 2008). The bathymetric profile of the bank shows two shallow peaks, then, below 50 m depth, the bank is connected with a steep NW side to the flat continental platform, while the other flanks gently dip along the continental slope (Rossi & Colantoni, 1976; Rossi & Gabbianelli, 1978; Romagnoli, 2004). The flanks of the Amendolara Bank are characterized by frequent slides and by zones with gas emissions along the active tectonic structures (Ceramicola et al., 2008). The top of the mount is sediment-starved and characterized by the production and accumulation of skeletal hash and finer bioclastic sediment. Coralligenous bioconcretioning, mainly composed of algae, bryozoans, sponges and interspersed coarse sediments, occurs from the top to about 50 m depth (Cecere & Perrone, 1988). Coarse sediments, becoming progressively finer with depth, are then replaced by mud below 80 m depth (Strusi et al., 1985). Annual oceanographic measurements document water transparency from about 10 to 16 m, temperature from 13.3 to 18.4°C around 100 m depth (Cecere & Perrone, 1988), and a predominant NS-flowing current (Grancini et al., 1969). More in detail, the site explored during the ROV survey is located on the bank SE flank at 39°50′17″N–16°48′52″E between 120–180 m Here, the area is characterized by a 'tabular' bedrock interrupted at places by rounded peaks draped by muddy sediment as for the adjacent sea bottom.

Bari Canyon is a remarkable, 30-km long and 10-km wide, WE-trending structure cutting the Apulian outer shelf (Fig. 1). It is characterized by multiple heads and two main branches (Trincardi et al., 2007). The northern branch is a narrow, straight incision with a slight steepness. The other branch is wider, asymmetric, U-shaped with a very steep southern wall and a more complex northern side (Turchetto et al., 2007). The Bari Canyon represents a sort of channel through which suspended fine sediment generated by the Po River (the primary fluvial system entering the Adriatic Sea) is conveyed into the deep southern Adriatic basin through cascading water masses (Ridente et al., 2007; Trincardi et al., 2007; Turchetto et al., 2007). It also conveys nutrients representing an important source of food nourishing the deep communities (Bianchelli et al., 2008). The explored area during our ROV survey is located at 41°17′15″N–17°16′38″E, between 380 and 500 m depth, along the canyon's southern branch.

Results

The studied species (Fig. 2)

Pachastrella monilifera and *Poecillastra compressa* are two demosponge species belonging to the Order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◀ **Fig. 2** ROV exploration of the sponge assemblages. **a-d** Amendolara Bank (120–180 m depth). **a** *Poecillastra compressa* and *Pachastrella monilifera* colonizing the upper side of a tabular bedrock. Below the rock, in the sheltered side, are visible numerous encrusting sponges and ophiuroids. **b** Cup-like and fan-like shaped specimens of *P. compressa* entrapping in a network the thin sediment over their surface. **c** Plate-like adult specimen of *P. compressa* surrounded by numerous juveniles visible on the muddy bottom. **d** Plate-like specimens of *P. monilifera* found on the detrital bottom. Cidarid sea urchins are visible between the specimens. **e–k** Bari Canyon (380–500 m depth). **e** Assemblage of fan-like specimens of *P. monilifera* and *Madrepora oculata*. **f** Close sponge-white coral association. **g** Cidarid sea urchin grazing sponge tissues. **h** Traces of the grazing activity of sea urchins on the tissues of *P. monilifera*. **i** Adult specimens of *P. monilifera* surrounded by juveniles (*white arrows*) growing on dead coral skeletons. **j** Zoanthid polyps (*white arrows*) growing on a specimen of *P. monilifera*. **k** Specimens of *P. compressa* in the assemblage. *Scale bar* **e** 20 cm; **a–d**, **h–i**, **k** 10 cm; **f–g**, **j** 3 cm

Astrophorida sharing a great morphological and dimensional plasticity, which allow them to grow in a wide variety of different environments and on several types of substrate. Usually, *P. monilifera* is described as massive or cup-like in shape, and generally white in colour, while *P. compressa* is reported as plate- or cup-like, and orange in colour when observed alive (Uriz, 1978, 1982; present study). The latter has been reported also white or grey, but exsiccation and fixation may cause colour loss, as observed in our samples.

The spicular complements were compared with literature data (Maldonado, 2002) to verify the taxonomic identification of the samples. On the base of the spicular complement, all white specimens collected on the Amendolara Bank are attributed to *P. monilifera* (Fig. 2a, d), while all orange specimens are described as *P. compressa* (Fig. 2a–c). Colour, therefore, represented a good identification character for both species in the analysis of the studied ROV images.

Average sizes of the sponges are very similar both between the two species and between the two sites. They range between 6.9–7.2 ± 0.2–0.6 × 10.3–11.7 ± 0.3–1.0 cm for *P. monilifera* and between 8.1–8.2 ± 1.2–0.4 × 11.9–12.6 ± 0.7–2.3 cm for *P. compressa*, for Amendolara Bank and Bari Canyon, respectively. Specimens of both species grow more slowly in height than in width. The equations describing the ratio between width and height of the specimens of increasing size are very similar in both species ($y = 1.28x + 1.24$ and $y = 1.38x + 0.77$ for *P. compressa* from the Amendolara Bank and *P. monilifera* from the Bari Canyon, respectively) (Fig. 3a, b). The size-frequency distributions of height and width of both species are unimodal with a mode in the classes 4–6 cm both for height and width and a long tail of rare large specimens (Fig. 3c–f).

In both sites, fan-shaped sponges may show the same orientation on large portions of the sea floor (Fig. 2a, d, e, i), and this habit, in the assemblage of Bari Canyon, is shared also by the coral colonies. In addition, due to silting, especially *P. compressa* shows a characteristic network of sediment deposited on its mucous ectosoma, around the oscula (Fig. 2b).

Large groups of close fan-like specimens (up to 10 individuals) of both species are commonly observed on the sea bottom. In both sites, younger specimens are found surrounding adults on the hard substrates or on the dead coral colonies (Fig. 2c, i). As soon as they grow in height, both species develop a fan-like shape (Fig. 2). However, *P. compressa* shows thicker and more regular laminae that are able to fold up forming cup-shaped specimens (Fig. 2a, b), while *P. monilifera* generally forms thinner laminae with a folded upper border (Fig. 2d, h).

Abundance, biomass and bathymetric distribution

Pachastrella monilifera and *Poecillastra compressa* are the major sponge components in the benthic assemblages settling the deepest reaches of the Amendolara Bank (Fig. 2a–d) and the Bari Canyon (Fig. 2e–k). In the Amendolara Bank, the most abundant species is *P. compressa* (70% of the 270 observed sponges), while in the Bari Canyon *P. monilifera* is dominant (97.2% of the 1,043 observed sponges). The total average sponge abundance is slightly lower in the Amendolara Bank respect to the Bari Canyon (7.3 ± 1.1 and 10.0 ± 0.7 specimens m^{-2}, respectively) (Fig. 3). The Amendolara assemblage shows a patchy distribution, with elevated rocky areas characterized by high sponge densities (up to about 27 specimens m^{-2}) interspersed with silted hard substrates deprived of visible epifauna. The assemblage is mixed, with both species contributing to the total abundance (2.6 ± 0.7 and 4.7 ± 0.9 specimens m^{-2} for *P. monilifera* and *P. compressa*, respectively) (Fig. 3). The assemblage of Bari Canyon shows very high maximal total densities (up to 50

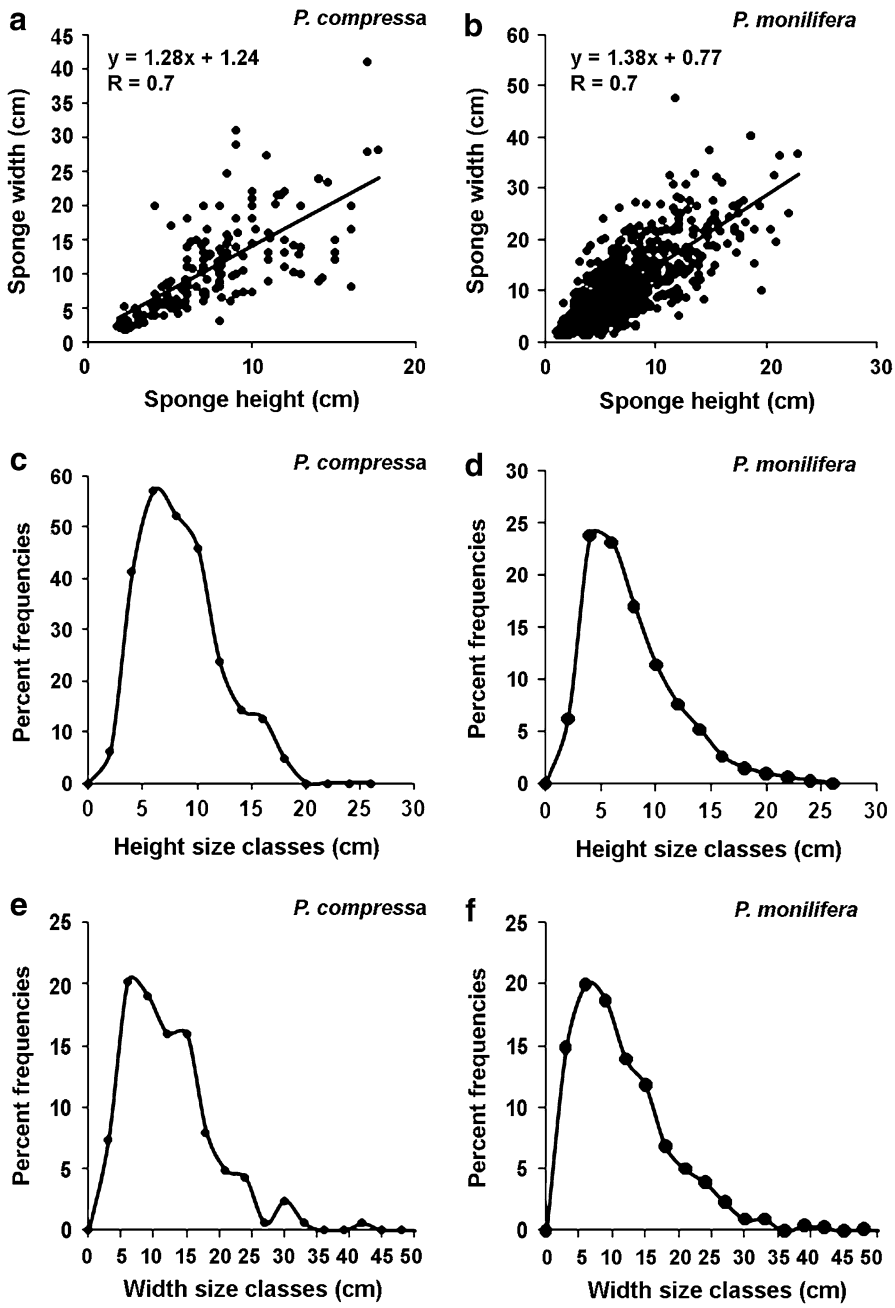

Fig. 3 Sponge morphometry. **a**, **b** Ratio between width and height of *Poecillastra compressa* and *Pachastrella monilifera*. **c–f** Size-frequency distributions of height and width of the two sponge species

specimens m^{-2}) and the sponges are widely distributed over the entire explored sea ground.

In the Bari assemblage, *P. monilifera* is dominant in the entire depth range considered (9.7 ± 0.7 specimens m^{-2}), while *P. compressa* occurs more occasionally (0.2 ± 0.07 specimens m^{-2}) (Fig. 5). On the Amendolara Bank (Fig. 4) instead, the maximum average abundance of *P. compressa* is recorded in the first considered depth range (120–140 m) (8.6 ± 1.6 specimens m^{-2}), then it decreases towards 1.9 ± 1.0 specimens m^{-2} in the deepest depth range (160–180 m). *P. monilifera*, instead, shows its maximum abundance (4.4 ± 1.5 specimens m^{-2}) in the intermediate depth range (140–160 m). Below 180 m depth, both species become extremely rare.

The total sponges biomass contribution is 230.3 and 315.5 gWW m^{-2}, respectively, in the Amendolara Bank and in the Bari Canyon. In the first site,

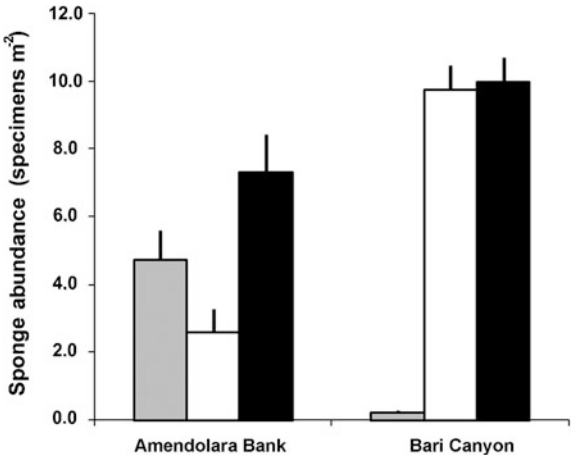

Fig. 4 Average sponge abundance distribution (specimens $m^{-2} \pm$ SE) of the studied sponge assemblages, Amendolara Bank and Bari Canyon. *Grey bars*: *Poecillastra compressa*, *white bars*: *Pachastrella monilifera*, *black bars*: all sponges

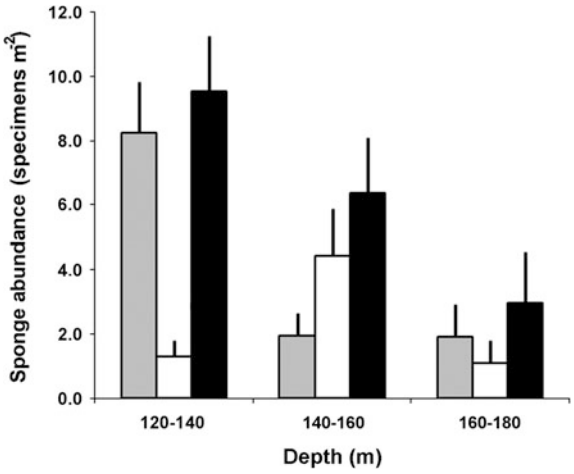

Fig. 5 Average sponge abundance at different depths (specimens $m^{-2} \pm$ SE) of the Amendolara Bank sponge assemblage. *Grey bars*: *Poecillastra compressa*, *white bars*: *Pachastrella monilifera*, *black bars*: all sponges

P. monilifera occurs with a biomass of 90.7 gWW m^{-2} and *P. compressa* with 132.6 gWW m^{-2}, while, in the second site, they occur with a biomass of 338.5 and 5.6 gWW m^{-2}, respectively.

Functional role of deep sponges

In both under study sites, the sponge grounds, comprehensive of other occasional massive and encrusting sponges species, represent centres of attraction of numerous other species. In the Amendolara Bank, sponges grow in patches, both on the rocks sparse on the muddy sea floor and on the tabular, heavily silted rocks, elevating about 1 m from the bottom. In particular, on the latter, the massive sponges occupy only the upper side of the rocks (Fig. 2a), together with other occasional species tentatively attributed to the genera *Mycale* and *Haliclona* and small colonies of the scleractinian *Dendrophyllia cornigera* (Lamarck, 1816). The sheltered, lower side of rocks, often featuring cavities and crevices, is occupied by the encrusting blue sponge *Hamacantha* (*Vomerula*) *falcula* (Bowerbank, 1874) and by other unidentified encrusting and massive sponges. Many other species are found living in this benthic assemblage, such as octopuses, polychaetes and echinoderms, for examples, cidarids, *Peltaster placenta* (Müller-Troschel, 1842) and numerous ophiuroids (Fig. 2a). Several fish species are observed in this habitat, such as *Helicolenus dactylopterus* (Delaroche, 1809), *Anthias anthias* (Linnaeus, 1758), *Callanthias ruber* (Rafinesque, 1810), *Merluccius merluccius* (Linnaeus, 1758), *Phycis blennoides* (Brünnich, 1768), *Aulopus filamentosus* (Bloch, 1792), *Macroramphosus scolopax* (Linnaeus, 1758) and *Scorpaena* sp.

In the Bari Canyon, the sponge assemblage settles on hard substrates and often is intimately associated with living *Madrepora oculata* colonies (occurring with an abundance of 4.1 ± 0.3 colonies m^{-2}, that corresponds to a biomass of 386 gWW m^{-2}) (Fig. 2e–k). At places, branches of *M. oculata* emerge from the holes and crevices of the sponge tissues (Fig. 2f). Here, *P. monilifera* and *P. compressa* show a peculiar association with a small white zoanthid, which resembles *Parazoanthus anguicomus* (Norman, 1868), although no precise taxonomic identification was possible in absence of actual samples was gathered. Zoanthids, usually observed along the outer margin of their host, appear embedded in the sponge ectosome, emerging with the distal portion of the column (Fig. 2j). Other massive and encrusting sponges (such as *Haliclona* sp.) are more rarely observed. The megabenthic community is composed of the species commonly associated with white corals (such as the solitary scleractinian *Desmophyllum dianthus* (Esper, 1794) and the commensal polychaete *Eunice norvegica* (Linnaeus, 1767)), hydroids, ceriantharians, colonial scleractinians such as *D. cornigera*, polychaetes [such as *Filograna* sp. and *Sabella pavonina*

(Savigny, 1818)], the echiurid *Bonellia viridis* (Rolando, 1821), crustaceans (such as *Munida* sp. and various shrimps), ascidians and echinoderms [such as cidarids, *Echinus melo* (Lamarck, 1816) and *P. placenta*]. Very few fish species are observed in the Bari Canyon assemblage: the rare *Gaidropsarus granti* (Regan, 1903) (Canese, pers. observ.), then *P. blennoides*, *A. filamentosus*, and *Pagellus bogaraveo* (Brünnich, 1768), the latter however, particularly abundant.

Sponges represent also a major source of organic carbon for some of the most abundant vagile organisms moving both on the soft bottom surrounding the rocks covered by the sponges and on the sponges themselves, namely cidarid sea urchins. At both sites, numerous specimens of *P. monilifera* and *P. compressa* show traces of predation ranging from ovoid erosions crossing the entire thickness of the sponge tissues to losses of large lobes of sponges. At the end of this process only some fragments of the sponge remain alive (Fig. 2d, f–i). Both species are affected by grazing, especially *P. monilifera*. In particular, in the Bari Canyon, where this species is dominant, the average percentage of sponges showing traces of grazing is 20.2 ± 2%. On average, the measured sponges may lose up to 70% of their surface because of grazing, while 12.1 ± 1.8 gWW is the average biomass loss caused by sea urchin predation (with a maximum of 56.3 gWW for a single specimen).

Discussion and conclusion

Sponges may represent one of the most important components of deep megabenthic communities, both in the oceanic environments (Rice et al., 1990; Conway et al., 1991; Klitgaard, 1995; Rogers, 1999; Gerdes et al., 2008; Hogg et al., 2010) and in semi-enclosed basins, such as the Mediterranean Sea (Vacelet, 1969; Pulitzer-Finali, 1983; Magnino et al., 1999; Longo et al., 2005, Bo et al., 2011b). Differently from corals, the biodiversity and the ecological role of sponges in deep assemblages is far less appreciated, probably as a result of the difficulty to classify species only through ROV images or to collect small or encrusting specimens.

Among the most interesting sponge grounds described so far, vast Antarctic shelf sponge assemblages are noticeable. They occur between 50 and 350 m depth and are mainly constituted of hexactinellids reaching impressive values of biomass (Barthel & Gutt, 1992; Gerdes et al., 2008). Other massive glass sponge reefs are found around 140–300 m depth along the northwestern continental margin of North America developing in areas of high sedimentation (Conway et al., 1991). Along the North Atlantic Ocean shelf edge, sponge grounds are mainly constituted by several demosponge species found at 600–1,000 depth (Hogg et al., 2010), while dense populations of the hexactinellid *Pheronema carpenteri* (Thomson, 1869) were discovered along the Mid-Atlantic Ridge, off Morocco and in the western Mediterranean basin between 500 and 1,600 m depth. This species is able to create grounds extending for kilometres and reaching in certain areas densities up to 6 specimens m^{-2} (Rice et al., 1990; Barthel, 1996). The existence of Mediterranean sponge assemblages, was recently reported from the white coral banks off Santa Maria di Leuca (Ionian sea) (Longo et al., 2005; Mastrototaro et al., 2010; Vertino et al., 2010) and also from the Bari Canyon, one of the areas here described (Freiwald et al., 2009). Finally, even if they do not constitute true sponge grounds, abundant assemblages of massive species were reported also on the rocks hosting wide mesophotic coral meadows along the Italian coast (Bo et al., 2011a). They represent the second most abundant component in these environments and generally live beneath the coral branching net, forming a sort of ground cover, exploiting the organic particulated matter sinking from above (Bo et al., 2011c).

This study represents the first description of dense paucispecific assemblages of *Poecillastra compressa* and *Pachastrella monilifera*, falling in the definition of sponge grounds in terms of abundance, extension, patchy distribution and ecological role (Hogg et al., 2010).

Despite the numerous literature records of these species, no ecological data exist on such dense assemblages, neither for the Mediterranean Sea nor for the Atlantic basin. Based also on unpublished records of these species in the Tyrrhenian Sea and on fishermen bycatch, the described assemblages may represent typical Mediterranean sponge grounds. The two studied sites differ in terms of composition of the benthic community, being the Amendolara Bank exclusively characterized by sponges (particularly *P. compressa*), while the Bari Canyon hosts a mixed assemblage of sponges (mainly *P. monilifera*) and *Madrepora oculata*. It is hypothesised that these assemblages are found in sites characterized by locally

accelerated currents, enhancing seabed food particle supply (Duineveld et al., 2007; Carlier et al., 2009), therefore favouring suspension feeders, such as sponges. In both sites studied during this research, the presence of unidirectional bottom currents is hypothesised on the base of the uniform orientation of the fan-shaped sponges and corals (Wainwright & Koehl, 1976; Warner, 1977; McDonald et al., 2003). Intensity of current is, however, moderate as supposed on the base of the heavy siltation evident on the specimens. In terms of biomass, the Bari assemblage shows the highest value. The oceanographic regime of the canyon together with the co-occurrence of corals, probably favour in this site higher levels of particulated organic matter, hence a more abundant community of suspension feeders. With respect to the Atlantic sponge ground described for the Porcupine Bank (an average of 1.5 specimens m^{-2} of *P. carpenteri*) (Rice et al., 1990), the Bari Canyon shows comparable values of biomass (315 and 372 gWW m^{-2}, respectively, for the Mediterranean and Atlantic site), but obviously no reef structure.

The co-occurrence of the two species seems a common feature. Both species show an Atlantic–Mediterranean overlapped distribution. According to Maldonado (2002) and Cardenas (2010), in fact, records of these taxa from outside this region have to be attributed to close congeneric species.

P. monilifera was recorded along the entire Tyrrhenian and Ionian Italian coastlines, from Liguria to Apulia, and along the Sicilian coasts, with the exception of the Strait of Messina, but including the Sicilian Channel (Pansini & Longo, 2003, 2008; Martinelli et al., 2007). Records are known also from the entire western Adriatic coastline, from Otranto to Istria. Concerning the rest of the Mediterranean Sea, the species was reported from the Gulf of Lions, from Catalunya and Balearic Islands, Alboran Sea and Algerian basin (Pansini & Longo, 2003, 2008). In the Atlantic Ocean, the species shows an affinity for the temperate-cold fauna, being present in the coasts of Ireland, Azores, Canaries, coasts of Marocco and South Africa (Topsent, 1894, 1904, 1928; Ferrer Hernández, 1914; Stephens, 1915; Burton, 1926; Levi, 1960, 1967; Uriz, 1988; Boury-Esnault et al., 1994). Along the Italian peninsula *P. compressa* was reported for the Tyrrhenian Sea, including Sardinia and Corsica, the Ionian and Adriatic coast (Pansini & Longo, 2003, 2008; Martinelli et al., 2007). In the rest of the Mediterranean Sea, the sponge was reported in the same places as *P. monilifera*, while, concerning the Atlantic Ocean, it shows a higher affinity for the boreal fauna, having been recorded along the South African coast, Canaries, Azores, Portugal, Atlantic coasts of Spain and France, coast of Ireland, United Kingdom, Norway and Iceland (Topsent, 1894, 1904, 1913, 1928; Ferrer Hernández, 1914; Stephens, 1915; Burton, 1930, 1959; Levi, 1967; Borojevic et al., 1968; Boury-Esnault et al., 1994).

The studied sites show slight differences of sponge abundance: the assemblage of Bari Canyon is more homogeneous than that of Amendolara Bank (97 and 3%, respectively, for *P. monilifera* and *P. compressa* for the Bari Canyon vs. 30 and 70% for the same species in the Amendolara Bank). Larval aggregation in a patchy rocky environment probably is among the most important factors influencing specimen distribution. In both sites, the settling of sponges is tightly connected with the presence of hard bottoms (both rocks or dead corals), therefore the species may show a variable distribution (as in the case of Amendolara Bank), with peaks of abundances alternated to areas characterized by heavy sedimentation that are not colonized.

Little information is available about the size structure of sponge populations (Turon et al., 1998). Size-frequency distributions, obtained for the two species in the studied localities, indicate unimodal trends with peaks for the intermediate size classes and long tails. This kind of trend is very common in modular organisms like sponges and corals (Linares et al., 2008; Salvati et al., 2010). We hypothesise that the sponges grow quickly to increase the filtering efficiency and to avoid the sedimentation while their maximal size is probably constrained by the friction with the current (and partially by predation). Tails are composed of few specimens, probably living in peculiar sheltered habitat, that are able to continuously grow.

Regarding their bathymetric distribution, in the explored areas, *P. compressa* seems less bathophilic than *P. monilifera* being the most abundant species in the shallower investigated depth ranges of the Amendolara Bank and almost absent in the deepest areas of the Bari Canyon. In general, both *P. compressa* and *P. monilifera* are eurybathic species commonly recorded also inside the shallow water coralligenous concretions (Bertolino, 2011). While in deep habitats (more than 50 m depth), they show a

characteristic massive fan-like or cup-like shape, in the coralligenous they are insinuating. This observation shows the importance of the coralligenous as centre of biodiversity (Ballesteros, 2006): this habitat, in fact, may host deep species characterized by a high phenotypic plasticity which allow them to live in the crevices of the concretions.

The numerous associated organisms found within the two under scrutiny communities indicate that *P. monilifera* and *P. compressa*, due to their massive, elevated morphology, may create three-dimensional habitats over homogeneous, sedimented hard bottoms, providing suitable refuges for other benthic organisms or for vagile fauna. Among the most specialized epibionts we found a small, white zoanthid tentatively attributed to the genus *Parazoanthus*, living as symbionts with both sponge species. Symbiotic relationships between sponges and zoanthids have been frequently observed in tropical and subtropical waters (Swain & Wulff, 2007; Reimer et al., 2008), from shallow to deep habitats (Beaulieu, 2001). In the Mediterranean Sea, it is well documented the recurrent relationship between *Parazoanthus axinellae* Schmidt, 1862 and several species of sponges belonging to the genus *Axinella* (Sarà & Vacelet, 1973; Previati et al., 2010), however, some species are more commonly found on hard bottom substrates (Salvati et al., 2010). As pointed out recently (Montenegro-González & Acosta, 2010), the sponge morphology is an important trait in zoanthid habitat selection. In our case, the zoanthid probably finds refuge into the tissues of the sponge and, contemporaneously, exploits the water flow from an elevated position respect to the sedimented bottom, where it was never observed.

From an ecological point of view, these sponge species may play the same engineering role held by colonial, arborescent corals in deep environments (Klitgaard & Tendal, 2004; Hogg et al., 2010; Bo et al., 2011c).

These sponges, moreover, represent an important step for the benthic–pelagic coupling in the studied assemblages. Even if many sponges avoid predation through the production of secondary metabolites (Clavico et al., 2006), the studied species are strongly exploited by the grazing activity of cidarid echinoids. The presence of sponge spicules in the gut content of these sea urchins was already observed in the Mediterranean Sea by Tortonese (1965) while several observations on the diet of these organisms, based on sponges and corals, were made in cold-water ecosystems (Jacob et al., 2003). Previous authors (Freiwald et al., 2009, Vertino et al., 2010) reported the grazing activity of sea urchins on the coral component of the Bari assemblage, suggesting that these grazers may represent the most important biotic constraint for these communities. The grazing activity has a considerable impact also on the sponge component. We have estimated, in fact, that about 1/5 of the sponge specimens living in the studied sites shows traces of foraging. Since cidarids are known to be widely distributed on the Mediterranean soft bottoms, it is plausible that the organic faecal pellets produced by these vagile predators are then released both within the assemblage and in its neighbourhood, fertilizing the area in the proximity of these habitats.

Due to the high local biodiversity supported by these ecosystems, to the important functional roles held by the sponge components and to their vulnerability towards trawling activities, it is strongly recommended to pursue, also for these deep habitats, protective measures, as suggested for the recently discovered mesophotic coral meadows and Atlantic sponge grounds (Hogg et al., 2010; Bo et al., 2011a).

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References

Babic, K., 1922. Monactinellida und Tetractinellida des Adriatischen Meeres. Zoologische Jahrbücher, Abteilung für Systematik, Geographie und Biologie der Tiere 46: 217–302.

Ballesteros, E., 2006. Mediterranean coralligenous assemblages: a synthesis of present knowledge. Oceanography and Marine Biology 44: 123–195.

Barthel, D., 1996. Fish eggs and pentacrinoids in Weddell Sea hexactinellids: further examples for the structuring role of sponges in Antarctic benthic ecosystems. Polar Biology 17: 91–99.

Barthel, D. & J. Gutt, 1992. Sponge associations in the eastern Weddell Sea. Antarctic Science 4: 137–150.
Beaulieu, S. E., 2001. Life on glass houses: sponge stalk communities in the deep sea. Marine Biology 138: 803–817.
Bertolino, M., 2011. Sponges of the Coralligenous Community in the Mediterranean Sea. Università Politecnica delle Marche, PhD Thesis: 1–162.
Beuck, L., A. Freiwald & M. Taviani, 2010. Spatiotemporal bioerosion patterns in deep-water scleractinians from off Santa Maria di Leuca (Apulia, Ionian Sea). Deep Sea Research II 57: 458–470.
Bianchelli, S., C. Gambi, A. Pusceddu & R. Danovaro, 2008. Trophic conditions and meiofaunal assemblages in the Bari Canyon and the adjacent open slope (Adriatic Sea). Chemistry and Ecology 24: 101–109.
Bo, M., G. Bavestrello, S. Canese, M. Giusti, M. Angiolillo, C. Cerrano, E. Salvati & S. Greco, 2011a. Coral assemblages off the Calabrian Coast (South Italy) with new observations on living colonies of *Antipathes dichotoma*. Italian Journal of Zoology 78: 231–242.
Bo, M., M. Bertolino, M. Borghini, M. Castellano, A. Covazzi Harriague, C. G. Di Camillo, G. P. Gasparini, C. Misic, P. Povero, K. Schroeder & G. Bavestrello, 2011b. Characteristics of the mesophotic megabenthic assemblage of the Vercelli Seamount (North Tyrrhenian Sea). PLoS One 6: e16357.
Bo, M., S. Canese, M. Giusti, C. Spaggiari, M. Angiolillo, E. Salvati, S. Greco & G. Bavestrello, 2011c. Mesophotic coral forests of the Italian seas. Proceedings of the World Conference on Marine Biodiversity, Scotland: 23.
Boehlert, G. W. & A. Genin, 1987. A review of the effects of seamounts on biological processes. In Keating, B. H., P. Fryer, R. Batiza & G. W. Boehlert (eds), Seamounts, Islands and Atolls. Geophysical Monograph Series, Vol. 43: 319–334.
Borojevic, R., L. Cabioc & C. Levi, 1968. Inventaire de la Faune Marine de Roscoff: Spongiares. Edition de la Station Biologique de Roscoff: 2–41.
Bourcier, M. & H. Zibrowius, 1973. Les "boues rouges" déversées dans le canyon de la Cassidaigne (région de Marseille). Observations en soucoupe plongeante SP 350 (juin 1971) et résultats de dragages. Téthys 4: 811–842.
Boury-Esnault, N., M. Pansini & M. J. Uriz, 1994. Spongiaires bathyaux de la Mer d'Alboran et du Golfe Ibéro-Marocain. Mémoires du Museum National D'Histoire Naturelle 160: 1–174.
Burton, M., 1926. Description of South African sponges collected in the South African Marine Survey. Part I. Myxospongia and Astrotetraxonida. Fisheries Bulletin. Fisheries and Marine Biological Survey Division, Union of South Africa Reports 4: 1–29.
Burton, M., 1930. Norwegian sponges from the Norman Collection. Proceedings of the Zoological Society of London 2: 487–546.
Burton, M., 1959. Spongia. The Zoology of Iceland 2: 1–71.
Cardenas, P., 2010. Phylogeny, Taxonomy and Evolution of the Astrophorida (Porifera, Demospongiae). University of Bergen, PhD Thesis: 1–80.
Carlier, A., E. Le Guilloux, K. Olu, J. Sarrazin, F. Mastrototaro, M. Taviani & J. Clavier, 2009. Trophic relationships in a deep Mediterranean cold-water coral bank (Santa Maria di Leuca, Ionian Sea). Marine Ecology Progress Series 397: 125–137.
Cecere, E. & C. Perrone, 1988. First contribution to the knowledge of macrobenthic flora of the Amendolara Sea-Mount (Ionian Sea). Oebalia 14: 43–67.
Ceramicola, S., D. Civile, A. Caburlotto, A. Cova, D. Accettella, M. Caffau, D. Cotterle, P. Diviacco, N. Wardell & R. Ramella, 2008. Analisi morfo-sedimentaria del margine calabro ionico settentrionale. Proceedings of the GNGTS 2008 Conference: 423–424.
Clark, M. R., A. A. Rowden, T. Schlacher, A. Williams, M. Consalvey, K. I. Stocks, A. D. Rogers, T. D. O'Hara, M. White, T. M. Shank & J. M. Hall-Spencer, 2010. The ecology of seamounts: structure, function, and human impacts. Annual Reviews of Marine Science 2: 253–278.
Clavico, E. E. G., G. Muricy, B. A. P. da Gama, D. Batista, C. R. R. Ventura & R. C. Pereira, 2006. Ecological roles of national products from the marine sponge *Geodia corticostylifera*. Marine Biology 148: 479–488.
Conway, K. W., J. V. Barrie, W. C. Austin & J. L. Luternauer, 1991. Holocene sponge bioherms on the western Canadian continental shelf. Continental Shelf Research 11: 771–790.
D'Addabbo Gallo, M., L. De Morone & S. Grimaldi de Zio, 1987. Heterotardigrada of the Amendolara Shoal, High Ionian Sea. In Bertolani, R. (ed.), Biology of Tardigrades. Proceedings of the Fourth International Symposium on the Tardigrada, September 1985, Modena, Italy. Selected Symposia and Monographs: 93–101.
Di Geronimo, I., R. La Perna, A. Rosso & R. Sanfilippo, 1998. Notes on two upper-circalittoral assemblages from the Amendolara Bank (Northern Ionian Sea). Bollettino Accademia Gioenia di Scienze Naturali 30: 243–262.
Duineveld, G. C. A., M. S. S. Lavaleye, M. J. N. Bergman, H. de Stigter & F. Mienis, 2007. Trophic structure of a cold-water coral mound community (Rockall Bank, NE Atlantic) in relation to the near-bottom particle supply and current regime. Bulletin of Marine Science 81: 449–467.
Ferrer Hernández, F., 1914. Esponjas del Cantábrico. Parte 2°. III. Myxospongida. IV. Tetraxonida. V. Triaxonida. Trabajos del Museo Nacional de Ciencias Naturales, serie Zoológica 17: 3–44.
Freiwald, A., L. Beuck, A. Rüggeberg, M. Taviani & D. Hebbeln, 2009. The white coral community in the Central Mediterranean Sea revealed by ROV surveys. Oceanography 22: 58–74.
Gerdes, D., E. Isla, R. Knust, K. Mintenbeck & S. Rossi, 2008. Response of Antarctic benthic communities to disturbance: first results from the artificial benthic disturbance experiment on the eastern Weddell Sea Shelf, Antarctica. Polar Biology 31: 1469–1480.
Grancini, G., A. Lavenia & F. Mosetti, 1969. Ricerche oceanografiche nel Golfo di Taranto (Indagini fisiche del luglio 1968). Atti dell'Istituto Veneto di Scienze, Lettere ed Arti 127: 309–326.
Hogg, M. M., O. S. Tendal, K. W. Conway, S. A. Pomponi, R. W. M. van Soest, J. Gutt, M. Krautter & J. M. Roberts, 2010. Deep-Sea Sponge Grounds: Reservoirs of Biodiversity. UNEPWCMC Biodiversity Series no. 32. UNEP-WCMC, Cambridge: 1–88.
Hooper, J. N. A., 2000. 'Sponguide'. Guide to Sponge Collection and Identification. http://www.qm.qld.gov.

au/organisation/sections/SessileMarineInvertebrates/spong.pdf.
Ilan, M., N. Ben-Eliahu & B. S. Galil, 1994. Three deep water sponges from the Eastern Mediterranean and their associated fauna. Ophelia 39: 45–54.
Ilan, M., M. Gugel, B. S. Galil & D. Janussen, 2003. Small bathyal sponge species from East Mediterranean revealed by a non-regular soft bottom sampling technique. Ophelia 57: 145–160.
Jacob, U., S. Terpstra & T. Brey, 2003. High-Antarctic regular sea urchins—the role of depth and feeding in niche separation. Polar Biology 26: 99–104.
Klitgaard, A. B., 1995. The fauna associated with outer shelf and upper slope sponges (Porifera, Demospongiae) at the Faroe Islands, northeastern Atlantic. Sarsia 80: 1–22.
Klitgaard, A. B. & O. S. Tendal, 2004. Distribution and species composition of mass occurrences of large-sized sponges in the northeast Atlantic. Progress in Oceanography 61: 57–98.
Levi, C., 1960. Spongiaires des côtes occidentals africaines. Bullettin de l'Institut Francais d'Afrique Noire 22: 743–769.
Levi, C., 1967. Spongiaires d'Afrique du Sud. (3) Tetractinellides. Transactions of the Royal Society of South Africa 37: 227–256.
Linares, C., R. Coma, J. Garrabou, D. Díaz & M. Zabala, 2008. Size distribution, density and disturbance in two Mediterranean gorgonians: *Paramuricea clavata* and *Eunicella singularis*. Journal of Applied Ecology 45: 688–699.
Longo, C., F. Mastrototaro & G. Corriero, 2002. Sponge fauna associated with white corals from the Western Ionian Sea. Bollettino dei Musei e degli Istituti Biologici dell' Università di Genova 66–67: 118.
Longo, C., F. Mastrototaro & G. Corriero, 2005. Sponge fauna associated with a Mediterranean deep-sea coral bank. Journal of the Marine Biological Association of the United Kingdom 85: 1341–1352.
Magnino, G., M. F. Gravina, P. Righini, F. Serena & M. Pansini, 1999. Due Demosponge Lithistidi nuove per i mari italiani. Biologia Marina Mediterranea 6: 391–393.
Maldonado, M., 1992. Demosponges of the red coral bottoms from the Alboran Sea. Journal of Natural History 26: 1131–1161.
Maldonado, M., 2002. Family Pachastrellidae Carter, 1875. In Hooper, J. N. A. & R. W. M. van Soest (eds), Systema Porifera: A Guide to the Classification of Sponges. Kluwer Academic, Plenum Publisher, New York, Boston, Dordrecht, London, Moscow: 141–164.
Martinelli, M., G. Bavestrello, B. Calcinai & M. Taviani, 2007. Poriferi associati ai banchi di Coralli bianchi del Canale di Sicilia a Sud di Malta. Proceedings of the 68° UZI Congress, Lecce.
Mastrototaro, F., G. D'Onghia, G. Corriero, A. Matarrese, P. Maiorano, P. Panetta, M. Ghepardi, C. Longo, A. Rosso, F. Sciuto, R. Sanfilippo, C. Gravili, F. Boero, M. Taviani & A. Tursi, 2010. Biodiversity of the white coral and sponge community off Cape Santa Maria di Leuca (Mediterranean Sea). Deep Sea Research II 57: 412–430.
McDonald, J. I., A. K. McGuinness & J. N. A. Hooper, 2003. Influence of re-orientation on alignment to flow and tissue production in a *Spongia* sp. (Porifera: Demospongiae: Dictyoceratida). Journal of Experimental Marine Biology and Ecology 296: 13–22.
Montenegro-González, J. & A. Acosta, 2010. Habitat preference of Zoantharia genera depends on host sponge morphology. Universitas Scientiarum 15: 110–121.
Panetta, P., B. Dell'Angelo & F. Fiordiponti, 1985. I Poliplacofori del Banco dell' Amendolara (Golfo di Taranto). Oebalia 11: 767–769.
Pansini, M., 1987. Report on a collection of Demospongiae from soft bottoms of the eastern Adriatic Sea. In Clifford Jones, W. (ed.), European Contributions to the Taxonomy of Sponges. Sherkin Island Marine Station: 41–53.
Pansini, M., & C. Longo, 2003. A review of the Mediterranean Sea sponge biogeography with, in appendix, a list of the demosponges hitherto recorded from this sea. Biogeographia, XXIV, Marine Biogeography of the Mediterranean Sea: Patterns and Dynamics of Biodiversity: 59–90.
Pansini, M. & C. Longo, 2008. Porifera. In La checklist della flora e della fauna dei mari italiani (a cura di G. Relini). Ministero dell'Ambiente e tutela del territorio, Roma. Biologia Marina Mediterranea 15: 42–66.
Pansini, M. & B. Musso, 1991. Sponges from trawl-exploitable bottoms of Ligurian and Tyrrhenian Seas: distribution and ecology. Marine Ecology 12: 317–329.
Perrone, A., 1985. Report on the biological survey of Amendolara Seamount: Nudibranchia of Amendolara Seamount. Journal of Molluscan Studies 51: 102.
Previati, M., M. Palma, G. Bavestrello, C. Falugi & C. Cerrano, 2010. Reproductive biology of *Parazoanthus axinellae* (Schmidt, 1862) and *Savalia savaglia* (Bertoloni, 1819) (Cnidaria, Zoantharia) from the NW Mediterranean coast. Marine Ecology 31: 555–565.
Pulitzer-Finali, G., 1983. A collection of Mediterranean Demospongiae (Porifera) with, in appendix, a list of the Demospongiae hitherto recorded from the Mediterranean Sea. Annali del Museo civico di storia naturale Giacomo Doria 84: 445–621.
Reimer, J. D., F. Sinniger & C. P. Hickman Jr., 2008. Zoanthid diversity (Anthozoa: Hexacorallia) in the Galapagos Island: a molecular examination. Coral Reefs 27: 641–654.
Rice, A. J., M. H. Thurston & A. L. New, 1990. Dense aggregations of a hexactinellid sponge, *Pheronema carpenteri* in the Porcupine Seabight (Northeast Atlantic Ocean) and possible causes. Progress in Oceanography 24: 179–196.
Ridente, D., F. Foglini, D. Minisini, F. Trincardi & G. Verdicchio, 2007. Shelf-edge erosion, sediment failure and inception of Bari Canyon on the south-western Adriatic margin (Central Mediterranean). Marine Geology 246: 193–207.
Rogers, A. D., 1999. The biology of *Lophelia pertusa* (Linnaeus, 1758) and other deep-water reef-forming corals and impacts from human activities. International Review of Hydrobiology 84: 315–406.
Romagnoli, C., 2004. Segnalazione di terrazzi deposizionali sommersi nelle Isole Eolie Occidentali (Alicudi-Filicudi). Memorie Descrittive della Carta Geologica d'Italia 58: 155–158.
Rossi, S. & P. Colantoni, 1976. Appunti sul Banco Amendolara nel Golfo di Taranto. Giornale di Geologia 2: 277–284.
Rossi, S. & G. Gabbianelli, 1978. Geomorfologia del Golfo di Taranto. Bollettino della Società Geologica Italiana 97: 423–437.

Rosso, A., A. Vertino, I. Di Geronimo, R. Sanfilippo, F. Sciuto, R. Di Geronimo, D. Violanti, C. Corselli, M. Taviani, F. Mastrototaro & A. Tursi, 2010. Hard- and soft-bottom thanatofacies from the Santa Maria di Leuca deep-water coral province, Mediterranean. Deep Sea Research II 57: 360–379.

Salvati, E., M. Angiolillo, M. Bo, G. Bavestrello, M. Giusti, A. Cardinali, S. Puce, C. Spaggiari, S. Greco & S. Canese, 2010. The population of *Errina aspera* (Hydrozoa, Stylasteridae) of the Messina Strait (Mediterranean Sea). Journal of the Marine Biological Association of the United Kingdom 90: 1331–1336.

Samadi, S., T. Schlacher & B. Richer de Forges, 2007. Seamount benthos. In Pitcher, T. J., et al. (eds), Seamounts: Ecology, Fisheries & Conservation. Blackwell, Oxford: 119–140.

Sarà, L. & J. Vacelet, 1973. Ecologie des Démosponges. In Grassé, P. P. (ed.), Traité de Zoologie. VIII, Spongiaires. Masson, Paris: 462–576.

Stephens, J., 1915. Sponges of the coasts of Ireland. I. The Triaxonidand and part of the Tetraxonida. Fisheries, Ireland Scientific Investigations 4: 1–43.

Strusi, A., A. Tursi, E. Cecere, C. Montanaro & P. Panetta, 1985. The Amendolara Seamount (High Ionian Sea): general description. Oebalia 11: 379–388.

Swain, T. D. & J. L. Wulff, 2007. Diversity and specificity of Caribbean sponge-zoanthid symbioses: a foundation for understanding the adaptive significance of symbioses and generating hypotheses about higher-order systematics. Biological Journal of the Linnaean Society 92: 695–711.

Taviani, M. & L. Angeletti, 2009. Un giardino sepolto nel Mar Adriatico. Darwin 32: 74–81.

Taviani, M., A. Freiwald & H. Zibrowius, 2005a. Deep coral growth in the Mediterranean Sea: an overview. In Freiwald, A. & J. M. Roberts (eds), Cold-Water Corals and Ecosystems. Springer-Verlag, Berlin Heidelberg: 137–156.

Taviani, M., A. Remia, C. Corselli, A. Freiwald, E. Malinverno, F. Mastrototaro, A. Savini & A. Tursi, 2005b. First geomarine survey of living cold-water *Lophelia* reefs in the Ionian Sea (Mediterranean basin). Facies 50: 409–417.

Topsent, E., 1894. Etude monographique des Spongiaires de France I. Tetractinellida. Archives de Zoologie expérimentale et générale 2: 259–400.

Topsent, E., 1904. *Spongiaires des Açores*. Resultats des Campagnes Scientifique du Prince de Monaco 25: 1–279.

Topsent, E., 1913. Spongiaires provenant des campagnes scientifiques de la «Princesse-Alice» dans les Mers du Nord (1898–1899, 1906–1907). Resultats des Campagnes Scientifique du Prince de Monaco 45: 3–67.

Topsent, E., 1928. Spongiaires de l'Atlantique et de la Méditerranée, provenant des croisières du Prince Albert I de Monaco. Resultats des Campagnes Scientifique du Prince de Monaco 74: 1–373.

Tortonese, E., 1965. Fauna d'Italia. Echinodermata. Edizioni Calderini, Bologna: 380.

Trincardi, F., F. Foglini, G. Verdicchio, A. Asioli, A. Correggiari, D. Minisini, A. Piva, A. Remia, D. Ridente & M. Taviani, 2007. The impact of cascading currents on the Bari Canyon System, SW Adriatic margin (Central Mediterranean). Marine Geology 246: 208–230.

Tunesi, L., G. Diviacco & G. Mo, 2001. Observations by submersible on the biocoenosis of the deep-sea corals off Portofino promontory (Northwestern Mediterranean Sea). In Willison, J. H. M., J. Hall, S. Gass, E. L. R. Kenchington, M. Butler & P. Doherty (eds), Proceedings of the First International Symposium on Deep-Sea Corals. Ecology Action Centre and Nova Scotia Museum, Halifax: 76–87.

Turchetto, M., A. Boldrin, L. Langone, S. Miserocchi, T. Tesi & F. Foglini, 2007. Particle transport in the Bari Canyon (southern Adriatic Sea). Marine Geology 246: 231–247.

Turon, X., I. Tarjuelo & M. J. Uriz, 1998. Growth dynamics and mortality of the encrusting sponge *Crambe crambe* (Poecilosclerida) in contrasting habitats: correlation with population structure and investment in defence. Functional Ecology 12: 631–639.

Uriz, M. J., 1978. Contribución a la Fauna de Esponjas (Demospongia) de Cataluña. Universitad de Barcelona. PhD Thesis: 1–367.

Uriz, M. J., 1981. Estudio sistemático de las esponjas Astrophorida (Demospongia) de los fondos de pesca de Arrastre, entre Tossa y Calella (Cataluña). Boletin del Instituto Español de Oceanografia 6: 8–58.

Uriz, M. J., 1982. Estudio sistemático de las esponjas del order Axinellida (Demosponga) de la Costa Brava (Cataluña). Actas II Simposio ibérico de estudios del Bentos Marino 2: 57–80.

Uriz, M. J., 1983. Présence de l'espèce *Esperiopsis fucorum* (Demospongia, Poecilosclerida) en Méditerranée. Vie Milieu 33: 237–240.

Uriz, M. J., 1984. Descripción de nuevas esponjas del litoral de Namibia (sudoeste de África). Resultados Expediciones Científicas 12: 107–116.

Uriz, M. J., 1988. Deep-water sponges from the continental shelf and slope off Namibia (Southwest Africa): classes Hexactinellida and Demospongia. Monografías de Zoología Marina 3: 9–157.

Uriz, M. J. & M. A. Bibiloni, 1984. Esponjas Homosclerofóridas (Demospongia) del Litoral Catalán. Miscellánea Zoológica 8: 7–12.

Uriz, M. J. & M. Maldonado, 2000. The genus *Acanthodendrilla* in the Mediterranean Sea with description of a new species. Zoosystema 22: 401–410.

Uriz, M. J. & D. Rosell, 1990. Sponges from bathyal depths (1000–1750 m) in the Western Mediterranean Sea. Journal of Natural History 24: 373–391.

Vacelet, J., 1960. Eponges de la Méditerranée Nord-Occidentale récoltées par le "Président-Théodore-Tissier" (1958). Recherches et Travaux de Institut des Pêches Maritimes 24: 257–272.

Vacelet, J., 1961. Spongiaires (Démosponges) de la région de Bonifacio (Corse). Recherches et Travaux de la Station marine d'Endoume 22: 351–354.

Vacelet, J., 1969. Éponges de la roche du large et de l'étage bathyal de Méditerranée. Mémoires du Muséum National d'Histoire Naturelle 59: 146–219.

Vacelet, J., 1996. Nouvelles signalisations d'éponges profondes en Méditerranée. Mésogée 55: 107–114.

Vertino, A., A. Savini, A. Rosso, I. Di Geronimo, F. Mastrotaro, R. Sanfilippo, G. Gay & G. Etiope, 2010. Benthic habitat characterization and distribution from two representative sites of the deep-water SML Coral Mound

Province (Mediterranean). Deep Sea Research II 57: 380–396.

Voultsiadou-Koukouras, E. & R. W. M. van Soest, 1993. Suberitidae (Demospongiae, Hadromerida) from the North Aegean Sea. Beaufortia-Institute of Taxonomic Zoology (Zoological Museum) University of Amsterdam 43: 176–186.

Wainwright, S. A. & M. A. R. Koehl, 1976. The nature of flow and the reaction of benthic cnidarian to it. In Mackie, G. O. (ed.), Coelenterate Ecology and Behavior. Plenum, New York: 5–21.

Warner, G. F., 1977. On the shape of passive suspension feeders. In Keegan, B. F., P. O'Ceidigh & P. J. S. Boaden (eds), Biology of Benthic Organisms. Pergamon, Oxford/New York: 567–576.

Zibrowius, H., 1985. Spongiaires hexactinellides vivant en mer Ionienne par 2000 m de profondeur. Rapports Commission Internationale pour l'Exploration Scientifique de la Mer Méditerraneé 29: 335–338.

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SPONGE RESEARCH DEVELOPMENTS

Review paper

Mangrove and coral reef sponge faunas: untold stories about shallow water Porifera in the Caribbean

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Abstract Sponge faunas from coral reefs and mangrove ecosystems in the Caribbean have mostly been studied from an ecological perspective, with researchers considering the effects of physical and biological factors on their species distribution. To discern evolutionary patterns, this study analyzed the systematic composition, taxonomic diversity, and ecological properties (reproductive strategies, size, shape, endosymbiosis) of mangrove and reef sponge assemblages from seven distant Caribbean localities. Species composition was compared by use of cluster analysis (Sørensen's), and taxonomic diversity by use of the biodiversity index average taxonomic distinctness (AvTD). Mangrove and reef-associated sponge faunas were found to be statistically dissimilar, with the AvTD values suggesting stronger taxonomic bias toward specific groups in mangroves, irrespective of geographic distance. Most Demospongiae orders have 30–50% more species in coral reefs than in mangroves. The richest reef genera (*Agelas*, *Aplysina*, *Callyspongia*, *Petrosia*, and *Xestospongia*) rarely colonize contiguous mangrove formations. The distribution and diversity of suprageneric taxa suggest that coral reef sponge assemblages might represent an older fauna. This historical interpretation would place mangrove subtidal habitats as the youngest marine ecosystem, rather than a below-optimum ecosystem. Life history traits support a biological split discussed here from the perspective of distinct evolutionary histories and different environmental conditions.

Keywords Mangroves · Coral reefs · Sponges · Evolution · Animal history

Guest editors: M. Maldonado, X. Turon, M. A. Becerro & M. J. Uriz / Ancient animals, new challenges: developments in sponge research

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Introduction

Coral reef and mangrove ecosystems are among the most important tropical marine communities. Coral reefs harbor 4–5% of all known species, and are responsible for the highest recorded oceanic productivity (1,500–5,000 gC/m^2/year). Mangrove forests line up to 60–75% of tropical coasts; they constitute "diversity hotspots" (Rützler et al., 2000) and have been demonstrated to increase reef fish productivity (Mumby et al., 2007).

Sponges may be among the most diverse components of benthic fauna in shallow marine Caribbean systems. Reef sponges may quadruplicate the diversity of hermatypic and soft corals (Díaz & Rützler, 2001). On the other hand, in mangrove islands (leeward side) at a variety of Caribbean sites, sponges equal or surpass the richest groups of macroalgae or ascidians,

representing from 10 to 70% of the total root epiphytic diversity (Díaz & Rützler, 2009). Furthermore, sponges share first or second place of dominance in terms of root and peat area coverage at a variety of Caribbean mangrove sites (Pérez-Vázquez, 2007; Díaz & Rützler, 2009; Guerra-Castro et al., 2011). Marine sponges are essential to the ecology of these ecosystems, partly because of their unique capacity for water filtration but also because of the transformation of nutrients through their metabolic processes and those of their microbial associates (Díaz & Rützler, 2001; Lesser, 2006; de Goeij et al., 2008).

Mangrove and coral reef ecosystems are geographically, and ecologically interconnected throughout the Caribbean (Rützler et al., 2000; Díaz, 2005; Mumby et al., 2007). Comparative studies between these two faunas suggest mangrove sponge distributions are predominantly controlled by abiotic factors (Bingham & Young, 1995; Farnsworth & Ellison, 1996; Pawlik et al., 2007), with intense competition for space leading to the evolution of high growth capability (Wulff, 2005, 2009) and chemical defense mechanisms (Engel & Pawlik, 2005a). Reef faunas are believed to be mostly controlled by biotic factors, mainly fish predation and space competition with the development of allelopathic defense mechanisms (Dunlap & Pawlik, 1996, 1998; Pawlik, 1997, 1998; Wulff, 1997, 2005; Engel & Pawlik, 2005b). Recent studies suggest that biological features such as growth rates, recruitment patterns, asexual reproduction, and root–sponge interactions may vary between sponge species, and determine the presence, abundance, and dynamics of sponge populations on mangrove roots (Farnsworth & Ellison, 1996; Díaz & Rützler, 2009; Hunting et al., 2010). However, this analysis of mangrove and coral reef sponge faunas lacks comprehensive systematic comparison. Several questions remain unanswered, including:

- how similar are these faunas taxonomically?
- are there other ecological strategies besides growth rate, secondary chemistry, antipredatory defenses, and competitive growth capabilities that diverge between these faunas?

This paper reviews and analyzes distributional and ecological data on the sponges of Caribbean mangroves and coral reefs with the objective of understanding novel aspects of the ecological and evolutionary relationships among these faunas. Five aspects of Caribbean sponge biology and ecology are discussed:

1. sponge species distributions within each ecosystem;
2. taxonomic composition;
3. reproductive strategies—viviparity versus oviparity;
4. morphology (size and shape); and
5. distribution of sponge-cyanobacteria symbioses.

Materials and methods

Taxonomic composition of sponge assemblages of Caribbean mangroves and coral reefs

Faunal surveys, taxonomic revisions, gray literature, and two unpublished surveys by the author and another colleague (Dr Pedro Alcolado, Cuba) recently conducted in either mangrove or coral reef habitats were reviewed to gather data on the distribution of species for each habitat. Validity of species names and their classification were checked against the World Porifera Database (Van Soest et al., 2010). The information was gathered into a species-by-country-by-habitat (either mangrove and/or coral reef) matrix with values of presence (1) or absence (0) for each species. Caribbean sites included seven countries: Belize (Rützler et al., 2000; Díaz & Rützler, 2009; Rützler unpublished data), Cuba (Alcolado, 2002, unpublished data), Curaçao and Bonaire (Van Soest, 1978, 1980, 1984, 2009), Jamaica (Hechtel, 1965; Lehnert & van Soest, 1996, 1998), Little Cayman (Diaz, unpublished data), Panama (Clifton et al., 1997; Díaz, 2005), and Venezuela (Álvarez & Díaz, 1985; Pauls, 2003; Amaro, 2005; Alvizu, 2006; Pérez-Vázquez, 2007; Ramirez, 2010; Diaz et al. unpublished data). Total numbers of species were used to interpret the distribution of sponge taxa (genera, families, and orders) among mangrove and open reef environments. Data from cryptic coral reef studies from Curaçao and Bonaire (Kobluk & Van Soest, 1989; Van Soest, 2009) were included as a comparative habitat reference. The species matrix was analyzed using Sørensen's similarity index and then projected using MDS ordination to identify similar faunas within and among habitats (Clarke, 1993). The null hypothesis of no relationship within and among habitats was tested by analysis of similarities (ANOSIM) using all possible permutations ($n = 464$).

The biodiversity index average taxonomic distinctness (AvTD) and its associated statistical test (taxonomic distinctness test or TAXDTEST) were used to evaluate taxonomic diversity for each habitat by locality (Clarke & Warwick, 1998; Warwick & Clarke, 2001). AvTD is a measure of the average extent to which individuals in an assemblage are related to each other, on the basis of a taxonomic hierarchy. This index was used to test the null hypothesis that the AvTD for a particular habitat in a locality (mangrove, open or cryptic coral reef) is within the expected range considering the local species richness and the regional pool of species. This test was carried out using 1,000 simulations of AvTD values calculated from random subsets of species from the regional pool. Comparison of the AvTD for each habitat within each country enables comparison of the evolutionary relatedness of species within each habitat. All statistical analyses were conducted using Primer v6 (Primer-E, Plymouth, UK) (Clarke & Gorley, 2006).

Species richness was compared at suprageneric levels to evaluate possible predominance of genera, families, or orders in mangroves or coral reefs at different Caribbean sites.

Ecological patterns

The incidence of specific biological traits, for example reproductive strategies (viviparous vs. oviparous larvae), and ecological patterns, for example the incidence of cyanobacteria–sponge associations, and predominance of shapes or size ranges, were reviewed using published data and observations by the author.

Results

Taxonomic composition of Caribbean mangrove and coral reef sponge faunas

A list of 414 species was gathered in a taxonomic matrix of species presence in (1) or absence from (0) mangrove and open reef habitats in six countries, and cryptic reef habitats in two countries. The list can be found at the World Porifera Database web site (http://www.marinespecies.org/porifera/porifera.php?p=sourcedetails&id=149269) and at the Porifera Tree of Life (PorToL) website (http://www.portol.org/resources). A complementary taxonomic list that contains classification and authorship information for all Caribbean species can be found at the World Porifera Database site (http://www.marinespecies.org/porifera/porifera.php?p=sourcedetails&id=149270) and at the PorToL site.

Sørensen similarity analysis (Fig. 1) showed that mangrove and coral reef faunas from distant Caribbean regions have significant dissimilarity in species composition (ANOSIM test, $R = 0.685$, $P < 0.05$). All mangrove sites studied share more species between them than with nearby reefs (i.e., the mangroves in Panama share the highest similarity with faunas from mangroves sites throughout the Caribbean than with Panama's reef sponge fauna). Two groups are distinguished: one from the mangroves, with the highest similarities in species composition comprising Belize, Cuba, Panamá and Venezuela, and another from the reef composed of all of the studied coral reef sites and maintained despite the disparity of their species richness values (80–236 species).

All of the reef sites have taxonomic diversity that falls within the expected range of values considering the reported species richness (Fig. 2). However, the richest mangrove sites (i.e. Panama, Venezuela, and Belize) had AvTD values that were lower than expected (95% confidence test). These results suggest that the predominance of specific sponge taxa (i.e. Chalinidae and Mycalidae) in mangrove systems is not a random pattern but that there is a taxonomic bias toward specific groups.

Suprageneric species distributions among Caribbean mangrove and coral reef sponge fauna

The habitat distributions of the species listed in the data matrix shows that 320 species have been reported from open reef habitats (61% not shared with the mangroves) and while 145 species have been reported from mangrove habitats (24% of species not shared with the reefs). Therefore the reef sponge fauna is twice as rich as that found in the mangrove habitats. The number of species within each of 13 orders of Demospongiae orders and two orders of Calcarea is shown in Fig. 3. Species richness within each order ranges from 1 to 65, but is consistently 30–50% higher in open coral reefs than in mangroves for almost all orders considered.

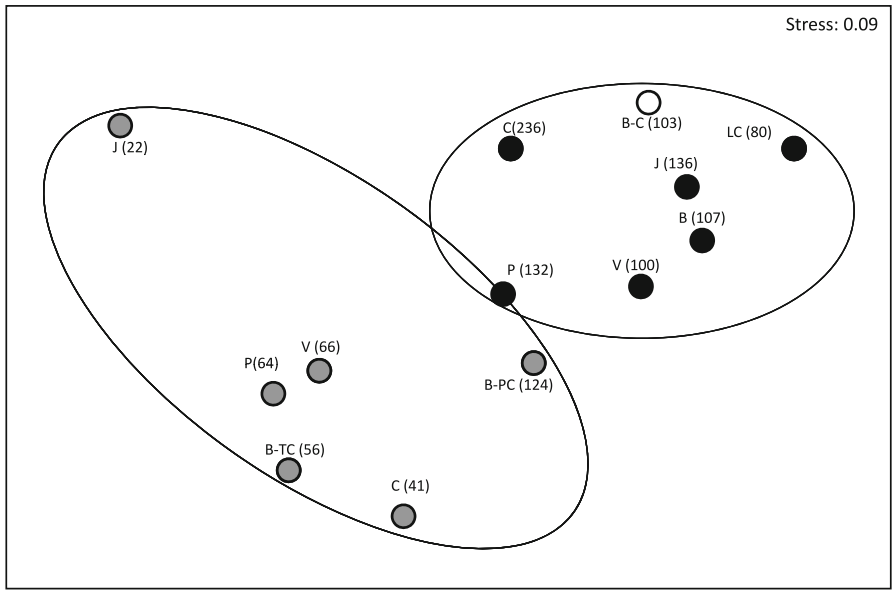

Fig. 1 Non-metric multidimensional scaling (nMDS) ordinations of Sørensen's similarities of sponge species composition among countries by habitat (species richness is indicated *in parentheses*). *Circles* represent data for mangroves (*gray*), open coral reefs (*black*), and cryptic reef habitats in Curacao and Bonaire (*white*). Included countries are Panamá (*P*), Jamaica (*J*), Cuba (*C*), Venezuela (*V*), Belize (Twin Cays: *B–TC*, Pelican Cays: *B–PC*), Little Cayman reefs (*LC*), and Curacao and Bonaire (*C–B*). The habitats were significantly dissimilar (ANOSIM test, $R = 0.685$, $P < 0.05$)

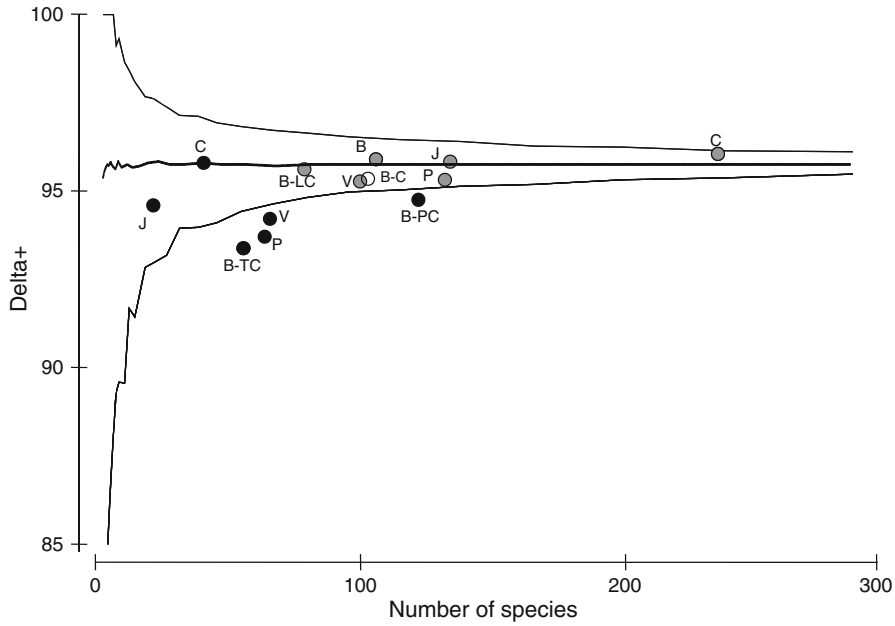

Fig. 2 Funnel plot for AvTD at each habitat by country. *Gray circles* correspond to reef habitats, *black circles* to mangrove habitats, and the *open circle* to cryptic habitats in Bonaire and Curacao. Funnels represent the simulated 95% confidence intervals for the expected AvTD values (TAXDTEST)

The species richness within the most abundant Demospongiae families reported from Caribbean coral reefs and mangrove habitats is summarized in Table 1. Most families among Demospongiae orders are richer in reef habitats than in mangrove habitats; within some orders, however, there is a clear distinction of which families diversify within each habitat. For instance, the families Mycalidae (Order Poecilosclerida), Chalinidae (Order Haplosclerida), and Dictyonellidae (Order Halichondriidae) are more diverse in the mangroves

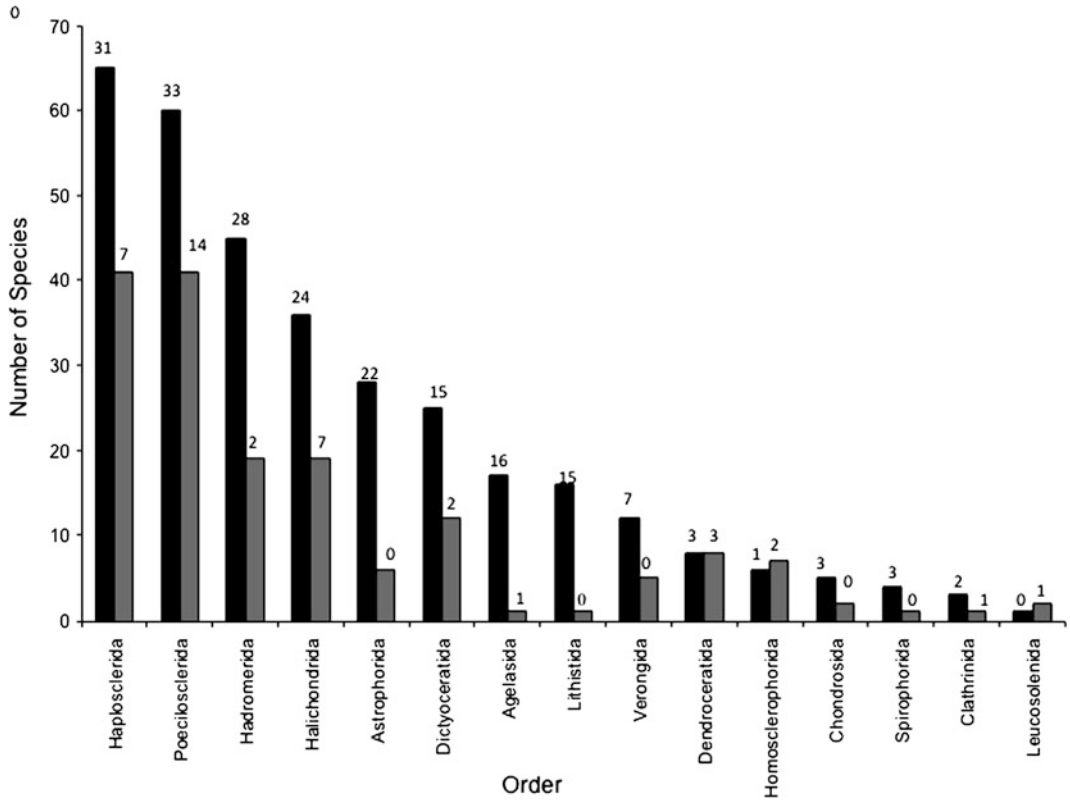

Fig. 3 Number of species for 13 Demospongiae and 2 Calcarea orders, for each habitat: *Black bars* represent coral reef species and *gray bars* represent mangrove species. The *numbers above the bars* represent species not shared between habitats

than in the reefs, and their relative importance becomes evident when we analyze their species richness as a percentage of the total richness reported for mangroves (Table 1). Some families and genera that are emblematic of coral reefs (Aplysinidae, Agelasiidae, Axinellidae, Ancorinidae) are almost absent from mangrove ecosystems. The genera of the various lithistid families have never been observed in mangrove ecosystems. Within the species-rich order Haplosclerida we find families with many more species on the reefs, for example the Petrosiidae, Callyspongiidae, and Niphatidae, and families radiating in the mangroves, for example the Chalinidae. This familial bias is repeated within the order Halichondrida, with the families Axinellidae and Halichondriidae radiating in the reefs, and the family Dictyonellidae radiating in the mangroves. If we express the species richness within each family as a percentage of the total diversity at each habitat we find a different picture. For the higher species numbers found on coral reefs among families such as Petrosiidae, Niphatidae, and Halichondriidae the percentage of the total species richness is very similar to that for both mangroves and reefs. Therefore, those families, despite having a large number of species on reefs, represent a similar proportion of species richness in both ecosystems. On the other hand, the taxonomic bias observed in mangroves for families such as Chalinidae, Dictyonellidae, Mycalidae, and Microcionidae becomes more evident because their species richness is a very high portion of the total diversity. In contrast, the families Agelasiidae, Axinellidae, and Clionaidae include most of the species on reefs.

Ecological patterns

Reproductive strategies

Most of the dominant demosponge genera (representing at least six demosponge orders) in Caribbean mangroves have viviparous larvae. These include *Halichondria*, *Hymeniacidon*, *Scopalina* (Halichondrida), *Haliclona*, *Chalinula* (Haplosclerida), *Clathria*,

Table 1 Relative representation of the most conspicuous Demospongiae families in Caribbean mangroves (MA) and coral reefs (CR), expressed as the number of species within a family (SPP) or as the percentage this number represents of the total species richness in each ecosystem (%)

Order	Family	MA–SPP	%	CR–SPP	%
Homosclerophorida	Plakinidae	6	5.4	6	2.3
	Oscarellidae	1	0.9	1	0.4
Spirophorida	Tetillidae	1	0.9	5	1.9
Astrophorida	Geodiidae	3	2.7	13	5
	Ancorinidae	1	0.9	11	4.2
Hadromerida	Clionaidae	3	2.7	19	7.3
	Suberitidae	6	5.4	9	3.4
	Tethyidae	1	0.9	7	2.7
Agelasida	Agelasiidae	1	0.9	15	5.8
Halichondriida	Axinellidae	2	1.8	16	6.2
	Dictyonellidae	8	7.2	6	2.3
	Halichondriidae	7	6.3	17	6.6
Lithistida	Siphonidiidae	0	0	4	1.5
	Discodermidae	1	0.9	5	1.9
	Scleritodermidae	0	0	4	1.5
Poecilosclerida	Mycalidae	12	10.8	9	3.4
	Microcionidae	9	8.1	16	6.2
Haplosclerida	Chalinidae	17	15.3	14	5.4
	Petrosiidae	7	6.3	17	6.5
	Niphatidae	9	8.1	16	6.2
	Callyspongiidae	3	2.7	12	4.6
Dictyoceratida	Spongiidae	2	1.8	8	3.1
	Irciniidae	3	2.7	6	2.3
	Thorectidae	4	3.6	12	4.6
Verongida	Aplysinidae	4	3.6	11	4.2
Total species		111		259	

Tedania, *Mycale* (Poeciosclerida), *Oscarella*, *Plakortis* (Homosclerophorida). The reported dominance of *Tedania ignis* in several Caribbean locations (Sutherland, 1980; Toffart, 1983; Díaz et al., 2004; Wulff, 2004) is probably related to its high and nearly year-around production of larvae (Rützler, unpublished data) and rapid growth (Wulff, 2005). On the reef there is not such a biased predominance of reproductive strategy; for most major species in the reefs, however, oviparous reproduction is observed (*Agelas*, *Aplysina*, *Verongula*, *Axinella*, *Chondrilla*, *Geodia*, *Cliona*, and most *Xestospongia* species) (Wapstra & van Soest, 1987; Maldonado, 2006).

Growth forms and size

Although most sponge growth forms (crusts, tubular, rods, massive, spherical, etc.) can be encountered at both mangroves and coral reefs, there is a clear dominance of tubular, ramose, and massive sponges among conspicuous coral reef species, whereas thin and massive crusts predominate among mangrove species. Díaz & Rützler (2009) found that thin encrusting sponges, for example *Mycale microsigmatosa*, *Haliclona manglaris*, *Halisarca* sp., *Clathria schoenus*, and *Spirastrella mollis* were among the most common species, only second or third in root-area coverage, after *T. ignis* (a massive to thick crust), which dominated in abundance at all four studied sites in Bocas del Toro (Panama). This result suggests that, at least in Bocas del Toro, encrusting species are highly successful competitors. On open reef habitats, the most conspicuous species are the massive large, ramose, tubular, fan, or vase sponges (Álvarez & Díaz, 1985; Diaz et al., 1991; Weil, 2006). Depending on the strength of water currents and the intensity of sedimentation, one or another form predominates. In

the Barrier reef at Belize, where the current is strong and constant, tube and vase shaped sponges dominate (Villamizar and Diaz, unpublished data). In reef walls the stringy or tubular sponges are the most conspicuous. In cryptic coral reef environments, most species are thin to thick encrusting sponges, or are very small tubular ones, for example Calcareous species (Kobluk & Van Soest, 1989; Diaz and Rützler, personal observation).

On mangrove roots, massive growth forms are common among the halichondrids (*Halichondria*, *Hymeniacidon*, and *Amorphinopsis*), poecilosclerids (*Tedania* spp., *Clathria* spp., *Mycale* spp.), and dictyoceratids (*Spongia*, and *Hyrtios* spp.) (Rützler et al., 2000; Díaz & Rützler, 2009). One might find rods, branches, and tubes, but usually in smaller sizes than in reefs. Size rather than shape might be a clear difference between these two faunas. Whereas mangrove sponges might reach sizes up to 10,000s of cm^3 (Díaz & Rützler, 2009), reef sponges reach sizes of up to 1,000,000s of cm^3, for example the massive *Xestospongia muta* and *Geodia neptuni*, with very high living volumes (Diaz and Villamizar unpublished data).

Distribution of cyanobacterial sponge associations

Coral reef open habitats, exclusively, contain 80% of the Caribbean cyanosponges (Díaz et al., 2007). All *Aplysina*, *Chondrilla*, *Erylus*, *Geodia*, *Ircinia*, *Neopetrosia*, *Petrosia*, *Spongia*, *Svenzea*, *Verongula*, *Xestospongia* species, and *Haliclona walentina* are among the most conspicuous reef species. Only eleven species in Caribbean mangroves have been reported with endobiotic cyanobacteria: *Geodia papyracea*, *G. tumulosa*, *G. gibberosa*, *Xestospongia wiedenmayeri*, *X. bocatorensis*, *Spongia pertusa*, *S. tubulifera*, *Hyrtios proteus*, *Chondrilla caribbaensis* f. *caribensis*, *Ircinia* sp., and *Haliclona* sp.

Discussion

The taxonomic and ecological divergence of sponge fauna inhabiting mangrove and open coral reef habitats is well supported by the current literature. First, statistically significant dissimilarities of sponge species composition between mangrove and open coral reef habitats were observed at a variety of Caribbean sites. The AvTD analysis further supports this distinction and indicates the existence of a strong taxonomic bias in the sponge fauna of mangroves compared with coral reefs, which suggests that a speciation process is associated with the mangrove habitat itself, operating independently of geographic distance. Second, throughout the comparison of ecological patterns of these faunas (reproductive strategies, growth forms, cyanobacterial symbioses) clear divergences are observed. Third, the reef fauna seems to be more diverse throughout most orders of Demospongiae, and consistent disjunctive radiation of families and genera is observed, at least in the five well studied Caribbean regions where these two ecosystems are contiguous (Belize, Cuba, Jamaica, Panama, and Venezuela). The possible effect of historical and environmental differences between mangrove and coral reefs are discussed below as potential causes of the taxonomic and ecological distinctiveness of their associated sponge fauna.

Distinct evolutionary histories

The first aspect to consider is that despite the geographic co-occurrence of these two ecosystems, it is highly probable that their "founding taxa" and the ecosystems themselves have very different evolutionary histories (geological periods in which each ecosystem and its major biological components evolved, evolutionary age of the lineages of sponge taxa that flourish at each type of ecosystem, etc.). Modern hermatypic coral reef ecosystems evolved since the beginning of the Cenozoic (preceded by the evolution of photobiosis in scleractinean corals 210 mya and the later evolution of hervibory and predation 60 mya) (Wood, 1998). On the other hand, the oldest mangrove record is of the genus *Nypa* 80 mya from the Indo-Pacific (Ellison et al., 1999) and the contemporary mangrove ecosystems in the Caribbean evolved 5 mya (Lacerda et al., 2002). Therefore, the evolution of mangroves as marine-terrestrial ecosystems is more recent than the development and evolution of coral reef ecosystems in the Caribbean basin. Furthermore, mangrove development around reef ecosystems is preceded by the growth of a coral reef structure, with subsequent geo-morphological processes leading to the environmental conditions (depth and sediment deposition) necessary for mangrove colonization. These geological and geo-morphological timing differences support the hypothesis that lineages of coral reef taxa evolved earlier than mangrove taxa, and that

these taxa are highly derived and adapted to a coral reef environment. This could in turn explain the lack of successful colonization of neighboring mangrove systems by major coral reef species (for example *Xestospongia*, *Agelas*, *Aplysina*, and *Axinellid*). Species of these common genera in the Caribbean probably evolved adaptations to exploit, survive in, and colonize coral reef habitats, when mangrove systems were not the conspicuous marine subtidal community they are today. The consistently higher diversity of sponge species, across most sponge orders, in open reef habitats as opposed to open mangrove habitats, supports the hypothesis of an older sponge fauna on the reef. The mangrove sponge fauna is probably younger and secondarily derived from previously existing faunas (open and cryptic coral reef habitats, deep waters, and rocky shores). The work discussed in this paper revealed significant dissimilarities among the prevailing forms observed in mangroves and those from open habitats in coral reefs. Furthermore, the sponge assemblages associated with cryptic habitats in Curaçao and Bonaire revealed higher similarity with open reef fauna. However, several genera which dominate in mangroves, for example *Haliclona*, *Clathria*, and *Mycale*, are common in reef sciophilous habitats (Kobluk & Van Soest, 1989), and some ecological patterns, for example the predominance of viviparity, are also common among cryptic species. Therefore, some of the genera that successfully colonized mangroves might have been derived from cryptic or sciophilous habitats.

This scenario of the history of these marine ecosystems would place mangrove subtidal habitats as the youngest marine ecosystem, rather than a below optimum marine ecosystem. Wulff (2005, 2009) transplanted reef and mangrove sponges on to each other's habitats in Belize, and found that some reef species thrive when placed on mangrove roots. Pawlik et al. (2007) repeated the same experiment but in the Florida Keys and obtained very different results, with all species dying after transplantation. It seems that a range of effects of abiotic and biotic factors may differently affect species survival in the Caribbean. However, it is probable that constraints in the early stages of development (recruitment and colonization mechanisms), and not adult survival, prevent these emblematic reef species from growing in most mangrove sites. A higher proportion of reef fauna on mangrove roots has been found in some mangrove areas adjacent to shallow reef patches (Rützler et al., 2000). Recruitment facilitation, spongivory pressure, and the existence of unique environmental conditions (clear and calm waters) have been suggested as explanations of this phenomenon (Rützler et al., 2000; Wulff, 2005).

Evolutionary relationships of particular taxonomic groups within mangrove ecosystems have been previously studied for other animal phyla. Ellison et al. (1999) depicted evolutionary relationships between a variety of gastropod groups and mangroves. When they compiled a fossil-occurrence database of gastropods and compared the distribution with that in their fossil mangrove database they encountered an ecological "nestedness" of gastropods and mangroves through time. Ellison et al. (1999) concluded, on the basis of the fossil distributions of both mangroves and gastropods, and the patterns of their species–area relationships, that these associations most likely share a Tethyan origin, with subsequent diversification patterns related to the tectonic reconfiguration of shorelines and continents.

Currently, we do not have a good sponge fossil record associated with mangrove ecosystems; however, one can trace phylogenetic relationships using various molecular markers to determine historical relationships between both faunas across the Caribbean. Duran & Rützler (2006) encountered "nested" populations of *Chondrilla* species (distinct phylotypes), either on reefs or on mangroves, all along the Caribbean. Although the results of their analysis suggested an ancestral origin of *Chondrilla* haplotypes from the mangroves, and a derived origin for the reef populations, their work is the first proof of the existence of distinct populations and low gene flow between the two habitats despite geographic continuity.

Other biological scenarios could explain the taxonomic distinctness and higher diversity of open coral reef sponge taxa compared with mangrove sponge taxa. A more heterogeneous marine system, for example a coral reef with more complex biological interactions, could promote a richer and distinct fauna, which argues against the historical distinctness proposed earlier. Higher species richness can also be related to the fact that coral reefs extend for several tens of meters in depth whereas mangroves occur only between 1 and 3 m deep. Only life-history studies demonstrating the existence of complex behavior in early life stages, different success in each habitat, and

the genetic historical association of specific groups will clarify these hypotheses.

Environmental conditions

Various environmental differences must be discussed as possible contributors to the taxonomic and ecological patterns presented here. A variety of environmental conditions in open and cryptic coral reef and mangrove habitats are characterized qualitatively in Table 2. Marked differences become evident when one compares general qualitative aspects among them. Abiotic factors are less likely to differently structure the reef sponge community because coral reef habitats have relatively constant, oligotrophic conditions (Pawlik et al., 2007). It is well known that few sponge species can overcome air exposure and high temperatures (Rützler, 1995). Daily temperature, tidal, and salinity variation found in mangrove systems must restrict the long term growth and survival of coral reef sponges that inhabit much more stable environments. The possible effect of changes in substrate availability on the dynamics of its associated benthic fauna has not yet been evaluated. Major coral reef "structure builders", hermatypic corals, grow very slowly, 1–2 cm per year, whereas mangrove roots grow at between 10 and 40 cm per year, adding continuously new substrate to the sub-tidal environment (Díaz & Rützler, 2009). These large differences in substrate dynamics must have important ecological consequences for the fauna inhabiting them. Strategies such as high growth rates, asexual reproduction, and the availability of viviparous recruitment stages would be favored in systems such as mangroves, where immediate colonization could be advantageous given the stochastic appearance of available substrate.

Oviparity and broadcasting strategies in sponges might be favored under conditions of strong unidirectional currents, with wider geographic distributions common for organisms inhabiting coral reefs. Shortened viviparous larval stages might be favored in slower current environments and in habitats with much restricted geographic distributions.

Cryptic reef species and several mangrove forms minimize volume to area coverage ratios. Limited space between roots and under reef crevices would favor smaller sizes in both environments. Open reef species tend to maximize volume-to-area ratios with bodies expanding in the water column, rather than on the substrate. Associations with symbiotic organisms are strikingly excluded from cryptic and mangrove species, where light conditions are less favorable for photosynthetic organisms.

The high rate of stochastic and predictable events in mangrove ecosystems (tides temperature change, drastic substrate change, upwelling, etc.), has probably caused a myriad of variable conditions that have provided an opportunity for the genera that reached this environment to evolve distinct micro niches at these sites. The availability of these niches might explain the sympatric occurrence of so many unique species within several genera (for example *Haliclona*, *Mycale*), where one can find two or three species of the same genus co-occurring on a single mangrove root.

Table 2 General characterization of environmental factors at Caribbean mangrove, and open and cryptic reef habitats

Condition	Mangrove	Open reef	Cryptic reef
Temperature daily/seasonal changes	High	No	No
Salinity daily/seasonal changes	High	No	No
Sedimentation	Low-high	Low-high	Low
Water circulation	Poor	Mid to high	Poor
Tides	Diurnal	No	No
Wave action	Very low	High	Low
Solar radiation	Low	High	Low
Nutrient levels	High	Low	Low
Substrate type	Living wood, peat, mud-sand	Sand, coral rock	Sand, coral rock
Substrate availability	Very low	Low	Very low
Rate of substrate growth	10–40 cm/year	1–2 cm year	NA
Predation	Low	High	Low

Conclusions

Sponge faunas from mangrove and open coral reef habitats have high distinctness at different taxonomic levels, in a variety of Caribbean sites, suggesting a different founding fauna in both marine ecosystems.

Distribution and diversity of suprageneric taxa suggests that the coral reef sponge fauna might be older, and that the mangrove sponge fauna might be younger and derived from coral reef cryptic, deep water, and rocky shores habitats.

The historical aspects of the evolution of coral reef and mangrove fauna must be further explored (e.g. genetically) to evaluate the hypothetical explanations proposed here for the strong taxonomic disjunction between these to co-occurring faunas.

Mangroves might be the latest evolutionary frontier, because they are among the youngest marine ecosystems.

The effect of various environmental conditions (temperature and salinity changes, tidal currents, substrate affinities) at various life stages of these sponge species must be studied to evaluate the contribution of environmental differences to the ecological distinctness of these two faunas.

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References

Alcolado, P. M., 2002. Catálogo de las esponjas de Cuba. Avicennia 15: 53–72.

Álvarez, B. & M. C. Díaz, 1985. Las esponjas de un arrecife coralino en el Parque Nacional Archipiélago de Los Roques: Taxonomía y Ecología. Universidad Central de Venezuela, Caracas, Venezuela. Trabajo Especial de Grado para optar al Título de Licenciado en Biología.

Alvizu, A., 2006. Estructura comunitaria de esponjas asociadas a dos parches coralinos en la Isla de Cubagua, Venezuela. Universidad de Oriente, Margarita, Venezuela. Trabajo presentado para optar al título de Licenciado en Biología Marina.

Amaro, M. E., 2005. Demospongiae (Porifera) asociadas a sustrato coralino, en la Bahía de Mochima, Estado Sucre, Venezuela, durante el año 2004–2005. Universidad de Oriente, Cumaná, Venezuela. Trabajo de grado para optar al título de MSc Ciencias Marinas.

Bingham, B. L. & C. M. Young, 1995. Stochastic events and dynamics of mangrove root epifaunal community. Marine Ecology 16: 145–163.

Clarke, K. R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18: 117–143.

Clarke, K. R. & R. N. Gorley, 2006. Plymouth Routines in Multivariate Ecological Research, Primer V6: User Manual/Tutorial. PRIMER-E Ltd, Plymouth, UK.

Clarke, K. R. & R. M. Warwick, 1998. A taxonomic distinctness index and its statistical properties. Journal of Applied Ecology 35: 523–531.

Clifton, K. E., K. Kim & J. Wulff, 1997. A field guide to the reefs of Caribbean Panamá with emphasis of Western San Blas. In Lessios, H. A. & I. G. Macintyre (eds), Proceedings of the 8th International Coral Reef Symposium, Vol. 1: 167–184.

de Goeij, J. M., H. van den Berg, M. Martine, E. van Oostveen, H. G. Epping & F. C. van Duyl, 2008. Major bulk dissolved organic carbon (DOC) removal by encrusting coral reef cavity sponges. Marine Ecology Progress Series 357: 139–151.

Díaz, M. C., 2005. Common sponges from shallow marine habitats from Bocas del Toro region, Panama. Caribbean Journal of Science 41: 465–475.

Díaz, M. C. & K. Rützler, 2001. Sponge: an essential component of Caribbean coral reefs. Bulletin of Marine Science 69: 535–546.

Díaz, M. C. & K. Rützler, 2009. Biodiversity and abundance of sponges in Caribbean mangrove: indicators of environmental quality. Smithsonian Contributions to the Marine Sciences 38: 151–172.

Diaz, M. C., B. Alvarez & R. A. Laughlin, 1991. The ecology of sponges in a fringing coral reef at the National park "Archipelago de los Roques", Venezuela II: Community structure. In Rüetzler, K. (ed.), New Perspectives in Sponge Biology. Smithsonian Institution Press, Washington, DC: 367–375.

Díaz, M. C., K. P. Smith & K. Rützler, 2004. Sponge species richness and abundance as indicators of mangrove epibenthic community health. Atoll Research Bulletin 518: 1–17.

Díaz, M. C., R. W. Thacker & K. Rützler, 2007. Haliclona (Soestella) walentinae (Chalinidae, Haplosclerida) and Xestospongia bocatorensis (Petrosiidae, Haplosclerida), two new sponge species from Caribbean Panamá with filamentous cyanobacterial symbionts. In Custódio, M. R., G. Lôbo-Hajdu, E. Hajdu & G. Muricy (eds), Porifera Research: Biodiversity, Innovation, and Sustainability, Vol. 1: 31–36.

Dunlap, M. & J. R. Pawlik, 1996. Video-monitored predation by Caribbean reef fishes on an array of mangrove and reef sponges. Marine Biology 126: 117–123.

Dunlap, M. & J. R. Pawlik, 1998. Spongivory by parrotfish in Florida mangrove and reef habitats. Marine Ecology 19: 325–337.

Duran, S. & K. Rützler, 2006. Ecological speciation in a Caribbean marine sponge. Molecular Phylogenetics and Evolution 40: 292–297.

Ellison, A. M., E. J. Farnsworth & R. E. Merkt, 1999. Origins of mangrove ecosystems and the mangrove biodiversity anomaly. Global Ecology and Biogeography 8: 95–115.

Engel, S. & J. R. Pawlik, 2005a. Interactions among Florida sponges. I. Reef habitats. Marine Ecology Progress Series 303: 133–144.

Engel, S. & J. R. Pawlik, 2005b. Interactions among Florida sponges. II. Mangrove habitats. Marine Ecology Progress Series 303: 145–152.

Farnsworth, E. J. & A. M. Ellison, 1996. Scale-dependent spatial and temporal variability in biogeography of mangrove root epibiont communities. Ecological Monographs 66: 45–66.

Guerra-Castro, E., P. Young, A. Pérez-Vázquez, S. Carteron & A. Alvizu, 2011. Spatial variability and human disturbance of sponge assemblages associated with mangrove roots in the southern Caribbean. Marine & Freshwater Research 62: 491–501.

Hechtel, G. J., 1965. A Systematic Study of the Demospongiae of Port Royal, Jamaica. Peabody Museum of Natural History. Yale University, New Haven, CT.

Hunting, E. R., H. G. van der Geest, A. M. Krieg, M. B. L. van Mierlo & R. W. M. van Soest, 2010. Mangrove–sponge associations: a possible role for tannins. Aquatic Ecology 44: 679–684.

Kobluk, D. R. & R. W. M. van Soest, 1989. Cavity-dwelling sponges in a southern Caribbean coral reef and their paleontological implications. Bulletin of Marine Science 44: 1207–1235.

Lacerda, L. D., J. E. Conde, B. Kjerfve, R. Alvarez-León, C. Alarcón & J. Polanía, 2002. American mangroves. In de Lacerda, L. D. (ed.), Mangrove Ecosystems: Function and Management. Springer-Verlag, Berlin: 1–62.

Lehnert, H. & R. W. M. van Soest, 1996. North Jamaican deep fore-reef sponges. Beaufortia 46: 53–81.

Lehnert, H. & R. W. M. van Soest, 1998. Shallow water sponges of Jamaica. Beaufortia 48: 71–103.

Lesser, M., 2006. Benthic–pelagic coupling on coral reefs: feeding and growth of Caribbean sponges. Journal of Experimental Marine Biology and Ecology 328: 277–288.

Maldonado, M., 2006. The ecology of the sponge larva. Canadian Journal of Zoology 84: 175–194.

Mumby, P. J., A. R. Harborne, J. Williams, C. V. Kappel, D. R. Brumbaugh, F. Micheli, K. E. Holmes, C. P. Dahlgren, C. B. Paris & P. G. Blackwell, 2007. Trophic cascade facilitates coral recruitment in a marine reserve. Proceedings of the National Academy of Sciences USA 104: 8362–8367.

Pauls, S. M., 2003. Esponjas. In Aguillera, M., A. Azócar & E. González (eds), Biodiversidad en Venezuela. Tomo I. Fundación Polar & Ministerio de Ciencia y Tegnología, Caracas: 210–219.

Pawlik, J. R., 1997. Fish predation on Caribbean reef sponges: an emerging perspective of chemical defenses. In Proceedings of the 8th International Coral Reef Symposium, Vol. 2: 1255–1258.

Pawlik, J. R., 1998. Coral reef sponge: do predatory fishes affect their distribution? Limnology and Oceanography 43: 1396–1399.

Pawlik, J. R., S. McMurray & T. P. Henkel, 2007. Abiotic factors control sponge ecology in Florida mangroves. Marine Ecology Progress Series 339: 93–98.

Pérez-Vázquez, A., 2007. Estudio de las comunidades de esponjas sociadas a las raíces del mangle rojo Rhizophora mangle en la Laguna de la Restinga entre julio 2004 y agosto 2005. Universidad de Oriente, Margarita, Venezuela. Trabajo presentado para optar al título de Licenciado en Biología Marina.

Ramirez, I. A., 2010. Taxonomía de esponjas marinas (Porifera) de la Isla de la Tortuga, dependencia federal, Venezuela. Trabajo de grado de Magister en Ciencias Marinas. Universidad de Oriente, Cumana: 114 pp.

Rützler, K., 1995. Low-tide exposure of sponges in a Caribbean mangrove community. Marine Ecology 16: 165–179.

Rützler, K., M. C. Díaz, R. W. M. van Soest, S. Zea, K. P. Smith, B. Álvarez & J. Wulff, 2000. Diversity of sponge fauna in mangrove ponds, Pelican Cays, Belize. Atoll Research Bulletin 476: 230–248.

Sutherland, J. P., 1980. Dynamics of the epibenthic community on roots of the mangrove *Rhizophora mangle*, at Bahia de Buche, Venezuela. Marine Biology 58: 75–84.

Toffart, J. L., 1983. Les Peuplements des racines de palétuviers en Guadeloupe (Antilles Françaises), I. Analyse floristique et faunistique; méthodologie et premier résultats. Bulletin d'Ecologie 14: 227–239.

van Soest, R. W. M., 1978. Marine sponges from Curaçao and other Caribbean localities. Part I. Keratosa. In Hummelinck, P. W. & L. J. van der Stee (eds), Studies on the Fauna of Curaçao and Other Caribbean Islands, Vol. 56. M. Nijhoff, The Hague: 1–94.

van Soest, R. W. M., 1980. Marine sponges from Curaçao and other Caribbean licalities, Part II. Haplosclerida. In Hummelinck, P. W. & L. J. van der Steen (eds), Studies on the Fauna of Curaçao and Other Caribbean Islands, Vol. 62. M. Nijhoff, The Hague: 1–173.

van Soest, R. W. M., 1984. Marine sponges from Curaçao and other Caribbean localities, Part III. Poecilosclerida. In Hummelinck, P. W. & L. J. van der Steen (eds), Studies on the Fauna of Curacao and Other Caribbean Islands, Vol. 66. M. Nijhoff, The Hague: 1–167.

van Soest, R. W. M., 2009. New sciophilous sponges from the Caribbean (Porifera: Demospongiae). Zootaxa 2107: 1–40.

van Soest, R. W. M., N. Boury-Esnault, J. N. A. Hooper, K. Rützler, N. J. de Voogd, B. Alvarez de Glasby, E. Hajdu, A. B. Pisera, R. Manconi, C. Schoenberg, D. Janussen, K. R. Tabachnick, M. Klautau, B. Picton & M. Kelly, 2010. World Porifera database. http://www.marinespecies.org/porifera.

Wapstra, M. & R. W. M. van Soest, 1987. Sexual reproduction, larval morphology and behaviour in Demosponges from the southwest of the Netherlands. In Vacelet, J. & N. Boury-Esnault (eds), Taxonomy of Porifera. Springer-Verlag, Berlin: 281–307.

Warwick, R. M. & K. R. Clarke, 2001. Practical measures of marine biodiversity based on relatedness of species. Oceanography and Marine Biology: An Annual Review 39: 207–231.

Weil, E., 2006. Diversidad y abundancia relativa de corales, octocorales y esponjas en el Parque Nacional Jaragua, República Dominicana. Revista de Biología Tropical 54: 423–443.

Wood, R., 1998. The ecological evolution of reefs. Annual Reviews of Ecology, Evolution, and Systematics 29: 179–206.

Wulff, J. L., 1997. Parrotfish predation on cryptic sponges of Caribbean coral reefs. Marine Biology 129: 41–52.

Wulff, J., 2004. Sponges on mangrove roots, Twin Cays, Belize: early stages of community assembly. Atoll Research Bulletin 519: 1–10.

Wulff, J. L., 2005. Trade-offs in resistance to competitors and predators, and their effects on the diversity of tropical marine sponges. Journal of Animal Ecology 74: 313–321.

Wulff, J. L., 2009. Sponge community dynamics on Caribbean mangrove roots: significance of species idiosyncrasies. Smithsonian Contributions to Marine Science 38: 501–514.

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SPONGE RESEARCH DEVELOPMENTS

Qualitative variation in colour morphotypes of *Ianthella basta* (Porifera: Verongida)

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Abstract Natural populations of marine invertebrates often exhibit measureable morphologic variation resulting in taxonomic confusion. This potentially has severe consequences for experimental design and data management. Species of the sponge genus *Ianthella* embody a number of different morphologies and a diverse range of secondary metabolites. Among them, *Ianthella basta* (Pallas, 1776), a common sponge in Papua New Guinea and the Great Barrier Reef (GBR), exhibits two dominant colour morphotypes: yellow and purple. Specimens collected from Orpheus Island on the GBR were investigated using phylogenetic (CO1, ITS-2 sequence analysis), chemical (mass spectrometry) and microbial (DGGE and 16S rRNA clone library) techniques in an effort to fully characterise the two colour morphs. Phylogenetic analyses indicated sharp genetic discontinuities within *I. basta* sensu lato independent of colour variation. The two morphotypes did, however, correspond to distinct DGGE profiles largely due to the presence of additional bands in the purple morpho-group. Further comparison of the microbial communities by 16S rRNA gene sequencing revealed that whilst both colour morphs were dominated by only two bacterial symbionts (residing within the *Gamma* and *Alphaproteobacteria*), the purple morph also contained minor representatives of the *Cyanobacteria*, *Chloroflexi* and *Verrucomicrobia*. Untargeted metabolic profiling by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) indicated two distinct clusters corresponding to the different sponge colours. A clear association was found between the araplysillin class of compounds and the purple morphotype of *I. basta*, indicating the utility of a metabolomic approach to assess differences between colour morphs. These results have important implications for ecological investigations in sponges and other invertebrate taxa whose morphology is fundamentally dynamic, stressing the need for precise taxonomic, chemical and microbial descriptions.

Keywords *Ianthella* · GBR · FTICR-MS · Colour morphotype · Metabolomic profile · Phenotype plasticity

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Introduction

When selecting a model for studies of ecology, biodiscovery or symbiosis, it is essential to define the taxonomic status of the organism and to uncover any hidden intra-species variation, especially in the case

of geographically distinct populations of so-called cosmopolitan species (Caputi et al., 2007; Sordino et al., 2008; Poore & Andreakis, 2011). The taxonomic identification of many marine invertebrates to the species level remains the task of specialists (Ackers & Moss, 1987; Bell & Barnes, 2001). However, difficulties in identification arise due to (1) a lack of fixed morphological features that can be used to delineate a species (Bond & Harris, 1988; Gaino et al., 1995) and (2) conspicuous levels of morphological plasticity which may cause the lines between genetically distinct taxa to become morphologically cryptic (Bond & Harris, 1988; Gaino et al., 1995). This phenotypic plasticity includes cases where individuals of the same species can appear less similar than those of different species; this is particularly true among marine sponges (Ackers et al., 1992).

Marine invertebrates commonly produce secondary metabolites with the largest incidence found within the phylum Porifera (Erwin & Thacker, 2007). The prevalence of such compounds in marine invertebrate taxa has not gone unnoticed taxonomically (Bergquist & Wells, 1983; Lee & Gilchrist, 1985) and has helped to identify new species and to clarify species complexes in the past (Thompson et al., 1987). In sponges, classes of compounds are used to delineate families rather than species (Lee & Gilchrist, 1985). The apparent unreliability of this taxonomic marker to distinguish individual species is largely due to our limited knowledge of the distribution and variability across taxa, the biosynthetic origin and the ecological role of the secondary metabolites. This problem hinders studies of potential biotechnological and pharmaceutical applications of these compounds, as well as systematics and taxonomy of marine invertebrates. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) has recently emerged as a powerful metabolomics tool (Aharoni et al., 2002; Want et al., 2007). It has the highest resolution among all spectrometric methods in revealing fine-scale diversity in complex mixtures and can therefore provide an insight into the chemical composition of organisms and how this composition may change between individuals within a species, with minimal sample preparation.

It is well documented that sponges and microbes often form intimate associations, with representatives of 28 bacterial phyla, including candidate phyla, and both major lineages of *Archaea* reported (Taylor et al., 2007; Webster & Taylor, 2011). Recent research effort has focused on whether these associated microbes may be responsible for the production of some of the biologically active secondary metabolites found in sponge taxa (Taylor et al., 2007). For instance, species of the family Ianthellidae are characterised by the presence of biologically active, colourless, brominated tyrosine-derived macrocyclic molecules, so-called bastadins (Calcul et al., 2010). Sponges of this family display a number of different morphologies and have a wide geographical distribution. In particular, individuals of *Ianthella basta* are found in a variety of colour morphotypes (yellow, brown, green, blue or purple), with the purple and yellow dominating on the inshore Great Barrier Reef. Recent investigations of the microbial communities within *I. basta* have found the community to be dominated by members of the *Alpha* and *Gammaproteobacteria*, although the more exhaustive 454 tag pyrosequencing study conducted by Webster and colleagues identified additional representatives considered to be part of the rare biosphere (Luter et al., 2010; Webster et al., 2010).

In the present study, we employed a multi-disciplinary approach based on (1) sequence analysis of the mitochondrial partial CO1 gene and the second internal transcribed spacer regions (ITS-2), (2) untargeted metabolomic profiling using FTICR-MS and (3) denaturing gradient gel electrophoresis (DGGE) patterns coupled with 16S rRNA clone library analysis. Our aim is to determine whether yellow and purple morphotypes collected in sympatry are associated with distinct levels of genetic divergence, morph-specific metabolomic profiles and microbial composition at such a level to be considered biologically distinct species. The underlying null-hypothesis implies that yellow and purple morphs represent morphological variants within an acceptable range of phenotypic plasticity in a genetically homogeneous species.

Materials and methods

Specimen collection

Purple and yellow individuals of *Ianthella basta*, occurring in sympatry, were collected at a depth of 12 m from the same site (within 50 m) at Orpheus Island

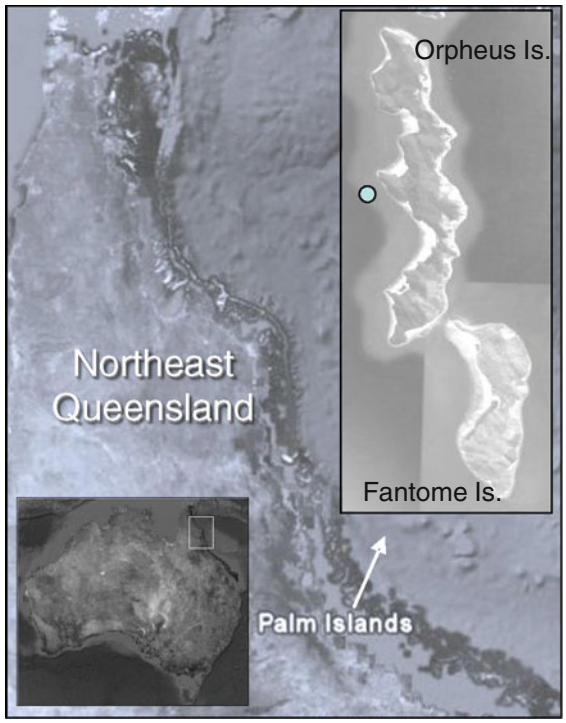

Fig. 1 Location of specimens analysed in this study

(Great Barrier Reef, Australia; latitude, 18°36.878′S; longitude, 146°29.990′E; Fig. 1). All specimens were photographed underwater prior to sampling. As previously observed, *I. basta* is susceptible to colour changes on exposure to air; therefore, sponge tissue samples were placed directly into plastic bags with seawater whilst still underwater and transported to land. Samples for genetic and microbial comparisons were excised from the biomass and transferred immediately into liquid nitrogen. These samples were stored at −80°C prior to analysis. Approximately 1 g wet weight of the remaining sponge tissue (8 yellow morphotypes and 17 purple morphotypes) was immediately immersed in 10 ml HPLC grade methanol (MeOH, Mallinckrodt), steeped overnight and stored at 4°C prior to mass spectral analysis. MeOH was the solvent of choice for the extraction based on previous chromatography analyses which yielded an abundance of chemistry (Motti et al., 2009).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from ca. 50 mg of tissue using the Power Plant DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA); DNA was subsequently gel purified using the NucleoSpin kit (Macherey-Nagel Inc., Bethlehem, PA), following manufacturer's protocol. Quantity and quality of DNA were examined by means of 1% agarose TAE buffer gel electrophoresis against known standards. The partial mitochondrial Cytochrome oxidase subunit 1 (CO1) and the nuclear second internal transcribed spacer (ITS-2) were PCR amplified using universal primers described in Folmer et al. (1994) and sponge-specific primers reported in Thacker & Starnes (2003), respectively. The ITS-2 marker is known to be suitable in resolving genealogical relationships at lower taxonomical levels (inter- and intra-specific). Standard PCR reactions were performed in 50 µl of medium containing approximately 10 ng of DNA template, 0.2 mM dNTPs, 1× PCR reaction buffer, 1 µM of forward and reverse primers each, 0.005% of bovine serum albumin (BSA), 3 mM $MgCl_2$ and 1 unit of Hot Star *Taq* polymerase (Qiagen, Dusseldorf, Germany). The amplification cycle for the ITS-2 PCR reaction included a cycle at 95°C for 15 min; 34 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 1.5 min; and a final elongation at 72°C for 2 min. The PCR conditions for the CO1 gene region were: 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 50 s, 42°C for 50 s, 72°C for 2 min; and a final elongation at 72°C for 10 min. Quantity and length of the PCR products were examined by 1% gel electrophoresis as described above. PCR reactions were sent to Macrogen Inc. (Korea, www.macrogen.com) for purification and direct sequencing in both directions with the same primers used for the PCR reactions. Sequences were submitted to GenBank, accession numbers JF915537–JF915551.

FTICR-MS analysis

The MeOH extract of each specimen was filtered and the solvent removed by gently heating the extract at 28°C under a stream of N_2 (g), followed by lyophilisation overnight. The weight of each extract was recorded. The MeOH extracts were desalted by washing with H_2O on a C18 sep pack (Phenomenex) followed by elution with 7 ml of MeOH, dried under N_2 (g) and resuspended in MeOH at 20 mg/ml. A 10 µl aliquot of each MeOH extract was diluted into 500 µl MeOH (0.5 mg/ml) ready for mass spectral analysis. FTICR-MS measurements (*m/z* 97.7015–2003.3738)

were performed on each extract using an unmodified Bruker BioAPEX 47e mass spectrometer equipped with an Analytica of Branford model 103426 (Branford, CT) electrospray ionisation (ESI) source in negative mode (cylinder = 1.9 kV, source = 4.0 kV and end cap = 3.5 kV, capillary exit = −160 V and skimmer potential = −10 V) (Motti et al., 2009). Direct infusion of each extract was carried out using a Cole Palmer 74900 syringe pump at a rate of 150 µl/h and the instrument externally calibrated using a methanolic solution of CF_3COONa (0.1 mg/ml MeOH). Data for each extract were collected with XMASS software (version 7.0.3, Bruker Daltonics). The resulting free induction decays (512,000 points, with 16 transients) were apodized, Fourier transformed and displayed in magnitude mode before internal calibration using *m/z* 155.0023 ± Δ 0.2 mmu which was present in all biological extracts negating the need to add an exogenous internal reference.

Chemometric analysis

FTICR-MS spectra were processed in the R environment (version 2.10, Comprehensive *R* Archive Network, http://www.r-project.org/), using the package FTICRMS (version 0.8, Barkauskas, 2009) in six steps: baseline correction, data transformation, peak location, peak selection, normalisation and statistical analysis. The default parameters for the R package FTICRMS were used except for the following: sm.par, the smoothing parameter for baseline calculation, which was experimentally determined to be approximately 1.0×10^{-11}; peak.method, method for locating a peak, was set to 'locmax' algorithm which returned a set of local maxima larger than a peak threshold; peak.thresh, which was experimentally determined to be 2.9; alignment algorithm used for peaks was align.method = 'affine'.

Statistical comparison of the MS peaks and their associated intensities was performed using the unsupervised pattern recognition method, principal component analysis (PCA), with the prcomp function in the R environment. Data were visualized using GraphPad Prism version 5.04 Windows (GraphPad Software, San Diego California USA, www.graphpad.com, 2010) by plotting principle component (PC) scores, where each point in the score plot represents an individual sample.

Microbial comparison

The 16S rRNA gene from 8 yellow and 17 purple *I. basta* morphotypes was amplified by PCR with universal bacterial DGGE primers following procedures outlined in Luter et al. (2010). Briefly, DNA was extracted using a modified version of (Wilson et al., 2002) and with a Power Plant DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene of samples from each extraction method was amplified by PCR with bacterial primers (1055f: 5′-ATGGCTGTCGTCAGC T-3′ and 1392r: 5′-ACGGGCGGTGTGTRC-3′) (Ferris et al., 1996). The reverse primer was modified to contain a 40-bp GC clamp (Muyzer et al., 1993). PCR conditions employed in Luter et al. (2010) were followed exactly. PCR products from both extraction methods were pooled, and 20 µl of each sample was added to an 8% (wt/vol) polyacrylamide gel containing a 50–70% denaturing gradient of formamide and urea. The gel was run at 60°C for 16 h in 1× TAE buffer at 75 V using the Ingeny D-Code system, stained with 1× SYBR gold for 10 min, visualized under UV illumination and photographed with the Viber Lourmat ChemiSmart 3000 system. Statistical comparison of the microbial community composition based on the presence (scored as 1)/absence (scored as 0) matrix of DGGE bands was performed using PCA with the prcomp function and visualized by plotting PC scores, where each point in the score plot represents an individual sample (as described above).

DNA from three purple individuals was pooled, and the 16S rRNA gene was amplified by PCR with universal bacterial primers, 63f 5′-CAGGCCTAACACATG CAA GTC-3′ (Marchesi et al., 1998) and 1492r 5′-GGT TACCTTGTTACGACT T-3′ (Lane, 1991), to construct a clone library. PCR conditions employed in Luter et al. (2010) were followed exactly, and PCR products were cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Plasmids were checked for inserts by PCR amplification using the M13 forward and reverse primers. Restriction digests were preformed on 100 clones using HhaI and HaeIII (New England Biolabs Inc.) to determine operational taxonomic units (OTU's) for each library. Duplicates from each representative OTU in each library were sent to Macrogen Inc. (Seoul, Korea) for

sequencing using 63f and 1492r as the sequencing primers. The clone sequences for the three replicate yellow sponges were previously published (Luter et al., 2010). These yellow sponges were collected at the same site and time as the purple sponges that were processed as part of this study.

Microbial phylogenetic analysis

Clone sequences were compared with available databases using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) to determine nearest relatives and percent similarity. Clone sequences from the purple morphotype were submitted to Genbank under the accession numbers HQ586955–HQ586964. Sequences were checked for chimera formation using Greengenes (DeSantis et al., 2006) and Bellerophon (Huber et al., 2004). Sequences were compiled, automatically aligned and manually edited in the ARB software package (http://www.arb-home.de (Ludwig et al., 2004). Initially, the tree was calculated with almost complete 16S rRNA (1,400 bp) sequences for all close relatives of target sequences using the neighbour-joining and maximum parsimony methods in ARB. Partial sequences were subsequently imported to the tree without changing branch topology using the ARB parsimony-interactive method. The robustness of inferred tree topologies was evaluated after 1,000 bootstrap pseudo replicates of the neighbour-joining data in the PHYLIP program (Felsenstein, 1993). *Escherichia coli* was used as an out group for the tree.

Sequence alignments and phylogenetic analysis

Electropherograms were assembled in Sequencher 4.9 (Gene Codes); sequences of the two markers were aligned manually in Bioedit v7.0.9 (Hall, 1999). Finally, three alignments (COI, ITS-2 and concatenated COI-ITS-2) were considered for phylogenetic reconstructions. The amount of phylogenetic information in each of the data sets was assessed by calculating g_1 statistics as a measure of the skewness of distribution of tree-lengths among 10,000 random parsimony trees (Hillis & Huelsenbeck, 1992) in PAUP*. The significance of the g_1 value was compared with critical values ($P = 0.01$) for four state characters, given the number of distinct sequences and the number of parsimony informative sites. Hierarchical likelihood ratio tests (hLRTs) were run in Modeltest Version 3.7 (Posada & Crandall, 1998) to identify the best-fitting model and parameters (gamma distribution, proportion of invariable sites, transition–transversion ratio) given the alignment. Maximum likelihood (ML) phylogenies were computed in PAUP* 4.0b10 version for Windows (Swofford, 2002) constrained with the best-fitting model of evolution identified by Modeltest and the heuristic search option or the exhaustive search option when the number of sequences was appropriate. Pairwise TrN-corrected comparisons of ITS-2 sequences were computed in MEGA5 (Tamura et al., 2007) to assess evolutionary divergence among Verongid sponges and *I. basta* clades. Bootstrap support for individual clades was calculated in the same software on 1,000 replicates using the same methods, options and constraints used for the ML tree-inferences (Felsenstein, 1985). Genealogical relationships among haplotypes were calculated using the median-joining algorithm ($\varepsilon = 0$, equally weighted characters) implemented in the software Network v4.6.0.0 (http://www.fluxus-technology.com). This method identifies groups of closely related haplotypes and uses 'median vectors' to connect sequences into a tree or network. Median vectors can be interpreted biologically as extinct individuals or haplotypes that have not been sampled yet (Bandelt et al., 1999).

Results

Chemometric analysis of FTICR-MS data

The negative mode FTICR-MS spectra of *I. basta* MeOH extracts (Fig. 2) were processed using the R FTICRMS package (Barkauskas, 2009). From each mass spectrum containing a total number of 997,481 *m/z* values, 49,081 were defined as 'strong' peaks, which were then compiled into a data matrix. The data matrix was analysed by PCA. The different colour morphotypes were easily resolved by PCA based on their metabolite profiles except for one anomalous purple sponge P8 which grouped with the yellow sponges (Fig. 3a).

Closer inspection of the FTICR-MS spectra revealed that the metabolite profile of the yellow morphotype contained peaks indicative of the bastadin class of compounds [peaks between *m/z* 900 and 1,300 with isotope distributions consistent with single and multiple brominated macrocyclic alkaloids

(Calcul et al., 2010)]. These same peaks were also detected in the purple morphotype along with two distinct isotope envelopes at *m/z* 795 and *m/z* 811, identified based on their accurate mass data as 19-hydroxy-araplysillin-I N^{20}-sulfamate (**1**) and araplysillin-I N^{20}-sulfamate (**2**), respectively (Motti et al., 2009). The metabolite profile for the anomalous sample P8 was found to resemble those of the yellow samples.

Microbial comparison

The PCA created using a presence and absence matrix of the DGGE revealed microbial differences between the purple and yellow morphotypes, with additional bands present in purple samples. However, consistent with the chemical analysis, sample P8 grouped closely with the yellow morphotypes (Fig. 3b). The 16S rRNA clone libraries of purple and yellow *I. basta* were both dominated by a single species of *Alphaproteobacteria* (comprised 79 and 64% of the yellow and purple, respectively) and a single species of *Gammaproteobacteria* (21 and 31% of the respective yellow and purple libraries) (Fig. 4). However, the purple morphotype also had minor representatives from the *Cyanobacteria*, *Verrucomicrobia* and *Chloroflexi*, which were absent in the yellow morphotype. The dominant *Alpha* and *Gammaproteobacteria* sequences showed ~1% difference between colour morph libraries indicating the possibility that different symbiotic strains inhabit the yellow and purple sponges. All *I. basta* sequences showed similarity to other sponge-specific bacteria (Fig. 5). Phylogenetic analysis revealed that clone HQ586957 was 94% similar to a *Chloroflexi* clone from a central Spain wetland (FJ517057); purple clone HQ586959 was 97% similar to a *Verrucomicrobia* strain from Chinese coastal waters

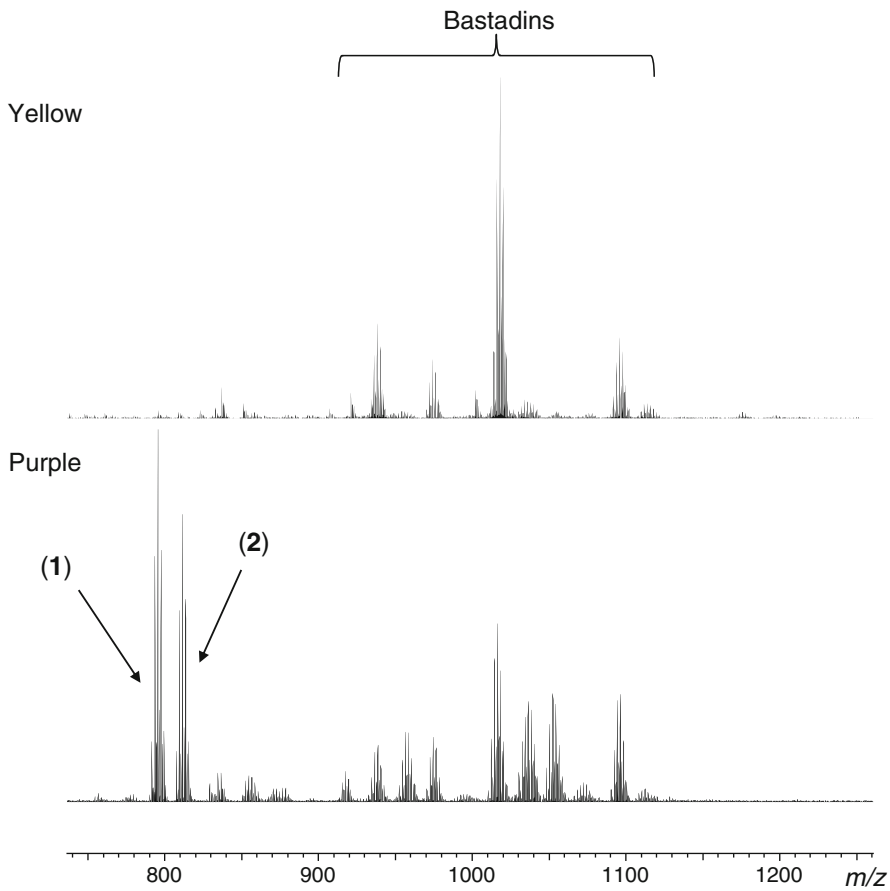

Fig. 2 Negative mode FTICR-MS (showing region *m/z* 740–1260) of a representative methanol extract from a purple and a yellow morphotype highlighting the presence of the bastadins and the two araplysillins, 19-hydroxy-araplysillin-I N^{20}-sulfamate (**1**) and araplysillin-I N^{20}-sulfamate (**2**)

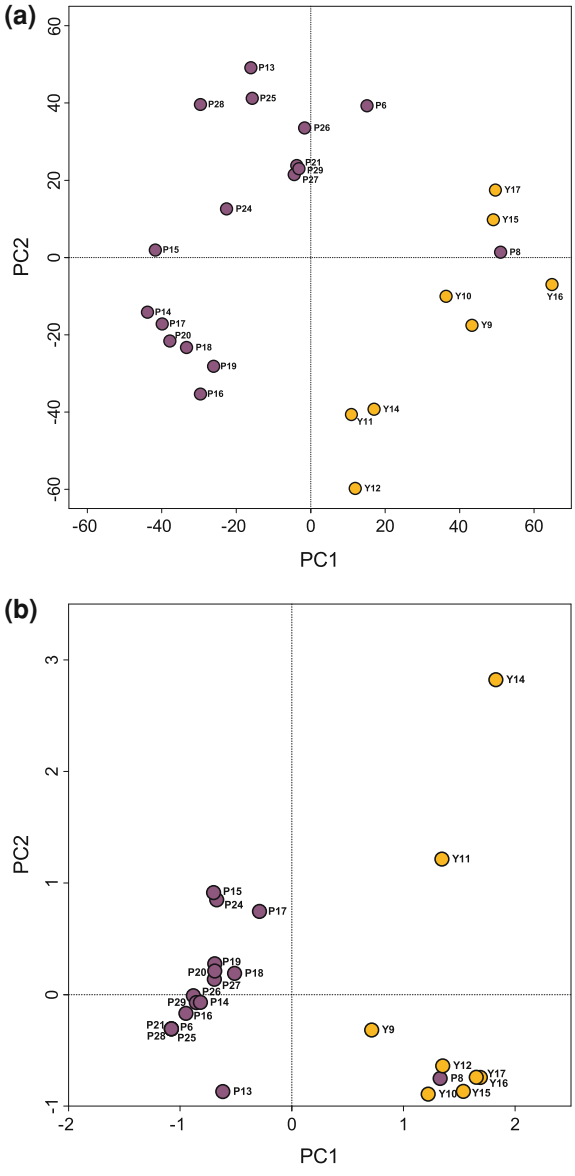

Fig. 3 Principal component analysis of **a** FTICR-MS profile data and **b** of sponge bacterial community composition using DGGE banding pattern data to construct a similarity matrix showing distinction between the yellow (Y) and purple (P) morphotypes

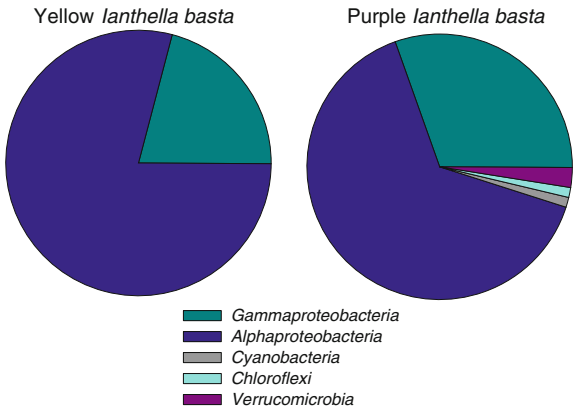

Fig. 4 Pie charts showing differences in bacterial community composition between purple and yellow *I. basta*. The graphs were constructed based on the frequency of clones from each library. 100 clones were screened from each library

(AY664013); and purple clone HQ586960 was identical to a *Cyanobacteria* clone from the Yellow Sea (HM057799) (Fig. 5). The purple *I. basta* library also contained a unique *Rhodobacterales* (HQ586964), not found in the yellow library, which was 99% similar to a *Siderastrea sidereal* clone from coral (EF123361).

Phylogenetic analysis and genealogical network reconstruction

Seven CO1 and eight ITS-2 sequences were obtained from four purple and four yellow individuals of *I. basta*, and these were merged with publically available Ianthellidae and outgroup species to create three sets of sequences: (1) a COI alignment for genealogical network reconstruction, (2) an ITS-2 alignment consisting of sequences from seven well-known species and the new ITS-2 sequences produced in this study and (3) a concatenated COI-ITS-2 alignment consisting of the newly produced sequences (see Table 1 for alignment length, model selection and summary statistics). The length distribution of 10,000 random trees computed for each of the alignments was considerably left-skewed indicating significant amount of phylogenetic signal in the datasets (Table 1). Median-joining networks inferred from the CO1 marker (Fig. 6a) revealed seven distinct haplotypes distributed in two haplo-groups. Model-constrained ML phylogenies inferred from the ITS-2 alignment resolved the taxa involved in the computation according to the taxonomic expectations and indicated three well-supported clades within *I. basta* (Fig. 7a). The same clades, named A, B and C, were strongly supported and recovered in ML phylogenies inferred from the concatenated data set (Fig. 6b). Pairwise comparisons corrected with the TrN model of evolution indicated high levels of evolutionary divergence between clades A–C and

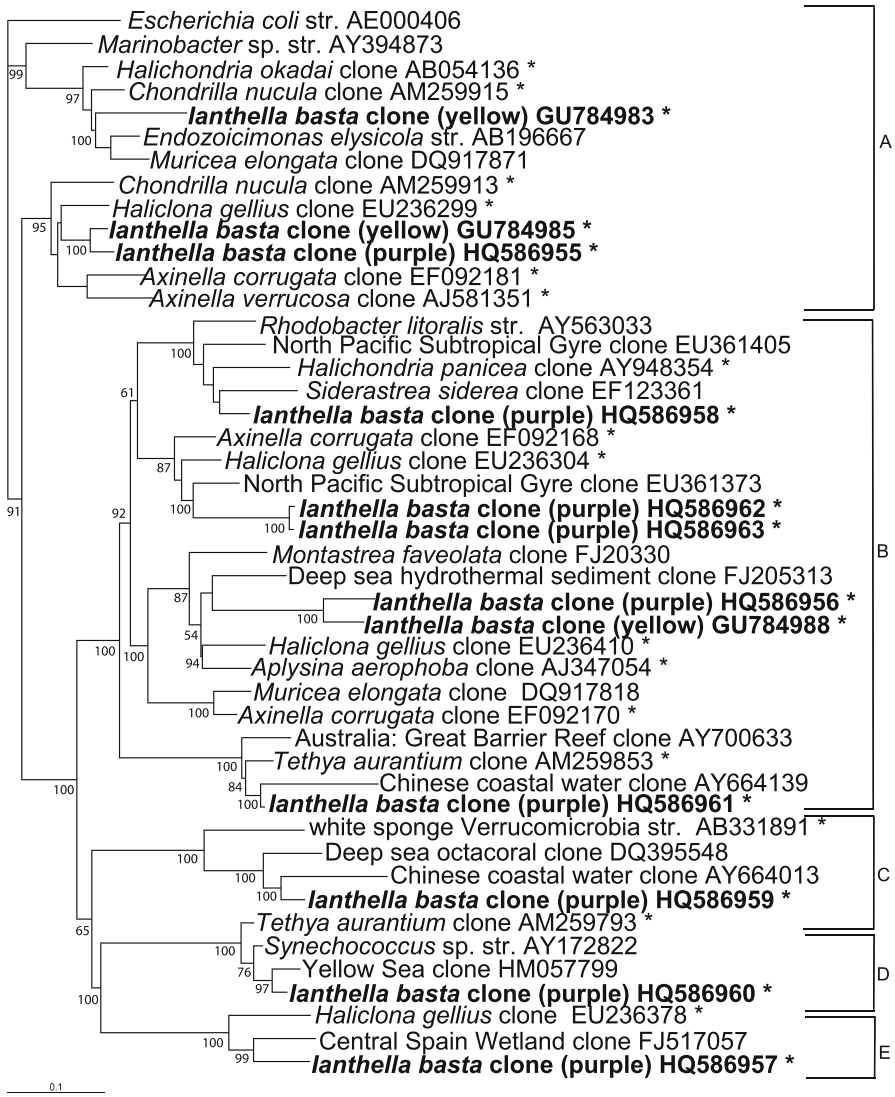

Fig. 5 Maximum-likelihood phylogeny of all 16S rRNA gene sequences retrieved from the clone library analysis. *I. basta* clones are indicated by *boldfacing*, with colour morphotype listed in parentheses and *asterisks* indicate clones isolated from marine sponges (A = *Gammaproteobacteria*, B = *Alphaproteobacteria*, C = *Verrucomicrobia*, D = *Cyanobacteria* and E = *Chloroflexi*). Numbers on nodes indicate bootstrap support for that node. Only values >50% are shown

Table 1 Alignment statistics

	l	*n*	*h*	g_1	*m*	*i*	*a*	*v*	*p*
COI	734	8	8	−1.41	K81uf	–	–	75	7
ITS	616	29	23	−0.93	TrN	–	0.4205	12	279
COI-ITS	1,208	8	8	−2.06	K80+G	–	0.0051	38	71

l alignment length, *n* number of sequences, *h* number of unique haplotypes, g_1 phylogenetic informativeness of the data, *m* evolutionary model selected by Modeltest, *I* proportion of invariable sites, *a* Gamma distribution shape parameter, *v* variable, parsimony uninformative sites, *p* parsimony informative sites

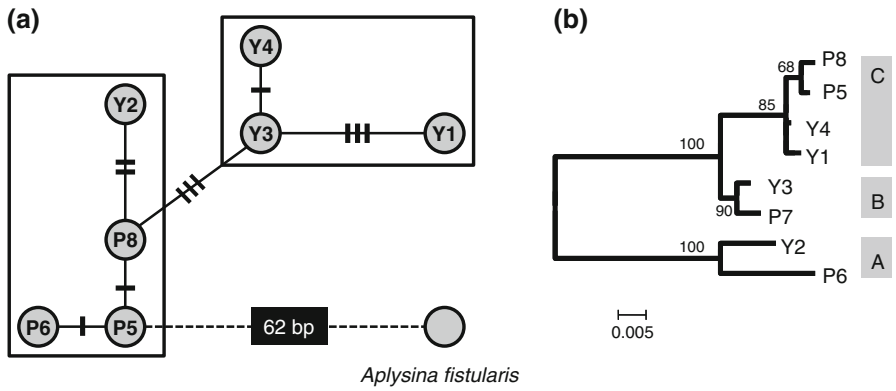

Fig. 6 **a** Median-joining network reconstruction based on partial COI gene sequences. *Circles* represent haplotypes; haplotype id is reported inside the cycles as Y (yellow morph) or P (purple morph) followed by the number of the specimen; *bars across lines* connecting haplotypes indicate base changes. **b** Model-constrained maximum likelihood phylogeny inferred from concatenated COI and ITS 2 sequences. Exhaustive searches retained one tree ($-ln = 2374.9985$); *numbers* on nodes denote ML bootstrap support for that node

A–B. Divergence values were recovered within the range reported for well-known, morphological distinct species. The lowest value of genetic divergence among clades was scored when B–C clades were compared, yet that value was still comparable to the levels of divergence encountered between *Verongula gigantea* and *Pseudoceratina arabica*. Incongruence in topology was observed between COI and ITS-2 markers and colour morph groups could not be associated with any of the aforementioned clades. Our discussion focuses on the results of the concatenated analyses, considered as the most substantial topology.

Discussion

In marine sponges, the limited number of useful morphological characters for taxonomic delineation and the wide range of phenotypic plasticity at the intra-species level are considered responsible for misidentifications (e.g. Blanquer & Uriz, 2007). In this study, metabolomic profiling and comparison of symbiont communities revealed consistent levels of chemical differentiation and disparities in bacterial assemblages between yellow and purple morphs of the sponge *I. basta*, a dominant component of marine communities at Orpheus Island, GBR. In this instance, FTICR-MS proved to be a quick and reliable tool for chemical profiling and enabled the rapid assessment of the colour morphs revealing chemotypic differences. These results, however, were not supported by the DNA sequence analysis. Mitochondrial and nuclear phylogenies indicated genetic discontinuities corresponding to cryptic lineages within this species. This hidden genetic diversity was not associated with variations of the live external sponge colour.

Dissimilarity in the chemical profiles of *I. basta* may reflect previously unrecognised genetically distinct or morphologically cryptic lineages of the host (Miller et al., 2001; Loukaci et al., 2004) or variability in symbiont populations (Bewley et al., 1996; McGovern & Hellberg, 2003). Verongid sponges are known to harbour symbionts, and the biosynthesis of many marine secondary metabolites is increasingly being identified as originating from symbiotic prokaryotes (Thomas et al., 2010). The bacterial community profile, determined from 16S rRNA clone libraries, differed slightly between the two colour morphs. The yellow morphotype, which contained the bastadin class of compounds, was dominated by two strains that were members of the *Alpha* and *Gammaproteobacteria*. The purple morphotype which contained the bastadins as well as two araplysillins was also dominated by the *Alpha* and *Gammaproteobacteria*, but the dominant strains in the purple sponge showed a slight (<1%) sequence variation to the dominant strains in the yellow sponges. In addition, the purple colour morphs contained minor representatives of the *Cyanobacteria*, *Verrucomicrobia* and *Chloroflexi*. In *I. basta*, it is

possible that these minor representatives are responsible for the production of araplysillins as these compounds are only found in the purple sponges. However, despite the existence of brominated molecules of microbial origin (Garson, 1993), there are no reports of the bastadin or araplysillin compounds being microbially derived.

The aforementioned chemical and microbial patterns were not associated with morphologically cryptic taxonomic units within *I. basta* sensu lato. In this study, unexpected yet consistent levels of genetic diversity were observed among *I. basta* individuals collected in sympatry (ca. 50 m; Fig. 7a, b); however, the recovered clades (Fig. 6b) did not fit the two colour morphs. Although chemical and microbial profiles support phenotypic differentiation in *I. basta*, they cannot be considered diagnostic characters for species delineation. The phylogenies inferred in this study clearly indicate that the taxonomic integrity and the genealogical relationships of the *I. basta* morpho-species complex require further assessment. Genetically cryptic lineages often occur in marine invertebrates assumed to be biologically distinct morpho-species and these are often associated with specific geographic patterns of distribution, morphological variation or ecophysiological and reproductive differences (Caputi et al., 2007; Sordino et al., 2008; Poore & Andreakis, 2011). Inconsistent identification of species may have severe implications in biodiversity assessments of

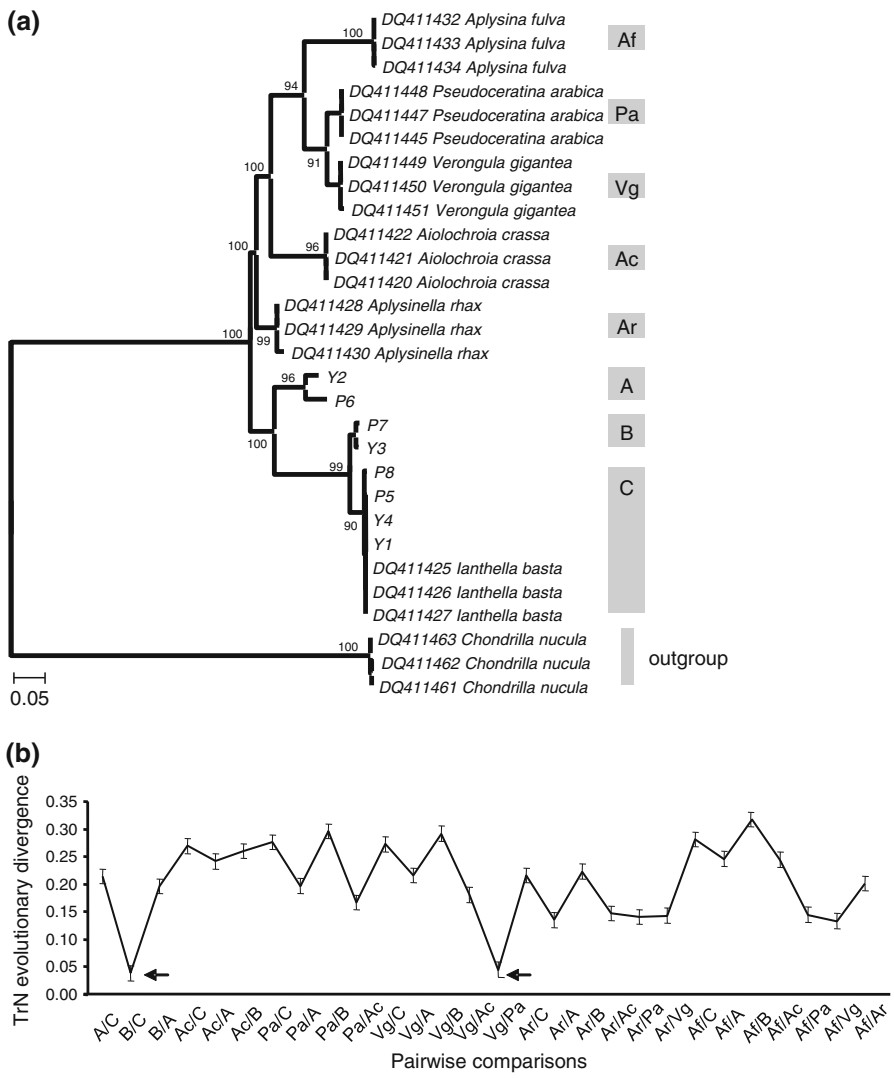

Fig. 7 **a** Model-constrained maximum likelihood phylogeny inferred from ITS-2 sequences. Heuristic searches retained one tree ($-ln = 2926.6396$); *numbers* on nodes denote ML bootstrap support for that node; *letters* in grey boxes indicate abbreviated names of *Ianthella basta* clades and other sponge species. The same abbreviations are used in **b** to indicate pairwise TrN-corrected evolutionary divergence comparisons among Verongid sponges and *I. basta* clades. *Vertical bars* indicate error margins calculated over 1,000 bootstrap replicates; *arrows* highlight similar evolutionary divergence levels recovered between clades B–C and morphologically distinct species *Pseudoceratina arabica* and *Verongula gigantea*

marine invertebrate communities ranging from inaccurate estimates of biological diversity to erroneous interpretations of ecological data (Bickford et al., 2007).

The presence of chemically and microbially distinct profiles in *I. basta* has further ecological implications as the araplysillin and bastadin classes of compounds are known to have strong biological activity. Previous studies examining the PPDK inhibitory activity of marine organisms (Doyle et al., 2005) showed that only the crude extract of the purple morphotype of *I. basta* was active; this activity was subsequently attributed to compounds **1** and **2** (Motti et al., 2009). It is also well documented that *I. basta* contains carotenoids, ingested and partially metabolised by the sponge (Hertzberg et al., 1989), and that the sponge tissue changes colour within minutes of collection from yellow and purple to a blackish purple due to pigment oxidation. This observation raises the ecological question as to why the yellow and purple morphotypes consistently produce different chemical profiles despite existing side-by-side and exposed to the same environmental conditions. Particularly relevant to this issue will be the identification and location of the cells responsible for the biosynthesis and/or storage of these compounds and the assessment of their ecological role. Rigorous comparative studies of the bioactivity of the different colour morphs, as well as of their potential metabolic costs, will be needed in order to adequately place our findings within an ecological framework. Underlying this remains the need for robust species identification essential for the accurate interpretation and assignment of the chemical and microbial patterns which may be involved in the production of secondary metabolites with interesting biological activity and possible clinical applications.

Previous studies on a range of taxa that present different colour morphs have assessed the variability of secondary metabolites over both biological and environmental ranges (Abdo et al., 2007 and references therein). Environmental variability is often associated with latitudinal, depth and/or location specific variability or gradients of light, temperature, fouling, wave action or currents. Biological variability on the other hand corresponds to genetic or symbiotic differences (Tarjuelo et al., 2004; Donia et al., 2011). As both *I. basta* colour morphotypes were collected from the same location and depth, environmental influences seem highly unlikely. A genetic influence seems equally unlikely since the cryptic lineages within *I. basta* do not fit the observed colour. It is possible that the variation in external colour is a result of the symbiont community structure. The morphotype and/or the chemotype may be controlled by the presence of very rare microbes within the *Verrucomicrobia*, *Cyanobacteria* or *Chloroflexi* or by strain-level (<1% sequence variation) differences in the dominant microbes. Another possibility is that the additional symbionts found in the purple morph are able to alter the chemical profile of the sponge, through metabolism of secondary metabolites and thereby affect the external colour. Indeed, this may explain the exception of sample P8 which was confirmed by field notes and photography to be purple in colour yet presented a chemical and microbial profile consistent with the yellow morphotype. This suggests that colour, while normally linked, may still be independent of the chemical profile and microbial community. The anomaly of sample P8 warrants further investigation as it may provide key insight into the biosynthetic origin and ecological role of the araplysillin compounds.

Conclusions

Morphological identification in sponges and other invertebrates can be problematic due to the limited number of fixed diagnostic characters available and the extent of phenotypic plasticity that may occur within a species. Studies focussing on the variation of secondary metabolites within species and communities are crucial for revealing the biological and ecological function of the secondary metabolite itself. The two dominant colour morphotypes in *I. basta* are characterised by distinct chemical and microbial profiles with as yet, unresolved genealogical relationships inferred at the molecular level. Despite a strong association between external living colour, secondary metabolite chemistry and microbial community, the details of this correlation remain unclear. This study has implications for the future supply and development of the bastadin class of compounds as pharmaceutical tools and for the management of *I. basta* as an exploitable resource. In addition, these results highlight the need for a multidisciplinary

approach in species whose morphology is fundamentally dynamic, in order to properly interpret morpho-species boundaries and ecological data. Future research will investigate the possible role of microbes in the putative production of both the bastadin and araplysillin classes of compounds in *I. basta*.

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References

Abdo, D. A., C. A. Motti, C. N. Battershill & E. S. Harvey, 2007. Temperature and spatiotemporal variability of salicylihalamide A in the sponge *Haliclona* sp. Journal of Chemical Ecology 33: 1635–1645.

Ackers, R. G. & D. Moss, 1987. Current problems with field recognition and species differentiation in shallow water British sponges. In Earll, R. & S. Gubbay (eds), Marine Recordings, Vol. 2. Marine Conservation Society, Herefordshire: 22–27.

Ackers, R. G., D. Moss & B. E. Picton, 1992. Sponges of the British Isles (Sponge V). Marine Conservation Society, Herefordshire. 175.

Aharoni, A., C. H. Ric de Vos, H. A. Verhoeven, C. A. Maliepaard, G. Kruppa, R. Bino & D. B. Goodenowe, 2002. Nontargeted metabolome analysis by use of Fourier transform ion cyclotron mass spectrometry. Omics 6: 217–234.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller & D. J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389–3402.

Bandelt, H. J., P. Forster & A. Röhl, 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16: 37–48.

Barkauskas, D., 2009. FTICRMS: programs for analyzing Fourier transform-ion cyclotron resonance mass spectrometry data. R package version 0.8. http://CRAN.R-project.org/package=FTICRMS.

Bell, J. J. & D. K. A. Barnes, 2001. Sponge morphological diversity: a qualitative predictor of species diversity? Aquatic Conservation: Marine and Freshwater Ecosystems 11: 109–121.

Bergquist, P. R. & R. Wells, 1983. Chemotaxonomy of the Porifera: the development and current status of the field. In Scheuer, P. (ed.), Chemical and Biological Perspectives, Vol. 5. Academic Press, New York: 1–50.

Bewley, C. A., N. D. Holland & D. J. Faulkner, 1996. Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. Experientia 52: 716–722.

Bickford, D., D. J. Lohman, N. S. Sodhi, P. K. L. Ng, R. Meier, K. Winker, K. K. Ingram & I. Das, 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology & Evolution 22: 148–155.

Blanquer, A. & M. J. Uriz, 2007. Cryptic speciation in marine sponges evidenced by mitochondrial and nuclear genes: a phylogenetic approach. Molecular Phylogenetics and Evolution 45: 392–397.

Bond, C. & A. K. Harris, 1988. Locomotion of sponges and its physical mechanism. Journal of Experimental Zoology 246: 271–284.

Calcul, L., W. D. Inman, A. A. Morris, K. Tenney, J. Ratnam, J. H. McKerrow, F. A. Valeriote & P. Crews, 2010. Additional insights on the Bastadins: isolation of analogues from the sponge *Ianthella* cf. *reticulata* and exploration of the oxime configurations. Journal of Natural Products 73: 365–372.

Caputi, L., N. Andreakis, P. Cirino, F. Mastrototaro & P. Sordino, 2007. Cryptic speciation in a model invertebrate chordate. Proceedings of the National Academy of Science of the USA 104: 9364–9369.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu & G. L. Andersen, 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 172: 5069–5072.

Donia, M. S., W. F. Fricke, J. Ravel & E. W. Schmidt, 2011. Variation in tropical reef symbiont metagenomes defined by secondary metabolism. PLoS One. 6:e17897.

Doyle, J. R., J. N. Burnell, D. S. Haines, L. E. Llewellyn, C. A. Motti & D. M. Tapiolas, 2005. A rapid screening method to detect specific inhibitors of pyruvate, orthophosphate dikinase as leads for C_4 plant-selective herbicides. Journal of Biomolecular Screening 10: 67–75.

Erwin, P. M. & R. W. Thacker, 2007. Phylogenetic analyses of marine sponges within the order Verongida: a comparison of morphological and molecular data. Invertebrate Biology 126: 220–234.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Felsenstein, J., 1993. PHYLIP (Phylogenetic Inference Package) Version 3.5c. Department of Genetics, University of Washington, Seattle, WA.

Ferris, M. J., G. Muyzer & D. M. Ward, 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Applied and Environmental Microbiology 62: 340–346.

Folmer, O., M. Black, W. Hoeh, R. Lutz & R. Vrijenhoek, 1994. DNA primers for amplification of mitochondrial cytochrome C oxidases subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–297.

Gaino, E., R. Manconi & R. Pronzato, 1995. Organisational plasticity as a successful conservative tactics in sponges. Animal Biology 4: 31–43.

Garson, M. J., 1993. The biosynthesis of marine natural products. Chemical Reviews 9: 1699–1733.

Hall, T. A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium 41: 95–98.

Hertzberg, S., P. Bergquist & S. Liaaen-Jensen, 1989. Further occurrence of sulphated carotenoids in *Ianthella* species (Demospongia). Biochemical Systematics and Ecology 17: 51–53.

Hillis, D. M. & J. P. Huelsenbeck, 1992. Signal, noise and reliability in molecular phylogenetic analysis. Journal of Heredity 83: 189–195.

Huber, T., G. Faulkner & P. Hugenholtz, 2004. Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20: 2317–2319.

Lane, D. J., 1991. 16S rRNA sequencing. In Stackerbrandt, E. & M. Goodfellow (eds), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, Inc, New York: 115–148.

Lee, W. L. & B. M. Gilchrist, 1985. Carotenoid patterns in twenty-nine species of sponges in the order Poecilosclerida (Porifera: Demospongiae): a possible tool for chemosystematics. Marine Biology 86: 21–35.

Loukaci, A., G. Muricy, J.-P. Brouard, M. Guyot, J. Vacelet & N. Boury-Esnault, 2004. Chemical divergence between two sibling species of Oscarella (Porifera) from the Mediterranean Sea. Biochemical Systematics and Ecology 32: 93–899.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Y. Kumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode & K. H. Schleifer, 2004. ARB: a software environment for sequence data. Nucleic Acids Research 32: 1363–1371.

Luter, H. M., S. Whalan & N. S. Webster, 2010. Exploring the role of microorganisms in the disease-like syndrome affecting the sponge *Ianthella basta*. Applied and Environmental Microbiology 76: 5736–5744.

Marchesi, J. R., T. Sata, A. J. Weightman, T. A. Martine, J. C. Fry, S. J. Hiom & W. G. Wade, 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology 64: 795–799.

Mcgovern, T. M. & M. E. Hellberg, 2003. Cryptic species, cryptic endosymbionts, and geographical variation in chemical defences in the bryozoans *Bugula neritina*. Molecular Ecology 12: 1207–1215.

Miller, K., B. Alvarez, C. Battershill, P. Northcote & H. Parthasarathy, 2001. Genetic, morphological, and chemical divergence in the sponge genus *Latrunculia* (Porifera: Demospongiae) from New Zealand. Marine Biology 139: 235–250.

Motti, C. A., M. L. Freckelton, D. M. Tapiolas & R. H. Willis, 2009. FTICR-MS and LC-UV/MS-SPE-NMR Applications for the rapid dereplication of a crude extract from the sponge *Ianthella flabelliformis*. Journal of Natural Products 72: 290–294.

Muyzer, G., E. C. de Waal & A. G. Uitterlinden, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59: 695–700.

Poore, C. B. G. & N. Andreakis, 2011. Morphological, molecular and biogeographic evidence support two new species in the *Uroptychus naso* complex (Crustacea: Decapoda: Chirostylidae). Molecular Phylogenetics and Evolution 60: 152–169.

Posada, D. & K. A. Crandall, 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Sordino, P., N. Andreakis & E. R. Brown, 2008. Natural variation of model mutant phenotypes in *Ciona intestinalis*. PLoS One 3: e2344.

Swofford, D. L., 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland.

Tamura, K., J. Dudley, M. Nei & S. Kumar, 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

Tarjuelo, I., D. Posada, K. A. Crandall, M. Pascual & X. Turon, 2004. Phylogeography and speciation of colour morphs in the colonial ascidian *Pseudodistoma crucigaster*. Molecular Ecology 13: 3125–3136.

Taylor, M. W., R. Radax, D. Steger & M. Wagner, 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiology and Molecular Biology Reviews 71: 295–347.

Thacker, R. W. & S. Starnes, 2003. Host specificity of the symbiotic cyanobacterium *Oscillatoria spongeliae* in marine sponges, *Dysidea* spp. Marine Biology 142: 643–648.

Thomas, T. R. A., D. P. Kavlekar & P. A. LokaBharathi, 2010. Marine drugs from sponge-microbe association – a review. Marine Drugs 8: 1417–1468.

Thompson, J. E., P. T. Murphy, P. R. Bergquist & E. A. Evans, 1987. Environmentally induced variation in diterpene composition of the marine sponge *Rhopaloeides odorabile*. Biochemical Systematics and Ecology 15: 595–606.

Want, E. J., A. Nordstro, H. Morita & G. Siuzdak, 2007. From exogenous to endogenous: the inevitable imprint of mass spectrometry in metabolomics. Journal of Proteome Research 6: 459–468.

Webster, N. S. & M. W. Taylor, 2011. Marine sponges and their microbial symbionts: love and other relationships. Environmental Microbiology (Online Early). doi: 10.1111/j.1462-2920.2011.02460.x.

Webster, N. S., M. W. Taylor, F. Behnam, S. Lücker, T. Rattel, S. Whalan, M. Horn & M. Wagner, 2010. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environmental Microbiology 12: 2070–2082.

Wilson, K., L. Yutao, V. Whan, S. Lenhnert, K. Byrne, S. Moore, S. Pongsomboon, A. Tassanakajon, G. Rosenberg, E. Ballment, Z. Fayazi, J. Swan, M. Kenway & J. Benzie, 2002. Genetic mapping of the black tiger shrimp *Panaeus monodon* with amplified fragment length polymorphism. Aquaculture 204: 297–309.

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Phenotypic variability in the Caribbean Orange Icing sponge *Mycale laevis* (Demospongiae: Poecilosclerida)

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Abstract Sponge species may present several morphotypes, but sponges that are morphologically similar can be separate species. We investigated morphological variation in *Mycale laevis*, a common Caribbean reef sponge. Four morphotypes of *M. laevis* have been observed (1) orange, semi-cryptic, (2) orange, massive, (3) white, semi-cryptic, and (4) white, massive. Samples of *M. laevis* were collected from Key Largo, Florida, the Bahamas Islands, and Bocas del Toro, Panama. Fragments of the 18S and 28S rRNA ribosomal genes were sequenced and subjected to phylogentic analyses together with sequences obtained for 11 other *Mycale* species and additional sequences retrieved from GenBank. Phylogenetic analyses confirmed that the genus *Mycale* is monophyletic within the Order Poecilosclerida, although the subgenus *Aegogropila* is polyphyletic and the subgenus *Mycale* is paraphyletic. All 4 morphotypes formed a monophyletic group within *Mycale*, and no genetic differences were observed among them. Spicule lengths did not differ among the 4 morphotypes, but the dominant megasclere in samples collected from Florida and the Bahamas was the strongyle, while those from Panama had subtylostyles. Our data suggest that the 4 morphotypes constitute a single species, but further studies would be necessary to determine whether skeletal variability is due to phentotypic or genotypic plasticity.

Keywords Porifera · 18S rRNA · 28S rRNA · Ribosomal DNA · Caribbean · Morphotype · Genotype

Introduction

Ecological studies conducted in areas of high biodiversity may sacrifice precision in lower-level taxon sampling in order to gain a broader understanding of large-scale ecosystem processes. In community surveys, marine invertebrates are usually not completely identified to the species-level due to difficulties in field identification or lack of resolution in available taxonomic keys (e.g., Chou et al., 2004; Micheli et al., 2005; Chapman et al., 2010). Sponges (Phylum Porifera) are a good example of a taxon sampled at low resolution in many community surveys. In the widely used benthic survey methodologies employed

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by the Global Coral Reef Monitoring Network (GCRMN, http://www.gcrmn.org) and Reef Check (http://www.reefcheck.org), coral reef sponges are classified into a single group (Wilkinson, 2008). However, this single phylum is comprised of ~7000 species (Hooper & Van Soest, 2002). Furthermore, sponges are important members of the aquatic ecosystems they inhabit, both in terms of abundance and ecosystem function, they compete for space with other benthic organisms, they are dominant suspension feeders, and they provide habitat for a large and diverse number of other invertebrates (Corredor et al., 1988; Pile et al., 1996; Diaz & Rutzler, 2001; Henkel & Pawlik, 2005; Southwell et al., 2008). Given their ecological importance, why are sponges seldom identified to the level of species in the field?

The process of identifying sponge species can be difficult. Sponge identification is based on morphological attributes such as color, size and shape, the presence, and arrangement of the spiculate and fibrous skeleton, and most particularly, spicule type and size. This presents a challenge as sponges are morphologically plastic, often have different color morphs, and can change in shape and size due to environmental conditions (Palumbi, 1986) or biotic factors such as predation (Loh & Pawlik, 2009). Spicule morphology, by far the most important character state, can also vary among different habitats and growing conditions (Hooper, 1985; McDonald et al., 2002). Furthermore, sponges can incorporate abiotic materials from the environment or spicules from other sponges into their skeletons (Sollas, 1908; Teragawa, 1986; Hooper & Van Soest, 2002). Conversely, sponges that look almost identical in the field may be separate species (Klautau et al., 1999; Miller et al., 2001; Duran & Rützler, 2006; Wulff, 2006a; Blanquer & Uriz, 2007; Blanquer et al., 2008).

The Orange Icing Sponge *Mycale laevis* (Carter 1882, Order Poecilosclerida, subgenus *Mycale*) ranks as one of the 10 most common sponges on Caribbean coral reefs (Pawlik et al., 1995), and has been reported as a common associate of scleractinian corals (Goreau & Hartman, 1966; Hill, 1998). As is often observed for other sponge species, *M. laevis* has more than one growth form. It has been described as both semi-cryptic, a thinly encrusting form with most of the biomass growing under coral or other hard substrata (Fig. 1a; Wulff, 1997), and massive, a fleshy, apparent form that grows on the upper surface of substrata (Fig. 1b; Randall & Hartman, 1968; Wulff, 2006b). On reefs off Bocas del Toro, Panama and the Bahamas Islands, a white morph of *M. laevis* has also been described (Fig. 1c, d; Collin et al., 2005). This white morphotype is more common than the orange on some reefs at Bocas del Toro, exhibits both encrusting and fleshy forms (observed at the Bahamas Islands and Panama, respectively), and frequently grows in association with corals in the same manner as the orange morphotype (Loh personal observation). The white morphotype is superficially similar to the orange, having a compressible texture, a rough external surface, and osculae ringed by thin membranous collars with vertical white lines (Collin et al., 2005).

In this study, we investigated genetic differences among 4 morphotypes of *M. laevis* using partial 18S and 28S rRNA ribosomal gene sequences. Samples

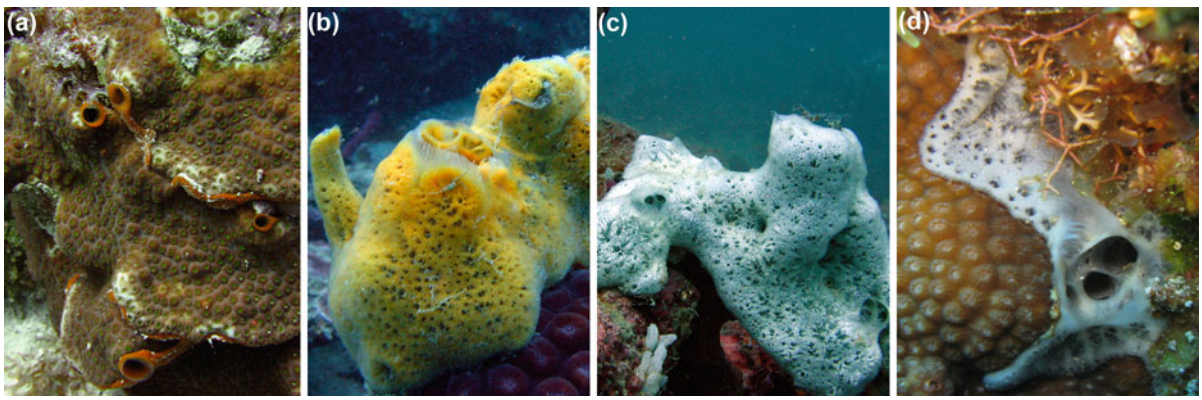

Fig. 1 The four morphotypes of *Mycale laevis*: **a** orange, semi-cryptic, **b** orange, massive, **c** white, massive, **d** white semi-cryptic

were collected from the coral reefs off Key Largo, Florida, Bocas del Toro, Panama, and the Bahamas Islands. In addition, we sequenced 11 species of *Mycale*, and retrieved 5 other sequences from Genbank to perform phylogenetic analyses and determine the taxonomic status of the 4 morphotypes of *M. laevis*. We also examined spicule morphology and diversity and calculated spicule dimensions to compare among the 4 morphotypes of *M. laevis*.

Materials and methods

Sample collection

When possible, three samples of each morphotype of *M. laevis* were collected from each of the study sites where they occurred (Table 1). The orange semi-cryptic morphotype was present at all the sites, while the orange massive morphotype was found only at Bocas del Toro. The white semi-cryptic morphotype was collected at Tuna Alley reef, Bahamas (2 samples), while the white massive morphotype was found only at Bocas del Toro. For our 3 sampling locations, the massive morphotypes (both orange and white), were only found at Bocas del Toro.

One to three samples of the following 11 *Mycale* species were collected and added to the analysis of samples of *M. laevis* to enhance phylogenetic tree resolution: *M. lingua* (Bowerbank 1866, collected in Norway by P. Cardenas) and *M. grandis* (Gray 1867, Singapore, S. C. Lim) from the subgenus *Mycale*; *M. sulevoidea* (Sollas 1902, Singapore, S. C. Lim), *M. adhaerens* (Lambe 1893, Hong Kong, Y. H. Wong and P. Y. Qian), and *M. carmigropila* (Hajdu & Rutzler, 1998, Panama, T. L. Loh) from the subgenus *Aegogropila*; *M. parishi* (Bowerbank 1875, Singapore, S. C. Lim) from the subgenus *Zygomycale*; *M. microsigmatosa* (Arndt 1927, Panama, T. L. Loh) and *M. fistulifera* (Row 1911, Israel, M. Ilan) from the subgenus *Carmia*; *M. laxissima* (Duchassaing & Michelotti 1864, Florida Keys, T. L. Loh) from the subgenus *Arenochalina*, and two unidentified species-*Mycale* cf. *lingua* from Norway and *Mycale* sp. J57 from Italy, both from the subgenus *Aegogropila* (subgenus identified by and collected by P. Cardenas). As an additional genus within the Poecilosclerida, *Desmapsamma anchorata* was collected by W. Leong from Key Largo, Florida. Samples were collected between 2007 and 2010 and immediately preserved in 95–100% ethanol, frozen at −20°C, or freeze-dried. All samples were then stored at −20°C until they were extracted.

Caribbean species were identified by comparing morphological and spicule characters with Hajdu & Rutzler (1998) and with the sponge voucher collection at the Bocas Research Station of the Smithsonian Tropical Research Institute (specimens submitted by C. Diaz and R. Thacker). Other *Mycale* species were identified by their respective collectors.

DNA extraction and sequencing

DNA was extracted using the Puregene kit (Gentra Systems). The most commonly genetic markers used to investigate taxonomic and phylogenetic issues in sponges are the 18S and 28s rRNA genes, and the mitochondrial gene cytochrome oxidase I (COI). Here, we designed the primer set 18sMycale01F 5′-ATAACTGCTCGAACCGTATGGCCT-3′ and 18SMycale01R 5′-AAACGCTAACATCCACCGATCCCT-3′ based on an 18S rRNA sequence of *M. fibrexilis* available from GenBank (AF100946) to amplify a fragment of 786 bp from the 18S rRNA gene. However, no amplification could be obtained for the species *M. lingua*, *M. parishi*, *Mycale* cf. *lingua*, *Mycale* sp. J57, 1 sample of the orange massive morphotype, and all white morphotypes of *M. laevis*, with the 18S rRNA primers described above. Amplification was finally obtained with the primer set 18sMycale02F 5′-CAACGGGTGACGGAGAATTA-3′ and 18sMycale02R 5′-TTTCAGCCTTGCGACCATACTC-3′, which was designed based on the consensus sequence of the poecilosclerid library available at GenBank. Amplification of a fragment from the 28S rRNA gene was performed using the forward primer 28sCallyF 5′-TGCGACCCGAAAGATGGTGAACTA-3′ and reverse primer 28sCallyR 5′-ACCAACACCTTTCCTGGTATCTGC-3′ (López-Legentil et al., 2010). No consistent amplification could be obtained for a fragment of the mitochondrial gene COI, although we used several universal primers and designed new ones based on poecilosclerid sequences retrieved from GenBank.

All amplifications were performed in a 25 µl total-reaction volume with: 1.25 µl of each primer (10 µmol), 12.5 µl GoTaq Colorless or Green Master Mix (Promega), and 0.5 µl DNA. A single soak at 94°C

Table 1 List of (a) 18S rRNA and (b) 28S rRNA sequences obtained from *M. laevis*, with replicate number for each morphotype-location, collection locations and sites, haplotype code, haplotype frequency, and GenBank accession numbers (Acc. No)

Morphotype	Replicate no.	Collection location	Collection site	Haplotype code	Haplotype frequency	Acc. No.
(a)						
Orange, semi-cryptic	1	Key Largo, Florida	Dixie Shoals	A03	0.0667	GU208832
Orange, semi-cryptic	2	Key Largo	Conch Wall	A15	0.6	HQ709340
	3		North Dry Rocks			HQ709341
Orange, semi-cryptic	2	Bahamas	Sweetings Cay			HQ709343
	3					HQ709344
Orange, semi-cryptic	2	Bocas del Toro, Panama	Adriana's Reef			HQ709346
	3					HQ709347
White, semi-cryptic	2	Bahamas	Tuna Alley			HQ709352
White, massive	2	Bocas del Toro	Old Point			HQ709350
Orange, massive	1		Punta Caracol			GU208833
Orange, semi-cryptic	1	Bahamas	Sweetings Cay	A06	0.0667	HQ709342
Orange, semi-cryptic	1	Bocas del Toro	Adriana's Reef	A09	0.0667	HQ709345
White, massive	1	Bocas del Toro	Hospital Point	A13	0.0667	HQ709349
Orange, massive	2	Bocas del Toro	Hospital Point	A16	0.0667	HQ709348
White, semi-cryptic	1	Bahamas	Tuna Alley	A26	0.0667	HQ709351
(b)						
White, massive	1	Bocas del Toro, Panama	Hospital Point	C01	0.882	HQ709333
	2		Old Point			HQ709335
	3					HQ709334
White, semi-cryptic	1	Bahamas	Tuna Alley			HQ709336
	2					HQ709337
Orange, semi-cryptic	2	Key Largo, Florida	Conch Wall			HQ709325
	3		North Dry Rocks			HQ709326
Orange, semi-cryptic	1	Bocas del Toro	Adriana's Reef			HQ709327
	2					HQ709328
	3					HQ709329
Orange, semi-cryptic	1	Bahamas	Sweetings Cay			HQ709330
	2					HQ709331
	3					HQ709332
Orange, massive	1	Bocas del Toro	Punta Caracol			GU324493
	3		Juan Point			HQ709339
Orange, semi-cryptic	1	Key Largo	Dixie Shoals	C06	0.0588	GU324492
Orange, massive	2	Bocas del Toro	Old Point	C16	0.0588	HQ709338

The replicate number refers to the replicate of each morphotype found at each location, and matches the labels in Fig. 2

for 5 min was followed by 40 amplification cycles (denaturation at 95°C for 30 s; annealing at 45°C for 28sCally and 18SMycale01 primer sets, and 50°C for 18SMycale02 primers, for 30 s; and extension at 68°C for 2 min), and a final extension at 72°C for 5 min for 28sCally and 18SMycale01 primer sets, and 10 min for 18SMycale02 primers in a Peltier PTC-200 gradient PCR.

PCR products were run in a 1% agarose gel to check for amplification results. The BigDye TM terminator v. 3.1 was used to carry out sequencing reactions with the same primers used in the

amplification step. Sequences were obtained in an ABI Prism 3100 automated sequencer. Nucleotide diversity for each gene fragment was estimated with DnaSP v. 4 (Rozas et al., 2003), and haplotype frequencies with Arlequin v. 2000 (Schneider et al., 2000).

Phylogenetic analyses

Sequences were aligned using BioEdit Sequence Alignment Editor v. 7.0.9 (Hall, 1999) and ClustalW (Larkin et al., 2007). Partial 18S and 28S rRNA sequences for *Mycale fibrexilis* (Wilson 1894; 18S: AF100946, 28S: AY026376) and two poecilosclerid species- *Iotrochota birotulata* (Higgin 1877; 18S: EU702421, 28S: AY561884) and *Tedania ignis* (Duchassaing & Michelotti 1864; 18S: AY737642, 28S: AY561878) were obtained from GenBank, as well as the 18S sequence for *Mycale* sp. 16 (AY737643). 18S and 28S rRNA sequences from the haplosclerid *Callyspongia plicifera* (Lamarck 1814; 18S: EU702412, 28S: AF441343) were used as outgroup sequences. The program jMODELTEST 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) was used to select the best model of DNA substitution. The transitional model with unequal base frequencies (Posada, 2003) was selected for the 18S rRNA region, with substitution rates varying among sites according to a invariant and gamma distribution (TIM1+I+G), while the variable-frequency Tamura-Nei evolution model (Tamura & Nei, 1993) was selected for the 28S rRNA region, with substitution rates varying among sites according to a gamma distribution (TrN+G).

Neighbor-joining (NJ) and maximum parsimony (MP) analyses were conducted using MEGA 4 (Tamura et al., 2007). Neighbor-joining analysis was conducted using the Maximum Composite Likelihood model, and data were re-sampled using 5,000 bootstrap replicates. For the MP analysis, the search method used was the close neighbor interchange with random addition trees at a replication level of 10, and data were re-sampled using 5,000 bootstrap replicates. A maximum likelihood analysis was performed in PAUP 4.0b10 (Swofford, 1998) for both 18S rRNA and for 28S rRNA sequences using the evolution models determined from jMODELTEST. Data were re-sampled using 100 bootstrap replicates. MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) was used to calculate the Bayesian posterior probabilities of branch nodes. The Monte Carlo Markov Chain length was initially set to 1 million generations with sampling every 100th generation and with a burn-in value of 2,500. The average standard deviation of split frequencies between two independent chains reached a value of less than 0.01 after 2,178,000 and 1,655,000 generations for 18S rRNA and 28S rRNA sequences, respectively.

Spicule morphology

For each morphotype of *M. laevis* present at each geographic location, spicules were analyzed from 2–3 individuals. From each sampled individual, a small piece of tissue that included both the ectosome and choanosome was immersed in a 50% solution of chlorine bleach (2.5% sodium hypochlorite in water) and left to oxidize overnight. The spicule mass was then rinsed twice with deionized water and stored in 100% ethanol. Spicules were mounted on a slide and viewed using a compound light microscope under 200–400× magnification. For each sponge sample, the lengths of up to twenty spicules for each spicule type observed were measured using a calibrated ocular micrometer. The lengths of the head and foot portions of the anisochelae were measured as well. Statistical analyses comparing the spicule lengths of the 4 morphotypes of *M. laevis* using one-way ANOVA nested by location-morphotype, and the subsequent Tukey's post-hoc analysis were conducted with JMP 7.0 (SAS Institute Inc.).

Results

Genetic data and phylogenetic analysis

Amplification using 18S and 28S rRNA primers resulted in consensus sequences of 786 and 439 bp, respectively. All sequences were deposited in GenBank (accession numbers are listed in Table 1). Partial 18S rRNA gene sequences obtained for all *Mycale* species sequenced in this study revealed 17 unique haplotypes, and an overall nucleotide diversity of 0.0126. The 15 samples of *M. laevis* had 7 haplotypes for 18S rRNA, with a nucleotide diversity

of 0.0054. The most common haplotype (A15) of *M. laevis* was recovered from 9 samples (relative frequency = 0.6), 2 orange semi-cryptic morphotypes from each of the 3 sampling locations, 1 for the orange massive morphotype from Bocas, 1 for the white massive morphotype from Bocas, and 1 for the white semi-cryptic morphotype from the Bahamas. The other 6 haplotypes of *M. laevis* were each represented by 1 sample only for the orange semi-cryptic and massive, and white massive morphotypes sampled from different locations (Table 1a). Partial 28S rRNA gene sequences revealed a total of 13 haplotypes, and an overall nucleotide diversity of 0.014 among all *Mycale* species analyzed. The 17 samples of *M. laevis* were represented by 3 haplotypes and a nucleotide diversity of 0.0045. The most common haplotype (C01) was obtained for 15 samples of *M. laevis* (relative frequency = 0.882), corresponding to all morphotypes analyzed, and from all sampled locations. The other 2 haplotypes obtained for *M. laevis* belonged to the orange semi-cryptic morphotype from Key Largo and the orange massive morphotype from Bocas del Toro (Table 1b). Haplotypes of *M. laevis* for both 18S and 28S rRNA did not appear to be grouped by geographic location or morphotype.

Regardless of geographic location or morphotype, the 4 phylogenetic analyses performed with 18S rRNA (Fig. 2a) and 28S rRNA (Fig. 2b) indicated that all individuals of *M. laevis* formed a monophyletic clade within the genus *Mycale* with bootstrap support values >50 for all analyses. Correspondingly, all the *Mycale* species analyzed except one formed a well-supported clade (Fig. 2). The sole exception was a sequence retrieved from GenBank for *M. fibrexilis*, which grouped most closely with the poecilosclerid *D. anchorata* for 18S rRNA and *T. ignis* for 28S rRNA.

Members of the subgenus *Aegogropila* represented in this study did not group in the same clade, but were interspersed with members from other *Mycale* subgenera, including *Mycale*, *Arenochalina*, *Zygomycale*, and *Carmia* (Fig. 2). *Mycale carmigropila* grouped most closely with *M. laxissima* from the subgenus *Arenochalina*. The subgenus *Mycale* appeared to be paraphyletic, with some species of the subgenus grouping with members from the subgenera *Arenochalina* and *Aegogropila* (Fig. 2).

Spicule morphology

All 4 morphotypes of *M. laevis* had the palmate anisochelae typical of the genus *Mycale*. Individuals either had only large anisochelae, or both large and small anisochelae, and the dominant megasclere for all samples was either the subtylostyle or the strongyle, depending on collection location (Fig. 3). Megascleres for *M. laevis* from Key Largo and the Bahamas were strongyles, while megascleres for individuals from Bocas del Toro were subtylostyles, regardless of morphotype. Other common microscleres found included raphides and sigmas (Table 2). Across all samples, the smaller anisochelae II (22.59 ± 0.67 μm, $n = 163$) were rarer, only observed in 11 of 17 samples, while the larger anisochelae I were found in all samples of *M. laevis* (Table 2), with a mean length of 80.27 ± 0.45 μm ($n = 295$). Mean lengths of subtylostyles and strongyles were 517.32 ± 2.76 μm ($n = 180$) and 456.62 ± 3.76 μm ($n = 160$), respectively. For the anisochelae I, significant differences in spicule length were observed between samples from different locations and morphotype ($P < 0.0001$), but the differences were not grouped by morphotype. The anisochelae I of all morphotypes of *M. laevis* from Bocas del Toro were significantly larger than those from the orange semi-cryptic morphotypes of *M. laevis* from Key Largo and the Bahamas, but no differences were found among the 3 morphotypes at Bocas del Toro. Anisochelae I from the Bahamian white morphotypes were intermediate in length between those from morphotypes from Key Largo and Bocas del Toro. No significant differences were found between strongyle lengths from Key Largo and Bahamas samples ($P = 0.4429$), and subtylostyle lengths among the 3 morphotypes found at Bocas del Toro ($P = 0.0766$).

Discussion

From the analyses of the 18S and 28S rRNA gene fragments, the morphological differences observed among the 4 morphotypes of *M. laevis* lack a genetic basis. In addition, no differences in spicule morphology were observed among the 4 morphotypes within a location. However, there were differences in spicule type between sampling locations. Populations of

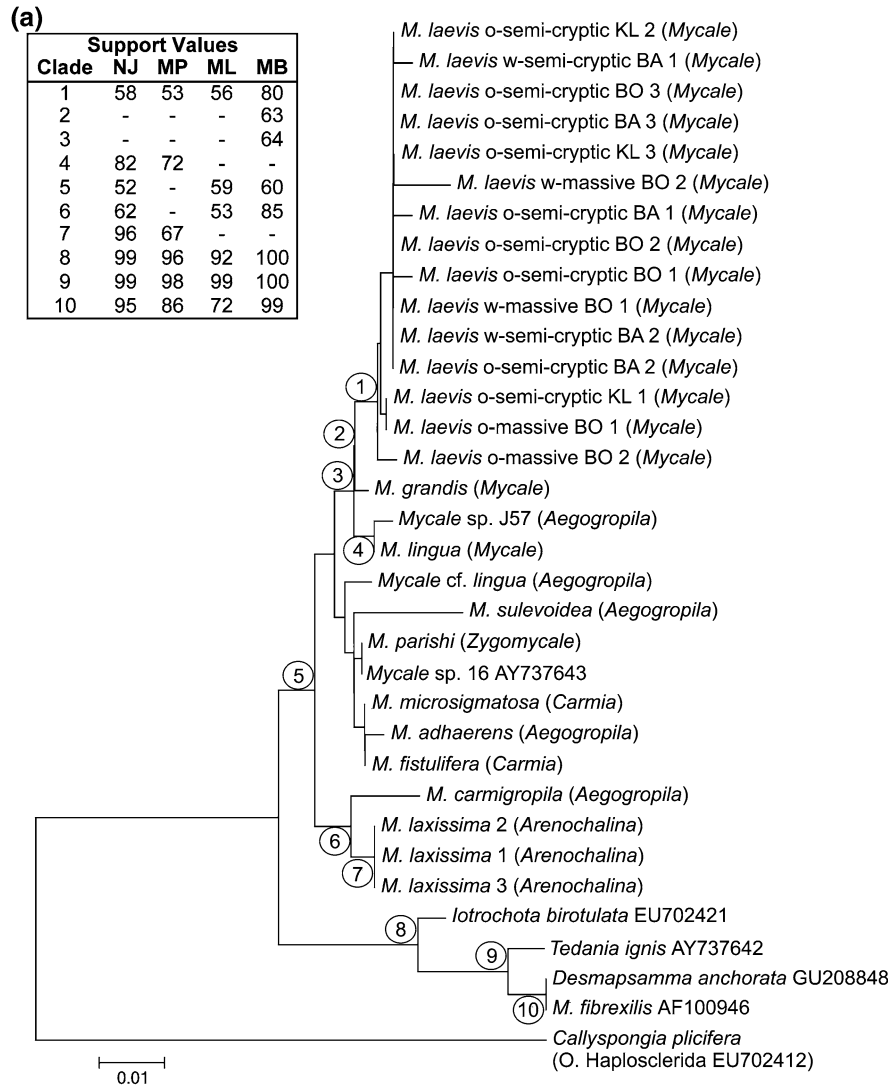

Fig. 2 Phylogenetic tree of fragments of **a** 18S and **b** 28S rRNA gene sequences of sponges from the order Poecilosclerida denoting the phylogenetic position of morphotypes of *M. laevis*. *Callyspongia plicifera* from the Order Haplosclerida was used as outgroup. Italicized labels in parentheses denote the subgenus of the *Mycale* species. Labels for *M. laevis* (o/w-semi-cryptic, o/w-massive) denote the morphotype of the sample analyzed with o: orange, w: white. Collection site is indicated by *KL* Key Largo, Florida; *BA* Bahamas; *BO* Bocas del Toro, Panama. The GenBank accession numbers for the 18S and 28S fragments, respectively, are provided for reference sequences. Tree topology was obtained from neighbor-joining (NJ) analysis. Well-supported clades are labeled, with the corresponding individual bootstrap values from NJ, maximum parsimony (MP), maximum likelihood (ML) analyses and the posterior probabilities from the MrBayes analysis (MB) listed in the *table inset*. The *scale bar* represents 0.01 and 0.02 substitutions for 18S and 28S, respectively

M. laevis in Key Largo and the Bahamas had strongyles as their dominant megasclere, while those from Bocas del Toro, Panama had substylostyles. Variation in the megascleres of *M. laevis* has been reported previously in the literature. Subtylostyles were the only megasclere observed in *M. laevis* from Belize (Hajdu & Rutzler, 1998), while styles and strongylostyles were reported for specimens from Curaçao and Puerto Rico (Van Soest, 1984). Although spicules, especially microscleres, can be present or absent in sponge individuals depending on the collection location (Zea, 1987), it is rare for

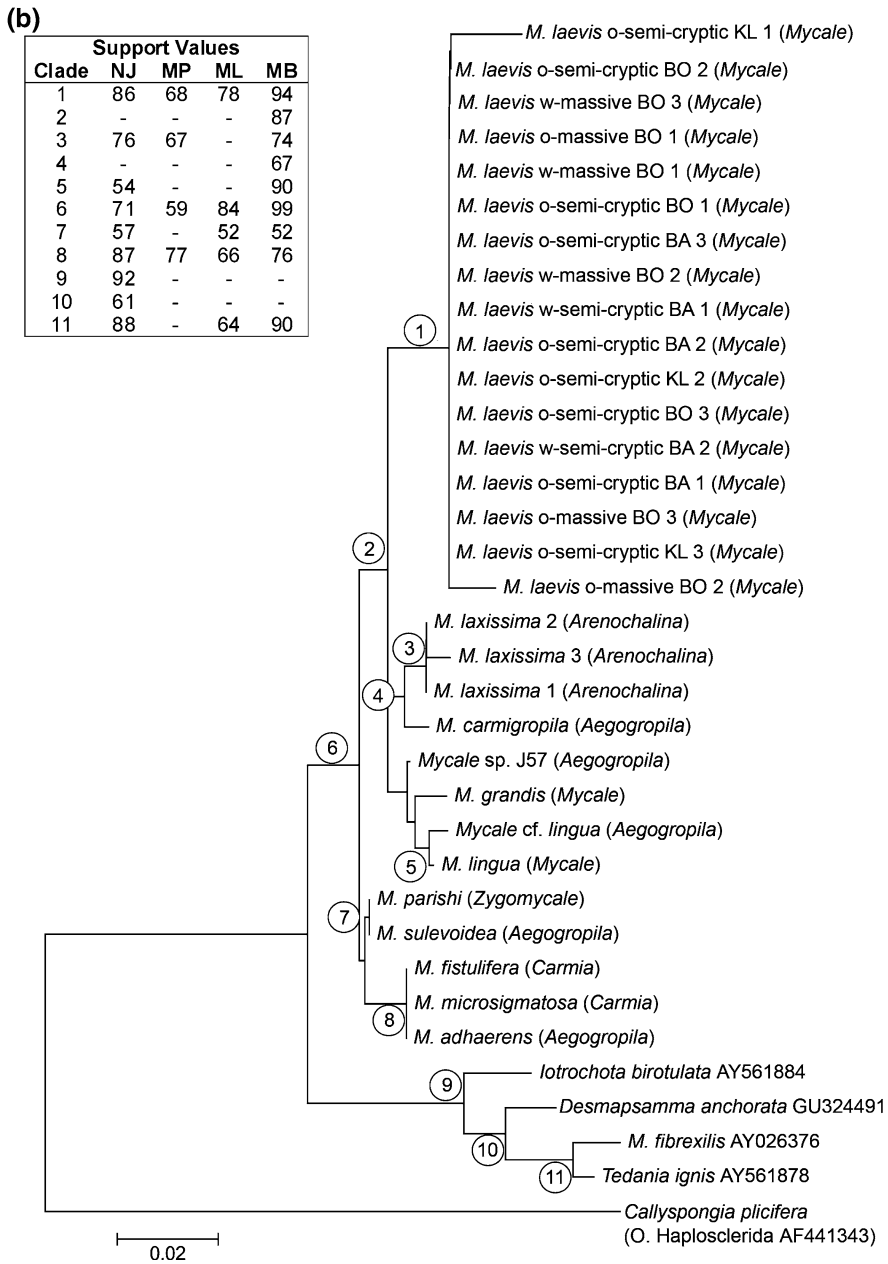

Fig. 2 continued

megasclere types to vary. Hartman (1967) argued for the designation of subspecies of *Neofibularia nolitangere* based on geographical variation in megascleres. Variation in spicule type and shape can be explained by silicon limitation (Maldonado et al., 1999), but seawater chemistry is unlikely to be the determining factor for the different spicule types found in *M. laevis*, as samples were all collected from shallow Caribbean coral reefs. Alternatively, for this species, megasclere type may not be a stable or valid taxonomic character. Further research, including samples from more specimens and populations, is needed to investigate these apparent geographic differences in megascleres among samples of *M. laevis.*

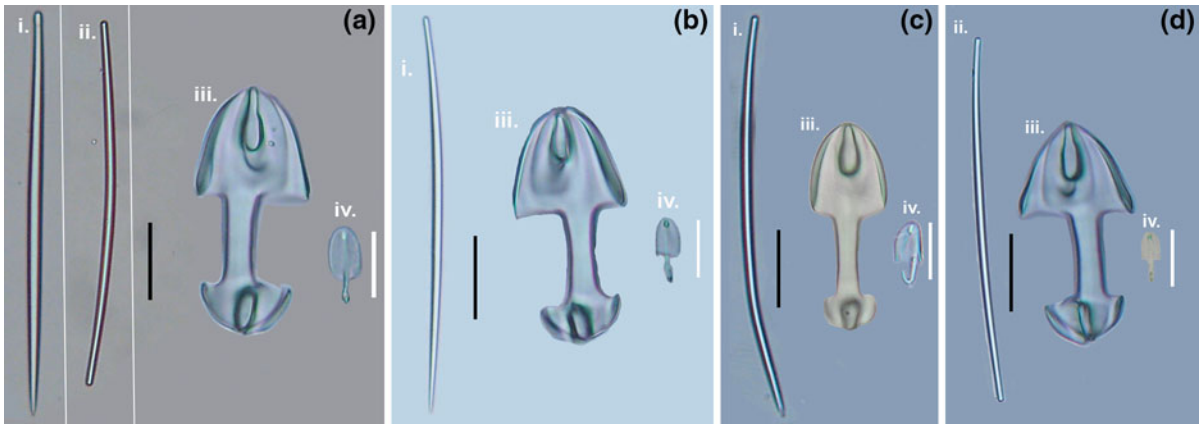

Fig. 3 The characteristic spicules of the morphotypes of *M. laevis* combining spicule types found in all geographic locations studied: **a** orange, semi-cryptic (Key Largo, Bahamas, Bocas del Toro), **b** orange, massive (Bocas del Toro), **c** white, massive (Bocas del Toro), **d** white, semi-cryptic (Bahamas). Samples from Key Largo and the Bahamas have strongyles as their dominant megasclere, while the dominant megasclere in samples from Bocas del Toro is the subtylostyle. *i* subtylostyle, *ii* strongyle, *iii* anisochela I, *iv* anisochela II. *Black scale bar* = 100 μm, *white scale bar* = 20 μm

Since this manuscript was accepted for publication, we visited islands in the southeastern Caribbean (April 2011), and observed both the orange and white forms of *M. laevis* growing on the same reefs, with an intermediate white-orange form on some reefs as well. Variation between the orange and white forms was also noted by Williams & Bunkley-Williams (1990), and attributed to sponge bleaching in response to temperature changes. Megascleres of *M. laevis* collected from Curaçao (orange, semi-cryptic) and Martinique (orange, white and intermediate, all massive) were all subtylostyles ~500 μm in length.

The different phylogenetic analyses using 18S and 28S rRNA gene sequences revealed the same basic genetic structure, and grouped all morphotypes of *Mycale laevis* in the same clade with the other sequenced species of *Mycale*. Thus, our results support the validity of using the presence of anisochelae, in combination with a monoaxonic megasclere, as a taxonomic character to distinguish the genus *Mycale*. The presence of chelae appears to be an autapomorphy for the poecilosclerids, as they have a complex structure and are unique to the order (Erpenbeck et al., 2007). The same study of the Poecilosclerida showed that species with chelae were monophyletic while those lacking chelae were polyphyletic.

Phylogenetic analyses with 18S and 28S rRNA also indicated that sponges of the subgenus *Aegogropila* were polyphyletic as they grouped with sponges from 4 other subgenera- *Arenochalina*, *Mycale*, *Zygomycale*, and *Carmia*. The sponge *M. carmigropila* has ectosomal features related to both subgenera *Aegogropila* and *Carmia*, and is provisionally assigned to *Aegogropila* (Hajdu & Rutzler, 1998). Despite its name, *M. carmigropila* was more closely related to *M. laxissima* (subgenus *Arenochalina*) than to either *Aegogropila* or *Carmia*. Finally, the subgenus *Mycale* appeared to by paraphyletic. Analysis of 18S rRNA sequences grouped *M. grandis*, *M. lingua* and all sequences of *M. laevis* in a clade with *Mycale* sp. J57 (*Aegogropila*). In the 28S rRNA analyses, the clade representing the subgenus *Mycale* included species from *Arenochalina* and *Aegogropila*. Our results suggest that a revision of the subgenus classification within *Mycale* combining both molecular and morphological data may be needed.

Intra-specific morphological diversity is often associated with genetic divergence or with differences in local environmental conditions. In our study, the observed differences in shape and color among the 4 morphotypes of *M. laevis* did not correlate with genetic data. Field observations have revealed that the massive growth form of *M. laevis* dominates on overfished, predator-scarce reefs like Bocas del Toro, but is largely absent on reefs protected from fishing and with high predator densities like those off Key Largo (Loh & Pawlik, 2009). As all the morphotypes of *M. laevis* are palatable to spongivorous fish, the

Table 2 Spicule types and lengths from the four morphotypes of *M. laevis*

Morphotype	Collection location	Replicate	Spicules			
			Megasclere	Length (μm)	Microsclere	Length (μm)
Orange and semi-cryptic	Key Largo, Florida	1	Strongyle	399.63–485.44–554.88 (20)	Anisochela I	55.00–78.31–147.50 (20), (40.94%; 20.75%)
					Anisochela II	37.38–55.70–71.88 (8), (52.90%; 21.94%)
					Sigma	30.00–41.00–50.00 (20)
					Raphide	39.9–54.72–68.4 (20)
		2	Strongyle	250.00–416.25–480.00 (20)	Anisochela I	68.75–75.19–81.25 (20), (41.90%; 18.37%)
					Anisochela II	20.00–23.75–27.50 (2), (63.16%; 23.68%)
					Sigma	18.75–24.84–30.00 (8)
					Raphide	40.00–52.13–80.00 (20)
		3	Strongyle	395.00–461.25–510.00 (20)	Anisochela I	67.50–73.33–77.50 (3), (40.91%; 20.45%)
					Sigma	25.00–35.83–45.00 (12)
					Raphide	38.75–52.88–90.00 (20)
	Bahamas	1	Strongyle	370.00–466.75–525.00 (20)	Anisochela I	67.50–78.81–85.00 (20), (44.57%; 20.94%)
					Raphide	37.50–52.31–75.00 (20)
		2	Strongyle	330.00–466.25–530.00 (20)	Anisochela I	66.25–74.69–82.50 (20), (40.67%; 19.41%)
					Raphide	45.00–59.50–67.50 (20)
		3	Strongyle	375.00–443.75–505.00 (20)	Anisochela I	65.00–75.38–85.00 (20), (41.96%; 17.74%)
					Anisochela II	17.50–19.17–20.00 (3), (60.87%; 13.04%)
					Sigma	20.00–29.75–35.00 (5)
					Raphide	45.00–55.75–67.50 (20)
	Bocas del Toro, Panama	1	Subtylostyle	450.00–527.75–585.00 (20)	Anisochela I	67.50–83.31–92.50 (20), (43.51%; 22.21%)
					Sigma	30.00–41.75–55.00 (20)
					Raphide	57.50–70.38–76.25 (20)
		2	Subtylostyle	450.00–528.25–565.00 (20)	Anisochela I	65.00–80.38–87.50 (20), (44.63%; 19.28%)
					Sigma	25.00–39.00–50.00 (20)
					Raphide	50.00–68.38–100.00 (20)
		3	Subtylostyle	470.00–521.50–565.00 (20)	Anisochela I	80.00–84.81–90.00 (20), (45.10%; 18.57%)
					Anisochela II	15.00–20.25–22.50 (20), (66.05%; 18.52%)
					Sigma	30.00–44.75–55.00 (20)
					Raphide	60.00–73.94–80.00 (20)

Table 2 continued

Morphotype	Collection location	Replicate	Spicules			
			Megasclere	Length (μm)	Microsclere	Length (μm)
Orange and massive	Bocas del Toro, Panama	1	Subtylostyle	442.75–501.40–529.00 (20)	Anisochela I	15.68–21.02–22.8 (20) (63.73%; 21.02%)
					Anisochela II	69–82.37–86.25 (20) (45.38%; 18%)
					Sigma	23–51.75–71.88 (20)
					Raphide	34.2–58.21–65.55 (20)
		2	Subtylostyle	425.00–507.00–560.00 (20)	Anisochela I	67.50–78.38–82.50 (20), (46.01%; 18.66%)
					Anisochela II	18.75–21.00–23.75 (20), (62.80%; 7.38%)
					Sigma	35.00–48.30–60.00 (20)
					Raphide	52.50–65.75–75.00 (20)
		3	Subtylostyle	475.00–525.00–660.00 (20)	Anisochela I	77.50–87.75–97.50 (20), (43.87%; 19.94%)
					Anisochela II	20.00–23.25–30.00 (20), (63.17%; 19.09%)
					Sigma	25.00–47.30–60.00 (20)
					Raphide	70.00–83.94–105.00 (20)
White and massive	Bocas del Toro, Panama	1	Subtylostyle	430.00–505.00–570.00 (20)	Anisochela I	77.50–83.13–92.50 (8), (38.72%; 16.73%)
					Anisochela II	15.00–19.5–23.75 (20) (66.03%; 18.59%)
					Sigma	25.00–36.31–47.50 (20)
					Raphide	70.00–79.00–97.50 (20)
		2	Subtylostyle	410.00–521.75–585.00 (20)	Anisochela I	67.50–86.25–97.50 (20), (43.19%; 19.28%)
					Anisochela II	20.00–22.38–25.00 (20), (64.80%; 19.55%)
					Sigma	30.00–44.50–60.00 (20)
					Raphide	50.00–68.56–120.00 (20)
		3	Subtylostyle	445.00–518.25–570.00 (20)	Anisochela I	65.00–80.44–90.00 (20), (42.35%; 18.10%)
					Anisochela II	15.00–18.81–22.50 (20), (63.79%; 21.93%)
					Sigma	20.00–37.75–50.00 (20)
					Raphide	62.50–68.44–77.50 (20)
White and semi-cryptic	Bahamas	1	Strongyle	405.00–485.75–555.00 (20)	Anisochela I	75.00–83.13–87.50 (4), (41.35%; 16.92%)
					Anisochela II	17.50–20.75–22.50 (9), (60.24%; 18.67%)
					Sigma	20.00–32.06–40.00 (17)
					Raphide	47.50–55.38–63.75 (20)
		2	Strongyle	375.00–445.50–510.00 (20)	Anisochela I	67.50–77.06–87.50 (20), (42.25%; 19.71%)
					Sigma	20.00–29.75–45.00 (20)
					Raphide	38.75–48.25–53.75 (20)

The spicule lengths are presented in the 'shortest-mean length-longest' format, with the number of spicule replicates in parentheses. The second set of parentheses for the anisochelae denote the proportional length of the anisochela head and foot, respectively

sponge is likely grazed down when predators are abundant, and thus restricted to a semi-cryptic growth form in refugia under coral colonies and other hard reef substrata (Loh & Pawlik, 2009). In the south-eastern Caribbean, massive forms of *M. laevis* dominated where spongivore density is low, such as on the island of Martinique, and overfished reefs off St Lucia (personal observation).

The results of this study suggest that, like other sponge species, *M. laevis* exhibits morphological plasticity under different environmental conditions. Sympatric color morphotypes of another common Caribbean sponge, *Callyspongia vaginalis*, also did not exhibit any significant differences in gene sequences despite clear differences in surface architecture (López-Legentil et al., 2010). A note of caution bears repeating, however, as the genetic analyses presented here were based on a single genetic marker (ribosomal RNA), and further studies with more specimens and additional genetic markers are necessary to assess whether morphological variability of *M. laevis* is due to phentotypic plasticity or genotypic variation.

Conclusion

Sponges are morphologically plastic, may have different color morphotypes, and can change in shape and size due to environmental conditions. In this study, we analyzed the variation in two fragments of the ribosomal genes 18S and 28S rRNA to assess the taxonomic status of 4 morphotypes of the Orange Icing sponge, *M. laevis*. Analysis of the gene fragments provided no evidence for differentiation among the morphotypes. Based on this study, the most parsimonious explanation for the observed morphological variability in *M. laevis* is differences in local environmental conditions, such as the abundance of sponge-eating predators.

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References

Blanquer, A. & M. J. Uriz, 2007. Cryptic speciation in marine sponges evidenced by mitochondrial and nuclear genes: a phylogenetic approach. Molecular Phylogenetics and Evolution 45: 392–397.

Blanquer, A., M. J. Uriz & G. Agell, 2008. Hidden diversity in sympatric sponges: adjusting life-history dynamics to share substrate. Marine Ecology Progress Series 371: 109–115.

Chapman, M. G., T. J. Tolhurst, R. J. Murphy & A. J. Underwood, 2010. Complex and inconsistent patterns of variation in benthos, micro-algae and sediment over multiple spatial scales. Marine Ecology Progress Series 398: 33–47.

Chou, L. M., J. Y. Yu & T. L. Loh, 2004. Impacts of sedimentation on soft-bottom benthic communities in the southern islands of Singapore. Hydrobiologia 515: 91–106.

Collin, R., M. C. Diaz, J. L. Norenburg, R. M. Rocha, J. A. Sanchez, A. Schulze, M. Schwartz & A. Valdez, 2005. Photographic identification guide to some common marine invertebrates of Bocas Del Toro, Panama. Caribbean Journal of Science 41: 638–707.

Corredor, J. E., C. R. Wilkinson, V. P. Vicente, J. M. Morell & E. Otero, 1988. Nitrate release by Caribbean reef sponges. Limnology and Oceanography 33: 114–120.

Diaz, M. C. & K. Rutzler, 2001. Sponges: an essential component of Caribbean coral reefs. Bulletin of Marine Science 69: 535–546.

Duran, S. & K. Rützler, 2006. Ecological speciation in a Caribbean marine sponge. Molecular Phylogenetics and Evolution 40: 292–297.

Erpenbeck, D., S. Duran, K. Rützler, V. Paul, J. N. A. Hooper & G. Wörheide, 2007. Towards a DNA taxonomy of Caribbean demosponges: a gene tree reconstructed from partial mitochondrial CO1 gene sequences supports previous rDNA phylogenies and provides a new perspective on the systematics of Demospongiae. Journal of the Marine Biological Association of the United Kingdom 87: 1563–1570.

Goreau, T. F. & W. D. Hartman, 1966. Sponge: effect on the form of reef corals. Science 151: 343–344.

Guindon, S. & O. Gascuel, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704.

Hajdu, E. & K. Rutzler, 1998. Sponges, genus *Mycale* (Poecilosclerida: Demospongiae: Porifera), from a Caribbean mangrove and comments on subgeneric classification. Proceedings of the Biological Society of Washington 111: 737–773.

Hartman, W. D., 1967. Revision of *Neofibularia* (Porifera, Demospongiae), a genus of toxic sponges from the West Indies and Australia. Postilla 113: 1–41.

Henkel, T. P. & J. R. Pawlik, 2005. Habitat use by sponge-dwelling brittlestars. Marine Biology 146: 301–313.

Hill, M. S., 1998. Spongivory on Caribbean reefs releases corals from competition with sponges. Oecologia 117: 143–150.

Hooper, J., 1985. Character stability, systematic, and affinities between Microcionidae (Poecilosclerida) and Axinellida. In Rützler, K. (ed.), New Perspectives in Sponge Biology. Smithsonian Institution Press, Washington: 284–294.

Hooper, J. N. A. & R. Van Soest, 2002. Systema Porifera: A Guide to the Classification of Sponges, Vol. 1. Kluwer Academic, New York.

Klautau, M., C. A. M. Russo, C. Lazoski, N. Boury-Esnault, J. P. Thorpe & A. M. Solé-Cava, 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. Evolution 53: 1414–1422.

Loh, T.-L. & J. R. Pawlik, 2009. Bitten down to size: fish predation determines growth form of the Caribbean coral reef sponge *Mycale laevis*. Journal of Experimental Marine Biology and Ecology 374: 45–50.

López-Legentil, S., P. M. Erwin, T. P. Henkel, T. -L. Loh & J. P. Pawlik, 2010. Phenotypic plasticity in the Caribbean sponge *Callyspongia vaginalis* (Porifera: Haplosclerida). Scientia Marina 74: 445–453.

Maldonado, M., M. Carmona & M. Uriz, 1999. Decline in Mesozoic reef-building sponges explained by silicon limitation. Nature 401: 785–788.

McDonald, J. I., J. N. A. Hooper & K. A. McGuinness, 2002. Environmentally influenced variability in the morphology of *Cinachyrella australiensis* (Carter 1886) (Porifera: Spirophorida: Tetillidae). Marine and Freshwater Research 53: 79–84.

Micheli, F., L. Benedetti-Cecchi, S. Gambaccini, I. Bertocci, C. Borsini, G. C. Osio & F. Romano, 2005. Cascading human impacts, marine protected areas, and the structure of Mediterranean reef assemblages. Ecological Monographs 75: 81–102.

Miller, K., B. Alvarez, C. Battershill & P. Northcote, 2001. Genetic, morphological, and chemical divergence in the sponge genus *Latrunculia* (Porifera: Demospongiae) from New Zealand. Marine Biology 139: 235–250.

Palumbi, S. R., 1986. How body plans limit acclimation: responses of a demosponge to wave force. Ecology 67: 208–214.

Pawlik, J. R., B. Chanas, R. J. Toonen & W. Fenical, 1995. Defenses of Caribbean sponges against predatory reef fish: I. Chemical deterrence. Marine Ecology Progress Series 127: 183–194.

Pile, A. J., M. R. Patterson & J. D. Witman, 1996. In situ grazing on plankton <10 μm by the boreal sponge *Mycale lingua*. Marine Ecology Progress Series 141: 95–102.

Posada, D., 2003. Using Modeltest and PAUP* to select a model of nucleotide substitution. In Baxevanis, A. D. D. B. Davison, R. D. M. Page, G. A. Petsko, L. D. Stein, & G. D. Stormo (eds), Current Protocols in Bioinformatics. Wiley, New York: 6.5.1–6.5.14.

Posada, D., 2008. jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25: 1253–1256.

Randall, J. & W. Hartman, 1968. Sponge-feeding fishes of the West Indies. Marine Biology 1: 216–225.

Ronquist, F. & J. P. Huelsenbeck, 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rozas, J., J. C. Sanchez-DelBarrio, X. Messeguer & R. Rozas, 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497.

Schneider, S., D. Roessli & L. Excoffier, 2000. Arlequin ver. 2000. A software for population genetics data analysis. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva.

Sollas, I., 1908. The inclusion of foreign bodies by sponges, with a description of a new genus and species of Monaxonida. Annals & Magazine of Natural History 1: 395–401.

Southwell, M. W., J. B. Weisz, C. S. Martens & N. Lindquist, 2008. In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. Limnology and Oceanography 53: 986–996.

Swofford, D. L., 1998. PAUP* 4.0, version b10. Sinuaer, Sunderland, MA.

Tamura, K. & M. Nei, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology & Evolution 10: 512–526.

Tamura, K., J. Dudley, M. Nei & S. Kumar, 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology & Evolution 24: 1596–1599.

Teragawa, C. K., 1986. Particle transport and incorporation during skeleton formation in a keratose sponge: *Dysidea etheria*. Biological Bulletin 170: 321–334.

Van Soest, R. M., 1984. Marine sponges from Curacao and other Caribbean localities. Part III Poecilosclerida. In Hummelinck, P. W. & L. J. Van Der Steen (eds), Studies on the Fauna of Curacao and Other Caribbean Islands No. 199. Foundation for Scientific Research in Surinam and the Netherland Antilles, Utrecht: 1–187.

Wilkinson, C., 2008. Status of Coral Reefs of the World: 2008. Global Coral Reef Monitoring Network and Reef and Rainforest Research Centre. Townsville, Australia.

Williams, E. H. & L. Bunkley-Williams, 1990. The worldwide coral reef bleaching cycle and related sources of coral mortality. Atoll Research Bulletin 335: 1–71.

Wulff, J. L., 1997. Parrotfish predation on cryptic sponges of Caribbean coral reefs. Marine Biology 129: 41–52.

Wulff, J. L., 2006a. Rapid diversity and abundance decline in a Caribbean coral reef sponge community. Biological Conservation 127: 167–176.

Wulff, J. L., 2006b. Sponge systematics by starfish: Predators distinguish cryptic sympatric species of Caribbean Fire Sponges, *Tedania ignis* and *Tedania klausi* n. sp. (Demospongiae, Poecilosclerida). Biological Bulletin 211: 83–94.

Zea, S., 1987. Esponjas del Caribe Colombiano, 1st ed. Catalogo Cientifico, Bogotá.

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SPONGE RESEARCH DEVELOPMENTS

Growth and regeneration of the elephant ear sponge *Ianthella basta* (Porifera)

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Abstract Sponges are an important component of the benthic community, especially on coral reefs, but demographic data such as growth, recruitment or mortality are notably limited. This study examined the growth of the elephant ear sponge *Ianthella basta*, the largest and in some areas one of the dominating sponge species on Guam and other pacific reefs. We measured growth rates of the natural population on Guam over the course of one year and identified intra-individual growth patterns. Initial sponge sizes ranged from 200 to 35,000 cm^2. Specific growth rates ranged from 0.08 to 6.08 with a mean specific growth rate of 1.43 ± 1.29 (SD) $year^{-1}$. Furthermore, specific growth decreased with sponge size. The age estimate for the largest sponge (1.7 m height × 9.5 m circumference) was ~8 years. Intra-individual growth was mostly apical. This study demonstrated high growth rates, which has notable implications for environmental assessments, management and potential biomedical applications.

Keywords Elephant ear sponge · *Ianthella basta* · Porifera · Demography · Growth

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Introduction

Sponges are an important component of benthic coral reef communities (Diaz & Rützler, 2001). On Caribbean reefs, their importance has begun to be recognized, where sponge assemblages reach similar diversities and abundances as scleractinian corals (Targett & Schmahl, 1984; Suchanek et al., 1985). However, on Pacific reefs, sponges have received much less attention despite their various ecological roles. Sponges are benthic filter feeders (Reiswig, 1971; Hadas et al., 2009; Riisgard & Larsen, 2010), serve as habitat for organisms (Duffy, 1992; Henkel & Pawlik, 2005; Hultgren & Duffy, 2010) and affect the benthic community composition by competitive interactions (Suchanek et al., 1985; Engel & Pawlik, 2000).

Despite their high diversity and abundance, research just started to investigate life history traits like growth, life span, or reproduction in more detail (e.g. Turon et al., 1998; De Caralt et al., 2008; Koopmans & Wijffels, 2008; McMurray et al., 2010). While there is no doubt that growth, size, or life span

affect and explain many ecological interactions and functions (Peters, 1983), more research needs to be done to reveal sponge life histories (McMurray et al., 2008).

There are many factors that may have contributed to this lack of studies. Sponges are often very slow growing organisms, which require long-time studies to estimate growth rates (Reiswig, 1973; Duckworth & Battershill, 2001). Growth can vary significantly among seasons, populations, or sites (Garrabou & Zabala, 2001; De Caralt et al., 2008), and many sponge species have a highly variable morphology, which complicates accurate size estimates (but see Koopmans & Wijffels, 2008). Sponges also lack distinct morphological structures that can be used as age indicators like otoliths in fishes or growth rings in trees.

Growth rates have been determined for a number of sponge species, including encrusting, rope-like, tubular, and massive growth forms (e.g. Turon et al., 1998; Garrabou & Zabala, 2001; Tanaka, 2002; De Caralt et al., 2008; Koopmans & Wijffels, 2008; McMurray et al., 2008). Hoppe (1988) investigated growth of the flabellate sponge *Agelas clathrodes*, but to our knowledge, no data are known from fan-like growing sponges.

When measuring growth in sponges it should be recognized that sponges often heal wounds much faster compared to their normal rate of growth (Ayling, 1983; Smith & Hildemann, 1986; Hoppe, 1988), and wound healing/tissue regeneration is therefore not a good predictor of normal growth rates.

Estimation of the age structure of benthic communities is essential for applied ecological assessments, which require knowledge of the age and growth of the community to assess recovery rates after habitat destruction or mitigation projects.

Ianthella basta (Pallas) is a conspicuous, fan- or funnel-like shaped sponge reaching heights of up to 2 m (Bergquist & Kelly-Borges, 1995, personal observation). *I. basta* is widely distributed in the Indo-Pacific (Bergquist & Kelly-Borges, 1995). Its distribution ranges from the Mascarene Islands to Vanuatu, the Philippines and Guam. However, *I. basta* is absent from all intervening Micronesian Islands including the Federated States of Micronesia and Palau (Kelly et al., 2003). Therefore, it has been speculated whether it colonized Guam by jump dispersal or became introduced through anthropogenic transport (Kelly et al., 2003). The fact that on Guam *I. basta* only occurs in Apra Harbor, where the port is situated, may support the latter. The restriction to Apra Harbor as documented by Paulay et al. (2002) has significant consequences for the population of *I. basta* on Guam. For construction of a new aircraft carrier wharf, large reef areas in Apra Harbor are planned to be dredged (Navy, 2010). This would reduce or potentially eliminate much of the actual habitat of *I. basta* on Guam. While *I. basta* might be an introduced species, it lacks invasiveness in that it does not seem to compete with other sessile invertebrates for limited space. It is mainly found along the edge of coral slopes where the hard substrate changes to soft bottom sediments with occasional rocky outcrops to which it is attached. In this habitat, *I. basta* adds to the rugosity and provides shelter for other invertebrates and fishes (Rohde and Schupp, personal observation). As one of the largest and most conspicuous sponges inside Apra Harbor, *I. basta* has also become an attraction for the local dive operators. Therefore, *I. basta* has become a biological and economical important species. Thus for both, assessments of ecological damage and potential mitigation demands, an assessment of the population characteristics (age, growth) of *I. basta* is essential and encouraged by local resource agencies (D. Burdick, personal communication).

Another reason to investigate the natural growth rate of *I. basta* is recent studies describing its unusual chitin skeleton and the potential of such skeletons in biomedical applications (Brunner et al., 2009; Ehrlich et al., 2010a, b). However, the use of marine natural products in general and of the identified chitin scaffold in particular is restricted by supply limitations (e.g. there is no synthesis available for chitin scaffolds). Consequently, a detailed knowledge of growth rates and growth patterns is necessary to evaluate if aquaculture could be viable to produce enough material for biomedical applications.

The aim of this study was to determine the growth and consequently the age of the natural population of *I. basta*. Beside the general lack of knowledge on sponge growth rates, the results could highlight the consequences of habitat destruction and explore whether *I. basta* constitutes a sustainable source for tissue harvest for extraction of chitin scaffolds.

Materials and methods

Growth of the natural *Ianthella basta* population

The study site was at Western Shoals, Apra Harbor, on the west coast of Guam (13°27.3′N, 144°39.2′E). This is a very sheltered location with a high density of *I. basta*. At depths between 8 and 11 m, we tagged 40 specimen of *I. basta* using numbered aluminum washers that were nailed to the reef next to the sponges. We chose specimen over the entire size range of the present population. In June 2009 and June 2010, circumference and slant height were measured by SCUBA using measuring tapes. The shape of a cone was used as a model to calculate the area of the sponge tissue, and growth was calculated as:

$$G = (A_2 - A_1)/A_1 \times (1/\mathrm{d}t),$$

where G is the specific growth rate (year^{-1}), A_1 is the initial area (cm^2), A_2 is the final area (cm^2), and t is the time (years).

To assess whether size affects the growth rates of the sponges, individuals were grouped into 4 size classes: 1 (from 195 to 1,000 cm^2, $n = 6$), 2 (from 1,001 to 3,000 cm^2, $n = 18$), 3 (from 3,001 to 10,000 cm^2, $n = 9$) and 4 (>10,000 cm^2, $n = 8$). Specific growth data were log10-transformed to obtain homogeneity of variances (Levene's test). Growth differences among size-class were analyzed by a one-way ANOVA and Tukey's posthoc test (SPSS 17).

Five commonly used growth models were fit to size-increment data to determine the best model describing growth of *I. basta* (McMurray et al., 2008): the general von Bertalanffy growth formula (gVBGF) (von Bertalanffy, 1938; Beverton & Holt, 1957; Pauly, 1981), specialized von Bertalanffy growth formula (sVBGF) (Richards, 1959; Pauly, 1981), Gompertz (Gompertz, 1825; Winsor, 1932), Richards (Richards, 1959; Ebert, 1980), and Tanaka (1982) growth functions. The square root of area estimates was used as an average linear size to model growth of *I. basta*. The difference equations of the models were fitted to final and initial linear sizes on a Walford plot by nonlinear regression (SOLVER, MS Excel 2007). To produce size-at-age curves, we used parameter estimates using the integrated forms of the growth functions, which were subsequently squared to obtain area-at-age plots. The sum of squared error (SSE), coefficient of determination and Akaike information criterion (AIC) (Akaike, 1973) were used to evaluate model fit. Because the AIC evaluates the trade-off between model biases that results from too few parameters versus variance that results from too many, it can be used to evaluate any difference between the 2- and 3-parameter models that may otherwise be neglected through use of the SSE as the sole fitting criteria (Burnham & Anderson, 2002). The model with the lowest SEE and AIC is sought to select the best fitting model. Because sample size (n) was small with respect to the number of model parameters, K, ($n/K < 40$), the second-order correction (AICc) was used (Burnham & Anderson, 2002). As a measure of each model relative to the best model, the model selection criteria AIC difference, Δi, was calculated.

Regeneration and intra-individual growth patterns

To observe the intra-individual growth and regeneration patterns, we tagged ten additional specimen of *I. basta* with slant heights of 60–90 cm at the same site at 12 m depth in May 2010. Of these sponges, all the tissue above 50 cm slant height was cut off. At every 10 cm height, cable ties were pierced through the sponge tissue to determine whether growth occurred throughout the entire sponge body, or whether apical regeneration of tissue dominated. Additionally, two round holes (3 cm diameter) were cut at 10 and 30 cm height to identify and measure regeneration in response to injuries.

Sponge growth was remeasured two and five months later by measuring the distances between the cable ties and the diameter of the holes to estimate growth at the different portions of the thalli.

Results

Initial size estimates of the sponges ranged from 200 to 35,000 cm^2 with a mean value of 5,702 cm^2. Specific growth rates ranged from 0.08 to 6.08 with a mean specific growth rate of 1.43 ± 1.29 (SD) year^{-1}. Comparisons among size-classes revealed that smaller sponges had higher growth rates (Fig. 1). Sponges in size-class one showed specific growth rates of 3.21 ± 2.16 year^{-1} (mean ± SD), sponges in size-class two

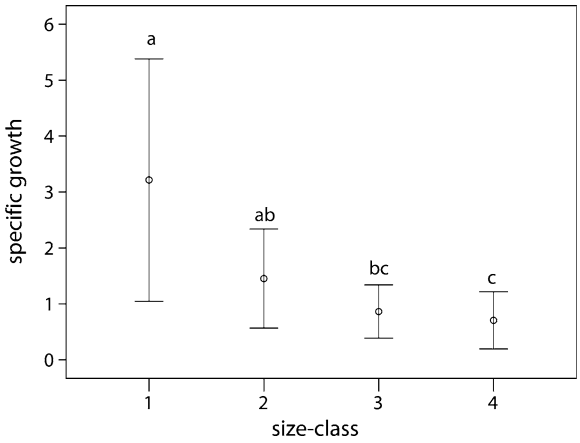

Fig. 1 Specific growth (±SD) of *I. basta* for the 4 size-classes. *Letters* indicate significant differences between size-classes (Tukey's posthoc comparison, $P < 0.05$)

1.45 ± 0.88 year^{-1} (mean ± SD), sponges in size-class three 0.86 ± 0.47 year^{-1} (mean ± SD) and sponges in size-class 4 grew 0.70 ± 0.51 year^{-1} (mean ± SD). Differences among size-classes were significant ($P < 0.001$, ANOVA). Figure 1 depicts the significant differences from the posthoc comparisons.

The parameter estimates of the growth models are presented in Table 1. All models showed a high model fit. The models in order of best fit are: Tanaka > Gompertz > Richards > gVBGF > sVBGF. The SSE values were in agreement with the AICc and revealed a similar fit of Tanaka, Gompertz, Richards and gVBGF with a slightly lower fit of the sVBGF model (Table. 2). According to the models, the largest measured sponges in this study were ~8 years old (Fig. 2).

Specimen of *I. basta* that were cut off at 50 cm height regenerated apical tissue at rates of 1.03 cm/month (Fig. 3). The basal and middle parts of the sponge body did either not grow at all, or only slightly (0.25 cm/month). The holes cut in the center of the sponge (3 cm diameter) healed within 8 weeks.

Table 2 Fitting criteria of growth functions

Model	K	r^2	SSE	AICc	Δi
Tanaka	3	0.922	8483.7	216.6	0.00
Gompertz	2	0.914	9303.9	217.8	1.25
Richards	3	0.914	9303.9	220.2	3.60
gVBGF	3	0.914	9310.0	220.2	3.63
sVBGF	2	0.892	11730.3	226.9	10.29

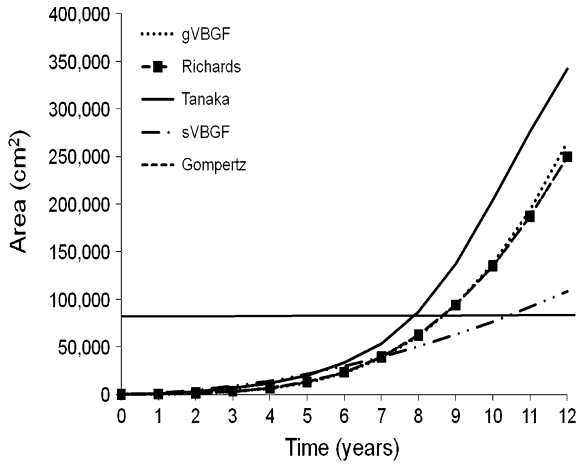

Fig. 2 Size at age curves from parameter estimates in Table 1. *Line* at 82,000 cm^2 refers to the largest measured sponge in this study

Discussion

Ianthella basta is a common member on reefs in Apra Harbor, Guam, and reefs in the tropical Western Pacific. While there has been renewed interest in this species for biomedical applications (Bergquist & Kelly-Borges, 1995; Brunner et al., 2009), little is known about its ecology and demography. Because the US NAVY has large scale dredging plans for Apra Harbor, more detailed knowledge of *I. basta* ecology and demography is needed to adequately assess mediation for destroyed coral reef areas.

Table 1 Parameter estimates for growth functions fitted to square root of final and initial areas; n = 40

gVBGF	Richards	Tanaka	sVBGF	Gompertz
$S\infty = 1950.8$	$S\infty = 1590.5$	$a = 0.0001$	$S\infty = 108684.6$	$S\infty = 1590.4$
$k = 0.109$	$k = 0.117$	$f = 0.00003$	$k = 0.0002$	$k = 0.117$
$d = 69166.6$	$d = -96618.5$	$d = 1981.4$		

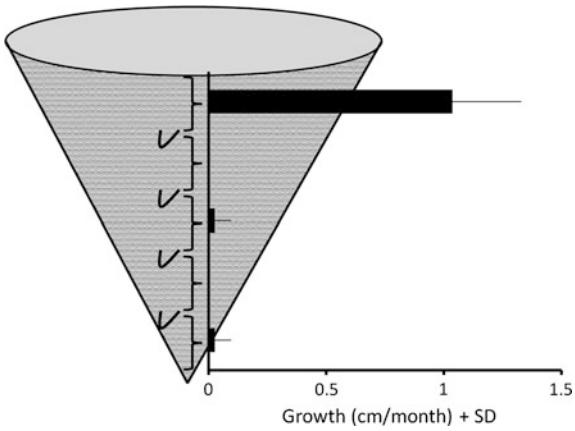

Fig. 3 Intra-individual growth and regeneration patterns of *I. basta*. The drawing shows which sections of the sponge body were tagged with cable ties and subsequently measured. *Bars* represent mean growth of the respective sponge section

This study demonstrated that *I. basta* is a remarkably fast growing sponge species, which reaches sizes of up to 2 m height in less than 10 years. To our knowledge, there is only one other study, which estimated growth and age of a sponge species that reaches similar sizes. The Caribbean sponge *Xestospongia muta* also grows to heights of 170 cm, but growth measurements indicated that specimens of this size are around 250 years old (McMurray et al., 2008). Even though *X. muta* is a massive species that needs to build up much more biomass to reach these large dimensions, it is an interesting fact that *X. muta* needs around 25 times longer to reach similar dimensions as *I. basta*.

The specific growth rate of *I. basta* was with 1.43 year^{-1} around threefold higher than that of *X. muta*, but similar to growth of four Mediterranean sponge species with mean rates from 1.08 to 2.4 year^{-1} (Garrabou & Zabala, 2001). However, individuals of size-class 1 grew as much as 3.21 year^{-1} leading to a relative fast transition in higher size-classes. Specific growth rates are a relative measure; therefore, the absolute biomass production is higher in large sponges compared to small sponges with similar specific growth rates. Consequently, growth rates of 0.7–1.45 in size classes 2 to 4 are comparable to Mediterranean sponge species (Garrabou & Zabala, 2001), but due to the large size of *I. basta* specimen, the biomass production is much higher. This could be one reason supporting the establishment of many large sponge individuals.

Specific growth rates decreased with increasing size. This pattern has also been found for many sponge species (e.g. Reiswig, 1973; Garrabou & Zabala, 2001; De Caralt et al., 2008; McMurray et al., 2008; but see Duckworth & Battershill, 2001). The negative correlation of size and specific growth of *I. basta* is especially not surprising, since absolute growth was similar among all sizes. Growth was apical with 1 (±0.69 SD) cm month^{-1} along the upper edge, resulting in lower specific growth with increasing size. All measured specimen showed positive growth, but the intraspecific variation was very high and this seems to be characteristic for many sponge species (Reiswig, 1973; Dayton et al., 1974; Wulff, 1985; Duckworth & Battershill, 2001; Garrabou & Zabala, 2001; McMurray et al., 2008).

Holes that were cut in the center of the sponge healed within 2 months. This rate is very similar to the apical regeneration rates and much higher than the growth measured at this part of uninjured sponge specimens. This further demonstrates that growth is almost entirely restricted to edges of the sponge, whether they are the apical edge, edges of holes from predation or, in this case artificial injury. Some sponge species showed wound healing processes that generated tissue much faster than their normal growth (Ayling, 1983; Smith & Hildemann, 1986; Hoppe, 1988). Assuming that wound healing exerts the highest physiologically possible growth rate and the fact that natural apical growth in *I. basta* showed similar rates, we suggest that the rate of 1 cm month^{-1} is the upper limit of growth under the conditions of this study.

The question arises, what limits the size and the age of *I. basta*? The largest specimens of the population were estimated to be around 8 years old, but still growing with average rates of 1 cm month^{-1}, indicating infinite growth for this species. Since no larger and therefore older specimen could be found on Guam, other biotic or abiotic factors must restrict the sponge to a maximal size and age.

Predation can significantly affect a sponge community (e.g. Wulff, 1997; Pawlik, 1998) and consequently also affect growth measurements by consuming sponge tissue. During our study, we observed no evidence of predation, such as bite marks or removal of significant amounts of biomass. The crude extract of *I. basta* deterred feeding by various predators (Becerro et al., 2003). Therefore, the observed growth seems not or only minimally restricted by predation.

The effect of abiotic factors on the growth and mortality of *I. basta* has not been studied. Apra Harbor is a geographically protected bay with low current and wave dynamics. These conditions have been described as similar to other locations where *I. basta* occurs (Bergquist & Kelly-Borges, 1995; Kelly et al., 2003). It therefore seems likely that high water movement restricts the distribution of *I. basta* and may also restrict its size. Large sponges with an area of over 80,000 cm^2 offer a large resistance to waves or currents. One possibility is that this size represents a threshold where water movements rip off the sponges and in this way limit their size and age distribution. Extreme wave action, as it occurs during typhoons, could topple and kill the sponges (when they subsequently decompose in the fine sediment). With the last typhoon occurring on Guam on December 8, 2002 it seems unlikely that typhoons are the sole event restricting the maximum size, as medium size sponges would have had over 8 years to grow and therefore should have had reached a total age of 10 to 15 years, with corresponding sizes (100,000 cm^2 to 500,000 cm^2, depending on the model). However, the maximum size we observed was 82,000 cm^2, making such a scenario unlikely. Another factor restricting the size could be the fiber dominated skeleton (chitin and spongin), which might be too flexible to support a larger (>82,000 cm^2) fan-shaped skeleton against currents and wave action.

Other sponge species have shown to regrow at their bases after detachment by storms or anthropogenic effects (Schmahl, 1999; McMurray et al., 2010). This effect has not been shown for *I. basta*, but could contribute to the preservation of the abundance of *I. basta*.

The population of *I. basta* in Apra Harbor is very isolated. The sponge does not occur on other reefs around Guam and can also not be found on the surrounding Micronesian Islands (Kelly et al., 2003). Consequently, the loss of the population in Apra Harbor would extinct this species from the entire region. The planned dredging of large reef areas in Apra Harbor to build an aircraft carrier berthing will reduce the habitat of *I. basta* to a great extent. Many surveys have been done to assess the marine community, to evaluate environmental consequences and to determine the appropriate quantity of the compensatory mitigation measures that will be recommended for the project (Navy, 2010). However, to evaluate how the loss of significant parts of the *I. basta* population could be compensated, one requires demographic data such as growth, recruitment and mortality. None of these data have been available so far. The results of this study allow the assessment of the population structure, i.e. age-distribution curves, or size-at-age analyses, which describe the present population. In order to estimate population recovery rates after disturbances, data on recruitment and mortality are essential but non-existent so far. However, the fact that *I. basta* reaches high abundances despite its relative ephemerality indicates that recruitment could be high and regular. Since *I. basta* tissue is relatively tough and does not tear or fragment easily, asexual reproduction by fragmentation seems less likely. If the recruitment is accomplished through self-seeding by the current population, large scale dredging could diminish the larval producing population to the point that recruitment is disrupted and the population would eventually die off.

Within the last years, several studies investigated *I. basta* with regard to its chitin-based skeleton (Ehrlich et al., 2007a, b, 2010a, b; Brunner et al., 2009). These chitin-based scaffolds are of high interest for many biomedical applications like tissue engineering and biomedicine (e.g., Maeda et al., 2008; Jayakumar et al., 2010). However, one major obstacle that also natural product chemists face is the supply problem (Faulkner et al., 2000). The source organisms of bioactive compounds often need to be collected in large quantities to supply the necessary amount for the industry. This can often not be justified ecologically (Munro et al., 1994). The farming of marine organisms is sometimes an alternative to collecting specimens from the wild. But this can only be an economically relevant alternative if the organisms lend themselves to a cost-effective cultivation (Schupp et al., 2009). Using *I. basta* as source for chitinous scaffolds requires the harvest of large amounts of sponge tissue. Both, ecologically sustainable wild harvest and farming therefore rely on high growth rates that provide a sufficient supply. The growth rates of *I. basta* are high compared to other sponge species and preliminary experiments revealed that cultivation of *I. basta* can be suitable (Rohde and Schupp, in preparation). However, this would only be suitable if a healthy natural population is present to support any aquaculture settings.

Further studies on the demography of *I. basta* (e.g. reproduction and mortality) together with this study could provide the necessary framework to enable effective management and potential mitigation.

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References

Akaike, H., 1973. Information theory and an extension of the maximum likelihood principle. In Petrov, B. N. & F. Csaki (ed.), Proceedings of the 2nd International Symposium on Information Theory. Akademiai Kiado, Budapest: 267–281.

Ayling, A. L., 1983. Growth and regeneration rates in thinly encrusting Demospongiae from temperate waters. Biological Bulletin, Marine Biological Laboratory, Woods Hole 165: 343–352.

Becerro, M. A., R. W. Thacker, X. Turon, M. J. Uriz & V. J. Paul, 2003. Biogeography of sponge chemical ecology: comparisons of tropical and temperate defenses. Oecologia 135: 91–101.

Bergquist, P. R. & M. Kelly-Borges, 1995. Systematics and biogeography of the genus *Ianthella* (Demospongiae: Verongida: Ianthellidae) in the South-West Pacific. The Beagle, Records of the Museums and Art Galleries of the Northern Territory 12: 151–176.

Beverton, R. J. H. & S. J. Holt, 1957. On the Dynamics of Exploited Fish Populations. Fisheries Investigations of the Ministry of Agriculture and Fisheries, Food in Great Britain, Series 2, Sea Fish, Vol 19. Facsimile reprint 1993. Chapman & Hall, London.

Brunner, E., H. Ehrlich, P. Schupp, R. Hedrich, S. Hunoldt, M. Kammer, S. Machill, S. Paasch, V. V. Bazhenov, D. V. Kurek, T. Arnold, S. Brockmann, M. Ruhnow & R. Born, 2009. Chitin-based scaffolds are an integral part of the skeleton of the marine demosponge *Ianthella basta*. Journal of Structural Biology 168: 539–547.

Burnham, K. P. & D. R. Anderson, 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretical Approach. Springer, New York.

Dayton, P. K., G. A. Robilliard, R. T. Paine & L. B. Dayton, 1974. Biological accommodation in the benthic community at McMurdo Sound, Antarctica. Ecological Monographs 44: 105–128.

De Caralt, S., M. J. Uriz & R. H. Wifffels, 2008. Grazing, differential size-class dynamics and survival of the Mediterranean sponge *Corticium candelabrum*. Marine Ecology Progress Series 360: 97–106.

Diaz, M. C. & K. Rützler, 2001. Sponges: an essential component of Caribbean coral reefs. Bulletin of Marine Science 69: 535–546.

Duckworth, A. R. & C. N. Battershill, 2001. Population dynamics and chemical ecology of New Zealand demospongiae *Latrunculia* sp nov and *Polymastia croceus* (Poecilosclerida : Latrunculiidae : Polymastiidae). New Zealand Journal of Marine and Freshwater Research 35: 935–949.

Duffy, J. E., 1992. Host use patterns and demography in a guild of tropical sponge-dwelling shrimps. Marine Ecology Progress Series 90: 127–138.

Ebert, T. A., 1980. Estimating parameters in a flexible growth equation, the Richards function. Canadian Journal of Fisheries and Aquatic Sciences 37: 687–692.

Ehrlich, H., M. Krautter, T. Hanke, P. Simon, C. Knieb, S. Heinemann & H. Worch, 2007a. First evidence of the presence of chitin in skeletons of marine sponges. Part II. Glass sponges (Hexactinellida: Porifera). Journal of Experimental Zoology Part B-Molecular and Developmental Evolution 308B: 473–483.

Ehrlich, H., M. Maldonado, K. D. Spindler, C. Eckert, T. Hanke, R. Born, C. Goebel, P. Simon, S. Heinemann & H. Worch, 2007b. First evidence of chitin as a component of the skeletal fibers of marine sponges Part I. Verongidae (Demospongia: Porifera). Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 308B: 347–356.

Ehrlich, H., M. Ilan, M. Maldonado, G. Muricy, G. Bavestrello, Z. Kljajic, J. L. Carballo, S. Schiaparelli, A. Ereskovsky, P. Schupp, R. Born, H. Worch, V. V. Bazhenov, D. Kurek, V. Varlamov, D. Vyalikh, K. Kummer, V. V. Sivkov, S. L. Molodtsov, H. Meissner, G. Richter, E. Steck, W. Richter, S. Hunoldt, M. Kammer, S. Paasch, V. Krasokhin, G. Patzke & E. Brunner, 2010a. Three-dimensional chitin-based scaffolds from Verongida sponges (Demospongiae: Porifera). Part I. Isolation and identification of chitin. International Journal of Biological Macromolecules 47: 132–140.

Ehrlich, H., E. Steck, M. Ilan, M. Maldonado, G. Muricy, G. Bavestrello, Z. Kljajic, J. L. Carballo, S. Schiaparelli, A. Ereskovsky, P. Schupp, R. Born, H. Worch, V. V. Bazhenov, D. Kurek, V. Varlamov, D. Vyalikh, K. Kummer, V. V. Sivkov, S. L. Molodtsov, H. Meissner, G. Richter, S. Hunoldt, M. Kammer, S. Paasch, V. Krasokhin, G. Patzke, E. Brunner & W. Richter, 2010b. Three-dimensional chitin-based scaffolds from Verongida sponges (Demospongiae: Porifera). Part II: Biomimetic potential and applications. International Journal of Biological Macromolecules 47: 141–145.

Engel, S. & J. R. Pawlik, 2000. Allelopathic activities of sponge extracts. Marine Ecology-Progress Series 207: 273–281.

Faulkner, D. J., M. K. Harper, M. G. Haygood, C. E. Salomon & E. W. Schmidt, 2000. Symbiotic bacteria in sponges: sources of bioactive substances. In Fusetani, N. (ed.), Drugs from the Sea. Karger, Basel: 107–119.

Garrabou, J. & M. Zabala, 2001. Growth dynamics in four mediterranean demosponges. Estuarine, Coastal and Shelf Science 52: 293–303.

Gompertz, B., 1825. On the nature of the function expressive of human mortality, and on a new mode of determining the value of life contingencies. Philosophical Transactions of the Royal Society of London, Series B 115: 513–585.

Hadas, E., M. Shpigel & M. Ilan, 2009. Particulate organic matter as a food source for a coral reef sponge. Journal of Experimental Biology 212: 3643–3650.

Henkel, T. P. & J. R. Pawlik, 2005. Habitat use by sponge-dwelling brittlestars. Marine Biology 146: 301–313.

Hoppe, W. F., 1988. Growth, regeneration and predation in 3 species of large coral reef sponges. Marine Ecology Progress Series 50: 117–125.

Hultgren, K. M. & J. E. Duffy, 2010. Sponge host characteristics shape the community structure of their shrimp associates. Marine Ecology Progress Series 407: 1–12.

Jayakumar, R., D. Menon, K. Manzoor, S. V. Nair & H. Tamura, 2010. Biomedical applications of chitin and chitosan based nanomaterials—a short review. Carbohydrate Polymers 82: 227–232.

Kelly, M., J. N. A. Hooper, V. Paul, G. Paulay, R. W. M. Van Soest & W. de Weerdt, 2003. Taxonomic inventory of the sponges (Porifera) of the Mariana Islands. Micronesica 35–36: 100–120.

Koopmans, M. & R. H. Wijffels, 2008. Seasonal growth rate of the sponge *Haliclona oculata* (Demospongiae: Haplosclerida). Marine Biotechnology 10: 502–510.

Maeda, Y., R. Jayakumar, H. Nagahama, T. Furuike & H. Tamura, 2008. Synthesis, characterization and bioactivity studies of novel beta-chitin scaffolds for tissue-engineering applications. International Journal of Biological Macromolecules 42: 463–467.

McMurray, S. E., J. E. Blum & J. R. Pawlik, 2008. Redwood of the reef: growth and age of the giant barrel sponge *Xestospongia muta* in the Florida Keys. Marine Biology 155: 159–171.

McMurray, S. E., T. P. Henkel & J. R. Pawlik, 2010. Demographics of increasing populations of the giant barrel sponge *Xestospongia muta* in the Florida Keys. Ecology 91: 560–570.

Munro, M. H. G., J. W. Blunt, R. J. Lake, M. Litaudon, C. N. Battershill & M. J. Page, 1994. From seabed to sickbed: what are the prospects? In Van Soest, R. W. M., T. Van Kempen & J. Braekman (eds), Sponges in Space and Time. AA Balkema, Rotterdam: 395–400.

Navy, U.S.D.o.t., 2010. Guam and CNMI Military Relocation: EIS, Vol. 4: Aircraft Carrier Berthing.

Paulay, G., L. Kirkendale, G. Lambert & C. Meyer, 2002. Anthropogenic biotic interchange in a coral reef ecosystem: a case study from Guam. Pacific Science 56: 403–422.

Pauly, D., 1981. The relationships between gill surface area and growth performance in fish: a generalization of von Bertalanffy's theory of growth. Meeresforschung 28: 251–282.

Pawlik, J. R., 1998. Coral reef sponges: do predatory fishes affect their distribution? Limnology and Oceanography 43: 1396–1399.

Peters, R. H., 1983. The Ecological Implications of Body Size. Cambridge University Press, Cambridge.

Reiswig, H. M., 1971. In situ pumping activities of tropical Demospongiae. Marine Biology 9: 38–50.

Reiswig, H. M., 1973. Population dynamics of three Jamaican Demospongiae. Bulletin of Marine Science 23: 191–226.

Richards, F. J., 1959. A flexible growth function for empirical use. Journal of Experimental Botany 10: 290–300.

Riisgard, H. U. & P. S. Larsen, 2010. Particle capture mechanisms in suspension-feeding invertebrates. Marine Ecology Progress Series 418: 255–293.

Schmahl, G. P., 1999. Recovery and growth of the giant barrel sponge (*Xestospongia muta*) following physical injury from a vessel grounding in the Florida Keys. Memoirs of the Queensland Museum 44: 532.

Schupp, P. J., C. Kohlert-Schupp, S. Whitefield, A. Engemann, S. Rohde, T. Hemscheidt, J. M. Pezzuto, T. P. Kondratyuk, E. J. Park, L. Marler, B. Rostama & A. D. Wright, 2009. Cancer chemopreventive and anticancer evaluation of extracts and fractions from marine macro- and micro-organisms collected from twilight zone waters around Guam. Natural Product Communications 4: 1717–1728.

Smith, L. C. & W. H. Hildemann, 1986. Allograft-rejection, autograft fusion and inflammatory responses to injury in *Callyspongia diffusa* (Porifera, Demospongia). Proceedings of the Royal Society of London, Series B: Biological Sciences 226: 445–464.

Suchanek, T. H., R. C. Carpenter, J. D. Witman & C. D. Harvell, 1985. Sponges as important space competitors in deep Caribbean coral reef communities. In Reaka, M. L. (ed.), The Ecology of Deep and Shallow Coral Reefs. Symposia Series for Undersea Research. NOAA, Rockville: 55–59.

Tanaka, K., 1982. A new growth curve which expresses infinitive increase. Publications of the Amakusa Marine Biology Laboratory Kyushu University 6: 167–177.

Tanaka, K., 2002. Growth dynamics and mortality of the intertidal encrusting sponge *Halichondria okadai* (Demospongiae, Halichondrida). Marine Biology 140: 383–389.

Targett, N. M. & G. P. Schmahl, 1984. Chemical Ecology and Ddistribution of Sponges in the Salt River Canyon, St. Croix, U.S.V.I. NOAA Technical Memorandum OAR NURP-1.

Turon, X., I. Tarjuelo & M. J. Uriz, 1998. Growth dynamics and mortality of the encrusting sponge *Crambe crambe* (Poecilosclerida) in contrasting habitats: correlation with population structure and investment in defence. Functional Ecology 12: 631–639.

von Bertalanffy, L., 1938. A quantitative theory of organic growth (inquires on growth laws II). Human Biology 10: 181–213.

Winsor, C., 1932. The Gompertz curve as a new growth curve. Proceedings of the National Academy of Science, USA 18: 1–8.

Wulff, J. L., 1985. Patterns and processes of size change in Caribbean demosponges of branching morphology. In Rutzler, K. (ed.), New Perspectives in Sponge Biology. Smithsonian Institution Press, Washington: 425–435.

Wulff, J. L., 1997. Parrotfish predation on cryptic sponges of Caribbean coral reefs. Marine Biology 129: 41–52.

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SPONGE RESEARCH DEVELOPMENTS

The marine sponge *Ianthella basta* can recover from stress-induced tissue regression

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Abstract Sponges often exhibit tissue regression in response to stressful conditions. This study investigated whether handling stress invoked tissue regression in *Ianthella basta* and assessed whether sponges could recover from this regressed tissue state. Six necrotic specimens and 12 healthy explants were collected at Orpheus Is. Australia and transported to aquarium facilities. Sponges were photographed daily and an integrated density (ID) measurement was used to quantify tissue regression. Histological samples were taken from sponge explants to compare cellular organization. Bacterial communities of regressed and recovered tissue were compared using Denaturing Gradient Gel Electrophoresis (DGGE). After 12 h both necrotic and healthy sponges displayed substantial tissue regression. However, within 72 h all sponges recovered to their original condition. The ID of the sponge tissue doubled, confirming tissue recovery in *I. basta*. Sponges affected by tissue regression had significantly fewer choanocyte chambers and more densely packed granulated cells than recovered sponges. DGGE revealed the same microbial symbionts in both regressed and recovered sponges. Handling stress associated with collection and transportation is sufficient to invoke tissue regression in this species, but sponges can rapidly recover. This study contributes to our understanding of how sponges respond to environmental pressures, influencing population resilience and persistence.

Keywords Choanocyte chambers · Histology · Symbiont · Porifera · Stress response

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Introduction

Sponges are among the oldest of the Metazoans with fossils dating back to the Precambrian, approximately 600 million years ago (Li et al., 1998). Sponges are often described as having a simplistic body plan because they were thought to lack distinct tissues and organs (Simpson, 1984). However, growing evidence suggests that sponges have a higher level of complexity than traditionally thought. For example, sponges are equipped with functional immune and apoptotic systems (Müller, 2003) typical of other integrated

animals. Some sponges also possess up to five different types of epithelia tissue (Leys et al., 2009) and others have displayed compartmentalization of cells (e.g., choanocytes; de Goeij et al., 2009), challenging the view that sponges lack distinct tissues and organization. In addition, some sponge cells (e.g., archaeocytes) are believed to be totipotent (Müller, 2006). The recently published genome of *Amphimedon queenslandica* confirms that although sponges lack structures such as neurons and muscles, they posses genes central to the form and function of higher animals (Srivastava et al., 2010). The complex cellular biology of sponges is likely to be a key contributor to their ability to occupy a wide range of environments and associated conditions (Fafanđel et al., 2003).

Sponges have the capacity to recover from damage caused by predation (Ayling, 1983), physical disturbances (Wulff, 2006, 2010), environmental stress (Leamon & Fell, 1990), and disease (Rützler, 1988). Indeed, some sponges can initiate cellular recovery of the pinacoderm within 24 h of injury, with complete recovery occurring within weeks (Louden et al., 2007), highlighting the ability of sponge cells (e.g., archaeocytes) to migrate and proliferate (Simpson, 1984). In addition, sponges also display high telomerase activity (Koziol et al., 1998), potentially aiding in rapid cell proliferation and further contributing to the success of cellular processes in recovery from sublethal damage. Although, sponges lack a true nervous system, they have the capacity to produce non-neural reflex behaviors (Meech, 2008). For instance, sponges can contract their tissues and canal systems in efforts to cope with sediment stress (Leys & Meech, 2006) and predator attacks (Nickel, 2004). Given the clear impacts of climate change on coral reef communities (Hughes et al., 2003), the ability of sponges to recover from environmental stressors will become even more important.

Although, some sponges undergo "choanocyte shedding" and cell loss under ambient environments (de Goeij et al., 2009), some species respond to environmental stress by contracting their tissues to skeletal structures leaving subdermal spaces (Knight & Fell, 1987; Imsiecke et al., 1996). This process reduces the area of tissue in contact with the surrounding water thereby providing a buffer to suboptimal water conditions (Francis et al., 1990) such as fluctuating salinity (Knight & Fell, 1987; Leamon & Fell, 1990). Our knowledge of how sponges respond to stressors (including the process of short-term tissue regression) continues to grow, particularly our understanding of the cellular pathways for this process (Fell, 1974; Imsiecke et al., 1996). In comparison, our knowledge of microbial symbiotic responses to this process is limited (but see Thoms et al., 2008).

Sponges often form intimate associations with microbes, with representatives of 25 bacterial phyla and both major lineages of *Archaea* being reported to date (Taylor et al., 2007; Webster & Taylor, 2011). In some cases, microbes can account for up to 40% of the total sponge biomass (Vacelet & Donadey, 1977), though some sponges also have low microbial abundances (Hentschel et al., 2006). It has been documented that sponges from geographically different locations can host similar microbial communities (Hentschel et al., 2002; Taylor et al., 2005). While this uniformity exists, microbial communities of sponges under stressful conditions such as elevated seawater temperatures (Lemoine et al., 2007; López-Legentil et al., 2008; Webster et al., 2008a), heavy metal stress (Webster et al., 2001; Selvin et al., 2009), and disease (Olson et al., 2006; Webster et al., 2008b; Angermeier et al., 2011) have been shown to change and this shift often correlates with a decline in host health.

This study explores tissue regression and recovery of the marine sponge *Ianthella basta*, documenting morphological and histological differences, as well as investigating the microbial symbionts in sponges with regressed and recovered tissues.

Materials and methods

Sponge collection

Six necrotic specimens (including substrate) of the marine sponge *I. basta* were collected at Orpheus Is., northeastern Australia (18°36.878′S, 146°29.990′E). All six specimens were categorized as health-compromised, displaying some level of tissue necrosis and brown spot lesions, as described in Luter et al. (2010). In addition, 12 healthy *I. basta* explants were collected. The explants had been previously cut from healthy donor sponges using a scalpel blade (approximately 10 cm^3), fixed to the reef in an Aquapurse basket (TTP plastics by design; Brisbane, Queensland, Australia), and allowed to heal for 12 weeks before collection. Whole necrotic sponges and healthy

sponge explants were transported to the Australian Institute of Marine Science and kept in a 1,000 l flow-through outdoor aquarium with unfiltered seawater, under a natural day/night light regime. Explants were contained in a plastic mesh basket within the aquarium. The flow rate was maintained at 600 ml min^{-1} and the temperature was kept at 28°C, the same temperature as Orpheus Is. at this time of year. All sponges were closely monitored and the six necrotic sponges photographed daily for 5 days. In addition to visual comparisons of the necrotic sponges, the images were compared using the integrated density (ID) measurement of Image Tool for Windows (UTHSCA), which calculates the mean gray level of the image and multiplies that by the number of pixels in the image.

Six *I. basta* explants displaying regressed tissues were sampled at 12 h. The remaining six explants were sampled at 72 h, when sponges had recovered. Explants were used in all histological and molecular analyses.

Histology

Samples from both regressed and recovered *I. basta* were fixed in 10% formalin with phosphate buffered saline (PBS) for 24 h at 4°C and then transferred to 70% ethanol and stored at −20°C for 4 weeks. Samples were dehydrated through a graded series of ethanol, embedded in paraffin, sectioned to 5 µm and stained using Mayer's Haematoxylin, and Young's Eosin Erythrosin (Bancroft & Stevens, 1990). Samples were viewed and photographed using the Olympus DP12 Microscope Digital Camera System (Melville, NY). All 12 sections examined ($n = 6$ regressed tissue, $n = 6$ recovered tissue) were cut through a similar plane of the sponge sample (longitudinal). Ten fields of view per section were examined to quantify the total number of choanocyte chambers at 400× magnification. For the purpose of this study, choanocyte chambers were defined as a circular ring of cells.

DNA extraction and DGGE

DNA from *I. basta* explants ($n = 6$ regressed tissue, $n = 6$ recovered tissue) was extracted according to manufacturer's protocol with a Power Plant DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene of all samples was amplified by PCR with universal bacterial primers (1055f, 5′-ATGG CTGTCGTCAGCT-3′; 1392r, 5′-ACGGGCGGTGT GTRC-3′) (Ferris et al., 1996). The reverse primer was modified to contain a 40-bp GC clamp (Muyzer et al., 1993). PCRs contained 5 µl of deoxynucleoside triphosphate (2.5 mM), 5 µl of 10× OptiBuffer, 0.15 µl of each primer (100 pmol μl^{-1}), 0.4 µl of bovine serum albumin (BSA; 10 mg ml^{-1}), 3 µl of $MgCl_2$ (50 mM), 0.5 µl of Bio-X-ACT *Taq* polymerase (Bioline, London, United Kingdom), and 1 µl of DNA template. Reactions were made up to 50 µl of total volume with Milli-Q water. The PCR conditions were as follows: 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final elongation at 72°C for 7 min. Fifteen microliters of each sample was added to an 8% (wt/vol) polyacrylamide gel containing a 50–70% denaturing gradient of formamide and urea. The gel was run at 60°C for 16 h in 1× TAE buffer at 75 V using the Ingeny D-Code system, stained with 1× SYBR gold for 10 min, visualized under UV illumination and photographed with the Viber Lourmat ChemiSmart 3000 system.

Statistical analysis

Assumptions of normality were unable to be met, despite transformation (Zar, 1999). Therefore, the non-parametric Kruskal–Wallis test was performed to determine if there was a significant difference in choanocyte chamber numbers between sponges with regressed and non-regressed tissues. Principal component analysis (PCA) using a presence (1)/absence (0) matrix created from the Denaturing Gradient Gel Electrophoresis (DGGE) bands was used to analyze microbial community composition between sponges with regressed and recovered tissue. All analyses were performed using Statistica 8 (StatSoft, 2002).

Results

Less than 12 h after being transferred to the outdoor flow-through aquarium, all six necrotic sponges and 12 healthy explants displayed reductions in tissue volume, where the tissue had contracted to central skeletal fibers leaving subdermal gaps between sponge fibers (Fig. 1a). Within 72 h, sponges had regained the lost tissue volume and recovered the subdermal gaps

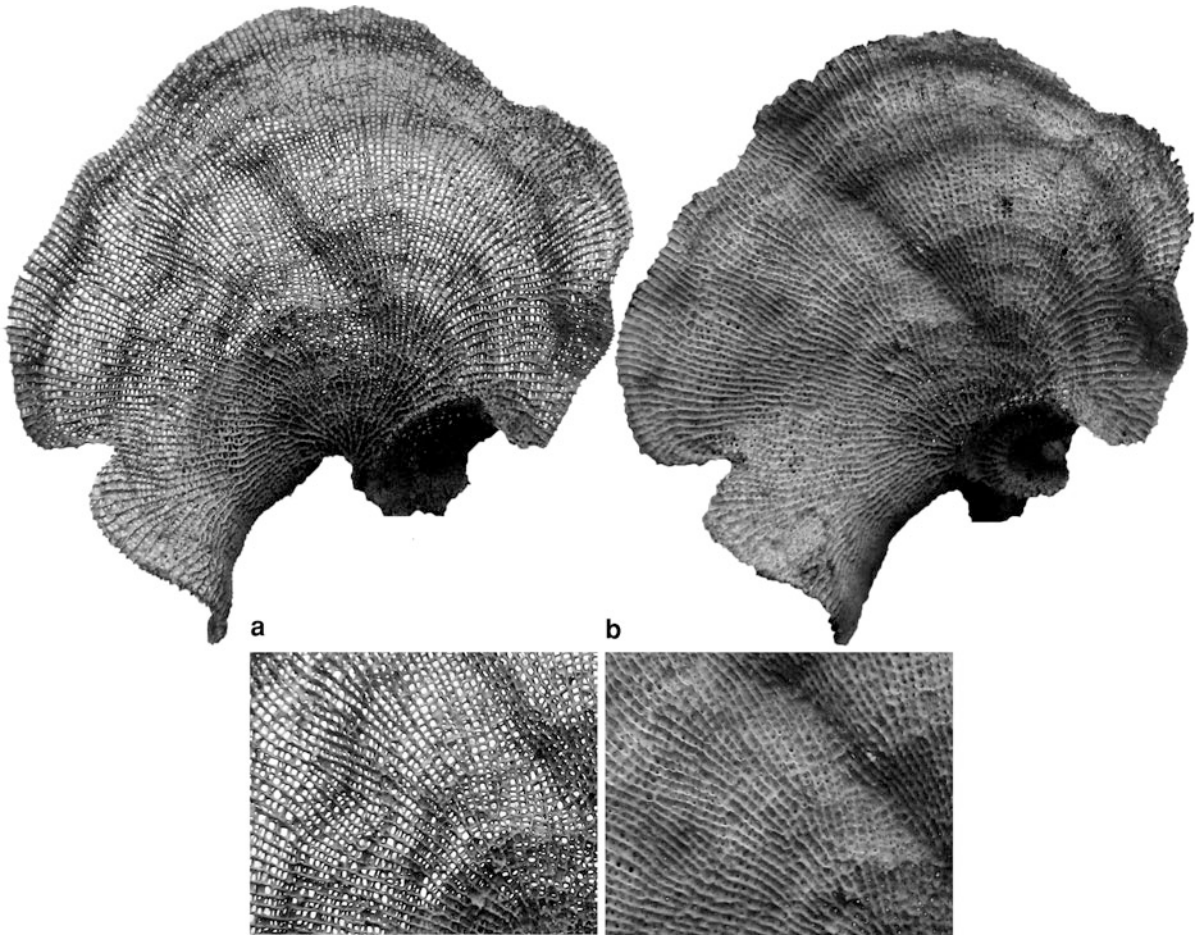

Fig. 1 Rapid tissue recovery exhibited by the same *I. basta* individual in a regressed state at time 0 (**a**) and a recovered state after 72 h (**b**)

between skeletal fibers. The ID of the sponge tissue effectively doubled within 72 h (increasing by 92%), confirming tissue recovery in *I. basta* (Fig. 1a, b).

Histological analysis

Sponges exhibiting regressed tissues had a markedly different histology than sponges with recovered tissues. The arrangement of cells in regressed samples was more compact and densely packed around the sponge fibers than in recovered tissues (Fig. 2a, b). In addition, regressed samples had a larger number of granulated cells (Fig. 2a, b). Sponges with regressed tissue also had significantly fewer apparent choanocyte chambers than samples with recovered tissue ($H = 79.6$, $P = 0.000$). For example, the mean number of choanocyte chambers per field of view in sponges with recovered tissues was 4.5 ($\pm$0.7 SE), compared to 0.4 ($\pm$0.1 SE) in sponges with regressed tissues. In addition, the choanocyte chambers observed in samples with regressed tissues often contained damaged and/or missing cells (Fig. 2b). In contrast, choanocyte chambers of samples with recovered tissues showed little cellular degradation or cell loss (Fig. 2c, d).

DGGE

Denaturing Gradient Gel Electrophoresis analysis comparing regressed and recovered *I. basta* revealed low microbial diversity overall, with only 22 unique bands observed (Fig. 3). Both regressed and recovered samples revealed the presence of three dense bands, which correspond to an *Alphaproteobacteria* (A),

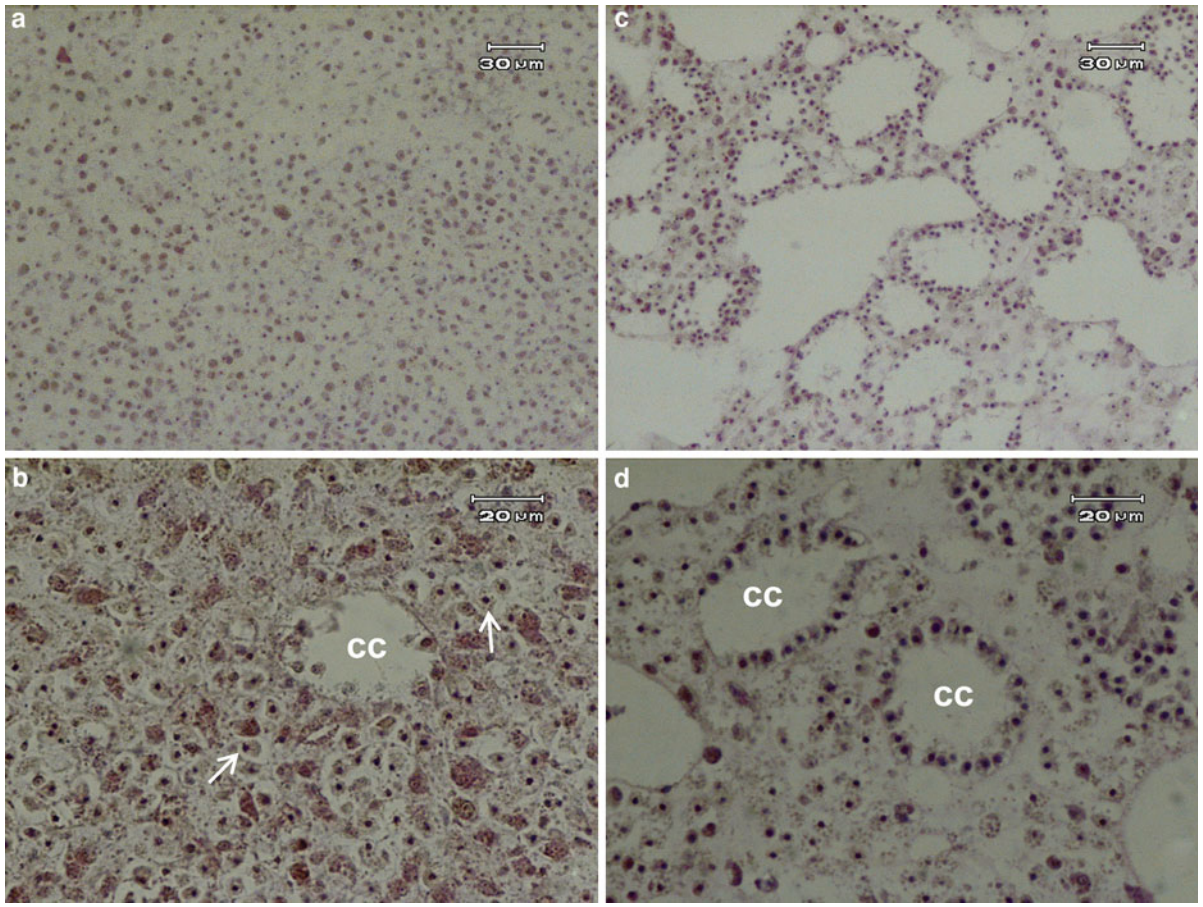

Fig. 2 Histological images of *I. basta* exhibiting regressed (**a**, **b**) and recovered tissues (**c**, **d**). *Arrows* indicate examples of granulated cells and *cc*: choanocyte chamber

Gammaproteobacteria (B), and *Thaumarchaeota* (C) previously reported as stable symbionts of *I. basta* (Luter et al., 2010). Band D (JN388034) is a cyanobacterium with 98% similarity to an uncultured cyanobacterium recovered from cold spring sediment (GQ302542). While, this band appeared brighter in some regressed tissue samples, DGGE is not a quantitative technique and band D had previously been detected at similar intensities in healthy *I. basta* (Luter et al., 2010). Principle Component Analysis based on the DGGE banding patterns of microbial communities between regressed and recovered tissues explained 79% of the variability in the first three factors, with the first two factors explaining 61%. The ordination showed no evidence of different microbial communities between regressed and recovered sponges (Fig. 4). While, most samples grouped close together irrespective of whether they were from regressed or recovered samples, there was a notable grouping of two samples which were distinct from all other samples; however, these represented a member from each of the tissue states. In addition, larger variation was observed between individuals with regressed tissues, evidenced by the increased separation of samples in the ordination, compared to those with recovered tissues (Fig. 4).

Discussion

Ianthella basta specimens in this study displayed notable tissue regression and recovery within a 72 h period. This phenomenon has been reported in other sponge species under adverse conditions (Imsiecke et al., 1996; Böhm et al., 2001; Thoms et al., 2008); however, unlike those studies, no reduction bodies

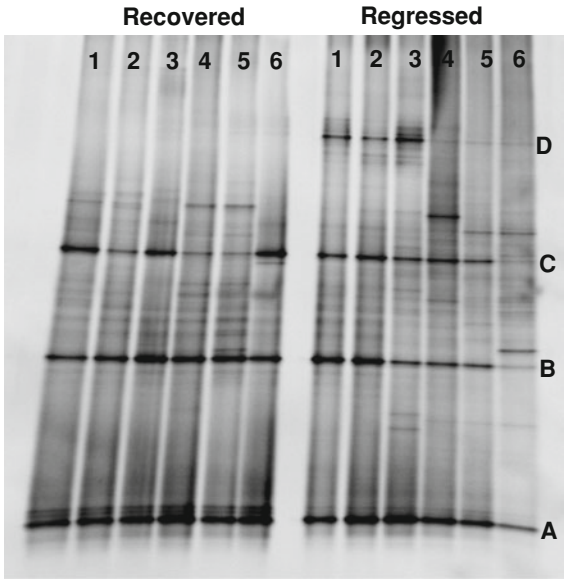

Fig. 3 DGGE image of 16S rRNA-defined bacterial populations from *I. basta* samples with regressed and recovered tissues. Bands labeled *A–C* represent the stable symbionts of *I. basta* previously reported. *Band D* is a cyanobacterium with 98% similarity to an uncultured cyanobacterium recovered from cold spring sediment (GQ302542), which has also been previously identified in healthy *I. basta* samples (Luter et al., 2010)

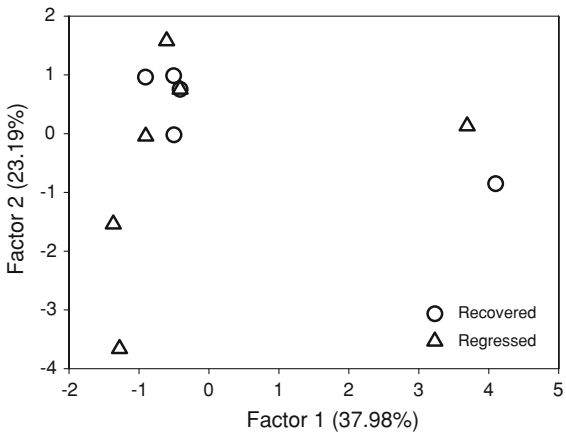

Fig. 4 Principal components analysis (PCA) of *I. basta* microbial community composition, using DGGE banding pattern data to construct a similarity matrix, for recovered and regressed tissues

and/or gemmules were observed in *I. basta*. In fact, no reproductive structures were noted for any of the samples, regardless of their tissue state. This study was consistent with the findings of Thoms et al. (2008) in respect to the absence of choanocyte chambers. *I. basta* with regressed tissues had reduced numbers of apparent choanocyte chambers and more densely packed cells. The loss of sponge tissue and/or cells without the formation of reproductive structures has also been documented in a few cases. For instance, in response to sediment exposure, *Halicohondira panicea* sloughs its outermost tissue layer, which also prevents damaging surface fouling (Barthel & Wolfrath, 1989). Interestingly, *Halisarca caerulea* is capable of constantly renewing its filtering system through the shedding and subsequent proliferation of choanocyte cells (de Goeij et al., 2009). Based on the techniques employed in this study, the mechanism of apparent choanocyte cell loss in *I. basta* cannot be ascertained.

In this study, we propose that *I. basta* may be going into a stress-induced "dormant" state following collection and transportation. A dormant state, characterized by loss of choanocyte chambers, has been reported in *Halichondria bowerbanki* in response to seasonal temperature stress (Hartman, 1958). In this study, temperature was maintained at approximately 28°C, mechanical damage was minimal and sponges were kept from air exposure during collection. However, considering the tissue necrosis and lesions identified before collection, it is likely that these sponges were already stressed and transportation or water quality change from their natural environment may have been sufficient to evoke this tissue regression response. Sponge explants also displayed regressed tissues, yet they had no visible signs of necrosis when collected. This further supports the proposal that handing stress associated with collection and transportation can invoke this stress response. While, in this proposed dormant state, it is possible that there is not an actual loss of cells, but rather a reorganization of the cells. Tanaka & Watanabe (1984) observed the differentiation of archaeocytes into choanocytes and eventually choanocyte chambers. During the early stages of archaeocyte differentiation, cells also appeared more densely packed.

To date, this type of tissue regression and subsequent recovery has yet to be observed in *I. basta* in its natural environment. However, it has been observed in another sponge of the same genus that is commonly found in the same habitat (Whalan, unpublished data). In this instance, all sponges sampled ($n = 30$) in September 2010 for reproductive biology displayed regressed tissue and choanocyte

arrangements consistent with regressed *I. basta*. Further study is required, but given tissue regression coincided with spawning of oocytes, there is a possibility that reproduction (e.g., spawning) may represent a stressful event that contributes to tissue regression.

The three dominant symbionts of *I. basta*, previously reported by Luter et al. (2010), were observed in all samples in this study, regardless of the tissue state. The PCA revealed the microbial community composition of two individuals were different to the remaining 10 sponges, although these samples were not exclusively from sponges displaying regressed tissues. This would suggest that the regressed state of the tissue is not driving the changes in the microbial community nor are microorganisms responsible for the tissue regression. Although, *I. basta* has a low diversity of microbes, the dominant community members (an *Alphaproteobacteria*, *Gammaproteobacteria*, and *Thaumarchaeota*) remain stable through space, time, and differing environmental conditions (Luter et al., 2010; Luter, unpublished data). Conversely, the microbial community of *Aplysinella* sp. was observed to shift under a regressed tissue state, with higher species richness in regressed samples (Thoms et al., 2008). The functional roles of the microbial symbionts in *I. basta* are not yet known but their specificity suggests that they may play a key role in sponge physiology, health, and survival.

Ianthella basta displayed tissue regression and subsequent recovery over the course of 72 h, indicating that tissue regression is a form of stress response in this species. However, the exact mechanisms behind the tissue loss and regeneration in *I. basta* remain unknown. Future research should attempt to observe the regression and recovery process as it is taking place to determine whether any cellular differentiation and/or cell shedding occurs in *I. basta*. It is evident that although sponges are considered "simple" they have the ability to carry out non-neural processes (e.g., contraction), which enable them to cope with various stressors (Nickel, 2004; Leys & Meech, 2006). Regardless of the stimuli responsible for the regression in *I. basta*, their ability to recover demonstrates the resilience capacity of sponges, which will be increasingly important in a changing climate.

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References

Angermeier, H., J. Kamke, U. R. Abdelmohsen, G. Krohne, J. R. Pawlik, N. L. Lindquist & U. Hentschel, 2011. The pathology of sponge orange band disease affecting the Caribbean barrel sponge *Xestospongia muta*. FEMS Microbiology Ecology 75: 218–230.

Ayling, A. L., 1983. Growth and regeneration rates in thinly encrusting Demospongiae from temperate waters. Biological Bulletin 165: 343–352.

Bancroft, J. D. & A. Stevens, 1990. Theory and Practice of Histological Techniques. Churchill Livingstone, Edinburgh.

Barthel, D. & B. Wolfrath, 1989. Tissue sloughing in the sponge *Halichondria panicea*: a fouling organism prevents being fouled. Oecologia 78: 357–360.

Böhm, M., U. Hentschel, A. B. Friedrich, L. Fieseler, R. Steffen, V. Gamulin, C. I. Müller & W. E. G. Müller, 2001. Molecular response of the sponge *Suberites domuncula* to bacterial infection. Marine Biology 139: 1037–1045.

de Goeij, J. M., A. de Kluijver, F. C. van Duyl, J. Vacelet, R. H. Wijffels, A. F. P. M. de Goeij, J. P. M. Cleutjens & B. Schutte, 2009. Cell kinetics of the marine sponge *Halisarca caerulea* reveal rapid cell turnover and shedding. The Journal of Experimental Biology 212: 3892–3900.

Fafanđel, M., W. E. G. Müller & R. Batel, 2003. Molecular response to TBT stress in marine sponge *Suberites domuncula*: proteolytical cleavage and phosphorylation of KRS_SD protein kinase. Journal of Experimental Marine Biology and Ecology 297: 239–252.

Fell, P. E., 1974. Diapause in the gemmules of the marine sponge, *Haliclona oculata*. Biological Bulletin 147: 333–351.

Ferris, M. J., G. Muyzer & D. M. Ward, 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Applied and Environmental Microbiology 62: 340–346.

Francis, J. C., S. Trabanino, R. J. Baerwald & F. W. Harrison, 1990. Analysis of the reduction-regeneration cycle in *Ephydatia fluviatilis* (Porifera: Spongillidae) with scanning electron microscopy. Transactions of the American Microscopical Society 109: 254–264.

Hartman, W. D., 1958. Natural history of the marine sponges of southern New England. Bulletin of the Peabody Museum of Natural History (Yale University) 12: 1–155.

Hentschel, U., J. Hopke, M. Horn, A. B. Friedrich, M. Wagner, J. Hacker & B. S. Moore, 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. Applied and Environmental Microbiology 68: 4431–4440.

Hentschel, U., K. M. Usher & M. W. Taylor, 2006. Marine sponges as microbial fermenters. FEMS Microbiology Ecology 55: 167–177.

Hughes, T. P., A. H. Baird, D. R. Bellwood, M. Card, S. R. Connolly, C. Folke, R. Grosberg, O. Hoegh-Gulberg, J. B. C. Jackson, J. Kleypas, J. Lough, P. Marshall, M. Nystrom, S. R. Palumbi, J. M. Pandolfi, B. Rosen & J. Roughgarden, 2003. Climate change, human impacts, and the resilience of coral reefs. Science 301: 929–933.

Imsiecke, G., J. Munkner, B. Lorenz, N. Bachinski, W. E. G. Müller & H. C. Schroder, 1996. Inorganic polyphosphates in the developing freshwater sponge *Ephydatia muelleri*: effect of stress by polluted waters. Environmental Toxicology and Chemistry 15: 1329–1334.

Knight, P. & P. E. Fell, 1987. Low salinity induces reversible tissue regression in the estuarine sponge *Microciona prolifera* (Ellis & Solander). Journal of Experimental Marine Biology and Ecology 107: 253–261.

Koziol, C., R. Borojevic, R. Steffen & W. E. G. Müller, 1998. Sponges (Porifera) model systems to study the shift from immortal to senescent somatic cells: the telomerase activity in somatic cells. Mechanisms of Ageing and Development 100: 107–120.

Leamon, J. & P. E. Fell, 1990. Upper salinity tolerance of and salinity-induced tissue regression in the estuarine sponge *Microciona prolifera*. Trasactions of the American Microscopical Society 109: 265–272.

Lemoine, N., N. Buell, A. Hill & M. Hill, 2007. Assessing the utility of sponge microbial symbiont communities as models to study global climate change: a case study with *Halichondria bowerbanki*. In Custódio, M. R., G. Lôbo-Hajdu, G. Hajdu & G. Muricy (eds), 7th International Sponge Symposium, Rio de Janeiro: 419–425.

Leys, S. P. & R. W. Meech, 2006. Physiology of coordination in sponges. Canadian Journal of Zoology 84: 288–306.

Leys, S. P., S. A. Nichols & E. D. M. Adams, 2009. Epithelia and integration in sponges. Integrative and Comparative Biology 49: 167–177.

Li, C. W., J. Chen & T. E. Hua, 1998. Precambrian sponges with cellular structures. Science 279: 879–882.

López-Legentil, S., B. Song, S. E. McMurray & J. R. Pawlik, 2008. Bleaching and stress in coral reef ecosystems: hsp70 expression by the giant barrel sponge *Xestospongia muta*. Molecular Ecology 17: 1840–1849.

Louden, D., S. Whalan, E. Evans-Illidge, C. Wolff & R. de Nys, 2007. An assessment of the aquaculture potential of the tropical sponges *Rhopaloeides odorabile* and *Coscinoderma* sp. Aquaculture 270: 57–67.

Luter, H. M., S. Whalan & N. S. Webster, 2010. Exploring the role of microorganisms in the disease-like syndrome affecting the sponge *Ianthella basta*. Applied and Environmental Microbiology 76: 5736–5744.

Meech, R. W., 2008. Non-neural reflexes: sponges and the origins of behavior. Current Biology 18: 70–72.

Müller, W. E. G., 2003. The origin of metazoan complexity: Porifera as integrated animals. Integrative and Comparative Biology 43: 3–10.

Müller, W. E. G., 2006. The stem cell concept in sponges (Porifera): metazoan traits. Seminars in Cell & Developmental Biology 17: 481–491.

Muyzer, G., E. C. de Waal & A. G. Uitterlinden, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59: 695–700.

Nickel, M., 2004. Kinetics and rhythm of body contractions in the sponge *Tethya wilhelma* (Porifera: Demospongiae). The Journal of Experimental Biology 207: 4515–4524.

Olson, J. B., D. J. Gochfeld & M. Slattery, 2006. *Aplysina* red band syndrome: a new threat to Caribbean sponges. Diseases of Aquatic Organisms 71: 163–168.

Rϋtzler, K., 1988. Mangrove sponge disease induced by cyanobacterial symbionts: failure of a primitive immune system? Diseases of Aquatic Organisms 5: 143–149.

Selvin, J., S. Shangmugha Priya, G. Seghal Kiran, T. Thangavelu & N. Sapna Bai, 2009. Sponge-associated marine bacteria as indicators of heavy metal pollution. Microbiological Research 164: 352–363.

Simpson, T. L., 1984. The Cell Biology of Sponges. Springer-Verlag New York Inc., New York, NY.

Srivastava, M., O. Simakov, J. Chapman, B. Fahey, M. A. E. Gauthier, T. Mitros, G. S. Richards, C. Conaco, M. Dacre, U. Hellsten, C. Larroux, N. H. Putnam, M. Stanke, M. Adamska, A. Darling, S. M. Degnan, T. H. Oakley, D. C. Plachetzki, Y. Zhai, M. Adamski, A. Calcino, S. F. Cummins, D. M. Goodstein, C. Harris, D. J. Jackson, S. P. Leys, S. Shu, B. J. Woodcroft, M. Vervoort, K. S. Kosik, G. Manning, B. M. Degnan & D. S. Rokhsar, 2010. The *Amphimedon queenslandica* genome and the evolution of animal complexity. Nature 466: 720–727.

StatSoft I, 2002. STATISTICA (Data Analysis Software System), Version 8. StatSoft, Tulsa.

Tanaka, K. & Y. Watanabe, 1984. Choanocyte differentiation and morphogenesis of choanocyte chambers in the freshwater sponge, *Ephydatia fluviatilis*, after reversal of developmental arrest caused by hydroxyurea. Zoological Science 1: 561–570.

Taylor, M. W., P. J. Schupp, R. de Nys, S. Kjelleberg & P. D. Steinberg, 2005. Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. Environmental Microbiology 7: 419–433.

Taylor, M. W., R. Radax, D. Steger & M. Wagner, 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiology and Molecular Biology Reviews 71: 295–347.

Thoms, C., U. Hentschel, S. Schmitt & P. Schupp, 2008. Rapid tissue reduction and recovery in the sponge *Aplysinella* sp. Marine Biology 156: 141–153.

Vacelet, J. & C. Donadey, 1977. Electron microscope study of the association between some sponges and bacteria. Journal of Experimental Marine Biology and Ecology 30: 301–314.

Webster, N. S., & M. Taylor, 2011. Marine sponges and their microbial symbionts: love and other relationships. Environmental Microbiology. doi:10.1111/j.1462-2920.2011.02460.x.

Webster, N. S., R. I. Webb, M. J. Ridd, R. T. Hill & A. P. Negri, 2001. The effects of copper on the microbial community of a coral reef sponge. Environmental Microbiology 3: 19–31.

Webster, N. S., R. E. Cobb & A. P. Negri, 2008a. Temperature thresholds for bacterial symbiosis with a sponge. ISME Journal 2: 830–842.

Webster, N. S., J. R. Xavier, M. Freckelton, C. A. Motti & R. Cobb, 2008b. Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. Environmental Microbiology 10: 3366–3376.

Wulff, J. L., 2006. Rapid diversity and abundance decline in a Caribbean coral reef sponge community. Biological Conservation 127: 167–176.

Wulff, J., 2010. Regeneration of sponges in ecological context: is regeneration an integral part of life history and morphological strategies? Integrative and Comparative Biology 50: 494–505.

Zar, J. H., 1999. Biostatistical Analysis. Prentice Hall, New Jersey.

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SPONGE RESEARCH DEVELOPMENTS

Nutrient utilisation by shallow water temperate sponges in New Zealand

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Abstract Major nutrients such as phosphate, nitrate, ammonium and silicate, are involved in the metabolic processes of marine organisms. Sponges take up and produce inorganic nutrients and the extent at which they affect the budgets available for other organisms has received little attention. For this reason, we investigated nutrient fluxes for several sponge species in order to estimate whether sponges were net producers or consumers of nutrients from the water column, and how these patterns changed over time. Nutrient fluxes were examined on the south coast of Wellington, New Zealand. For the nutrient analysis (nitrate, nitrite, ammonium, phosphate and silicate), water samples were collected in situ from the inhalant and exhalant water of different sponge species. Samples were analysed both in a multi-species survey and over a two-year period for three other species to determine any temporal changes in fluxes. Our results yielded significant differences in nutrient concentrations between the inhalant and exhalant water for some of the species, but there was no clear pattern associated with the time of year. The levels of dissolved inorganic nutrients in the ambient water varied considerably over the 2-year study period. It is possible that a lack of a clear pattern of nutrient uptake/release of nutrients in some of the study species, and the fact that not all species showed significant uptake/release at different times of the year, may be related to high levels of temporal and spatial variation in the ambient nutrient availability, as well as other temporal fluctuations in parameters, such as water temperature, sponge size, and concentration of food in the water column. Finally, we believe that the activity of specific microbial communities associated with these sponges may be important in explaining the fluxes we have reported.

Keywords Nutrient fluxes · In situ · Sponges · Temporal · Variation · Temperate ecosystem

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Introduction

Marine organisms utilise major nutrients including phosphate, nitrate, ammonium and silicate for metabolic functions. Nitrogen has been intensively studied due to its importance in nutrient fluxes and transformations that occur in the marine environment

(e.g. Zehr & Ward, 2002; Zehr & Kudela, 2011); it influences the trophic biology and ecology of marine organisms, and ultimately their ecological distribution and abundance (Fiore et al., 2010). Nitrogen can potentially be taken up as dissolved inorganic nitrogen (DIN), dissolved organic nitrogen (DON) and particulate organic nitrogen (PON) (Davy et al., 2002). In seawater, there are three main forms of DIN: nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+). The level of DIN in marine environments mainly depends on biological processes that constitute sinks and sources for these nutrients (Valiela, 1984). Nitrate is the most important form of DIN in the oceans, and both ammonium and nitrate are generally considered to be the most important sources of regenerated and newly produced nitrogen for biological processes (Karl, 2002; Suratman et al., 2008). Soluble and PON compounds resulting from decaying organisms, together with nitrogen excreted by plants and animals, are rapidly broken down to ammonia (NH_3) by different types of bacteria, and ammonia is also excreted directly by animals together with urea and peptides (Grasshoff et al., 1999). Understanding ammonium consumption (e.g. by phytoplankton and nitrifying bacteria) and its generation by major food web components is very important, since estimates of the flux of ammonium through food webs from the biomass and metabolic activities of bacteria, phytoplankton and zooplankton suggest that heterotrophic micro-organisms are responsible for most of the ammonium that is regenerated and assimilated by phytoplankton (Koike et al., 1986). Nitrification is the process of ammonium transformation to nitrite and subsequently to nitrate (Schlappy et al., 2010).

Phosphorus (P) is an essential nutrient utilised by all organisms for energy transport and growth (Benitez-Nelson, 2000), and it enters the oceans predominantly through rivers (Benitez-Nelson, 2000). In the ocean, total dissolved phosphorus is partitioned between a dissolved inorganic phosphorus pool (also referred to as soluble reactive phosphorus or phosphate, PO_4^{3-}), and a dissolved organic phosphorus pool (DOP) (Orrett & Karl, 1987). Dissolved nitrate and phosphate are important for ocean productivity; both nutrients become exhausted in the surface layers of the oceans through uptake by phytoplankton, and are vertically transported by upwelling or mixing processes (Michaels et al., 1996; Tyrrell, 1999). Silicate, a dissolved form of silicon (Si), is another major oceanic nutrient and constitutes a fundamental nutrient for diatoms, silicoflagellates, radiolarians and many sponges (De la Rocha et al., 2000; Maldonado et al., 2005). These organisms are silicon biomineralisers that polymerise silica to build skeletons of biogenic silica (BSi) (De la Rocha et al., 2000; Maldonado et al., 2005). Recent studies have provided data suggesting that siliceous sponges play an important role in Si-cycling in diverse marine environments, with substantial contributions to the processes of BSi production and dissolution (Maldonado et al., 2005, 2010).

Suspension-feeders are very important components in marine ecosystems because of the role they play through their filtering activities in controlling and maintaining water quality. Recent studies have demonstrated that sponges with large amounts of sponge-associated bacteria can utilise dissolved organic matter (DOM), but that in these cases this 'food source' supplies the majority of the carbon and energy needs of the sponge (Yahel et al., 2003; de Goeij et al., 2008). Sponges also feed on suspended micro-organisms, such as heterotrophic and photosynthetic bacteria, which represent another source of nutrients, and constitute the >2–5 μm fraction of the planktonic community. These picoplanktonic organisms are involved in the transformation and processing of dissolved inorganic nutrients before they become available to other marine organisms (Azam & Hodson, 1977; Partensky et al., 1999). As a result of sponge feeding and metabolism of particles, sponges excrete dissolved inorganic and organic waste back into the water column, and thus are major contributors to the cycling of essential elements (Dame & Olenin, 2005).

The large biomass of sponges, their high filtration rates, and their associations with micro- and macro-organisms, strongly suggest that they have an important role in the balance and dynamics of carbon and nutrients, and links between the water column and benthos (Diaz & Rutzler, 2001). Hence, in addition to a passing seawater microbial population serving as a food source (Friedrich et al., 2001; Hentschel et al., 2002); sponges harbour large amounts of bacteria in their tissues that can comprise as much as 40% of their biomass (Vacelet, 1975; Vacelet & Donadey, 1977). Various micro-organisms have evolved to reside in sponges, forming sponge–microbe symbioses.

These micro-organisms include cyanobacteria, a diverse range of heterotrophic bacteria, facultative anaerobes and unicellular algae (Hentschel et al., 2002; Taylor et al., 2007; Mohamed et al., 2008). Some studies have suggested that the symbiotic organisms can be a source of energy to sponges (Reiswig 1971, 1975; Ribes et al., 1999a) and that they can contribute to the sponge's' health and nutrition (Mohamed et al., 2010). Indeed, several lines of evidence indicate that some sponges obtain a significant portion of their nutrients from the bacterial symbionts (Wang, 2006).

In this study, we measured five nutrient fluxes: nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_2). These nutrients were measured because they constitute important sinks and sources in the marine environment, and because sponges are involved in the transformation and cycling process of these dissolved inorganic nutrients. We addressed three main questions related to the utilisation of inorganic nutrients by 10 sponge species: (1) Do nutrient concentrations in the water column change over time? (2) Are there differences in the ambient nutrient concentration compared with the exhalant water for different sponge species? and (3) Does the flux identified in (2) show temporal variation for the different sponge species? Water samples were analysed both in a single season multi-species survey and over time for three other species over a 2-year period.

Methods

Study site and species description

Nutrient fluxes of sponge assemblages were examined at two sites located within the Taputeranga Marine Reserve on the south coast of Wellington, New Zealand (Fig. 1). Two nearby (<150 m) reef sites

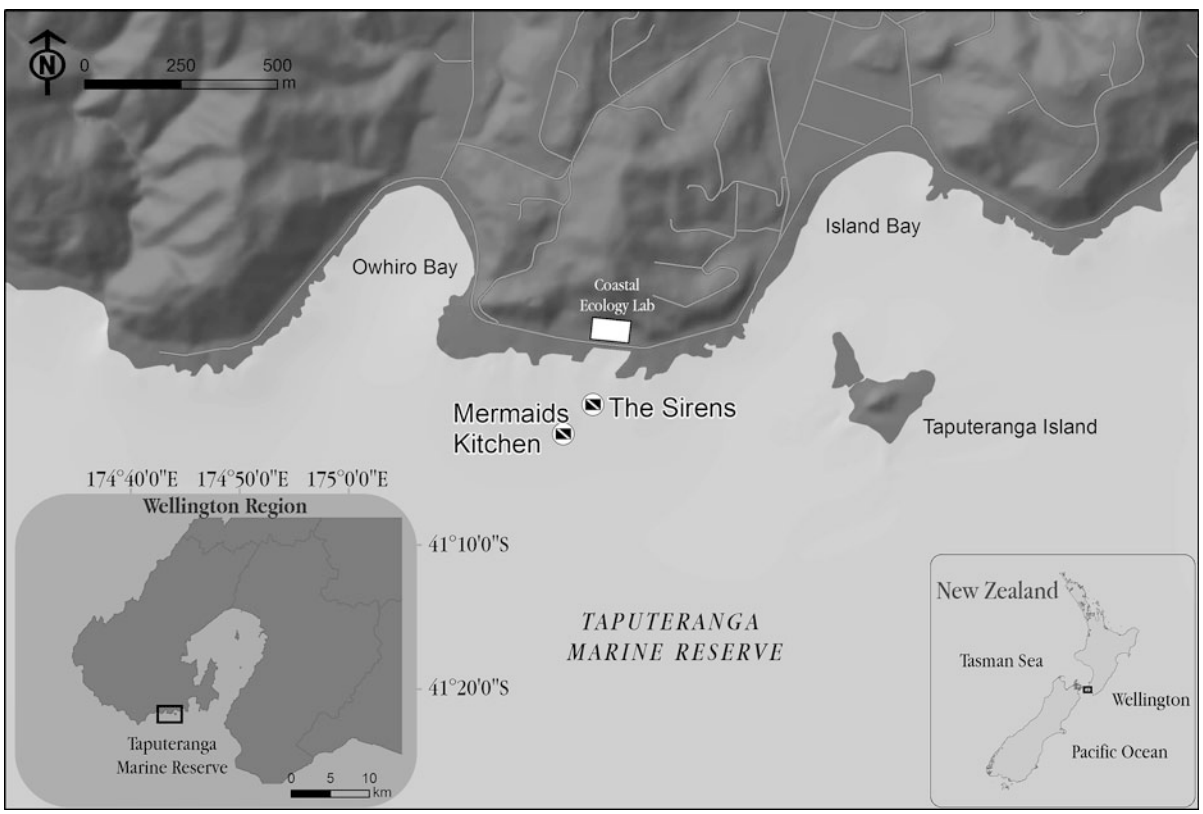

Fig. 1 Map of the south coast of Wellington showing the two study sites, Mermaids Kitchen and The Sirens, within Taputeranga Marine Reserve, Wellington New Zealand. Data sources: New Zealand Territorial Authority data, Wellington Digital Elevation Model image. Projection and Datum: NZMG 1949

were sampled as it was not possible to collect enough samples from a single site; both sites experience very similar environmental conditions. The study area is characterised by being a very dynamic environment where there is a high sponge abundance and diversity, particularly on the sides of channels and crevices, and underneath rocks, boulders and overhangs in the rocky subtidal ecosystems that characterise this coast (Berman et al., 2008). We quantified the nutrient fluxes for seven sponge species in a multi-species survey. These species were *Dysidea* sp., *Haliclona* sp., *Plakina* sp., *Polymastia* sp., *Tethya bergquistae*, *Leucetta* sp., and *Leucosolenia echinata*. In addition, three common and conspicuous demosponges were selected to examine seasonal changes in these fluxes (*Haliclona venustina*, *Strongylacidon* sp., and *Crella incrustans*). All the species were chosen because they are very common in the study area, and their well defined exhalant oscula reduced the risk of sampling error and made sampling in situ easier to conduct.

Dysidea sp. is often found on rock walls or on the sides of channels; *Haliclona* sp. is commonly found on rock walls and overhangs. *Plakina* sp. is a very conspicuous encrusting species found on boulders, rock walls, crevices and overhangs; *Polymastia* sp. is commonly found on rock walls and channels and has a very distinctive papillate surface with an orange-yellow colour, *T. bergquistae* is found in a range of rocky habitats. *L. echinata* is a very common calcareous sponge mostly found on rock walls and crevices in the subtidal zone; *Leucetta* sp. is another calcareous sponge that is widely distributed and mainly found on rock walls and overhangs. The seasonal study species *H. venustina*, *C. incrustans* are *Strogylacidon* sp. are all common on vertical rock walls on the sides of channels.

Sampling

We collected two separate sets of seawater samples: samples for a multi-species survey and samples collected at three different times per year (winter, spring and autumn) over a 2-year period (2009 and 2010). Both surveys were collected across the two sites. It was not possible to collect samples during winter time in the second year due to bad weather conditions. No summer data was included in the present study due to methodological problems encountered with some samples during collection. The multi-species survey was carried out during the high tide of the sampling days in: November 2008, January, February and March 2009 and included the seven sponge species listed above. Due to unfavourable/poor weather conditions that limit dive time in the study area, three sponge specimens from each species were used for this study. All samples were collected in situ using SCUBA from sponge specimens found at 7 to 10 m depth. Ambient and exhalant water samples were collected from three specimens of all the study species using 10-ml sterile plastic syringes with blunt-ended needles. Sample collection consisted of water being slowly drawn (over several minutes) from the inhalant water at a distance of $\sim$3 cm from the sponge ostia, and then from the exhalant water inside the oscular aperture; care was taken to avoid contact with sponge tissue. Immediately after collection, water samples were transferred to 15-ml sterile polypropylene test tubes, kept in the dark, transported on ice and frozen (-20°C) in an upright position until analysis. Samples were not filtered prior to freezing (see Barr & Rees, 2003); for the purposes of this study we assumed that nutrient concentration from the haphazardly sampled osculum was the same for all other oscula for that specimen.

Nutrient analysis

On the day of analysis, samples were thawed at room temperature, with the exception of those for silicate analysis that were kept in the dark at 4°C for 24 h prior to analysis. This was because dissolved silicate polymerises or crystallises during the freezing process; so samples had to be given sufficient time for depolymerisation to occur (Kirkwood, 1994). All glass and plasticware used for the analyses was washed with a phosphate-free detergent and acid rinsed (10% diluted HCl). The precision of each analysis depends on measuring the exact weight of the chemical/reagent to make up the working solutions, so considerable care was taken during this process. The working stock solution, as well as the working standards, were prepared fresh daily. The determination of ammonium is very sensitive to traces of this nutrient in the laboratory, so all the samples were covered with parafilm during the analysis. All samples were measured for inorganic nutrient content with a SAN^{plus} Segmented Flow Analyser following SKALAR methods (Skalar, Breda, The Netherlands) for the determination of

each nutrient. Unfortunately, the seasonal silicate samples could not be measured due to an unresolved technical failure of the machine. An increase or decrease in the exhalant concentration of nutrients (compared to the ambient concentration) was interpreted as the uptake/use or excretion/release, respectively, of that particular nutrient by a sponge.

Data analysis

For each of the seven sponge species from the multi-species survey, we used a paired *t* test to evaluate differences between inhalant and exhalant water nutrient levels (data met assumptions of normality and equal variance). This was done separately for the five different nutrients (NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}, SiO_2), where nutrient concentrations were expressed in μM. The Shapiro–Wilk test was used to examine the assumption of normality. To analyse the samples collected over the 2-year period, we also conducted a paired *t* test where each sponge species was analysed separately for each nutrient per season. Statistical differences were determined at the 5% level and all statistical analyses were conducted by R ver. 2.7.2 (R Development Core Team, 2011).

Results

Changes in nutrient levels in the water column over time

The levels of dissolved inorganic nutrients in the ambient water that was collected from around the sponges (samples averaged from all the samples; $n = 9$) varied considerably over the 2-year study period (Fig. 2). Nitrate concentrations ranged from 0.39 to 1.81 μM (spring and winter of the first year, respectively). Nitrite generally showed very low levels over time (ranging from 0.05 to 0.46 μM); the highest levels of nitrite measured in the ambient water were found in spring of the first year (0.64 ± 0.48 μM). Ammonium levels ranged from 2.08 to 9.50 μM (spring of the first year and autumn of the second year, respectively). Phosphate concentrations remained low during the study period, ranging from 0.25 and 0.34 μM, except in autumn of the second year, when the levels of this nutrient were very high (9.02 μM). The highest concentration of nitrate measured from the ambient water was in winter of the first year (2.07 ± 0.23 μM), and the highest levels of ammonium and phosphate from the ambient water were detected during the autumn of the second year (10.56 ± 1.34 and 10.47 ± 0.79 μM, respectively). The average nutrient concentrations across the 2-year study period in the ambient seawater were: 1.16 μM nitrate, 0.17 μM nitrite, 6.03 μM ammonium and 2.06 μM phosphate (Fig. 2).

Nutrient fluxes for the multi-species survey

The results from the multi-species survey yielded significant differences in the nutrient levels between the ambient and exhalant water for some, though not all, of the species (Table 1). Ambient-exhalant differences were detected in the ammonium levels for *Leucetta* sp., which showed an increase in this

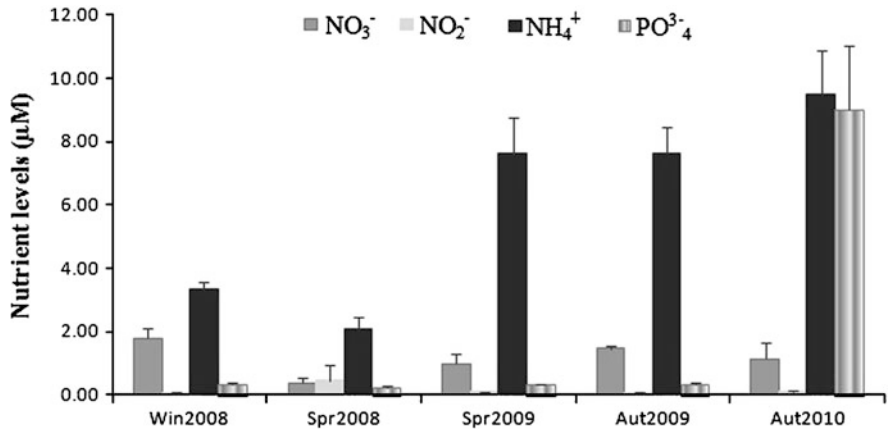

Fig. 2 Seasonal variation in the ambient nutrient levels (μM) over the 2-year study period

Table 1 Nutrient levels of nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_2) in the inhalant and exhalant water of the sponge species

Species	*Inh*	*Exh*	Statistic/paired t test
NO_3^-			
Tethya bergquistae	1.27 ± 0.05	0.48 ± 0.02	$t = 20.57$; $P < 0.01$**
Haliclona sp.	0.59 ± 0.05	0.55 ± 0.07	$t = 2.99$; $P = 0.09$
Plakina sp.	0.64 ± 0.10	0.66 ± 0.04	$t = -0.41$; $P = 0.72$
Dysidea sp.	0.65 ± 0.08	0.69 ± 0.05	$t = -0.49$; $P = 0.66$
Polymastia sp.	1.30 ± 0.05	1.32 ± 0.11	$t = -0.16$; $P = 0.88$
Leucosolenia echinata	0.41 ± 0.05	0.39 ± 0.01	$t = 0.57$; $P = 0.62$
Leucetta sp.	0.42 ± 0.01	0.43 ± 0.03	$t = -0.81$; $P = 0.49$
NO_2^-			
Tethya bergquistae	0.09 ± 0.00	0.13 ± 0.02	$t = -3.86$; $P = 0.06$
Haliclona sp.	0.07 ± 0.01	0.08 ± 0.01	$t = -2.11$; $P = 0.16$
Plakina sp.	0.03 ± 0.01	0.02 ± 0.02	$t = 0.14$; $P = 0.89$
Dysidea sp.	0.02 ± 0.00	0.03 ± 0.02	$t = -0.54$; $P = 0.63$
Polymastia sp.	0.07 ± 0.01	0.12 ± 0.06	$t = -1.16$; $P = 0.36$
Leucosolenia echinata	0.01 ± 0.00	0.01 ± 0.00	$t = -2.61$; $P = 0.12$
Leucetta sp.	0.01 ± 0.00	0.02 ± 0.01	$t = -0.75$; $P = 0.53$
NH_4^+			
Tethya bergquistae	7.33 ± 1.76	9.77 ± 1.72	$t = -2.59$; $P = 0.12$
Haliclona sp.	6.78 ± 0.72	15.99 ± 2.77	$t = -4.57$; $P < 0.05$*
Plakina sp.	5.95 ± 0.34	8.76 ± 0.84	$t = -8.26$; $P < 0.05$*
Dysidea sp.	6.05 ± 0.41	0.44 ± 2.01	$t = -2.36$; $P = 0.14$
Polymastia sp.	5.73 ± 0.93	6.01 ± 1.25	$t = -0.22$; $P = 0.84$
Leucosolenia echinata	6.96 ± 0.42	6.8 ± 0.68	$t = 0.32$; $P = 0.77$
Leucetta sp.	6.06 ± 0.78	8.15 ± 0.88	$t = -5.98$; $P < 0.05$*
PO_4^{3-}			
Tethya bergquistae	0.44 ± 0.01	0.45 ± 0.02	$t = -0.51$; $P = 0.65$
Haliclona sp.	0.05 ± 0.02	1.86 ± 0.58	$t = -5.44$; $P < 0.05$*
Plakina sp.	0.37 ± 0.05	0.39 ± 0.07	$t = -1.77$; $P = 0.21$
Dysidea sp.	0.37 ± 0.00	0.4 ± 0.05	$t = -1.18$; $P = 0.35$
Polymastia sp.	0.82 ± 0.10	0.78 ± 0.04	$t = 0.89$; $P = 0.46$
Leucosolenia echinata	0.56 ± 0.03	0.58 ± 0.01	$t = -0.98$; $P = 0.42$
Leucetta sp.	0.58 ± 0.01	0.57 ± 0.01	$t = 1.66$; $P = 0.23$
SiO_2			
Tethya bergquistae	0.96 ± 0.02	0.92 ± 0.05	$t = 1.51$; $P = 0.26$
Haliclona sp.	1.36 ± 0.01	0.32 ± 0.11	$t = 14.3$; $P < 0.01$**
Plakina sp.	1.32 ± 0.02	1.18 ± 0.01	$t = 8.73$; $P < 0.05$*
Dysidea sp.	0.96 ± 0.03	0.96 ± 0.01	$t = -0.02$; $P = 0.98$
Polymastia sp.	0.93 ± 0.01	0.92 ± 0.01	$t = 1.83$; $P = 0.20$
Leucosolenia echinata	0.70 ± 0.01	0.65 ± 0.03	$t = 1.80$; $P = 0.21$
Leucetta sp.	0.64 ± 0.00	0.61 ± 0.02	$t = 2.69$; $P = 0.11$

Nutrient levels are expressed in μM and data presented are averages (±SD). Results from the paired t test are also shown.
* Significant at the 5% level, df = 2

nutrient in its exhalant water, along with *Plakina* sp. and *Haliclona* sp. In addition, the nitrate levels from the ambient water around *T. bergquistae* decreased. The phosphate levels were higher in the exhalant water from *Haliclona* sp., and silicate levels decreased in the exhalant water from *Haliclona* sp. and *Plakina* sp. (Table 1). There were no further significant differences.

Changes in nutrient fluxes over time

The nutrient fluxes for different sponge species varied with time of year. For *C. incrustans*, the paired *t* test indicated significantly higher nitrite levels in exhalant waters in winter, but lower exhalant levels in spring and autumn of the second year. Also, a significantly higher level of ammonium was detected in the exhalant water compared to ambient water in the spring of the first year. Finally, the analysis for this species showed significantly higher phosphate levels in the exhalant water in spring of the first year and significantly lower exhalant levels in spring of the second year (Fig. 3; Table 2). For *H. venustina*, the paired *t* test indicated significantly higher exhalant levels of nitrate in autumn of the first year and significantly lower exhalant nitrite levels in spring and autumn of the second year. For this species significantly higher exhalant ammonium levels were observed in spring and autumn of the first year. The analysis also showed significantly higher exhalant phosphate levels in spring and autumn of the first year (Fig. 4; Table 3). For *Strongylacidon* sp., the paired *t* test indicated significantly higher exhalant nitrate levels in spring and autumn of the first year. This species also exhibited significantly lower exhalant nitrite levels in spring of the second year and during autumn of both years. Significantly lower exhalant levels of ammonium were detected in winter and significantly higher exhalant levels of ammonium were detected in spring and autumn of the first year. Finally, the analysis for *Strongylacidon* sp. showed significantly lower phosphate levels during winter and significantly higher levels in spring of the second year and autumn of the first year (Fig. 5; Table 4). No other significant differences were observed between ambient and exhalant nutrient levels.

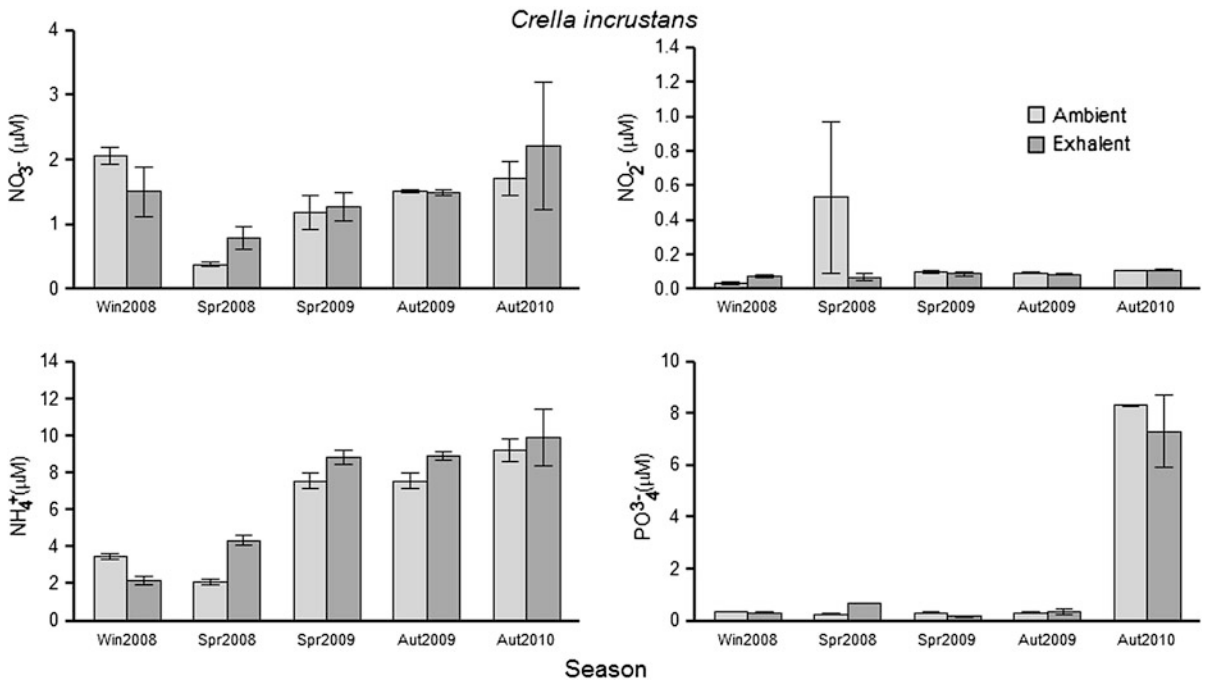

Fig. 3 Dissolved nutrient values (µM) from ambient (inhalant) and exhalant water samples taken from *Crella incrustans* showing increase or decrease (±SD) of each nutrient over time

Table 2 Paired *t* test showing differences between inhalant and exhalant water nutrient levels (μM) for each season and each of the inorganic nutrients studied for *Crella incrustans*

Crella incrustans					
Nutrient	Season	Inh	Exh	*t*	*P*
NO_3^-	Win2008	2.07 ± 0.23	1.51 ± 0.67	1.11	0.38
	Spr2008	0.39 ± 0.05	0.80 ± 0.30	−2.42	0.13
	Spr2009	1.20 ± 0.45	1.28 ± 0.38	−1.94	0.19
	Aut2009	1.52 ± 0.04	1.50 ± 0.06	1.32	0.31
	Aut2010	1.72 ± 0.45	2.22 ± 1.71	−0.68	0.56
NO_2^-	Win2008	0.04 ± 0.01	0.08 ± 0.02	−7.00	$P < 0.05$*
	Spr2008	0.54 ± 0.76	0.07 ± 0.04	1.03	0.40
	Spr2009	0.10 ± 0.01	0.09 ± 0.02	4.58	$P < 0.05$*
	Aut2009	0.09 ± 0.01	0.09 ± 0.00	5.23	$P < 0.05$*
	Aut2010	0.11 ± 0.00	0.11 ± 0.01	−0.19	0.86
NH_4^+	Win2008	3.49 ± 0.23	2.18 ± 0.40	3.58	0.06
	Spr2008	2.11 ± 0.32	4.36 ± 0.42	−11.94	$P < 0.01$**
	Spr2009	7.58 ± 0.76	8.84 ± 0.63	−2.59	0.12
	Aut2009	7.58 ± 0.76	8.94 ± 0.43	−3.81	0.06
	Aut2010	9.24 ± 1.08	9.94 ± 2.69	−0.52	0.64
PO_4^{3-}	Win2008	0.34 ± 0.01	0.31 ± 0.04	1.06	0.39
	Spr2008	0.27 ±0.02	0.69 ± 0.01	−22.03	$P < 0.01$**
	Spr2009	0.33 ± 0.04	0.17 ± 0.03	4.62	$P < 0.05$*
	Aut2009	0.33 ± 0.04	0.36 ± 0.19	−0.30	0.78
	Aut2010	8.34 ± 0.03	7.32 ± 2.43	0.71	0.54

Data presented are averages (±SD). * Significant at the 5% level, df = 2

Discussion

In this study, we treated sponges as a 'black-box' and were interested in the nutrient fluxes for individual sponges rather than total amounts released/taken-up or the mechanisms by which this happens. These will be a focus of future studies. We found significant differences in the uptake and/or release of some of the dissolved nutrients for most of the species in the multi-species survey, and differences in the uptake and release of nutrients were found for the three sponge species studied seasonally across the two-year period. However, no general patterns of uptake/release of a particular nutrient could be detected across all sponge species or time intervals.

Sponges ingest nitrogen from their food and, like many other marine invertebrates they usually excrete ammonium as a metabolic waste product (Brusca & Brusca, 1990). Considering the large amount of food particles (picoplankton) that sponges remove from the water column (Pile, 1997; Ribes et al., 1999a, b; Pile & Young, 2006), a large efflux or release of ammonium was expected. However, only *Haliclona* sp., *Plakina* sp. and *Leucetta* sp. from the multi-species survey had significantly higher exhalant ammonium levels compared to ambient water. From the seasonal study, *C. incrustans* showed a significant release of this nutrient during spring of the first year, *H. venustina* showed a significant release of ammonium during spring and autumn of the first year, while *Strongylacidon* sp. only excreted ammonium during spring and autumn of the first year. Photosynthetically active micro-organisms, such as cyanobacteria and eukaryotic algae, in addition to other micro-organisms, are often located in the outer-light-exposed tissue layers of sponges (Rüztler, 1985; Wilkinson, 1992); we believe the activity of these organisms may be important in explaining the fluxes we have reported. The lack of ammonium excretion by some of our study species, or at certain times of

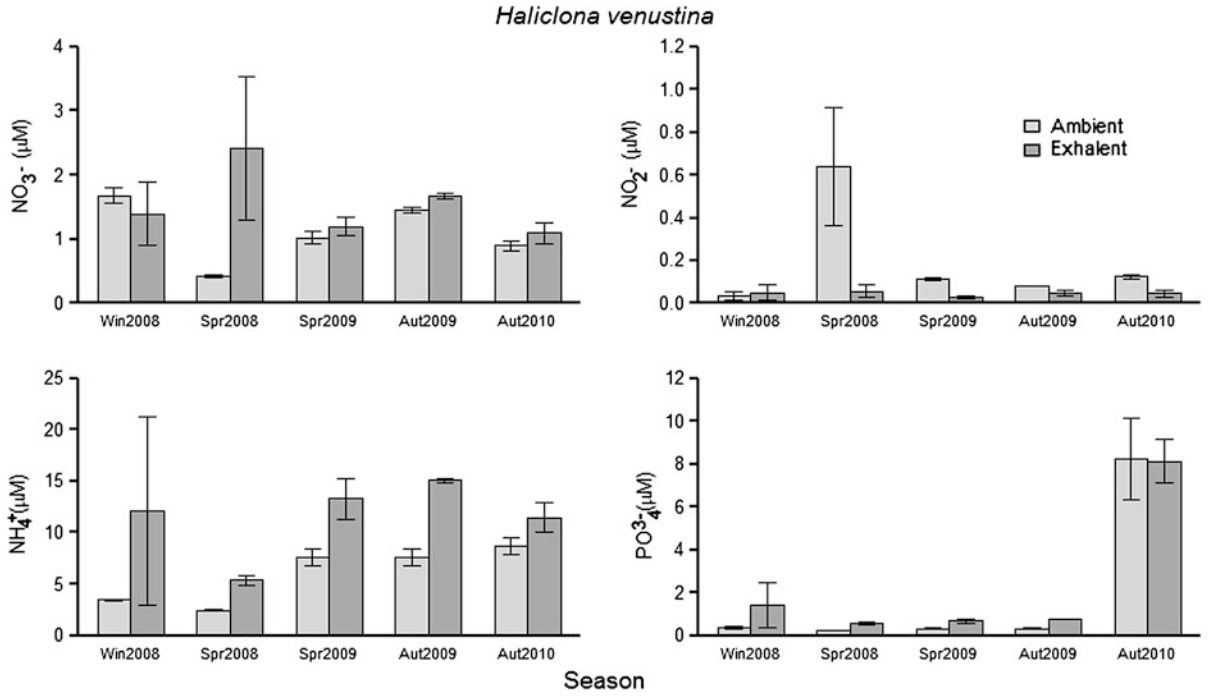

Fig. 4 Dissolved nutrient values (µM) from ambient (inhalant) and exhalant water samples taken from *Haliclona venustina* showing increase or decrease (±SD) of each nutrient over time

Table 3 Paired t test showing differences between inhalant and exhalant water nutrient levels (µM) for each season and each of the inorganic nutrients studied for *Haliclona venustina*

Haliclona venustina					
Nutrient	Season	Inh	Exh	*t*	*P*
NO_3^-	Win2008	1.68 ± 0.20	1.39 ± 0.85	0.51	0.65
	Spr2008	0.42 ± 0.03	2.42 ± 1.94	−1.75	0.22
	Spr2009	1.02 ± 0.17	1.20 ± 0.24	−2.01	0.18
	Aut2009	1.45 ± 0.07	1.67 ± 0.08	−4.92	$P < 0.05$*
	Aut2010	0.90 ± 0.14	1.09 ± 0.28	−2.43	0.13
NO_2^-	Win2008	0.04 ± 0.03	0.05 ± 0.06	−0.84	0.48
	Spr2008	0.64 ± 0.48	0.06 ± 0.05	2.20	0.15
	Spr2009	0.12 ± 0.01	0.03 ± 0.01	88.46	$P < 0.001$***
	Aut2009	0.08 ± 0.00	0.05 ± 0.02	2.32	0.14
	Aut2010	0.12 ± 0.01	0.05 ± 0.03	10.35	$P < 0.01$**
NH_4^+	Win2008	3.45 ± 0.06	12.12 ± 15.93	−0.93	0.44
	Spr2008	2.44 ± 0.13	5.37 ± 0.80	−6.74	$P < 0.05$*
	Spr2009	7.55 ± 1.42	13.32 ± 3.42	−2.65	0.11
	Aut2009	7.55 ± 1.42	15.08 ± 0.37	−7.46	$P < 0.05$*
	Aut2010	8.69 ± 1.33	11.49 ± 2.46	−3.17	0.08
PO_4^{3-}	Win2008	0.37 ± 0.14	1.43 ± 1.80	−1.00	0.42
	Spr2008	0.24 ± 0.01	0.59 ± 0.11	−5.48	$P < 0.05$*
	Spr2009	0.32 ± 0.03	0.67 ± 0.17	−3.78	0.06
	Aut2009	0.32 ± 0.03	0.77 ± 0.01	−20.59	$P < 0.01$**
	Aut2010	8.27 ± 3.32	8.14 ± 1.78	0.04	0.969

Data presented are averages (±SD). * Significant at the 5% level, df = 2

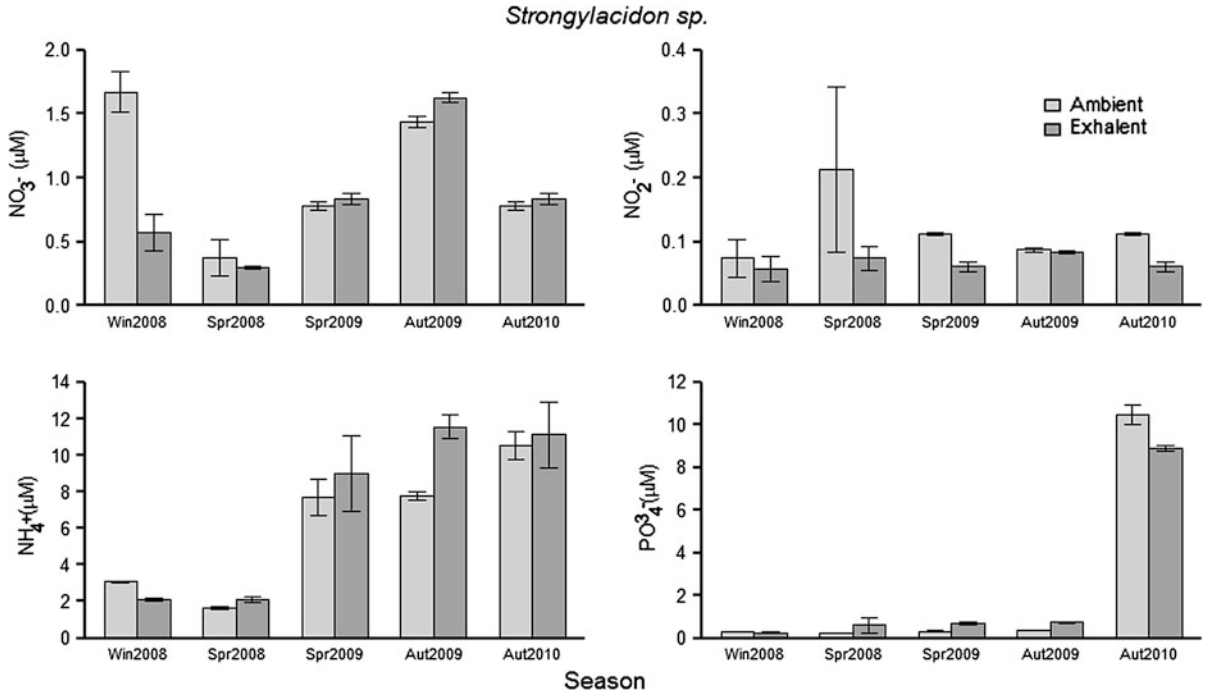

Fig. 5 Dissolved nutrient values (µM) from ambient (inhalant) and exhalant water samples taken from *Strongylacidon* sp. showing increase or decrease (±SD) of each nutrient over time

Table 4 Paired *t* test showing differences between inhalant and exhalant water nutrient levels (µM) for each season and each of the inorganic nutrients studied for *Strongylacidon* sp.

Strongylacidon sp.					
Nutrient	Season	Inh	Exh	*t*	*P*
NO_3^-	Win2008	1.67 ± 0.28	0.58 ± 0.25	52.26	$P < 0.001$***
	Spr2008	0.38 ± 0.25	0.30 ± 0.02	0.47	0.67
	Spr2009	0.79 ± 0.06	0.84 ± 0.08	−1.73	0.22
	Aut2009	1.44 ± 0.08	1.63 ± 0.06	−6.88	$P < 0.05$*
	Aut2010	0.79 ± 0.06	0.84 ± 0.08	−1.14	0.36
NO_2^-	Win2008	0.07 ± 0.05	0.06 ± 0.03	0.32	0.77
	Spr2008	0.21 ± 0.22	0.07 ± 0.03	0.94	0.44
	Spr2009	0.11 ± 0.00	0.06 ± 0.01	7.91	$P < 0.05$*
	Aut2009	0.09 ± 0.01	0.08 ± 0.00	6.30	$P < 0.05$*
	Aut2010	0.11 ± 0.00	0.06 ± 0.01	6.03	$P < 0.05$*
NH_4^+	Win2008	3.09 ± 0.09	2.09 ± 0.15	10.43	$P < 0.01$**
	Spr2008	1.67 ± 0.13	2.09 ± 0.24	−4.25	$P = 0.05$*
	Spr2009	7.69 ± 1.73	9.01 ± 3.64	−0.45	0.69
	Aut2009	7.80 ± 0.37	11.56 ± 1.11	−5.65	$P < 0.05$*
	Aut2010	10.56 ± 1.34	11.14 ± 3.10	−0.24	0.83
PO_4^{3-}	Win2008	0.31 ± 0.01	0.25 ± 0.02	5.14	$P < 0.05$*
	Spr2008	0.24 ± 0.02	0.62 ± 0.62	−1.02	0.41
	Spr2009	0.33 ± 0.04	0.68 ± 0.14	−4.38	$P < 0.05$*
	Aut2009	0.38 ± 0.02	0.74 ± 0.04	−19.40	$P < 0.01$**
	Aut2010	10.47 ± 0.79	8.91 ± 0.27	3.61	0.06

Data presented are averages (±SD). * Significant at the 5% level, df = 2

year, could be indicative of different microbial populations in different sponge species and/or changes in the activities of these microbial consortia in response to changes in the environment (e.g. seasonal temperature). In those cases where no ammonium release was detected, it may be that these associated microbes are taking up all the waste ammonium and either assimilating it or converting it to nitrite/nitrate. However, we did not find evidence of release of nitrite/nitrate for species that did not produce ammonium. This is perhaps surprising and it is not currently possible for us to explain this result without further consideration of the specific microbial communities associated with these sponges.

Tethya bergquistae and *Strongylacidon* sp. were the species that showed lower nitrate levels in the exhalant water compared to the inhalant water. Again this is likely to be explained by the activity of microbes assimilating this nutrient. *C. incrustans* was the only species that exhibited release of nitrite. Given that bacteria and archaea are the only organisms able to oxidise ammonium (Francis et al., 2005; You et al., 2009); the significant excretion of nitrite by *C. incrustans* suggests that this species may harbour micro-organisms that are responsible for the nitrite flux observed (Jiménez & Ribes, 2007). However, it remains to be elucidated if the three species mentioned above are photosynthetic sponges or not, although there are studies that have determined the presence of phototrophic micro-organisms in other sponges (see Gaino et al., 2006; Lemloh et al., 2009; Sipkema & Blanch, 2010). In contrast, *H. venustina* released nitrate during autumn of the first year, while *Strongylacidon* sp. released nitrate in autumn of the first year indicating the presence of nitrifying bacteria. This release of nitrate and nitrite can be interpreted as evidence that these sponges may be active sites of nitrification (Corredor et al., 1988; Díaz & Ward, 1997). Nitrite levels were lower in the exhalant water of *C. incrustans*, *H. venustina*, and *Strongylacidon* sp. at different times of the year (spring and autumn 2009 and autumn 2010), while *Strongylacidon* sp. released nitrate during autumn of the first year. In this case, it is possible that nitrite might be removed and used by nitrifying bacteria to produce nitrate. While it is possible to explain the lack of differences in N levels between inhalant and exhalant water by sponges stopping pumping, which in turn may have reduced the oxygen concentrations in the sponge creating an anaerobic environment suitable for denitrification (see Hoffmann et al., 2009; Schlappy et al., 2010), this is unlikely. We have conducted extensive measurements of exhalant flow rates for most of these species with no evidence of regular pumping inactivation (Perea-Blázquez 2011).

Symbiotic nitrifying bacteria have been shown to metabolise ammonium excreted by sponges and other symbionts by oxidizing it to nitrate (Corredor et al., 1988). In a recent study, Mohamed et al. (2010) demonstrated that nitrification in sponges is a metabolic capacity of the sponges' symbionts. In this study the symbiotic associations were not examined; nevertheless, the significant uptake and release of DIN compounds, and especially nitrate and nitrite, by some of the study species likely indicate microbial activity inside the sponges.

Our analyses detected silicate uptake only for *Plakina* sp. and *Haliclona* sp., which is perhaps surprising. It is, however, important to mention that our samples were not filtered (to remove diatoms from seawater) prior to storage, therefore it is possible that our silicate analyses failed to detect silicate uptake by other species that are heavily skeletonised sponges such as *Tethya* sp. and *Polymastia* sp., because the amount they consumed was very low as to compensate the silicate increase in the stored water samples resulting from diatom dissolution. Very little is known about silicate uptake rates and retention by sponges, although a recent study showed that the retention by a dense bathyal population of hexatinellid sponges was substantial, and that silicate retention by a dense temperate sponge population was far from negligible (Maldonado et al., 2005). One of our aims was to examine the natural fluctuations in silica in the water column over time. However, due to complications with the auto-analyser (see "Methods" section), this analysis could not be performed. Nevertheless, the results obtained from the multi-species survey suggest that some of the species are using silicate, which is most likely to be for spicule synthesis. As expected the calcareous sponges did not show evidence of silica uptake.

There were no general patterns in the changes in nutrient levels in the water column (ambient water) over time. For phosphate, the levels of this nutrient in the water column were significantly higher in autumn of the second year than at other times. Also levels of ammonia in the water column were significantly lower

during the spring and winter of the first year and significantly higher during autumn of the second year, while the levels of nitrate in the water column were significantly lower during spring of the first year than at other times. It is possible that a lack of a clear pattern of nutrient uptake/release of nutrients in some of the study species, and the fact that not all species showed significant uptake/release at different times of the year, may be related to high levels of temporal and spatial variation in the ambient nutrient availability, as well as other temporal fluctuations in parameters such as water temperature, sponge size, concentration of food and oxygen in the water column (Ayling, 1983; Cloern, 1996; Ribes, et al. 1999b).

To conclude, the nitrate and nitrite excretion fluxes reported here for some sponge species, suggest organic nitrogen mineralisation by the sponge's metabolism and the presence of micro-organisms inside the sponges that may play a role in nitrification (Díaz & Ward, 1997). However, further research is needed to examine the putative symbiotic associations using microscopy and molecular techniques in order to verify their presence. Other studies have suggested that temperate sponges are important nitrifiers in other regions. If the sponge species studied here do harbour microbial symbionts, it is likely that they too might be nitrifiers, and therefore contribute to nutrient recycling in temperate subtidal rocky reefs of New Zealand.

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References

Ayling, A. L., 1983. Factors affecting the spatial distributions of thinly encrusting sponges from temperate waters. Oecologia 60: 412–418.

Azam, F. & R. E. Hodson, 1977. Size distribution and activity of marine microheterotrophs. Limnology and Oceanography 22: 492–501.

Barr, N. G. & T. A. Rees, 2003. Nitrogen status and metabolism in the green seaweed *Enteromorpha intestinalis*: an examination of three natural populations. Marine Ecology-Progress Series 249: 133–144.

Benitez-Nelson, C. R., 2000. The biogeochemical cycling of phosphorus in marine systems. Earth Science Reviews 51: 109–135.

Berman, J., A. Perea-Blázquez, M. Kelly & J. Bell, 2008. Sponges of the Wellington south coast. In Gardner, J. & J. Bell (eds), The Taputeranga Marine Reserve. First Edition Ltd, Wellington.

Brusca, R. C. & G. J. Brusca, 1990. Phylum Porifera: The Sponges. Sinauer Press, Sunderland.

Cloern, J. E., 1996. Phytoplankton bloom dynamics in coastal ecosystems: a review with some general lessons from sustained investigation of San Francisco Bay, California. Reviews of Geophysics 34: 127–168.

Corredor, J. E., C. R. Wilkinson, V. P. Vicente, J. M. Morell & E. Otero, 1988. Nitrate release by Caribbean reef sponges. Limnology and Oceanography 33: 114–120.

Dame, R. F. & S. Olenin, 2005. Comparative Roles of Suspension-Feeders in Ecosystems. Kluwer Academic Publishers, Dordrecht.

Davy, S. K., D. A. Trautman, M. A. Borowitzka & R. Hinde, 2002. Ammonium excretion by a symbiotic sponge supplies the nitrogen requirements of its rhodophyte partner. The Journal of Experimental Biology 205: 3505–3511.

de Goeij, J. M., L. Moodley, M. Houtekamer, N. M. Carballeira & F. C. van Duyl, 2008. Tracing C-13-enriched dissolved and particulate organic carbon in the bacteria-containing coral reef sponge *Halisarca caerulea*: evidence for DOM feeding. Limnology and Oceanography 53: 1376–1386.

De la Rocha, C. L., M. A. Brzezinski & M. J. DeNiro, 2000. A first look at the distribution of the stable isotopes of silicon in natural waters. Geochimica Et Cosmochimica Acta 64: 2467–2477.

Diaz, M. C. & K. Rutzler, 2001. Sponges: an essential component of Caribbean coral reefs. Bulletin of Marine Science 69: 535–546.

Díaz, M. C. & B. B. Ward, 1997. Sponge-mediated nitrification in tropical benthic communities. Marine Ecology Progress Series 156: 97–107.

Fiore, C. L., J. K. Jarett, N. D. Olson & M. P. Lesser, 2010. Nitrogen fixation and nitrogen transformations in marine symbioses. Trends in Microbiology 18: 455–463.

Francis, C. A., K. J. Roberts, J. M. Beman, A. E. Santoro & B. B. Oakley, 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proceedings of the National Academy of Sciences of the United States of America 102: 14683–14688.

Friedrich, A. B., I. Fischer, P. Proksch, J. Hacker & U. Hentschel, 2001. Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. FEMS Microbiology Ecology 38: 105–113.

Gaino, E., M. Sciscioli, E. Lepore, M. Rebora & G. Corriero, 2006. Association of the sponge *Tethya* orphei (Porifera, Demospongiae) with filamentous cyanobacteria. Invertebrate Biology 125: 281–287.

Grasshoff, K., M. Ehrhardt, K. Kremling & L. G. Anderson, 1999. Methods of Seawater Analysis. Wiley, VCH.

Hentschel, U., J. Hopke, M. Horn, A. B. Friedrich, M. Wagner, J. Hacker & B. S. Moore, 2002. Molecular evidence for a

uniform microbial community in sponges from different oceans. Applied and Environmental Microbiology 68: 4431–4440.

Hoffmann, F., R. Radax, D. Woebken, M. Holtappels, G. Lavik, H. T. Rapp, et al., 2009. Complex nitrogen cycling in the sponge *Geodia barretti*. Environmental Microbiology 11: 2228–2243.

Jiménez, E. & M. Ribes, 2007. Sponges as a source of dissolved inorganic nitrogen: nitrification mediated by temperate sponges. Limnology and Oceanography 52: 948–958.

Karl, D. M., 2002. Nutrient dynamics in the deep blue sea. Trends in Microbiology 10: 410–418.

Kirkwood, D. S., 1994. The SANplus Segmented Flow Analyzer. Seawater Analysis. Skalar Analytical B.V., Breda: 51.

Koike, I., O. Holm-Hansen & D. C. Biggs, 1986. Inorganic nitrogen metabolism by Antarctic phytoplankton with special reference to ammonium cycling. Marine Ecology Progress Series 30: 105–116.

Lemloh, M.-L., J. Fromont, F. Brummer & K. M. Usher, 2009. Diversity and abundance of photosynthetic sponges in temperate Western Australia. BMC Ecol 9: 4.

Maldonado, M., C. Carmona, Z. Velasquez, A. Puig, A. Cruzado, A. Lopez & C. M. Young, 2005. Siliceous sponges as a silicon sink: an overlooked aspect of benthopelagic coupling in the marine silicon cycle. Limnology and Oceanography 50: 799–809.

Maldonado, M., A. Riesgo, A. Bucci & K. Rüztler, 2010. Revisiting silicon budgets at a tropical continental shelf: Silica standing stocks in sponges surpass those in diatoms. Limnology and Oceanography 55: 2001–2010.

Michaels, A. F., D. Olson, J. L. Sarmiento, J. W. Ammerman, K. Fanning, R. Jahnke, A. H. Knap, F. Lipschultz & J. M. Prospero, 1996. Inputs, losses and transformations of nitrogen and phosphorus in the pelagic North Atlantic Ocean. Biogeochemistry 35: 181–226.

Mohamed, N. M., A. S. Colman, Y. Tal & R. T. Hill, 2008. Diversity and expression of nitrogen fixation genes in bacterial symbionts of marine sponges. Environmental Microbiology 10: 2910–2921.

Mohamed, N. M., K. Saito, Y. Tal & R. T. Hill, 2010. Diversity of aerobic and anaerobic ammonia-oxidizing bacteria in marine sponges. ISME Journal 4: 38–48.

Orrett, K. & D. M. Karl, 1987. Dissolved organic phosphorus production in surface seawaters. Limnology and Oceanography 32: 383–395.

Partensky, F., J. Blanchot & D. Vaulot, 1999. Differential distribution and ecology of Prochlorococcus and Synechococcus in oceanic waters: A review. Bulletin de l'Institut Oceanographique (Monaco) 0: 457–475.

Perea-Blázquez A (2011) Interactions between Sponges and the Water Column: Nutrient Utilisation and Feeding By New Zealand Subtidal Sponges. PhD Dissertation. School of Biological Sciences. Victoria University of Wellington, New Zealand.

Pile, A. J., 1997. Finding Reiswig's missing carbon: Quantification of sponge feeding using dual-beam flow cytometry. In: Proceedings of the 8th international Coral Reef Symposium, pp. 1403–1410.

Pile, A. J. & C. M. Young, 2006. The natural diet of a hexactinellid sponge: Benthic-pelagic coupling in a deep-sea microbial food web. Deep-Sea Research Part I-Oceanographic Research Papers 53: 1148–1156.

R Development Core Team, 2011. R: A language and environment for statistical computing. Vienna, Austria. http://www.R-project.org.

Reiswig, H. M., 1971. Particle feeding in natural population of 3 marine demosponges. Biological Bulletin 141: 568–591.

Reiswig, H. M., 1975. Bacteria as food for temperate-water marine sponges. Canadian Journal of Zoology-Revue Canadienne De Zoologie 53: 582–589.

Ribes, M., R. Coma & J. M. Gili, 1999a. Natural diet and grazing rate of the temperate sponge *Dysidea avara* (Demospongiae, Dendroceratida) throughout an annual cycle. Marine Ecology Progress Series 176: 179–190.

Ribes, M., R. Coma & J. M. Gill, 1999b. Seasonal variation of particulate organic carbon, dissolved organic carbon and the contribution of microbial communities to the live particulate organic carbon in a shallow near-bottom ecosystem at the Northwestern Mediterranean Sea. Journal of Plankton Research 21: 1077–1100.

Rüztler, K., 1985. Associations between Caribbean sponges and photosynthetic organisms. In Rüztler, K. (ed.), New Perspectives in Sponge Biology. Smithsonian Institution Press, Washington.

Schlappy, M. L., S. I. Schottner, G. Lavik, M. M. M. Kuypers, D. de Beer & F. Hoffmann, 2010. Evidence of nitrification and denitrification in high and low microbial abundance sponges. Marine Biology 157: 593–602.

Sipkema, D. & H. W. Blanch, 2010. Spatial distribution of bacteria associated with the marine sponge *Tethya* californiana. Marine Biology 157: 627–638.

Suratman, S., T. Jickells, K. Weston & L. Fernand, 2008. Seasonal variability of inorganic and organic nitrogen in the North Sea. Hydrobiologia 610: 83–98.

Taylor, M. W., R. Radax, D. Steger & M. Wagner, 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiology and Molecular Biology Reviews 71: 295–347.

Tyrrell, T., 1999. The relative influences of nitrogen and phosphorus on oceanic primary production. Nature 400: 525–531.

Vacelet, J., 1975. Electron-microscope study of association between bacteria and sponges of genus Verongia (Dictyoceratida). Journal De Microscopie Et De Biologie Cellulaire 23: 271–288.

Vacelet, J. & C. Donadey, 1977. Electron-microscope study of association between some sponges and bacteria. Journal of Experimental Marine Biology and Ecology 30: 301–314.

Valiela, I., 1984. Marine Ecological Processes. Springer-Verlag, New York.

Wang, G. Y., 2006. Diversity and biotechnological potential of the sponge-associated microbial consortia. Journal of Industrial Microbiology & Biotechnology 33: 545–551.

Wilkinson, C. R., 1992. Symbiotic interactions between marine sponges and algae. In Reisser, W. (ed.), Algae and Symbioses: Plants, Animals, Fungi, Viruses, Interactions Explored. Biopress Ltd, Bristol.

Yahel, G., J. H. Sharp, D. Marie, C. Hase & A. Genin, 2003. In situ feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei*: bulk DOC is the major source for carbon. Limnology and Oceanography 48: 141–149.

You, J., A. Das, E. M. Dolan & Z. Q. Hu, 2009. Ammonia-oxidizing archaea involved in nitrogen removal. Water Research 43: 1801–1809.

Zehr, J. P. & R. M. Kudela, 2011. Nitrogen cycle of the open ocean: from genes to ecosystems. Annual Review of Marine Science 3: 197–225.

Zehr, J. P. & B. B. Ward, 2002. Nitrogen cycling in the ocean: new perspectives on processes and paradigms. Applied and Environmental Microbiology 68: 1015–1024.

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SPONGE RESEARCH DEVELOPMENTS

Experimental silicon demand by the sponge *Hymeniacidon perlevis* reveals chronic limitation in field populations

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Abstract Dissolved silicon (DSi) is a key marine nutrient. Sponges and diatoms are relevant DSi consumers, but sponges appear to have a less efficient uptake system that requires higher ambient DSI concentrations for maximum uptake. We experimentally tested whether a sponge adapted to live at the intertidal (*Hymeniacidon perlevis*) also shows such a need for high DSi. Under laboratory conditions, sponges were exposed to both the natural DSi concentration (10 μM) and much higher levels (25, 40, and 70 μM) for 36 h, being water samples taken at 6 h intervals to infer DSi uptake. Uptake rates shifted over time (particularly in high DSi treatments) and showed moderate inter-individual variability. Average DSi uptake rate at 70 μM was twice higher than those at 40 and 25 μM, which in turn were not significantly different from each other, but were twice higher than the uptake rate at 10 μM. Therefore, *H. perlevis* needs, for efficient uptake, ambient DSi concentrations two to four times higher than the maximum available in its natural habitat. From an eco-physiological point of view, it means that the skeletal growth in the populations of *H. perlevis* is chronically limited by DSi availability, a limitation that may favor sponge evolution toward skeletal slimming.

Keywords Silicate · Sponge · Porifera · Nutrient limitation · Benthic-pelagic coupling

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Introduction

Silicic acid, a biologically assimilable form of dissolved silicon (DSi), is an important ocean nutrient, being strongly involved in the control of primary production (e.g., Sarmiento & Gruber, 2006). Recent studies have suggested the idea that siliceous sponges are relevant DSi users at a large ecological scale, a role traditionally neglected by nutrient ecologists and biogeochemists and that is biasing the advance toward a realistic understanding of silicon cycling in marine systems, and particularly on continental margins

(Reincke & Barthel, 1997; Maldonado et al., 2005, 2010a, 2011; Chu et al., 2011).

One of the problems currently preventing an adequate assessment of the ecological implications of DSi utilization by sponges is the scarcity of data relative to their uptake rate. Studies are available only for the seasonal populations of the North-Atlantic demosponge *Halichondria panicea* (Fröhlich & Barthel, 1997; Reincke & Barthel, 1997), and for some sublittoral species in the genus *Axinella* (Maldonado et al., 2011). The scarce available evidence suggests that sponges appear to have a less efficient uptake system than that of diatoms, because it requires much higher ambient DSI concentrations for maximum uptake. To contribute to this issue, we have experimentally tested whether individuals of a common demosponge—*Hymeniacidon perlevis* (Montagu, 1818)—adapted to live at the intertidal zone also show such a need for high DSi.

Materials and methods

The studied species

Laboratory experiments were conducted on individuals of the halichondrid *Hymeniacidon perlevis*, a nearly cosmopolitan species, the geographical distribution of which may require further reconsideration with the help of molecular techniques for accurate discrimination of potential cryptic species and misidentifications. Previous studies on *H. perlevis* have suggested that this sponge is extremely plastic, being able to grow at either the intertidal or within the sublittoral, to feed on a wide variety of particulate sources, and to heal wounds and regenerate from small pieces at extremely rapid rates (Fu et al., 2006, 2007; Maldonado et al., 2010b). Individuals used in the laboratory experiments grew at the intertidal zone of Lingshui (38°32′33.77″N, 121°32′33.77″E; Yellow Sea, Dalian, China), where they become periodically exposed to air, also to temperatures as high as 40°C during summer low tides and as low as −10°C during winter. Nevertheless, this sponge is not a strict intertidal organism, for the largest part of the Dalian population living permanently submerged at sublittoral depths. Most of the body biomass of the intertidal individuals regresses during winter, probably by the combined effects of low temperatures and much fresh-water from heavy rainfall. The sponges are able to regrow rapidly each spring, producing appreciable biomass through summer and autumn until a new regression. They reproduce sexually each year, brooding parenchymella larvae that are released in summer (Xue & Zhang, 2009).

The uptake experiment

Upon collection from the intertidal zone during low tide (July 2008), sponges were transported to the laboratory in seawater and transferred to a 100-l aquarium of recirculating, filtered seawater with no food addition for 6 days. This acclimation period allowed healing of potential wounds and epithelium breakages caused to these cushion-shaped sponges when detaching them from the rocks. Tissue regeneration was a surprisingly rapid process in this species. For the experiment, sponges were distributed in four sets and individuals in each set exposed to a given DSi concentration (10, 25, 40, or 70 μM). DSi uptake by each individual was examined at 6 h intervals, during a 36 h period. Average DSi concentration in the sponge habitat the time of the experiments (July–August 2008) was 10 μM. Experimental DSi concentrations were obtained by using natural coastal seawater and adding sodium hexafluorosilicate (SHF) as a silicon source. Therefore, the 10 μM treatment required no SHF addition, unlike the 25, 40, and 70 μM DSi treatments. Prior to any SHF addition, seawater was filtered through 3-μm polycarbonate membranes to eliminate diatoms but allowing the pass of bacterioplankton and the smallest picoplankton, which are the main food source to these sponges (e.g., Ribes et al., 1999; Maldonado et al., 2010b).

The experiment was conducted in 5 l alimentary PVC incubation buckets that had been washed with 3% HCl and rinsed with deionized water twice prior to be filled with 4 l of seawater. For each of the four assayed concentrations, we intended to have five incubation buckets, each containing a single sponge. Nevertheless, when preparing the 40 μM DSi buckets, we added to one of the buckets the corresponding amount of SFS twice (i.e., it became 70 μM rather than 40 μM), leaving another bucket without its SFS addition (i.e., it did not become 40 μM but stayed at 10 μM). As a result of this error, which we noticed only when the results of our seawater analyses were processed 2 days later, we ended with an unequal

sample size for our DSi treatments, as it follows: $N_{10\ \mu M} = 6$, $N_{25\ \mu M} = 5$, $N_{40\ \mu M} = 3$, $N_{70\ \mu M} = 6$. To prevent that partial starvation during the experiment could negatively affect DSi uptake rates (Fröhlich & Barthel, 1997), the natural bacterioplankton coming with the seawater was supplemented by adding 10 mg (i.e., 2.5 mg l^{-1}) of freeze-dried, single-strain *Escherichia coli* culture every 18 h. This bacterium is known to be rapidly ingested and digested by this particular sponge (Fu et al., 2006; Maldonado et al., 2010b). During the experiment, the seawater in the culturing buckets was oxygenated by pumping air for 15 min every hour. Seawater temperature during the experiment varied from 19 to 21°C. As controls, we used an additional bucket for each DSi concentration treatment, which was filled with seawater at its corresponding DSi concentration but received no sponge. Control buckets were subjected to the same oxygen and food additions as the treatment buckets.

Once the sponges were transferred to the incubation buckets, we allowed them to acclimate for about 6 h, then pipetted off 10 ml of seawater from each bucket after mixing with a PVC rod for 5 min. This first set of seawater samples was used to determine DSi concentration at time "0" of experiment. Water sampling was repeated periodically every 6 h for a total of 36 h. Seawater samples were stored into sterile, acid-washed vials at 4°C for 12–48 h before analysis. Determination of DSi concentrations were conducted using a Bran-Luebbe Autoanalyzer III, following the standard colorimetric method for measuring molybdate-reactive silicate (Grasshoff et al., 1983). Chemical reactions were maintained under 37°C and the coloration change measured under 820 nm wavelength (Method: MT19 for seawater). Individual uptake at each concentration treatment and time interval was estimated by subtracting the final DSi concentration from the initial one in each bucket and correcting by the concentration change in the control bucket, caused typically by Si adsorption to bucket walls.

After the experiment, we determined the size of each assayed individual by measuring (±<5%) its displacement volume (ml), following standard protocols described elsewhere (e.g., Wilkinson & Vacelet, 1979; Maldonado et al., 2011). Subsequently, we weighed the sponges wet (g), freeze-dried them at 60°C to constant dry weight (g), and finally combusted them at 540°C for 10 h and weighed the ashes to estimate ash-free dry weight (AFDW). Significant regression equations were obtained to estimate dry weight and AFDW from volume, so that destructive sampling is no longer needed. Hourly uptake rates measured for each sponge individual were then normalized by its volume (ml) and AFDW weight (g). We have given uptake rates preferentially normalized by the volume (ml) of living sponge, as such an approach will allow subsequent inference of field DSi demands following non-destructive measurements of sponge volume through photography (Shortis et al., 2009), rulers (Maldonado et al., 2010a) or any other method avoiding specimen collection. Nevertheless, one of the two available studies to date provided DSi uptake kinetics as AFDW normalized values. Therefore, we have been compelled to implement such an AFDW normalization to make possible a reliable between-species comparison. Because uptake rate data were both normally distributed and homoscedastic, differences in mean uptake rate (integrated over the 36 h period) as a function of DSi treatments (10, 25, 40, and 70 µM) were examined using a one-way ANOVA on untransformed data, followed by sets of a posteriori pairwise Holm-Sidak tests.

Results

Our analyses indicated that DSi concentrations decreased progressively in treatment buckets over time (Fig. 1a), while concentrations were nearly steady in control buckets (Fig. 1b). Such a pattern difference between treatment and control buckets revealed both active DSi consumption by the sponges during the experiment, and minimum DSi adsorption to bucket walls.

Mean (±SD) DSi uptake rate (averaged over the 36 h period) increased with increasing DSi availability in the culturing bucket (Fig. 2), being 0.06 ± 0.02 µmol Si ml^{-1} sponge h^{-1} at the 10 µM DSi concentration, 0.15 ± 0.07 at 25 µM DSi, 0.18 ± 0.04 at 40 µM DSi, and 0.39 ± 0.07 at 70 µM DSi (see also Table 1). A 1-way ANOVA ($df = 19$, $F = 33.9$, $P < 0.001$) and subsequent Holm-Sidak pairwise comparisons revealed that average DSi uptake rate at 70 µM was significantly higher than those at 40 and 25 µM, which in turn were not significantly different from each other but higher than the uptake rate at 10 µM (Fig. 2). Differences in

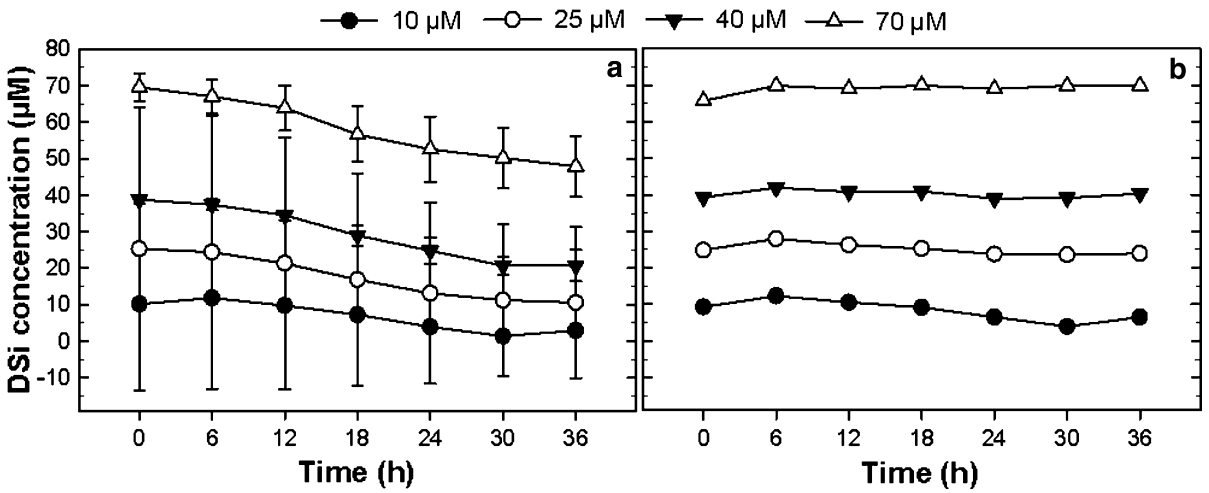

Fig. 1 Variation of mean (±SD) DSi concentrations measured at 6 h intervals within treatment (**a**) and control (**b**) culturing buckets during the 36 h experiment. Note that controls show no substantial DSi decrease over time

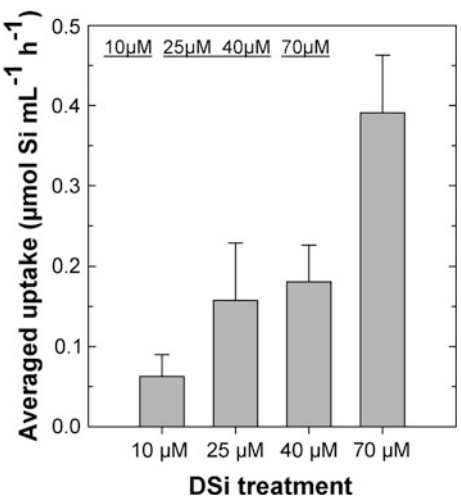

Fig. 2 Average (±SD) uptake rates (integrated over the 36 h period of experiment) in the different DSi concentration treatments. Uptake is given as µmol Si per ml of living sponge tissue and hour. At the upper left corner, DSi treatments (10, 25, 40, and 70 µM) are placed in ascending order according to the magnitude of the mean uptake responses. Treatments *underlined* by a same line are not significantly different from each other according to Holm-Sidak pairwise comparisons ($P > 0.05$) following a significant one-way ANOVA

uptake rate as a function of DSi concentration, caused the sponges to consume different Si amounts over the 36 h experiment, averaging 2.2 ± 0.9 µmol Si per ml of sponge at the 10 µM treatment, 5.6 ± 2.5 at 25 µM, 6.5 ± 1.6 at 40 µM, and 14 ± 2.5 at 70 µM.

When the time course of uptake rates was examined (Fig. 3), it was noticed that the mean rate at all four treatment concentrations varied over time, though more markedly at the higher concentrations (i.e., 40 and 70 µM). Figure 3 also shows that periods of high uptake rates alternated with periods of lower consumption rates, indicating that after a high-consumption pulse, the sponges needed to "metabolize" the taken-up DSi before engaging themselves in a new pulse of intense uptake. Such a pattern also corroborates that DSi is not entering the sponges by passive diffusion but by active transport. The magnitude of error bars (SD) also indicated moderate to large inter-individual variability in uptake rates during the experiment, irrespective of DSi concentration treatment (Fig. 3).

Discussion

To date, information on DSi uptake kinetics was available for only the demosponges *H. panicea* (Reincke & Barthel, 1997) and *Axinella* spp. (Maldonado et al., 2011). Both studies have reported DSi uptake to follow a Michaelis–Menten kinetics with saturation around 100 and 200 µM DSi, respectively. Although our experimental design cannot reveal the concentration at which *H. perlevis* reaches saturation, the data indicate that saturation shall be reached at concentrations higher than 70 µM. Interestingly, while the studies by Reincke & Barthel (1997) and Maldonado et al. (2011) suggested that the uptake rate of the sponges increases progressively with DSi

Table 1 Summary of DSi uptake rates (average ± SD) of *Hymeniacidon perlevis* in the various DSi concentration treatments

[DSi] (μM)	*Hymeniacidon* uptake rate (μmol Si g^{-1} AFDW h^{-1})	*Halichondria* uptake rate (μmol Si g^{-1} AFDW h^{-1})	*Hymeniacidon* uptake rate (μmol Si ml^{-1} h^{-1})	*Axinella* spp. uptake rate (μmol Si ml^{-1} h^{-1})
10	1.16 ± 0.50	3.42	0.06 ± 0.02	0.016
25	2.91 ± 1.33	6.76	0.15 ± 0.07	0.033
40	3.33 ± 0.84	8.94	0.18 ± 0.04	0.047
70	7.25 ± 1.34	11.62	0.39 ± 0.07	0.065

Uptake is expressed as either "μmol Si per AFDW sponge g and h" or "μmol Si per ml living sponge and h". This dual version of units allows comparison with the uptake rates predicted by the Michaelis–Menten models previously proposed for *Halichondria panicea* by Reincke & Barthel (1997) and *Axinella* spp. by Maldonado et al. (2011)

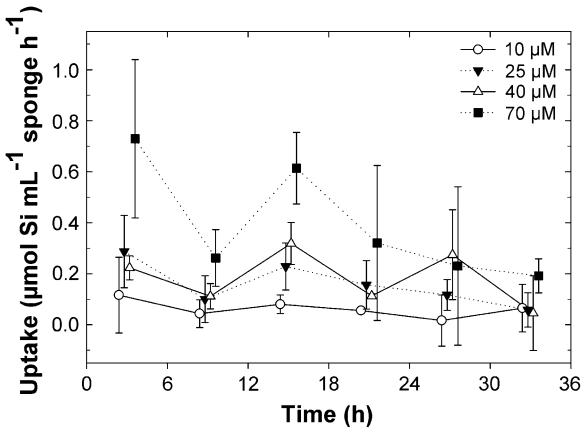

Fig. 3 Mean (±SD) uptake rates measured at 6 h intervals in each of the four DSi treatments (10, 25, 40, and 70 μM). Uptake is given as μmol Si per ml of living sponge tissue and hour

availability until reaching saturation, our data preliminarily suggest that in *H. perlevis* there could be a stepwise process, controlled by threshold concentrations above which the efficiency of the uptake system changes significantly. This suggestion is based on the realization that a DSi increase from 10 to 25 μM induced a significant increase in uptake performance, while an increase of identical magnitude between 25 and 40 μM elicited no significant acceleration in the uptake rate. On the one side, this type of response would be consistent with the idea that only above certain DSi thresholds, new groups of Si-responsive genes become activated and new types of spicule types can be produced (Maldonado et al., 1999; Krasko et al., 2000). Nevertheless, given that our sampling size was relatively low (*N* ranging from 3 to 6), we cannot discard the possibility that the ANOVA analysis lacked the power to detect differences between the 25 and 40 μM DSi treatment.

When mean uptake rates of *H. perlevis* at the different DSi treatments were compared with rates estimated for *H. panicea* (Table 1), values fell within the same range of magnitude, but being those measured in *H. perlevis* about 55% lower on average. The opposite pattern is found when *H. perlevis* uptake rates are compared with those known for some for *Axinella* species (Table 1). Consequently, all three sponge genera investigated to date appear to have uptake systems with different affinity by DSi. The reasons for these differences remain unclear. It is well known that spicule production varies over the year cycle (e.g., Bavestrello et al., 1993) and it could be that these sponges were not at the same stage of their annual growth cycle, therefore having very different DSi demands at the time of the experiments. Differences in DSi affinity could also be related to the fact that these sponges have different spicule (BSi) content (as calculated from dry weight/ash weight ratio) relative to the bulk of their respective soft tissues, accounting for about 34.4% of dry weight in *H. panicea* (e.g., Thomassen & Riisgård, 1995), 46.5 ± 8.6% in *H. peerlevis,* and 54.9 ± 11.5% in *Axinella* spp. (Maldonado et al., 2011). Another potential explanation for between-species differences in uptake rates could also be that some sponges are suspected to combine silica deposition with addition of organic skeletal materials, such as chitin (Ehrlich, 2011), in a way that is not understood yet. The formation of this skeletal composite could affect the speed at which DSi is processed and accounts for between-species uptake rate differences.

One of the most interesting aspects of the study is the fact that DSi uptake rates measured at 6 h interval showed marked shifts over time, alternating high-rate with low-rate periods (Fig. 3). The pattern was more obvious in the sponge individuals exposed to the

higher concentrations (i.e., 40 and 70 μM). Such a short-term alternation in DSi consumption rates rejects the possibility that the DSi concentration decline observed in the culturing buckets overtime could be due to passive diffusion of DSi within the sponge body. Rather, the observed pattern suggests that after a period of elevated uptake, sponges transiently reduce their consumption for some hours, probably to allow the taken-up DSi to be converted into biogenic silica. A more detailed study based on higher replication would be required to reveal patterns of periodicity, if any, and the intensity in uptake pulses. Previous studies on fresh-water sponge have shown that a 200 μm × 6–8 μm spicule can be completed in about 40 h (Weissenfels & Landschoff, 1977), although it remains unclear whether such a silicification process is either uninterruptedly continuous or stepwise. Likewise, it remains unclear how dissolved silicon is internalized by the sponges. Suggestions have been made that a sodium-bicarbonate co-transporting system could somehow be involved in taking up silicic acid from seawater (Schröder et al., 2004), which would rather be consistent with a transport enzymatic kinetic.

Our results reveal that, despite *H. perlevis* being a species evolved to growth at the intertidal zone, its uptake system appears to have been designed to consume daily much higher amounts of Si than are allowed by the natural DSi concentrations in the sublittoral zone. Our current measurements and previous work (Zhao et al., 2004) indicate that natural DSi concentration in the Dalian coastal area of the Yellow Sea range from 3 to 10 μM. South of the Dalian coast, DSi concentrations are known to range from 0.5 to 15 μM over the year, as a result of heavy seasonal rains and important runoffs by Yangtze River (about 1,000 km South from the Dalian study site) being transported North by the Kuroshio warm current (Zhang et al., 2005; 2007). Our seawater analyses ($n = 7$) indicated that DSi concentration in the sponge habitat at the time of the experiments was 10.09 ± 0.14 μM. Surprisingly, we have found that the uptake system of *H. perlevis* performs with significantly higher transport rates when DSi concentrations are at least twice to four times higher than the DSi maximum expected in their natural habitat under the most conservative approach (i.e., 15 μM). Therefore, skeletal growth in the sublittoral populations of *H. perlevis* is chronically limited by DSi availability.

Because in the genus *Halichondria* (as well as in many other halichondrids), the number of spicule types is very limited (often one or two types only), between-species taxonomic discrimination relies much on differences in spicule size. Given that optimal silicification in this species appears to require higher DSi concentration than are available in the coastal system, it cannot be ruled out that average spicule size varies drastically between populations subjected to different DSi regimes over the year, introducing an additional difficulty to correct species identification through the traditional skeletal criteria. Altogether the results of this study are consistent with previous claims of sublittoral sponges being strongly limited by DSi availability (Reincke & Barthel, 1997; Maldonado et al., 1999, 2011). The results also support the view that such a chronic limitation probably arises from the persistence in modern sponges of ancestral uptake systems that evolved in ancient oceans characterized by DSi concentrations being at least an order of magnitude higher than the maxima available in Recent oceans (Maldonado et al., 2011). It also reinforces the notion that the "current" DSi limitation is not a modern ecological process, since it probably started with the ecological expansion of diatoms during the Early Tertiary (Harper & Knoll, 1975; Maldonado et al., 1999; Lazarus et al., 2009). This process has been and it is still operating as an important environmental pressure. It has likely been forcing both the skeletal evolution of sublittoral siliceous sponges toward silica slimming and the spatial distribution of the species with high silicon needs toward high latitudes and/or aphotic zones of Recent oceans characterized by high DSi concentrations (Maldonado, 2009).

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References

Bavestrello, G., M. Bonito & M. Sarà, 1993. Silica content and spicular size variation during an annual cycle in *Chondrilla*

nucula Schmidt (Porifera, Demospongiae) in the Ligurian Sea. Scientia Marina 57: 421–425.

Chu, J. W. F., M. Maldonado, G. Yahel & S. P. Leys, 2011. Glass sponge reefs as a silicon sink. Marine Ecology Progress Series 441: 1–14.

Ehrlich, H., 2011. Silica biomineralization in Sponges. In Reitner, J. & V. Thiel (eds), Encyclopedia of Geobiology. Springer, Berlin: 796–808.

Fröhlich, H. & D. Barthel, 1997. Silica uptake on the marine sponge *Halichondria panicea* in Kirl Bight. Marine Biology 128: 115–125.

Fu, W., L. Sun, X. Zhang & W. Zhang, 2006. Potential of the marine sponge *Hymeniacidon perleve* as a bioremediator of pathogenic bacteria in integrated aquaculture ecosystems. Biotechnology and Bioengineering 93: 1112–1122.

Fu, W., Y. Wu, L. Sun & W. Zhang, 2007. Efficient bioremediation of total organic carbon (TOC) in integrated aquaculture system by marine sponge *Hymeniacidon perleve*. Biotechnology and Bioengineering 97: 1387–1397.

Grasshoff, K., M. Ehrhardt & K. Kremling, 1983. Methods of Seawater Analysis. Wiley, Nürnberg.

Harper, H. E. & A. H. Knoll, 1975. Silica, diatoms and Cenozoic radiolarian evolution. Geology 3: 175–177.

Krasko, A., B. Lorenz, R. Batel, H. C. Schröder, I. M. Müller & W. E. G. Müller, 2000. Expression of silicatein and collagen genes in the marine sponge *Suberites domuncula* is controlled by silicate and myotrophin. European Journal of Biochemistry 267: 4878–4887.

Lazarus, D. B., B. Kotrc, G. Wulf & D. N. Schmidt, 2009. Radiolarians decreased silicification as an evolutionary response to reduced Cenozoic ocean silica availability. Proceedings of the National Academy of Sciences, USA 106: 9333–9338.

Maldonado, M., 2009. Embryonic development of verongid demosponges supports independent acquisition of spongin skeletons as alternative to the siliceous skeleton of sponges. Biological Journal of the Linnean Society 97: 427–447.

Maldonado, M., M. C. Carmona, M. J. Uriz & A. Cruzado, 1999. Decline in Mesozoic reef-building sponges explained by silicon limitation. Nature 401: 785–788.

Maldonado, M., M. C. Carmona, Z. Velásquez, A. Puig, A. Cruzado, A. López & C. M. Young, 2005. Siliceous sponges as a silicon sink: An overlooked aspect of the benthopelagic coupling in the marine silicon cycle. Limnology and Oceanography 50: 799–809.

Maldonado, M., A. Riesgo, A. Bucci & K. Rützler, 2010a. Revisiting silicon budgets at a tropical continental shelf: Silica standing stocks in sponges surpass those in diatoms. Limnology and Oceanography 55: 2001–2010.

Maldonado, M., X. Zhang, X. Cao, L. Xue, H. Cao & W. Zhang, 2010b. Selective feeding by sponges on pathogenic microbes: a reassessment of potential for abatement of microbial pollution. Marine Ecology Progress Series 403: 75–89.

Maldonado, M., L. Navarro, A. Grasa, A. González & I. Vaquerizo, 2011. Silicon uptake by sponges: a twist to understanding nutrient cycling on continental margins. Nature. Scientific Reports 1: 1–8.

Reincke, T. & D. Barthel, 1997. Silica uptake kinetics of *Halichondria panicea* in Kiel Bight. Marine Biology 129: 591–593.

Ribes, M., R. Coma & J. M. Gili, 1999. Seasonal variation of particulate organic carbon, dissolved organic carbon and the contribution of microbial communities to the live particulate organic carbon in a shallow near-bottom ecosystem at the Northwestern Mediterranean Sea. Journal of Plankton Research 21: 1077–1100.

Sarmiento, J. & N. Gruber, 2006. Ocean Biogeochemical Dynamics. Princeton University Press, Princeton.

Schröder, H.-C., S. Perović-Ottstadt, M. Rothenberger, M. Wiens, H. Schwertner, R. Batel, M. Korzhev, I. M. Müller & W. E. G. Müller, 2004. Silica transport in the demosponge *Suberites domuncula*: fluorescence emission analysis using the PDMPO probe and cloning of a potential transporter. Biochemical Journal 381: 665–673.

Shortis, M., E. Harvey & D. Abdo, 2009. A Review of Underwater Stereo-image Measurement for Marine Biology and Ecology Applications Oceanography and Marine Biology Oceanography and Marine Biology—An Annual Review. CRC Press, Boca Raton: 257–292.

Thomassen, S. & H. U. Riisgård, 1995. Growth and energetics of the sponge *Halichondria panicea*. Marine Ecology Progress Series 128: 239–246.

Weissenfels, N. & H. W. Landschoff, 1977. Bau und Funktion des Süsswasserschwamms *Ephydatia fluviatilis* L. (Porifera). IV. Die Entwicklung der monaxialen SiO2-Nadeln in Sandwich-Kulturen. Zoologische Jahrbücher 98: 355–371.

Wilkinson, C. R. & J. Vacelet, 1979. Transplantation of marine sponges to different conditions of light and current. Journal of Experimental Marine Biology and Ecology 37: 91–104.

Xue, L. M. & W. Zhang, 2009. Growth and survival of early juveniles of the marine sponge *Hymeniacidon perlevis* (Demospongiae) under controlled conditions. Marine Biotechnology 11: 640–649.

Zhang, J., G. S. Zhang & S. M. Liu, 2005. Dissolved silicate in coastal marine rainwaters: Comparison between the Yellow Sea and the East China Sea on the impact and potential link with primary production. Journal of Geophysical Research 110: D16304.

Zhang, J., S. M. Liu, J. L. Ren, Y. Wu & G. L. Zhang, 2007. Nutrient gradients from the eutrophic Changjiang (Yangtze River) Estuary to the oligotrophic Kuroshio waters and re-evaluation of budgets for the East China Sea Shelf. Progress in Oceanography 74: 449–478.

Zhao, Q. Y., M. C. Deng, C. Y. Qu, X. J. Yu, M. F. Jin & W. Zhang, 2004. Elemental and total amino acid composition of two intertidal sponges in Yellow Sea. Marine Science 28: 27–31.

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SPONGE RESEARCH DEVELOPMENTS

Biosilica deposition in the marine sponge *Petrosia ficiformis* (Poiret, 1789): the model of primmorphs reveals time dependence of spiculogenesis

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Abstract Biomineralization is a phenomenon that spreads across all taxonomic kingdoms and has numerous potential applications in biotechnology. Using cell cultures (primmorphs) of Porifera as a model to study biosilicification, we hypothesised that different culture media can modulate siliceous spicule production both quantitatively and qualitatively. Long-term primmorph cultures of *Petrosia ficiformis* allowed comparing four experimental conditions: (1) natural seawater (SW) medium containing 5 μM silicate as control; (2) SW-Si 120 μM; (3) SW-Fe 5 μM; (4) SW-Si 60 μM Fe 2.5 μM evidencing several patterns of spiculogenesis. Here we have provided the first demonstration of how spiculogenesis processes are time-dependent and how spicules increase in number and size over time. The addition of dissolved silicon and low iron concentrations to the culture media produces larger spicules in greater numbers, affecting the proportions among spicule types as well. In particular, silicate seems to facilitate the production of fusiform oxeas, while iron stimulates the production of strongyloxeas. Considering the key role of spicules in taxonomic studies, our results point out the importance of environmental conditions in skeletal phenotypic plasticity, modulating the norm of reaction of the species.

Keywords Porifera · Cell culture · Silica deposition · Biomineralization

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Introduction

In Metazoa, there are several kinds of skeleton, but their architectural structure is always the most important feature as it provides mechanical support. In Porifera, the class Demospongiae builds a skeleton that is mainly formed of siliceous spicules and a collagen/spongin extracellular matrix (Nickel et al., 2006). Spicules serve to give Demospongiae a taxonomic character and are divided into microscleras (sigmas or chelas) and megascleras (monactines), reaching sizes of over 300 μm (Uriz et al., 2003).

Microscleres are mainly secreted intracellularly in a specific cell type called sclerocyte, while megascleres are produced in both intracellular and extracellular pathways (Müller et al., 2005; Uriz, 2006). In

Homosclerophida, silicification has also been described to involve epithelial cells along with sclerocytes (Maldonado & Riesgo, 2007). The biosilicification process starts from the formation of an axial filament assembled in organelles on which silica deposits itself in concentric layers (Uriz et al., 2000; Müller et al., 2005). A mechanism of Na–Si exchange (Coradin & Lopez, 2003) has been suggested to be involved in silica deposition, but in sponges it still needs to be definitely demonstrated. Spicule formation is a rapid in vivo process that can increase upto 5 μm/h (Weissenfel, 1989). In *Suberites domuncula*, when spicules reach a length of about 5–10 μm, they are extruded from sclerocytes into the mesohyl where their formation is complete (Eckert et al., 2006).

The shape and size of spicules are the most important taxonomic characteristics for sponge identification. However, spiculogenesis is not constant. It can vary depending on environmental parameters, above all on silica availability (Stone, 1970; Maldonado et al., 1999), affecting even the pattern of spicule types/shape (Maldonado et al., 1999). Spiculogenesis is also influenced by temperature that seems to enhance silica uptake when it is at a low level, as suggested by variation observed along latitudinal (Topsent, 1917; Hentschel, 1929; Hartman, 1958; Hooper, 1991) and bathymetric gradients (Bavestrello et al., 1993a; Mercurio et al., 2000). Environmental factors can, therefore, cause variations in sponge spicular size. As a result, there is high variability in the phenotypic characters of sponges due to the environmental modulation (Smith-Gill, 1983) of their genetic base body-plan.

Studies in this field could be improved with the use of the 3D sponge systems, known as primmorphs (Custodio et al., 1998), whose structure represents the intermediate stage between a cell and a sponge individual. Because of this peculiar characteristic, their use as a model has allowed researchers to investigate physiological processes at the initial stage of a sponge, without using adult individuals, but using better standardizing experiments.

The investigation on spicule formation patterns, not only from a descriptive but also from a quali-quantitative point of view, remains an important step to comprehend the steps in the construction of skeletal elements. Spiculogenesis is considered to be a key step in the development of a sponge (Müller et al., 2000; Krasko et al., 2002), and keeping this in mind, primmorphs could provide a great model to study the biological processes involved in the first steps of spicule formation.

In this work, we hypothesize that different culture media can affect spicule production in cell cultures. For this reason, we cultivated primmorphs obtained from the sponge *Petrosia ficiformis* (Poiret, 1789). We analysed spicules produced in long-term cultures (Nickel et al., 2001) and evaluated the influence of enriched sea water medium with silica and iron, alone or combined, which according to previous reports, affect spicule production (Krasko et al., 2002; Le Pennec et al., 2003; Valisano et al., 2007a, b). Based on previous results (Valisano et al., 2007a, b), sea water medium cultures were enriched with silica and iron at concentrations of 120 and 5 μM, respectively. Because of the negative influence of iron when combined with silica on cultures, both of them were supplemented at low concentrations. The survival of cultures was evaluated in different conditions and the analysis of spiculogenesis was carried out during this time. The size of primmorphs and the number and size of spicules were recorded. A correlation between the number of spicules and the size of primmorphs was hypothesised, and because of that missing correlation, the actual time-dependent spiculogenesis was analysed throughout this time by evaluating the trend of the number and size of spicules formed in the primmorphs, and the effect of supplementing sea water media at different experimental conditions on those parameters. Moreover, the analysis was conducted to identify the spicular type produced in the primmorphs and to evaluate the number and size of spicular types in all conditions. Finally, we also analysed the distribution of spicules throughout this time as frequency values of size classes to identify cohorts of spicules evidencing mode values and variability of spicules.

Materials and methods

Specimens collection

Specimens of *P. ficiformis* were collected along the Marine Protected Area of Portofino (Ligurian Sea, Italy) between 15 and 20 m depth. Sponges were immediately carried to the laboratory and maintained in aquaria at 12°C and salinity at 38‰.

Cell dissociation

The day after sampling, sponges were processed for dissociation of cells according to a previously established protocol (Müller et al., 1999). Sponge samples of 4–5 cm^3, continuously submersed in sea water, were cut into small pieces and transferred into 50 ml conical plastic tubes filled with calcium- and magnesium-free sea water supplemented with EDTA (CMFSW-EDTA). After shaking gently for 20 min, the solution was discarded and new CMFSW-EDTA was added. After shaking continuously for 40 min, the supernatant was collected and filtered through a 40 μm mesh nylon net, and the process was repeated once again. Samples were centrifuged (1,600 rpm, 458 g for 5 min) and washed twice in CMFSW. Cells were re-suspended in natural filtered sea-water in final pellets, and the dissociated cells were put into tissue culture plastic plates on an oscillating table in order to prevent them from getting attached to the bottom of the plates.

Experimental conditions and data analysis

Primmorphs were incubated in seawater-based media enriched in silicate (as Na-silicate) and/or iron (as ferric citrate), in the following conditions: (1) natural sea water (SW) medium containing 5 μM silicate as control; (2) SW-Si 120 μM; (3) SW-Fe 5 μM; (4) SW-Si 60 μM Fe 2.5 μM. The concentration of dissolved silica in the natural sea water of the experimental sets was assessed with the Silicate (Silicic Acid) Test (Merck).

Five-day-old primmorphs were put into plastic plates with 6 ml of medium, with 6 replicates per treatment, containing 30 primmorphs each. Culture media were replaced every 3 days with fresh new ones according to each experimental condition to maintain the concentrations stable. The cultures were monitored for 1 month.

Regarding the survival of primmorphs, data were compared with Analysis of Variance and analysed with Friedman Test because of the dependence of measures over time.

To investigate spiculogenesis, four primmorphs per dish treatment ($n = 4 * 6 = 24$) were collected prior to each culturing medium replacement and subsequently analysed. In this way, we collected the primmorphs at 8 different times for a total of 128 primmorphs. Each primmorph was laid on a slide and observed under an optic microscope. The radii of the primmorphs were measured, and spicules were identified because of their bright light reflection properties (Schröeder et al., 2005). Each time spicules were studied, their number in the primmorphs was recorded and their length and thickness were measured. Moreover, the type of spicule was identified, and analysis was conducted separately on each spicular type. In all, 749 spicules were measured. The collected data were elaborated in means and standard deviations.

Between-treatment differences in the average number of spicules in the primmorphs (log-transformed data) were examined using ANOVA and "a posteriori" Tuckey test after corroborating that data were normally distributed (Bartlett's test) and homoscedastic (Levene's Test). Data of length and thickness of spicules were checked with Cochran's Test for heteroscedasticity and analysed with Analysis of Variance and SNK Test for post hoc comparisons.

Data concerning the number of spicules for spicular type were elaborated in percentage of occurrence, arcsin square root transformed and analysed with Analysis of Variance and post hoc SNK Test; data concerning the size of spicules for spicular type were checked with Cochran's Test for heteroscedasticity, $\log(x + 1)$ transformed and analysed with Analysis of Variance and SNK Test for post hoc comparisons.

Finally, the number of each spicule type (oxea, strongyloxea, strongyle) produced in the primmorphs during each time interval was depicted graphically by distributing spicules in morphological types and size classes over time.

Results

Enrichment of sea water culture media affected the survival of cultures: the ANOVA test evidenced a significant difference in primmorph survival in the four experimental conditions (Fig. 1) (Table 1A). Primmorphs survived better in sea water enriched with Si 120 μM, showing about 75% survival during the first 6th time intervals (Table 1A). In contrast, the remaining culturing treatments showed only 25% survival over the same time period (Table 1A).

Besides the monitoring of primmorph survivorship, we performed an analysis of spicules which were located in the central region of the primmorphs and

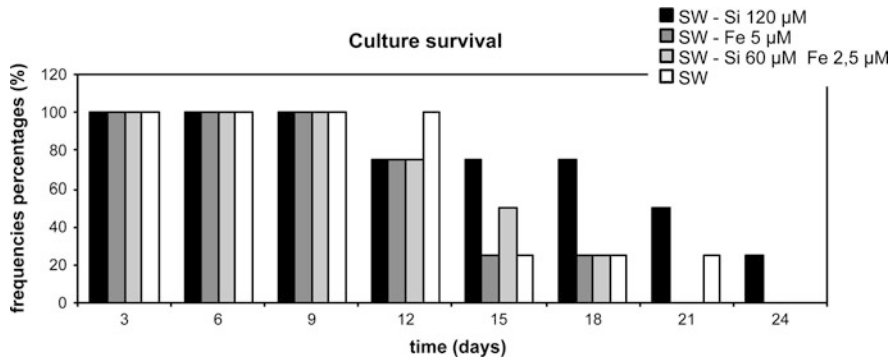

Fig. 1 Frequency percentages of the survival of primmorph cultures in four experimental conditions: SW, SW-Si 120 µM, SW-Fe 5 µM, SW-Si 60 µM Fe 2.5 µM

easily visible under a light microscope because of their translucent sponge tissues.

We hypothesised a correlation between the size of the primmorphs and the number of spicules produced in them, but none of the analysed conditions showed a correlation between the two parameters (data not shown). Because of that missing correlation, we concluded that spiculogenesis in primmorphs could be a time-dependent process.

At the beginning of the experiments, primmorphs without spicules were observed. As such, the datum was considered as zero. After some time, some fragments of spicules together with intact spicules were observed in the primmorphs, but they were not considered in the analysis of the newly-formed spicules.

The temporal trend in the number of spicules (Fig. 2) indicated a "time" effect on spicule production by the primmorphs, irrespective of treatment (Table 1B), demonstrating the actual time-dependent spiculogenesis within primmorphs. Recorded data, in all experimental conditions, showed a general increase in the number of spicules over time, even if not always linear. There was a decrease in their number within the first 10 days. Then, there was an increase in the number of spicules with a significant increase at the beginning (3rd and 6th day) and then again towards the end of the experiment (21 and 24 days) (Table 1B). Moreover, we evidenced between-treatment differences (Table 1B), with a higher number of spicules Si 120 µM and SW-Si 60 µM Fe 2.5 µM with respect to SW and SW-Fe 5 µM (Table 1B) during this time.

It turned out that the spicules increased over time in terms of both number and size.

They (Fig. 3a, b) also increased in size over time in all the analysed conditions with variability in the different experimental conditions (Table 1B), mainly due to time effect. At the beginning of the experiments we observed that the spicules were very short and thin, from 46.2 ± 1.73 to 62.4 ± 2.50 µm long and from 4.38 ± 0.15 to 5.34 ± 0,19 µm thick, depending on culture conditions (average values). Within the first 12 days, the size of the spicules in SW-Si 60 µM Fe 2.5 µM was significantly higher than that in SW-Si 120 µM, SW and SW-Fe 5 µM (Table 1), while no differences were recorded in their sizes in SW and SW-Fe 5 µM. In SW-Si 120 µM, we observed a long-time effect, which after 12 days allowed us to record an increase in the length of spicules up to 119.68 ± 3.05 µm, significantly higher than all the other conditions (Table 1B). Spicules increased their size in all conditions analysed, but at the end of the experiment, the largest spicule size was recorded in SW-Si 120 µM, which was higher than that in the other media (Table 1B). The average spicule sizes were 224.58 ± 3.15 µm long at the 21st day, and the difference recorded at the first time with respect to their sizes SW-Si 60 µM Fe 2.5 µM being lost. The increase in the size of the spicules is shown in Fig. 4.

Furthermore, we analysed spicular types produced in the primmorphs: we observed fusiform and curved oxeas, strongyles and strongyloxeas. Oxeas were found to be either acerate, hastate, sometimes mucronate or stepped. Styles were occasionally observed within primmorphs cultured with silicate. Spicular types observed in primmorphs are shown in Fig. 5; the trends in time of the number of spicular types expressed in frequencies percentages and the trends of their sizes are shown respectively in Figs. 6 and 7.

The production of the four different spicular types was influenced by the media used for cultures with variability over time; the results were evidenced by analysing both the number (Table 1C) and size of the spicular types produced (Table 1C). Short-time

Table 1 Statistical analysis: significant results in order of mention in the text

Data analysed	Details of significant results and comparison	Statistical tests	*P* values
A			
Survival of primmorphs	Experimentals conditions	ANOVA	$P < 0.001$
	Time within SW-Si	Friedman Test	$0.001 < P < 0.01$
	Time within the other conditions	Friedman Test	$P < 0.001$
B			
Number of spicules	Time	ANOVA	$P < 0.005$
	Begin versus end of experiments	Tukey Test	$P < 0.05$
	Experimental conditions	ANOVA	$P < 0.05$
	SW-Si and SW-SiFe versus SW and SW-Fe	Tukey Test	$P < 0.05$
Dimension of spicules	Time	ANOVA	$P = 0.000$
	SW-SiFe versus the other onditions within 12 days	SNK Test	$P < 0.05$
	SW-Si versus the other conditions after 12 days	SNK Test	$P < 0,05$
	SW-Si versus the other conditions at end of experiments	SNK Test	$P < 0,05$
C			
Number of spicules per Spicular types	Experimental conditions	ANOVA	$P = 0.0011$
Size of spicules per Spicular types	Experimental conditions	ANOVA	$P = 0.0000$
Fusiform oxeaes	SW-Si versus the other media	SNK Test	$P < 0.05$
	SW-SiFe versus SW-Si and SW	SNK Test	$P < 0.05$
	SW versus SW-Fe	SNK Test	$P < 0.001$
Strongyloxeaes	SW-Si Fe versus the other conditions	SNK Test	$P < 0.001$
Curved oxeaes	SW-Si Fe versus the other conditions	SNK Test	$P < 0.001$
	SW versus SW-Fe	SNK Test	$P < 0.05$
Strongyles	SW versus SW-Fe	SNK Test	$P < 0.001$
Spicular types within conditions	Curved oxeaes, fusiform oxeaes and strongyloxeae versus strongyles	ANOVA	$P = 0.0101$
SW-Si	Curved oxeaes, fusiform oxeaes and strongyloxeae versus strongyles	SNK Test	$P < 0.001$
	Fusiform versus strongyles and curved oxeaes	SNK Test	$P < 0.05$
SW-Fe	Strongyle, versus strongyloxeaes and oxeaes	SNK Test	$P < 0.001$
	Strongyloxeaes versus curved oxeaes	SNK Test	$P < 0.001$
SW-Si Fe	Strongiloxeaes versus other spicule types	SNK Test	$P < 0.001$
	Fusiform and curved oxeaes and strongiloxeaes versus strongyles	SNK Test	$P < 0.001$
SW	Strongyles versus the other spicular types	SNK Test	$P < 0.001$

effects were very evident in the first 15–18 days of monitoring, which were lost in the long run. Spicular types showed no differences at the beginning of the experiments and were always present with similar frequencies. Subsequently, the effect of different culture media began to be observed after 6 days.

There was more production of fusiform oxeas in SW-Si 120 μM than in the other media (Table 1C). In cultures with both silicon and iron, there was less production of fusiform oxeas than in the culture with exclusive addition of silicate, but its number was higher than that in the SW culture medium (Table 1C). Again, there was a higher number of fusiform oxeas in SW medium than in the medium with iron alone (Table 1C). The production of strongyloxeas showed higher efficiency of SW-Si 60 μM Fe 2.5 μM medium

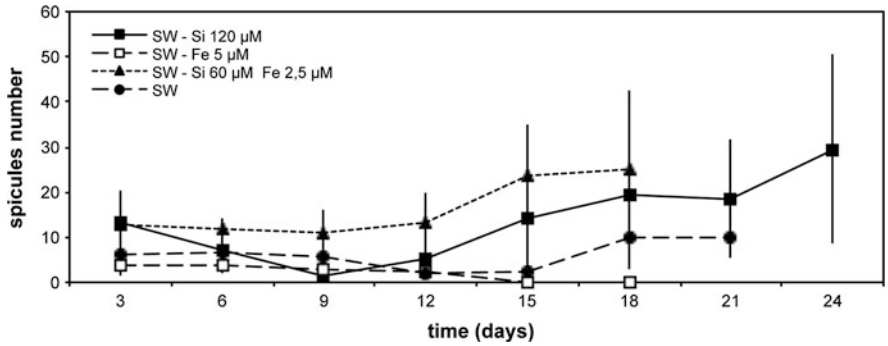

Fig. 2 Number of spicules formed within primmorphs during the time of monitoring in four experimental conditions: SW, SW-Si 120 µM, SW-Fe 5 µM, SW-Si 60 µM Fe 2.5 µM

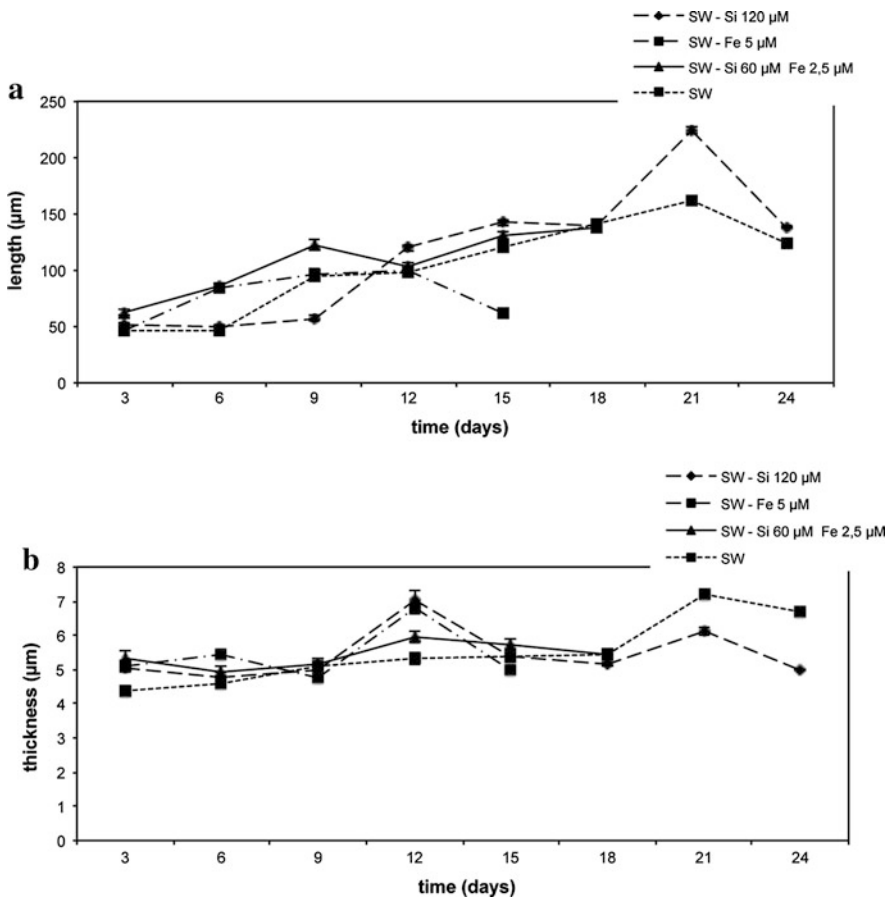

Fig. 3 Length (**a**) and thickness (**b**) of spicules within primmorphs (SW, SW-Si 120 µM, SW-Fe 5 µM, SW-Si 60 µM Fe 2.5 µM)

than that in other cultures as stronyloxeas were produced in higher quantities (Table 1C).

There was a higher production of curved oxeas in cultures with SW-Si 60 µM Fe 2.5 µM than in the other culture media (Table 1C), and their number was higher in SW than that in the cultures with the iron alone (Table 1C).

As in the case of fusiform and curved oxeas, the production of strongyles proved to be higher in SW than that in the cultures with the iron alone (Table 1C), but lack of abundant data recorded for strongyles did not allow us to go deeper into this type of spicule. It turned out that curved oxeas, fusiform oxeas and strongyloxeae had a significantly higher production than strongyles (Table 1C).

Also, in cultures with SW-Si 120 µM, there was a significantly higher production of curved oxeas, fusiform oxeas and strongyloxeas than strongyles

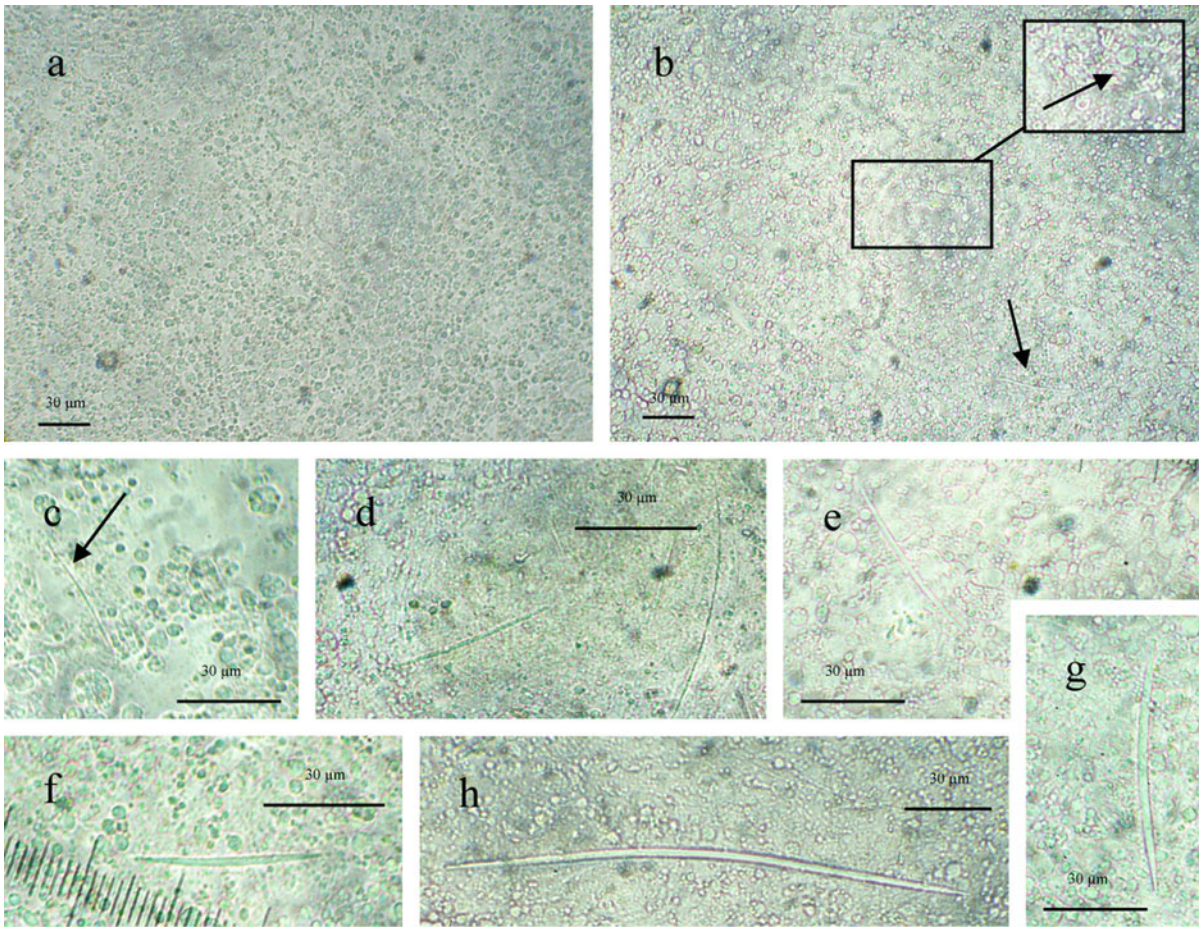

Fig. 4 Spicules within primmorphs during the 3 (**a**–**b**), 6 (**c**–**d**), 9 (**e**–**f**) and 12 (**g**–**h**) days of experiments

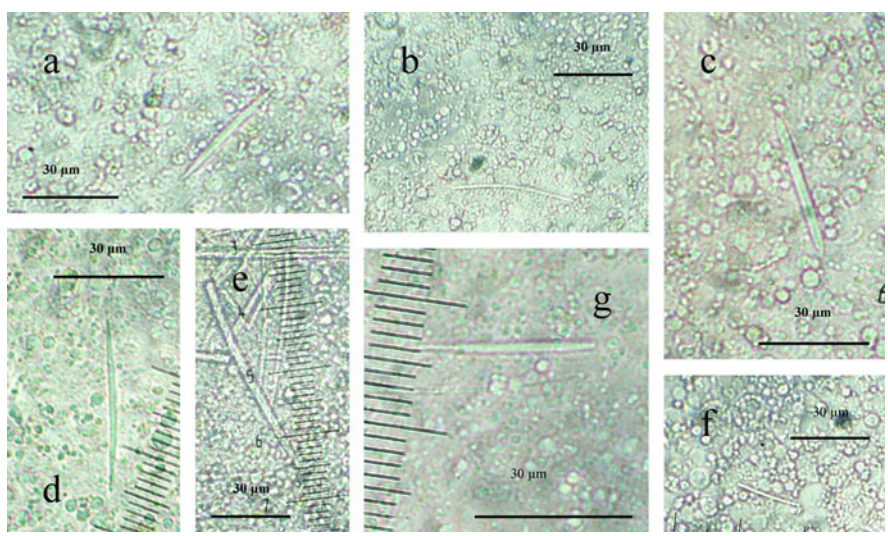

Fig. 5 Spicular types observed within primmorphs: fusiform (**a**) and curved (**b**) oxeae; acerate (**c**) and hastate (**d**) points; style (**e**); strongyle (**f**); strongyloxea (**h**)

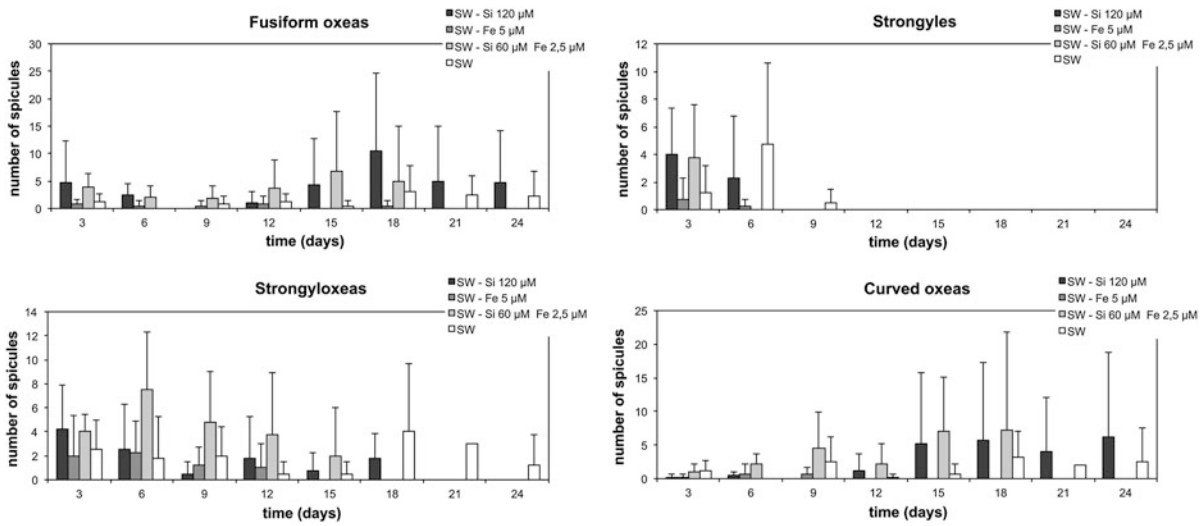

Fig. 6 Frequency percentages of the spicule number of the different spicular types formed within primmorphs (SW, SW-Si 120 µM, SW-Fe 5 µM, SW-Si 60 µM Fe 2.5 µM)

(Table 1C), while fusiform oxeas turned out to be more numerous than both strongyles and curved oxeas (Table 1C).

In cultures with iron alone, the spicule type that had the lowest production was strongyles, which had a lower frequency of production than strongyloxeas and oxeas (Table 1C). Moreover, strongyloxeas had a higher production than curved oxeas (Table 1C).

At the beginning of the experiments, in cultures with both silicon and iron, all spicule types showed similar frequencies, but after 6 days, differences began to show up: strongiloxeas had the highest frequency of production, and fusiform and curved oxeas and strongiloxeas had a higher frequency of production than strongyles (Table 1C).

In cultures with SW, spicular types were always present with similar values, In addition, in this case there was a lower production of strongyles than other spicular types (Table 1C).

Finally, we studied the frequency distribution of the types of spicules over time (Fig. 8), trying to identify cohorts of spicules.

At the beginning of the experiment, spicules of sizes ranging between 50 and about 100 µm were produced in all cultures. The majority of them measured between 50 and 60 µm. However, we identified differences among culture conditions, which became more accentuated in the course of time. In cultures with silicate alone, spicules produced were shorter than 80 µm, while in cultures with silicon and iron, spicules up to 110 and 130 µm showed up suddenly.

After 6 days of study, while the majority of spicule size ranged between 50 and 60 µm but shorter than 80 µm in cultures with silicate alone, the mode values of spicule size in cultures with silicon and iron moved up from 50–60 µm to 80–90 µm, reaching lengths of even 150 µm.

At the 9th day, the variability of size classes dramatically increased. We did not find small-size spicules any more, but an evenly represented distribution with size classes between 60 and 130 µm, especially for cultures enriched with both silicon and iron.

After 12 days, the modal classes moved up to 110 µm, with high values ranging from 95 to 115 µm, with the highest lengths in cultures with both silicon and iron. However, the frequencies in cultures with silicate were higher than those in cultures with SW. Then, after 15 days, a general increase in spicule size classes was evident with values ranging between 115 and 145 µm. Moreover, we began to observe the effect of silicate on spicule production: in cultures with SW-Si 120 µM, we identified a bimodal distribution with classes at 140 and 165 µm having a higher frequency. Modal values with 115 µm spicule size turned out to be lower in cultures with both silicon and iron than those in cultures with silicate alone. However, the

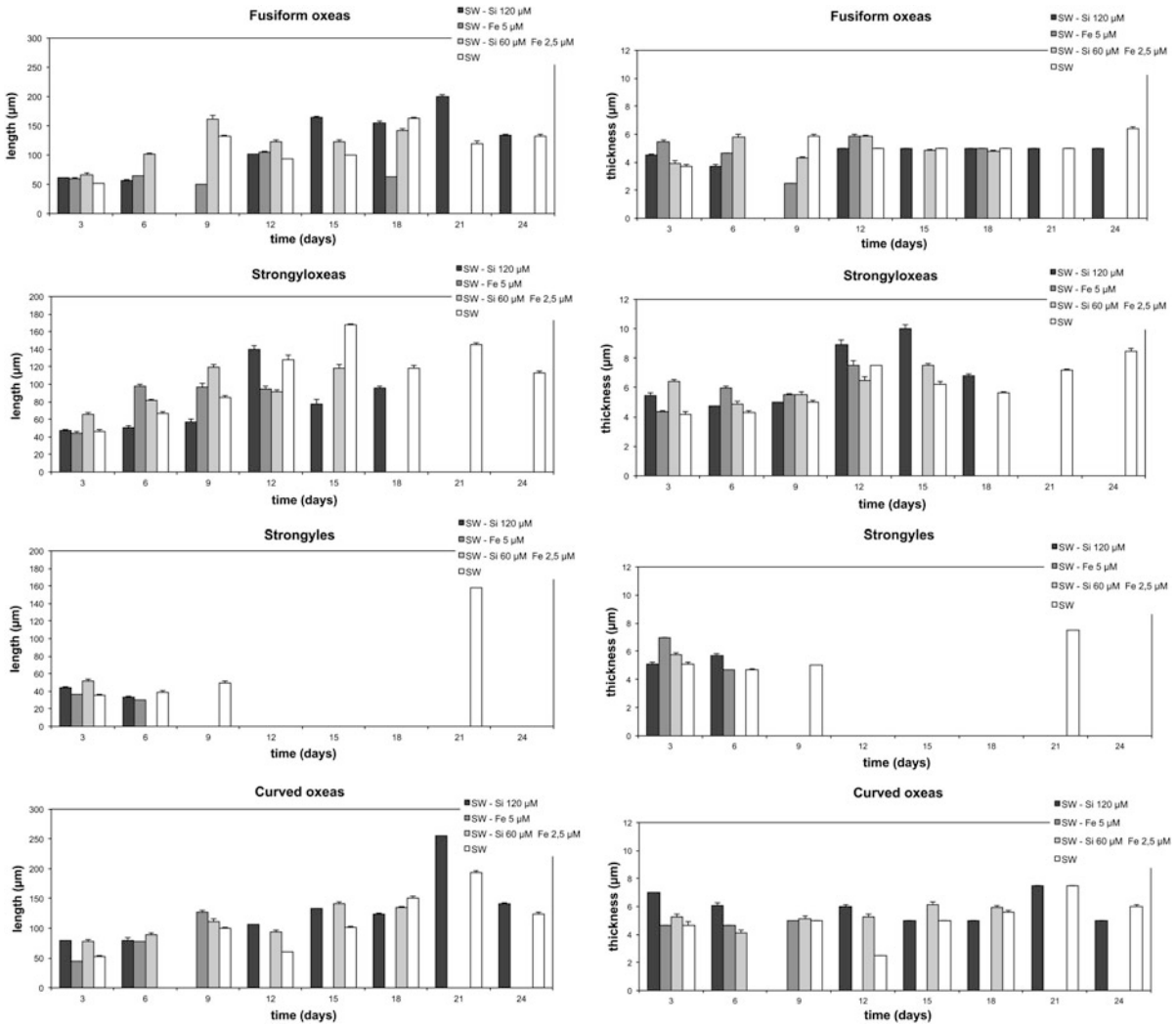

Fig. 7 Length (**a**) and thickness (**b**) of different spicular types (SW, SW-Si 120 µM, SW-Fe 5 µM, SW-Si 60 µM Fe 2.5 µM)

modal values were more variable with high frequencies from 105 to 180 µm.

After 18 days, the size classes of the spicules gradually increased up to 145–180 µm, even if sizes ranging between 110 and 130 µm stood out. Cultures with silicate alone again showed a bimodal distribution with similar values, ranging from 105 to 130 µm in the first case and the highest values ranging from 170 to 180 µm in the second one. However, in cultures with both silicon and iron, size classes that stood out more were those ranging between 140 and 145 µm.

After 21 days, the classes of major frequencies not only went up to a range of 155–165 µm but also reached the values up to 265 µm. The effect of cultures with both silicon and iron was gone because of the difficulty of cultures in the presence of iron in the long period. While in the SW medium, classes of major frequencies ranged between 155 and 165 µm, in cultures with silicate, we again saw a bimodal distribution with peaks between 180 and 210 µm and 240 and 265 µm.

At the end of the experiments in cultures with silicate, the class having the highest frequency was the one with145 µm, and those classes that ranged between 125 and 155 µm were more prominent. However, we found lower values in cultures with SW alone. Even if the effect on cultures due to enrichment seemed to have faded in the long run, in

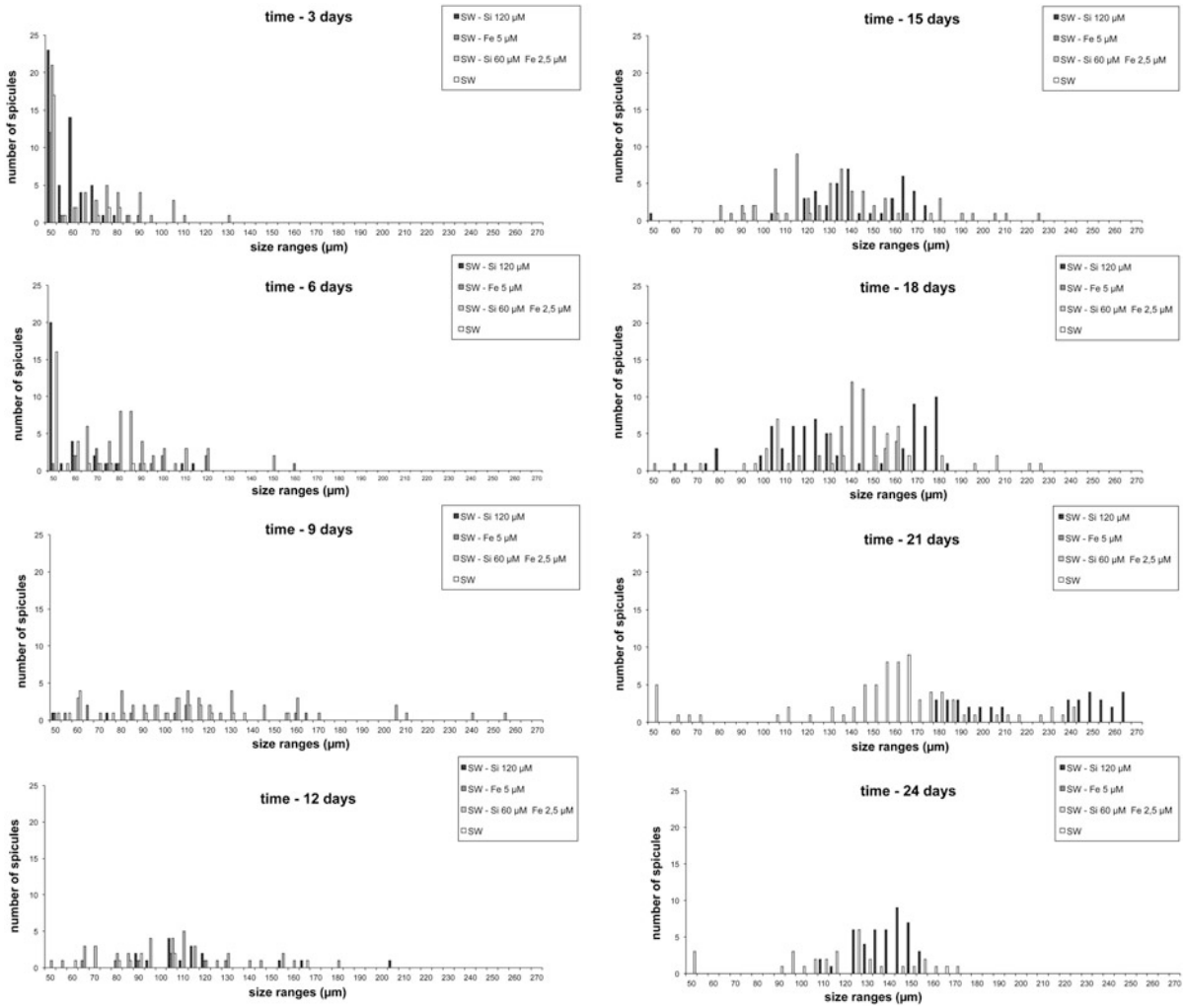

Fig. 8 Distribution analysis of spicule production into size classes over time (SW, SW-Si 120 µM, SW-Fe 5 µM, SW-Si 60 µM Fe 2.5 µM)

the short term, enriched cultures showed higher values and more variability than the simple cultures with SW.

Discussion

The incredible phenotypic plasticity of sponges can be studied not only at macroscopic level but also on a microscopic scale, thanks to the deep influence that the chemical features of sea water can have on biosilicification processes. While the formation process of spicules in primmorphs has been addressed in detail from a descriptive point of view, (Müller et al., 2005, 2006; Eckert et al., 2006), the scientific literature provides fragmentary data on them from a quantitative aspect, often coming from semi-quantitative approaches, conducted with only few primmorphs and for short-term periods (Krasko et al., 2000; Zhang et al., 2003; Cao et al., 2007a, b).

Data presented in this work characterized the production of spicules in primmorphs, together with an analysis of the influence of supplementing seawater-based media with both silicon and iron also, which are notably suggested to play an important role in biosilification processes (Valisano et al., 2007a, b). The survival analysis of primmorphs in different culture media showed a positive effect of Si 120 µM. Lack of correlation between the size of primmorphs and the number of spicules, and the significant effect of time in the analysis of the variability of spicule

number confirmed from a quantitative point of view that spiculogenesis is a time-dependent phenomenon, reinforcing the thought based on qualitative observations (Elvin, 1971; Pe', 1973). Both the number and size of the spicules showed a positive trend over time.

Spicules observed at the beginning of the experiments were very small and thin and very different from the dimensions of spicules of adult individuals, just as the sponges processed for cells preparation. This led us to consider that these spicules were newly formed. Moreover, the trend that showed up during the experiments demonstrated a significant increase in the size of spicules in primmorphs, going from small values to very high ones. Even if they were less in number and size, our results confirm that spicules are produced, also in the absence of further addition of silicate to seawater-based culture media (Valisano et al., 2007b). However, the enrichment of seawater-based media led to an increment in spicule production, and silicate proved to have a positive effect on spiculogenesis, which is in accordance with Maldonado et al. (1999) who documented a dramatic increase in the total number of spicules produced when silicate concentration in water was increased. The rapid response of primmorphs to silica enrichment may confirm that sponges are suffering from a severe and chronic limitation of DSi (<2 μM) in their natural habitat, as documented by Maldonado et al. (2011).

Despite the negative effect of enriching the culture media with iron that led to a decrease in the survival of cultures, our results suggest its possible role in spicule production at low concentrations also for primmorphs of the sponge *P. ficiformis*. The number of spicules increased with time, but not always in a linear relationship. However, a more stable trend was obtained in the presence of iron in primmorph culture. Therefore, if at high concentrations iron caused damage to the cultures (Valisano et al., 2007a, b), at low concentrations it seemed to influence silica deposition on spicules, as asserted by previous reports (Müller et al., 2006), and according to our previous results, we validated that supplementing of silicate leads to an increment in spicule production (Valisano et al., 2007a, b).

This pattern was also confirmed by spicule size. The addition of silicate not only produced a higher number of spicules but also bigger size spicules with respect to the ones produced in seawater alone. Results showed no difference in short-time observations between the addition of silicate alone and the addition of both silica and iron, even if the effect was lost in the long run. Therefore, at low concentrations, iron seems to have a role in spicule formation and silica deposition, while silicate gave us the confirmation of its influence on spicule production for primmorphs of the sponge *Hymeniacidon perleve*, as reported by other authors, even if the analysis was conducted with only two sets of measurements (Cao et al., 2007a).

Our results moreover confirm previous findings that silicon and iron affect the type of spicules (Maldonado et al., 1999) produced in primmorphs. Enriched cultures showed a variety of spicular types: silicate seemed to be important for the production of fusiform oxeas, while iron for the production of strongyloxeas.

However, generally speaking, in all conditions there was a significantly higher production of curved oxeas, fusiform oxeas and strongyloxeas than strongyles. Therefore, as previously suggested, the enrichment of seawater-based media leads to the formation of a variety of spicular types.

While for wild *P. ficiformis* (Poiret), Pulitzer–Finali (1983) describes that spicules from oxeas to strongyles are not separable in size classes, in primmorphs we noted cohorts of spicules and modal frequencies of sizes that compose the spicular assemblage over a temporal trend, contributing to the comprehension of how a sponge builds its skeleton.

Moreover, the bimodal distributions that came up during the experiments underlined the presence of newly-formed spicules that are subsequently produced in primmorphs in the temporal analysis.

In fact, the study of the distribution of spicules allowed us to point out the trend of all spicule types in the course of time, together with the analysis of the effects of the enrichment of the culture media.

Size variations of spicules in relation to variations in chemical and physical parameters, such as water temperature or silicate availability, or biological ones, such as the reproductive period of the sponge, have been studied for a long time (Stone, 1970; Sarà & Vacelet, 1973; Jones 1987a, b), and in particular, silicate availability has long since been considered an important factor affecting sponge spiculation. Factors affecting spicule formation are resumed in Table 2.

The final size of spicules is determined by silicic acid concentration in *E. fluviatilis* (Pe', 1973) and by temperature and silicate concentration in *Ephydatia*

muelleri (Elvin, 1971), which showed in cultured individuals an increase in the growth rate of spicules with increasing temperature up to 25°C, and a decrease in their growth rate with increasing silicic acid concentration (until 25 μg silicon/ml). Spicule size was found to be correlated with silicate concentration and inversely correlated with water temperature in *Hymeniacidon perleve* (Stone, 1970), and Mercurio et al. (2000) found the same evidence (spicule size varying according to water silicate concentration and inversely correlated with water temperature) in the sponge *Pellina semitubulosa.*

Temperature and silicate availability in the medium are, therefore, the most important characteristics that influence sponge spiculation; Simpson (1978) found an increase in spicule width with a decrease in temperature in the demosponge *Microciona prolifera* and advised that low temperatures stimulate the formation of wider spicules, suggesting a more efficient uptake and transport of silica.

Table 2 Environmental parameters affecting spicule plasticity and relative references

Environmental parameters	Effect	Species	References
Temperature	Low t ⇒ ↑ width	*M. prolifera*	Simpson (1978)
	(polymerization)	*H. perleve*	Stone (1970)
	Low t ⇒ ↑ width	*E. muelleri*	Elvin (1971)
	Enhance deposition	*H panacea*	Frölich & Barthel (1997)
	No correlation with silica uptake		
	Low t ⇒ ↓ Sponge silica content	*Pellina semitubulosa*	Mercurio et al. (2000)
High $Si0_2$ concentration	⇒ ↑ width	*H. canaliculata*	Hartman (1958)
	⇒ ↑ width	*E. muelleri*	Elvin (1971)
	⇒ ↑ width	*H. perleve*	Stone (1970)
	⇒ ↑ width–length	*E. fluviatilis*	Pe' (1973)
	⇒ ↓ no. spicules		
	⇒ ↑ width	*S. lacustris*	Jørgensen (1944), (1947)
	⇒ ↑ silica uptake	*H. panicea*	Frölich & Barthel (1997)
			Reincke & Barthel (1997)
Waves action	⇒ ↑ dimensions		Palumbi (1986)
Depth	Anomalies	Fresh-water sponges	Tuzet & Connes (1962)
			Connes (1963)
	⇒ ↑ size	?	Schröder (1936)
	⇒ ↑ size	*P. ficiformis*	Bavestrello et al. (1993a)
High latitudes	⇒ ↑ size		Topsent (1917)
	⇒ ↑ size	*Gellius*	Hentschel (1929)
			Hartman (1958)
	⇒ ↑ size		Hooper (1991)
	⇒ ↑ size	*Ophlitaspongia seriata*	Fry (1970)
Reproductive period	⇒ ↔ spiculogenesis	*H. elegans*	Jones (1987a, b, 1991)
		H. panicea	Barthel (1986)
	⇒ ↓ silica uptake	*H. panicea*	Frölich & Barthel (1997); Witte et al. (1994)
	⇒ ↓ spicule size	*C. nucula*	Bavestrello et al. (1993b)
	⇒ ↑ spiculogenesis (after release)	*P. semitubulosa*	Mercurio et al. (2000)
Body size		*H. panicea*	Frölich & Barthel (1997)
Starvation	⇒ ↓ silica uptake	*H. panicea*	Frölich & Barthel (1997)
		E. fluviatilis	Pe' (1973)

Frölich & Barthel (1997) demonstrated for the sponge *Halicondria panicea* that silica uptake depends on silicate availability and that higher temperatures result not only in higher transport rates but also in higher diffusive losses because of reduced polymerization at higher temperatures, clarifying the two different roles of the two parameters.

Variations in spicule size and/or spicular types have also been observed in *Chondrilla nucula* (Bavestrello et al., 1993b) and in *P. ficiformis* in relation to seasonality (Bavestrello & Pansini, 1994; Bavestrello, 1991), suggesting their possible relationship with variations of parameters linked to seasonal cycle, which could act as activators by stimulating the production of spicules/spicular types or by depressing other ones. These results suggest that such evidences could also be due to variations during the annual cycle of silicate availability in the water medium.

The variability in types, shapes and sizes of spicules depending on variations in physical and chemical parameters, such as temperature and ion concentrations in the water column (Maldonado et al., 1999; Frölich and Barthel, 1997; Bavestrello et al., 1993a; Valisano et al., 2007a, b), could therefore represent an important implication concerning the use of spicules for taxonomic identification.

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References

Barthel, D., 1986. On the ecophysiology of the sponge *Halicondria panicea* in Kiel Bight. I. Substrate specificity, growth and reproduction. Marine Ecology Progress Series 32: 291–298.

Bavestrello, G., M. Bonito & M. Sarà, 1993a. Influence of depth on the size of sponge spicules. Scientia Marina 57: 415–420.

Bavestrello, G., M. Bonito & M. Sarà, 1993b. Silica content and spicular size variation during an annual cycle in *Chondrilla nucula* Schmidt (Porifera, Demospongiae) in the Ligurian Sea. Scientia Marina 57: 421–425.

Bavestrello, G.,M. Pansini & M.Sarà, 1994. The variability and taxonomic status of different *Petrosia*-like sponges in the Mediterranean Sea. In Vans Soest, R. W. M., T. M. G. Van Kempen & J. C. Braekman (eds) Sponge in Time and Space, Balkema, Rotterdam: 83–92.

Bavestrello, G., 1991 Aspetti ecologici della variabilità intraspecifica dei Poriferi. PhD Thesis, pp. 1–69.

Cao, X., W. Fu & W. Zhang, 2007a. Dynamics of spicule production in the marine sponge *Hymeniacidon perlevis* during in vitro cell culture and seasonal development in the field. Cell & Tissue Research 329: 595–608.

Cao, X., X. Yu & W. Zhang, 2007b. Comparison of spiculogenesis in in vitro ADCP-primmorphs and explants culture of marine sponge *Hymeniacidon perleve* wit 3-TMOSPU Supplementation. Biotechnology Progress 23: 707–714.

Connes, R., 1963. La spiculation des Spongillidae est-elle un critère valable de systématique? Bulletin de la Société Zoologique de France 88: 388–392.

Coradin, T. & P. J. Lopez, 2003. Biogenic silica patterning: simple chemistry o subtle biology? ChemBioChem 4: 251–259.

Custodio, M. R., I. Prokic, R. Steffen, C. Koziol, R. Borojevic, F. Brummer, M. Nickel & W. E. G. Muller, 1998. Primmorphs generated from dissociated cells from the sponge Suberites domuncula: a model system for studies of cell proliferation and cell death. Mechanisms of Ageing and Development 105: 45–59.

Eckert, C., H. C. Schroeder, D. Brandt, Perovic-Ostadt & W. E. G. Müller, 2006. Histochemical and electron microscopic analysis of spiculogenesis in the demosponge *Suberites domuncula*. Journal of Histochemistry & Cytochemistry 54(9): 1031–1040.

Elvin, D., 1971. Growth rates of the siliceous spicules of the fresh-water sponge *Ephydatia muelleri* (Lieberkun). Transactions of the American Microscopical Society 90: 219–224.

Frölich, H. & D. Barthel, 1997. Silica uptake of the marine sponge *Halicondria panicea* in Kiel Bight. Marine Biology 128: 115–125.

Fry W. G., 1970 The sponge as a population: a biometric approach. In Symposium of the Zoological Society of London. Vol 25: 135–162.

Hartman, W. D., 1958. Natural history of marine sponges of southern New England. Bulletin of Peabody Museum of Natural History 12: 1–155.

Hentschel, E., 1929. Die Kiesel–und Hornschwämme des Nördlichen Meeres. Fauna Arctica Jena 5: 859–1042.

Hooper, J. A., 1991. Revision of the family Raspailiidae (Porifera: Demospongiae) with description of Australian species. Invertebrate Taxonomy 5: 1179–1418.

Jones, W. C., 1987a. Seasonal variations in the skeleton and spicule dimensions of *Haliclona elegans* (Bowerbank) sensu Topsent (1887) from two sites in North Wales. In Jones, W. C. (ed.), European contributions to the taxonomy of sponges. Litho Press, Middleton: 109–129.

Jones, W. C., 1987b. Skeletal variation in embryo-containing specimens of *Haliclona rosea* (Bowerbank) from Anglesey, North Wales. In Vacelet, J. & N. Boury-Esnault (eds) Taxonomy of Porifera. NATO ASI Series, 13. Springer–Verlag, Berlin, Heildeberg: 101–124.

Jones, W. C., 1991. Monthly variations in the size of spicules of the Haplosclerid sponge *Haliclona rosea* (Bowerbank). In Reitner, J. & H. Keupp (eds), Fossil and recent sponges. Springer Verlag, Berlin: 404–420.

Jørgensen, C. B., 1944. On the spicule formation of *Spongilla lacustris* (L.). I. The depence of the spicule formation on the content of dissolved and solid silicic acid of the milieu. Kongelinke Danske Videnskabernes Selskab Biologiske Meddelande 19: 1–45.

Jørgensen, C. B., 1947. On the spicule formation of *Spongilla lacustris* (L.). II. The rate of growth of the spicules. Kongelinke Danske Videnskabernes Selskab Biologiske Meddelande 20: 1–21.

Krasko, A., B. Lorenz, R. Batel, H. C. Schroeder, I. Müller & W. E. G. Müller, 2000. Expression of silicatein and collagen genes in the marine sponge *Suberites domuncula* is controlled by silicate and myotrophin. European Journal of Biochemistry 267: 4878–4887.

Krasko, A., H. C. Schroeder, R. Batel, V. A. Grebenjuk, R. Steffen, I. Müller & W. E. G. Müller, 2002. Iron induces proliferation and morphogenesis in primmorphs from the marine Sponge *Suberites domuncula*. DNA and Cell Biology 21: 67–80.

Le Pennec, G., S. Perovic, M. S. A. Ammar, V. A. Grebenjuk, R. Steffen, F. Brummer & W. E. G. Müller, 2003. Cultivation of primmoprhs from the marine sponge Suberites doumuncula: morphogenetic potensial of silicon and iron. A review. Journal of Biotechnology 100: 93–108.

Maldonado, & Riesgo, 2007. Intraepithelial spicules in a homosclerophorid sponge. Cell and Tissue Research 328: 639–650.

Maldonado, M., M. C. Carmona, M. J. Uriz & A. Cruzado, 1999. Decline in Mesozoic reef building sponges explained by silicon limitation. Nature 401: 785–788.

Maldonado M.,L. Navarro, A. Grasa, A. Gonzalez, I. Vaquerizo, 2011. Silicon uptake by sponges: a twist to understanding nutrient cycling on continental margins. NPG Scientific Reports 1(30).

Mercurio, M., G. Corriero, L. Scalera-Liaci & E. Gaino, 2000. Silica content and spicule size variations in *Pellina semitubulosa* (Porifera: Demospongiae). Marine Biology 137: 87–92.

Müller, W. E. G., M. Wiens, R. Batel, R. Steffen, H. C. Schroeder, R. Borojevic & M. R. Custodio, 1999. Establishment of a primary cell culture from a sponge: primmorphs from *Suberites domuncula*. Marine Ecology Progress Series 178: 205–219.

Müller, W. E. G., M. Bohm, R. Batel, S. De Rosa, G. Tommonaro, I. M. Müller & H. C. Schröeder, 2000. Application of cell culture for the production of bioactive compounds from sponges: synthesis of avarol from *Dysidea avara*. Journal of Natural Products 63: 1077–1081.

Müller, W. E. G., M. Rothenberger, A. Boreiko, W. Tremel, A. Reiber & H. Schröeder, 2005. Formation of siliceous spicules in the marine demosponge *Suberites domuncula*. Cell & Tissue Research 321: 285–297.

Müller, W. E. G., S. I. Belikov, W. Tremel, C. Perry, W. W. C. Gieskes, A. Boreiko & H. Schröeder, 2006. Siliceous spicules in marine Demosponges (example *Suberites domuncula*). Micron 37: 107–120.

Nickel, M., S. Leininger, G. Proll & F. Brümmer, 2001. Comparative studies on two potential methods for the biotechnological production of sponge biomass. Journal of Biotechnology 92(2): 169–178.

Nickel, M., T. Donathet, M. Schweikert & F. Beckmann, 2006. Functional morphology of *Tethya* species (Porifera): 1. Quantitative 3D-analysis of *Tethya wilhelma* by synchrotron radiation based X-ray microtomography. Zoomorphology 125: 209–223.

Palumbi, S. R., 1986. How body plan limit acclimation: responses of a demosponge to wave force. Ecology 67: 208–214.

Pe', J., 1973. Etude quantitative de la regulation du squelette chez une éponge d'eau douce. Archives de Biologie 84: 147–173.

Poiret, J.L.M. 1789. Voyage en Barbarie, ou Lettres Ecrites del'Ancienne Numidie pendant les Années 1785 et 1786, avec un Essai sur l'Histoire naturelle de ce Pays. Deuxième Partie. 1. (Paris): 1–315.

Pulitzer–Finali, G., 1983. A collection of Mediterranean Demospongiae (Porifera) with, in appendix, a list of the Demospongiae Hitherto recorded from the Mediterranean Sea. Annali del Museo Civico di Storia Naturale, Genova 84: 445–621.

Reincke, T. & D. Barthel, 1997. Silica uptake kinetics of *Halicondria panicea* in Kiel Bight. Marine Biology 129: 591–593.

Sarà, M & J. Vacelet, 1973. Ecologie des Demosponge. In Grassé, P. P. (ed.) Traite de Zoologie. Anatomie. Sistématique Biologie. vol III, 462–564.

Schröder, K., 1936. Beitrdge zur Kenntis der Spiculabildung, der Larvenspiculation und der Variationsbreiten der Gerlstnadeln von Slsswasserschwämmen. Z Morphol Okol Tiere 31: 245–267.

Simpson, T. L., 1978. The biology of the marine sponge *Microciona prolifera* (Ellis and Sollander). III. Spicule secretion and the effect of temperature on spicule size. Journal of Experimental Marine Biology and Ecology 35: 31–42.

Smith-Gill, S. J., 1983. Development plasticity: developmental conversion versus phenotypic modulation. American Zoologist 23: 47–55.

Stone, A. R., 1970. Seasonal variations of the spicule size in *Hymeniacidon perleve*. Journal of the Marine Biological Assocition of United Kingdom 50: 343–348.

Topsent, E., 1917. Spongiaires. Deuxième expedition antarctique française (1908–1910) commandée par le Dr. Jean Charcot. Sciences physiques: documents scientifiques 4: 1–88.

Tuzet, O. & R. Connes, 1962. Spicules anormaux d'une varieté écologique d' *Ephydatia fluviatilis* L. Vie et Milieu 13: 467–470.

Uriz, M. J., 2006. Mineral skeletogenesis in sponges. Can J Zool 84: 322–356.

Uriz, M. J., X. Turon & M. A. Becerro, 2000. Silica deposition in demospongiae: spiculogenesis in *Crambe crambe*. Cell & Tissue Research 301: 299–309.

Uriz, M. J., X. Turon, M. Becero & G. Agell, 2003. Siliceous spicules and skeleton frameworks in sponges: origin, diversity, ultrastructural patterns and biological functions. Microscopy Research and Technique 62: 279–299.

Valisano, L., G. Bavestrello, M. Giovine, A. Arillo & C. Cerrano, 2007a. Effect of iron and dissolved silica on primmorphs of *Petrosia ficiformis* (Poiret, 1789). Chemistry and Ecology 23: 233–241.

Valisano, L., A. Arillo, G. Bavestrello, M. Giovine & C. Cerrano, 2007b. Influence of temperature on primmorphs production in *Petrosia ficiformis* (Porifera, Demospongiae). In Porifera Research–Biodiversity, Innovation and Sustainability. Museu Nacional, Série Livros, Vol. 28, 7–10 May 2006, Armacao de Buzios, Rio de Janeiro: 639–643.

Weissenfel, N., 1989. Biologie und Mikrosckopische Anatomie der Sußwasserschwamme (Spongillidae). Gustav Fischer Verlag, Stuttgart.

Witte, U., D. Barthel & O. Tendal, 1994. The reproduction cycle of the sponge *Halicondria panicea* Pallas (1766) and its relationship to temperature and salinity. Journal of Experimental marine Biology and Ecology 183: 41–52.

Zhang, X., X. Cao, W. Zhang, X. Yu & M. Jin, 2003. Primmorphs from archaeocytes-dominant cell population of the sponge *Hymeniacidon perleve*: improved cell proliferation and spiculogenesis. Biotechnology and Bioengeneering 84: 583–590.

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SPONGE RESEARCH DEVELOPMENTS

Trace metal concentrations in the tropical sponge *Spheciospongia vagabunda* at a sewage outfall: synchrotron X-ray imaging reveals the micron-scale distribution of accumulated metals

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Abstract Major and trace elements were measured in sponges, seawater and sediment in Darwin Harbour (Australia) to test the hypothesis that metals are elevated in sponges closer to a sewage outfall compared with unimpacted sites. Seawater and sediment at the sewage discharge site contained high, but localised, concentrations of phosphorus (P), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As) and lead (Pb) compared with background sites. Metal concentrations in the sponge *Spheciospongia vagabunda* were highly elevated compared with other sponges and, although site specific, high metal concentrations were unrelated to the presence of sewage effluent. X-ray fluorescence microprobe imaging was used to investigate the metal distribution pattern in *S. vagabunda*. High Fe, Ni and Zn concentrations were either localised in circular patches (100–200 μm size) near water canals or in the pinacoderm, or scattered in spots (approximately 10 μm) throughout the tissue. This supports a microflora-mediated metal bioaccumulation hypothesis. In contrast, Co and Mn were highly dispersed and probably associated with aluminium- and iron-oxide rich sediment inclusions. Although the lack of association between sewage effluent and metal accumulation precludes the use of *S. vagabunda* as a biomonitor, the apparent differential mechanisms of metal accumulation warrants further investigation.

Keywords Sponge · Metals · Biomonitoring · Bioconcentration · *Spheciospongia vagabunda* · X-ray imaging

Guest editors: M. Maldonado, X. Turon, M. A. Becerro & M. J. Uriz / Ancient animals, new challenges: developments in sponge research

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Introduction

Darwin Harbour is a large (1,000 km^2) macrotidal estuary situated in tropical northern Australia and is renowned for its pristine nature and high biodiversity associated with abundant coral and sponge reefs. Darwin was one of the top ten fastest growing cities in Australia in 2008–2009 (Australian Bureau of Statistics, http://www.abs.gov.au/ausstats) and the past decade has seen a rapid increase in urbanisation, industrialisation and marine activity. Although urban areas represent a small proportion of the catchment

area, they contribute significantly to pollutant loads into Darwin Harbour (Skinner et al., 2009). Significant growth in mining and the oil and gas industry has seen the development of large new commercial port facilities.

To date the overall water quality of Darwin Harbour has remained near pristine largely as a result of the high rate of tidal water exchange. However, there are areas of localised impact from sewage (Skinner et al., 2009), stormwater (Welch et al., 2008) and loading of ore at the iron ore wharf (Woods, 1998). At Larrakeyah (Fig. 1) macerated but untreated sewage is pumped into Darwin Harbour at a depth of approximately 20 m. Waste waters from the Larrakeyah outfall carry domestic and light commercial discharge from Darwin city and contains appreciable amounts of ammonia, suspended solids and trace toxic metals (Power and Water Corporation, 2006). Reports have shown that there has been little measurable impact in nutrient and metal levels in surface seawater in the vicinity of the outfall (Moir, 1995; Dettrick & Schlusser, 2006). However, there have been no assessments of seawater, sediment or biota in the immediate area of the sewage outfall where biota, including sponges, are abundant.

More than 260 species of sponges have been identified in Darwin Harbour (Museum and Art Gallery of the Northern Territory records), and several studies elsewhere have identified sponges as useful biomonitors for metal contamination (Patel et al., 1985; Verdenal et al., 1985; Hansen et al., 1995; Perez et al., 2005; Rao et al., 2006, 2009; Cebrian et al., 2007). Many sponges support extensive prokaryotic and eukaryotic communities including bacteria (Vacelet & Donadey, 1977; Hentschel et al., 2002), archaea (Preston et al., 1996; Holmes & Blanch, 2007), cyanobacteria (Wilkinson, 1980; Rützler, 1990), dinoflagellates (Sará & Liaci, 1964; Rützler, 1990; Garson et al., 1998) and diatoms (Cox & Larkum, 1983). Sponges produce a range of compounds, presumably for protection against predators and antifouling (Becerro et al., 1997), and determining whether the compounds originate from endosymbiotic microorganisms or the sponge cells themselves has been the focus of much research (Garson et al., 1992; Flowers et al., 1998; Turon et al., 2000).

The aim of this study was to determine the contamination level near the Larrakeyah sewage effluent outfall using sponges as a biomonitor by measuring element levels in the marine sponge

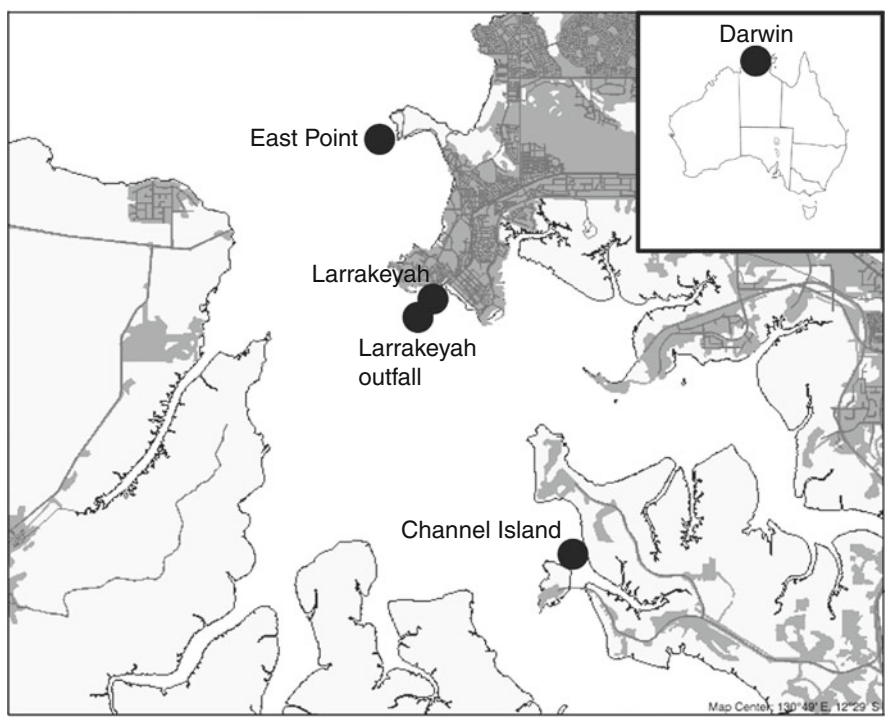


Fig. 1 Location of study sites in Darwin Harbour, Australia

Spheciospongia vagabunda (Demospongiae: Hadromerida: Clionaidae) at impacted and reference sites. We chose this species because it was present at a range of locations near the outfall and at reference sites and is very common through the Indo-Pacific. We measured key pollutant trace metals (cadmium, copper, iron, lead, nickel and zinc) as well as several other elements. Element levels in sediment and seawater were measured to further characterise the sample sites. In this study we tested the hypothesis that element levels in water, sediment and sponges are elevated at impacted sites compared with unimpacted sites. We further evaluated the micron-scale distribution of sequestered metals in the sponge to provide insight into metal bio-accumulation mechanisms.

Materials and methods

Site selection

At the Larrakeyah outfall site (12°28.046′S 130°49.77′E) (Fig. 1) sewage effluent is pumped along a 700-m pipe on the seafloor with the final 70 m of pipe at a depth of 20 m containing seven diffusers approximately 6 m apart. In addition, several reference sites in Darwin Harbour were sampled in this study: East Point (12°25.103′S 130°48.876′E), Channel Island (12°33′ 5.02″S 130°52′30″E) and Larrakeyah (12°27.790′S 130°49.765′E), shown in Fig. 1. The Larrakeyah reference site was at the shore end of the outfall pipe approximately 500 m from the closest diffuser. The influence of sewage at this site was not known.

Sponge selection

Initial dives at the Larrakeyah sewage outfall revealed the presence of at least 13 species including *S. vagabunda* growing on the discharge pipe. The observed species are common throughout Darwin Harbour including the selected reference sites (Alvarez, unpublished data). *Spheciospongia vagabunda* (Ridley, 1884) is a very common species through the Indo-Pacific, with known records from Indian Ocean, northern Australia Torres Strait, Great Barrier Reef, Sri Lanka, Papua New Guinea, Philippines and Central Pacific Ocean (Sutcliffe et al., 2010). Specimens from Darwin Harbour are generally thickly encrusting, following the shape of substrate and semi-buried, with short and irregular chimney like projections (2–5 cm long by 1–2 cm thick) protruding from the base of the sponge. Oscula (when seen) are located at top of chimneys. The surface is smooth but the consistency is firm.

Sampling

Specimens of *S. vagabunda* were collected by SCUBA diving at the Larrakeyah and East Point sites during neap tides in the Dry Season (May–August) 2009 and 2010. At the Larrakeyah outfall pipe not enough individual sponges were found at the diffusers so specimens were also taken from between the diffusers and kept separate. Sponges at Channel Island were collected by hand during a low spring tide (<0.3 m) in December 2009. Sponge samples were placed into WhirlPak bags (Nasco, USA) containing seawater and kept cool. Seawater (2 l) and sediment (200 g) (from areas adjacent to where sponges were growing) were collected in triplicate except for the outfall site where duplicates were collected and stored on ice. All containers used for collection were acid washed to trace metal standard.

Chemical analyses of water, sediment and sponges

In the laboratory, seawater collected at the outfall (diffusers and between diffusers) was centrifuged at 5,000 rpm for 10 min to pellet large particles. Seawater samples were then filtered using a 0.45 μm cellulose acetate syringe filter prior to acidification with HNO_3 (Merck SupraPur) to a concentration of 0.13% HNO_3 and stored at 4°C. Sediment was wet sieved to 2 mm, dried for 3 days in a 60°C oven and then ground to a powder using an automated agate mortar and pestle which was cleaned with acid washed sand between samples. Approximately 0.5 g of sediment was digested using 5 ml of HNO_3/$HClO_4$ (1 + 4) for 6 h at 200°C. Replicate samples of selected seawater and sediment samples were spiked with known amounts of metals during sample preparation for quality assessment. Seawater and dilute sediment digest solutions were analysed on an Agilent 7500ce Inductively Coupled Plasma Mass Spectrometer (ICP-MS) at Charles Darwin University. Digest blanks, replicate sample digests and certified reference materials were analysed for quality control and included seawaters CASS-4 and CASS-5 and sediment MESS-3 (Canadian Research Council).

Sponges were rinsed using 0.45 μm filtered seawater to remove sediment, macroinvertebrates, pebbles and other debris, placed into clean plastic bags and freeze-dried before grinding to a powder using an automated agate mortar and pestle. Powdered sponges were digested in 5 ml HNO_3 + 2 ml H_2O_2 for 2 h at 130°C. Dilute solutions of digested sponge tissue were then analysed by ICP-MS. Digest blanks, replicates and certified reference materials (NIST 1566 and 1575, IAEA 407) were included in each digestion batch. This method provides low detection limits (parts per billion range) but does not provide total digestion for all elements in silicate-rich matrices. In order to assess the recovery of total element concentrations by this method, four representative sponge tissue samples were digested in hot HF + HNO_3 + $HClO_4$ + H_2SO_4 followed by ICP-MS (ALS, Brisbane) (referred to as four-acid ICP-MS) and were also analysed by X-ray Fluorescence Spectrometry (XRF) on fused glass discs (ALS, Brisbane).

Environmental data analysis

The PRIMER & PERMANOVA package (Primer-E Ltd, Plymouth, UK) was used to display and examine relationships in bulk element concentrations in seawater, sediment and sponges between sites. Initially, ordination by principal components analysis (PCA) was performed on normalised data (element concentrations) from seawater and sediment to generate two dimensional plots displaying the relationships among the sites. For the sponge data, canonical analysis of principal coordinates (CAP) was performed to illustrate the differences among the samples detected by Permutational ANOVA (PERMANOVA). We used CAP because, according to Anderson et al. (2008) "sometimes there are real differences among a priori groups in multivariate space that cannot be easily seen in an unconstrained ordination (such as a PCA, PCO or MDS plot)".

PERMANOVA analysis was performed, using unrestricted permutations of raw data, to test for significant differences among sites in the patterns of elemental concentrations in the sponges. Analyses were done on all the variables together and on each of the individual variables. If the overall test was significant, pair wise tests were done to determine which sites were significantly different. For the sponge data, *P* values were adjusted using the sequential Bonferroni procedure (Quinn & Keough, 2002) to control Type I error rates.

Bioconcentration factors (BCF) for each site were calculated by dividing the average element concentration of sponges across a site by the average element concentration of sediment (<2 mm fraction) or seawater (dissolved phase) at that site.

Spearman's rank correlation coefficients were calculated for each element concentration in sponges versus sediment and sponges versus seawater using JMP 7.0 software (SAS Institute Inc., Cary, NC, USA). Average values were used for each site.

X-ray fluorescence microprobe sample preparation and analysis

Tissue samples of *S. vagabunda* were analysed by X-ray fluorescence microprobe (XFM) beamline at the Australian Synchrotron, Melbourne, Australia. Freshly collected sponge tissue was rinsed briefly in filtered seawater and small sections ($\sim$0.5 cm^3) were cut and placed in 2.5% gluteraldehyde in filtered seawater for at least 24 h. Tissue was washed three times (2 h each) in filtered seawater and then dehydrated through an ethanol series (70%/4 h; 80%/4 h; 96%/4 h; 100%/4 h; 100%/4 h) and washed three times in xylene ethanol (each wash was 2, 4 and 4 h). Samples were then embedded in paraffin wax, sectioned into 4 mm × 4 mm × 30 μm thin sections using a microtome and placed onto silicon nitride membranes (Silson Ltd, UK). A total of 14 sections were prepared covering external and internal sponge tissue. Elemental X-ray imaging was performed using a monochromatic primary X-ray beam of 12.7 keV focussed by a Kirkpatrick–Baez mirror pair to provide a beam spot size of approximately 2 μm (Australian Synchrotron, 2010). An automated sample stage was used to scan samples at 2 μm increments in the *x* and *y* dimensions with a dwell time of approximately 4 ms per pixel. Fluorescent X-rays were collected with the Maia 384 silicon detector array which spans a large solid angle allowing for high count rates and collection of large images in a relatively short time (approximately 1 M pixels per hour in this experiment) (Ryan et al., 2010). Spectral fitting and quantitative mapping was carried out using the Dynamic Analysis Method as implemented in the Geo-PIXE II software (Ryan, 2000; CSIRO, 2010). Fluorescent X-ray yield calculations were based on analysis of metal-doped

standard foils (Australian Synchrotron, 2010). In the absence of a more accurate matrix model, yield calculations were performed assuming a matrix of a single 30 μm cellulose layer. However, it should be noted that the analysed tissue sections were highly porous on a μm–mm scale and contained dispersed silica spicules.

Results

Seawater and sediment chemistry

Seawater from all reference locations showed little variation in the concentration of the elements measured (Table 1) and were within published ranges for Darwin Harbour seawater with the exception of zinc concentrations that were higher than would be expected for unimpacted waters (Munksgaard & Parry, 2001; Padovan, 2002). The Larrakeyah diffuser site was characterised by high concentrations of phosphorus, manganese, cobalt, iron, nickel, copper, zinc, arsenic and lead, with almost 70% of the variability explained by these elements (Fig. 2). A further 10% of the variation was due to differences in cadmium and chromium concentrations (Fig. 2). There were significant differences ($P < 0.01$) in seawater chemistry between the Larrakeyah diffuser and all other sites (Table 1) and element levels from the 'between diffuser' samples were highly variable, most likely due to different loads of the discharged sewage at the time of sampling.

Sediments collected at different sites in Darwin Harbour could be distinguished by their elemental composition (Table 2). Sediment from Channel Island was manganese rich compared with other sites, sediment from East Point had relatively low concentrations of iron and sediment from Larrakeyah had higher levels of nickel than the other sample sites. Sites could be spatially differentiated, with sediment at the Larrakeyah diffuser having elevated concentrations of phosphorus, iron, cobalt, nickel, copper, zinc, arsenic and lead, with almost 76% of the variability explained by these elements (Fig. 3). There were significant differences between the Larrakeyah diffuser and East Point ($P < 0.01$). The single sample from Larrakeyah was insufficient to resolve its relationship to the other sites.

Table 1 Element concentrations (ppb) in filtered (<0.45 μm) seawater collected from study sites

Site	P	Mn	Fe	Co	Ni	Cu	Zn	As	Cd	Pb
East Point[a]	<	1.75 ± 0.01	0.52 ± 0.03	0.02 ± 0.0	0.25 ± 0.01	0.26 ± 0.03	2.94 ± 0.52	1.59 ± 0.04	0.02 ± 0.0	0.03 ± 0.0
Channel Island[a]	<	2.18 ± 0.28	1.67 ± 0.42	0.04 ± 0.0	0.31 ± 0.03	0.58 ± 0.15	7.40 ± 3.83	1.15 ± 0.03	0.02 ± 0.0	0.03 ± 0.01
Larrakeyah[a]	<	1.14 ± 0.04	0.69 ± 0.15	0.02 ± 0.01	0.26 ± 0.03	0.25 ± 0.04	7.46 ± 4.07	1.37 ± 0.13	0.02 ± 0.0	0.04 ± 0.01
Between diffusers[ab]	486 ± 279	4.28 ± 2.20	7.50 ± 4.90	0.05 ± 0.02	0.39 ± 0.11	5.68 ± 3.15	30.54 ± 17.9	1.45 ± 0.15	0.03 ± 0.01	0.12 ± 0.05
Diffusers[b]	3,080 ± 281	23.5 ± 3.13	24 ± 0.81	0.10 ± 0.03	1.32 ± 0.24	22.1 ± 2.87	54.3 ± 13.3	2.31 ± 0.14	<	0.16 ± 0.02
Detection limit	32.00	0.05	0.85	0.01	0.10	0.08	0.35	0.25	0.02	0.02

Data are mean ± standard error. < Indicates values below detection limit. Superscript letters a, b refer to significantly different sites ($P < 0.01$) based on PERMANOVA using all elements

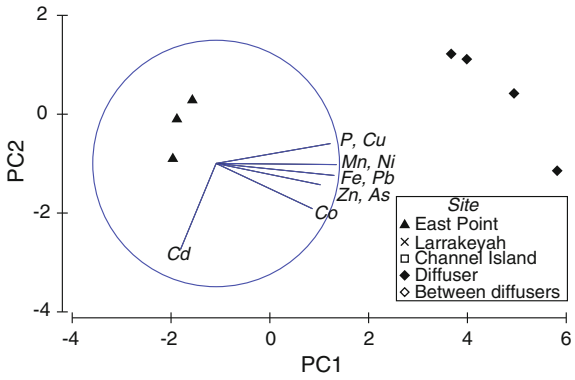


Fig. 2 Principal component analysis of elemental data from seawater. The first axis (PC1) accounts for 69.7% of the variability while PC2 accounts for 10.2% of the variability. The vectors show which elements are responsible for driving differences between sites

The metal concentrations obtained for seawater and sediment reference materials using the two-acid digestion method were within a range of 84–103% of the certified values except for chromium in sediment where the recovery was 20%. Recoveries of most metals from biota reference materials were between 80 and 100% except for aluminium (23–70%), copper (48–71%), chromium (27–48%), iron (50–54%) and nickel (45–67%). Recoveries from metal spiked samples of seawater, sediment and sponge were in the range 86–108%.

Sponge chemistry

Cadmium, cobalt, iron, manganese, nickel and zinc concentrations measured by two-acid digest ICP-MS were high in all *S. vagabunda* samples (Table 3). BCF calculated using sediment element concentrations were greater than one for cadmium, cobalt, nickel and zinc, and were particularly high for nickel (104–375) and cadmium (111–1,850) (Table 4). When seawater (dissolved fraction) concentrations were used in the calculation, bioconcentration values were in the order of millions for cadmium, cobalt, iron, nickel and zinc at all sites (Table 4).

Comparison of the two-acid/ICP-MS elemental concentration data for bulk samples of dried sponge tissue to data from the two alternative analytical methods (four-acid digest/ICP-MS and XRF) showed that four-acid digest and XRF had higher recoveries of aluminium, chromium, iron and lead. Results for all

Table 2 Element concentrations (ppm dry weight) in <2 mm fraction sediment collected from study sites

Site	P	Mn	Fe	Co	Ni	Cu	Zn	As	Cd	Pb
East Point[a]	364 ± 25.5	254 ± 27.5	7,790 ± 1,070	1.90 ± 0.33	3.26 ± 0.63	1.39 ± 0.24	5.80 ± 1.07	11.7 ± 0.89	0.05 ± 0.0	3.11 ± 0.36
Channel Island[ab]	367 ± 74.4	411 ± 84.3	13,100 ± 3,900	3.52 ± 0.93	4.84 ± 1.21	2.76 ± 0.68	18.9 ± 5.76	27.5 ± 8.64	0.12 ± 0.05	4.10 ± 0.88
Larrakeyah	499.3	298.7	14,500	4.17	7.49	3.68	18.2	10.1	0.12	6.12
Diffusers[b]	873 ± 45.2	332 ± 19.7	58,200 ± 1,780	7.65 ± 0.05	11.1 ± 0.05	14.9 ± 6.58	28.4 ± 4.67	56.4 ± 0.87	0.06 ± 0.0	13.0 ± 0.40
Detection Limit	0.80	0.20	6.00	0.02	0.06	0.20	0.30	0.30	0.02	0.60

Data are mean ± standard error. Superscript letters a, b refer to significantly different sites ($P < 0.01$) based on PERMANOVA using all elements

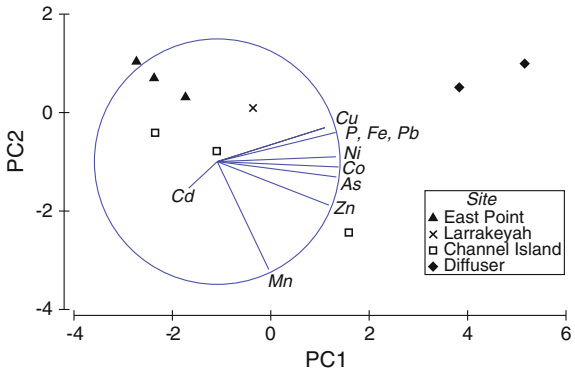


Fig. 3 Principal component analysis of element concentrations in sediments from the different sites. The first axis (PC1) accounts for 75.8% of the variability while PC2 accounts for 11.0% of the variability. The vectors show which elements are responsible for driving differences between sites

other analytes were similar for the three analytical methods (data not shown). This confirmed that the elevated metals of main interest in *S. vagabunda* (Cd, Co, Ni and Zn) were quantitatively recovered by the $HNO_3 + H_2O_2$ digestion method. Analysis by four-acid digest ICP-MS showed that *S. vagabunda* contained 0.62–0.80% sulphur ($n = 4$) and XRF determination showed a range of SiO_2 content from 18.5 to 23.8% ($n = 4$).

Sponge community level associations

In the CAP ordination of the metal concentrations in sponges, the two axes accounted for 76.2% of the variation (Fig. 4). *S. vagabunda* samples from Channel Island, East Point and Larrakeyah each formed spatially isolated clusters. Sponges from the Larrakeyah diffuser and from between diffusers had more variable elemental concentrations. For some elements at some sites, considerable variability in concentrations was observed. When communities were compared on the basis of their metal concentrations using PERMANOVA, *S. vagabunda* individuals from Channel Island, East Point and Larrakeyah were significantly different from each other ($P < 0.01$), with Channel Island sponges also different from sponges at the Larrakeyah diffuser and from between diffusers (Table 3). No statistical differences were observed between sponges from the Larrakeyah reference site, from between diffusers or from diffusers. PERMANOVAs performed on individual

Table 3 Average element concentrations (ppm dry weight) in *Spheciospongia vagabunda* at all study sites (two-acid digest)

Site	P	Mn	Fe	Co	Ni	Cu	Zn	As	Cd	Pb
East Point^{a} ($n = 3$)	1,120 ± 108^{a}	87.7 ± 30.6	1,720 ± 167	128 ± 4.60^{ae}	1,220 ± 46.4^{a}	2.30 ± 0.24^{a}	210 ± 8.48^{a}	7.52 ± 1.48	82.9 ± 2.63^{ac}	0.79 ± 0.18
Channel Island^{b} ($n = 3$)	703 ± 50.8^{a}	50.9 ± 30.2	956 ± 195	65.0 ± 0.87^{bc}	575 ± 6.32^{b}	2.48 ± 2.48^{ab}	121 ± 7.60^{b}	2.06 ± 0.24	13.3 ± 11.0^{b}	0.26 ± 0.11
Larrakeyah^{c} ($n = 8$)	1,760 ± 116^{b}	82.4 ± 8.50	3,390 ± 455	126 ± 9.81^{ace}	1,400 ± 104^{a}	3.94 ± 0.39^{a}	883 ± 64.9^{c}	12.7 ± 1.29	82.7 ± 6.16^{ac}	1.69 ± 0.27
Between diffusers^{ad} ($n = 16$)	1,670 ± 85.7^{b}	161 ± 21.0	6,620 ± 2,280	102 ± 7.06^{ce}	1,150 ± 85.8^{ab}	9.80 ± 1.22^{b}	572 ± 58.3^{d}	18.1 ± 2.09	67.4 ± 5.63^{a}	2.55 ± 0.55
Diffuser s^{ad} ($n = 4$)	1,540 ± 64.0^{b}	87.0 ± 20.1	2,400 ± 276	156 ± 19.6^{abd}	1,670 ± 210^{ab}	3.64 ± 0.40^{ab}	864 ± 108 ^{cd}	15.0 ± 2.44	111 ± 14.9^{bc}	0.85 ± 0.18

Data are mean ± standard error. Superscript letters a, b, c, d refer to significantly different sites based on PERMANOVA using all elements (site column) and individual elements (individual element columns)

Table 4 Bioconcentration values for sponge samples compared with sediment (<2 mm fraction) and seawater (filtered (<0.45 μm)

	Fe	Co	Ni	Zn	Cd
Sponge/sediment					
East Point	0.22	66.8	375	36.2	1,660
Channel Island	0.25	18.5	119	6.39	111
Larrakeyah	0.23	30.1	187	48.3	689
Between diffusers	0.11	13.4	104	20.1	1,120
Diffusers	0.04	20.4	150	30.3	1,850
Sponge/seawater					
East Point	3.32×10^6	2.56×10^6	4.89×10^6	0.07×10^6	4.14×10^6
Channel Island	0.57×10^6	1.61×10^6	1.85×10^6	0.02×10^6	0.66×10^6
Larrakeyah	4.91×10^6	6.25×10^6	5.38×10^6	0.12×10^6	4.14×10^6
Between diffusers	0.88×10^6	2.04×10^6	2.95×10^6	0.02×10^6	2.25×10^6
Diffusers	0.10×10^6	1.56×10^6	1.26×10^6	0.02×10^6	11.4×10^6

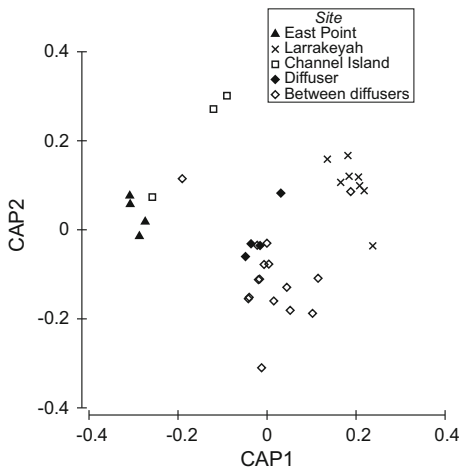


Fig. 4 Canonical analysis of principal coordinates showing elemental data from *S. vagabunda* at all sites. The first axis (CAP1) accounts for 50.6% of the variability while CAP2 accounts for 25.6% of the variability

elements showed that P, Co, Ni, Cu, Zn and Cd were significantly different between some sites (Table 3). Phosphorus concentrations were significantly higher in sponges from Larrakeyah, the Larrakeyah diffuser and between diffusers. Channel Island sponges had significantly lower concentrations of cobalt, nickel, zinc and cadmium compared with sponges from other sites.

The only correlation which was significant was for arsenic concentrations in sponges and sediment ($\rho = 1.00$, $P < 0.05$).

Metal concentration and distribution maps in sponges

Arsenic, chromium, iron, manganese, nickel and zinc concentration maps in *S. vagabunda* were obtained using XFM imaging. Cadmium could not be analysed as Cd K lines were not available at the primary beam energy of 12.7 keV and Cd L lines could not be resolved due to elemental interference. Concentrations of most elements determined by XFM were substantially lower than those determined by ICP-MS, except for copper which was higher than the copper concentrations obtained by all three bulk tissue methods. Subsequent ICP-MS analysis of the dehydration solutions used in the preparation of the XFM tissue sections revealed copper contamination of the ethanol used in those solutions; no other metals or metalloids were found in the dehydration solutions. Consequently, reliable XFM copper maps could not be obtained.

Although cobalt was readily detected in sponges by ICP-MS analysis, it was not easily detectable by XFM. Peak fitting and background estimation in the spectral region of cobalt and nickel peaks were affected by iron interference which lead to uncertainty in the derivation of both concentrations and detection limits for cobalt and nickel. Zinc and iron had the best spectral fit by XFM and their ratios were consistent with ICP-MS concentrations.

Quantitative elemental maps were obtained by XFM from thin sections of 11 samples of *S. vagabunda*. Although a variety of spatial patterns of metal distribution were seen in the mapped sections,

common features consisted of veins and semi-circular or circular 'hot spots' of elevated metal concentrations. Commonly these 'hot spots' were located near water canals and along the pinacoderm. Figure 5 shows a section of *S. vagabunda* (sample AS8206, Larrakeyah diffuser) with circular 'hot spots' of high metal concentrations and with a similar spatial distribution of areas with elevated iron, nickel and zinc concentrations. Typical concentrations found in these 'hot spots' were Fe $\approx$ 800 ppm, Ni $\approx$ 60 ppm, Zn = 150 ppm. Note that these concentrations represent in situ cellular metal concentrations on a μm scale and they should not be directly compared with the concentrations obtained for bulk samples of dried tissue as determined by ICP-MS. Circular 'hot spots' were mainly in the size range of 10–200 μm (Fig. 5). In contrast to the distribution of iron, nickel and zinc, areas with high chromium and manganese concentrations were smaller and more widely distributed throughout the tissue section (Fig. 5).

Discussion

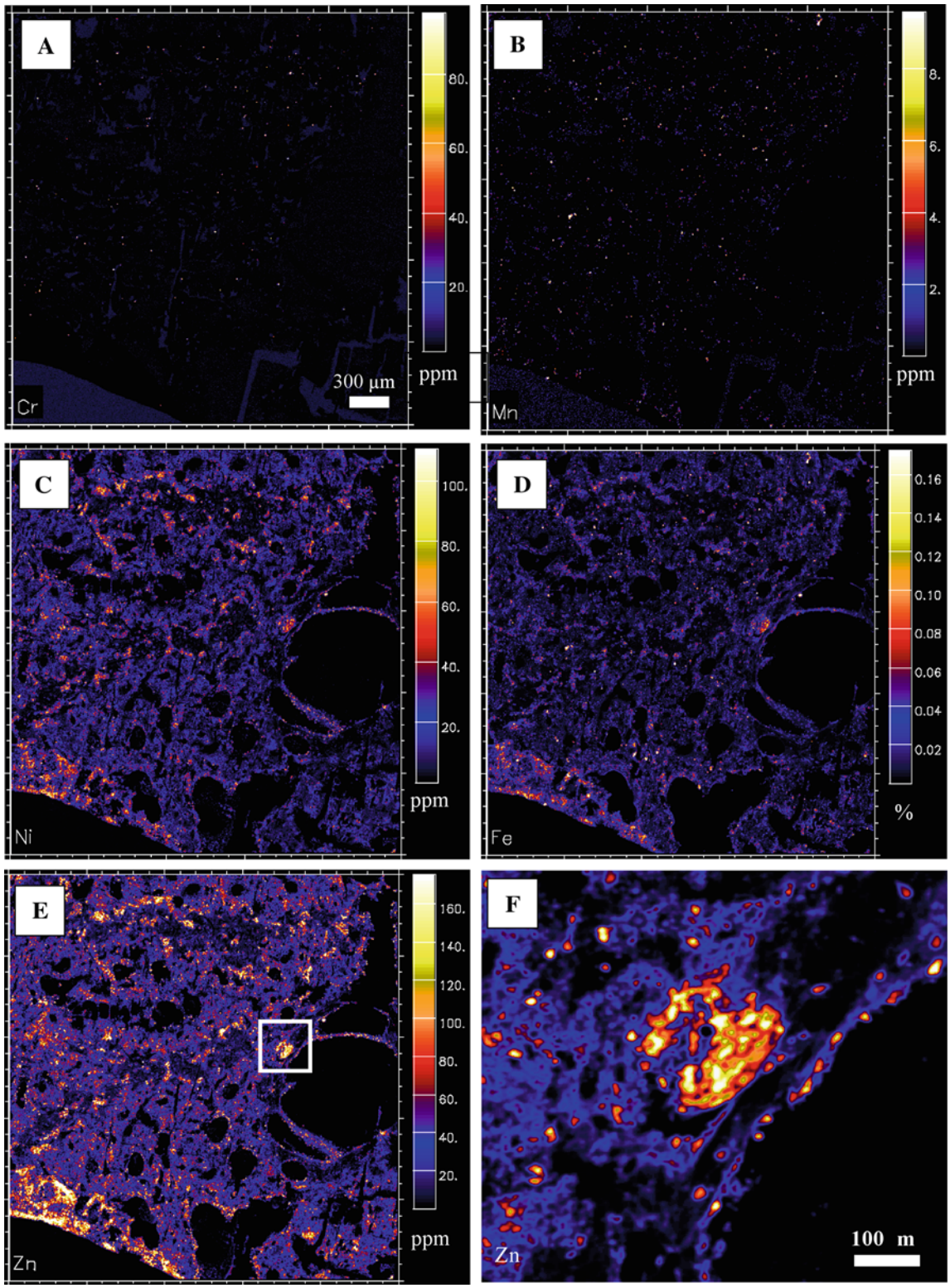
Sample sites were selected for sponge collection on the basis of their location relative to the sewage effluent outfall, which was assumed to give a range of impact levels. Element concentrations in filtered (0.45 μm) seawater and sediment (<2 mm) at each location confirmed that concentrations of most elements were substantially elevated in the immediate vicinity of the Larrakeyah sewage outfall. However, this effect was localised because element concentrations were near background levels within a few hundred metres of the diffuser. Element concentrations in seawater at reference sites in Darwin Harbour were typical of the levels previously reported in Darwin Harbour (Munksgaard & Parry, 2001; Padovan, 2002).

Sediment from the vicinity of the Larrakeyah sewage outfall was enriched with several elements, particularly iron, cobalt, nickel, copper and zinc but the levels were well below the effective range-medium guideline values (Long et al., 1995) and ANZECC sediment guidelines (ANZECC, 2000). Although this implies low toxicity at the outfall site, sediment with elevated metal contents may become resuspended during large tidal movements and contribute to the metal load accumulated by sponges and other filter feeders in the vicinity of the sewage outfall. Element concentrations in seafloor sediment from background reference sites were within the range previously reported for Darwin Harbour (Munksgaard & Parry, 2002).

Sampling in this study was opportunistic so while most sampling was performed in the Dry Season (May–August), samples from Channel Island were collected in the Wet Season (December). This study did not attempt to address changes in metal concentrations in sponges, sediment or seawater over different seasons. Previous studies have shown that there are only small differences in heavy metals and arsenic concentrations in Darwin Harbour sediments between seasons (Woods, 1998; Padovan, 2003). Water quality varied between seasons with respect to conductivity, salinity, temperature and turbidity, but remained the same with respect to nutrients (total and reactive phosphorus, nitrate/nitrite), dissolved oxygen, euphotic depth, total and volatile suspended solids and chlorophyll *a* (Padovan, 2002).

In contrast to the water and sediment chemistry, cadmium, cobalt, iron, manganese, nickel and zinc concentrations were elevated in all *S. vagabunda* individuals tested. The concentrations measured were high compared with previously published ranges of element concentrations in other sponge species (Verdenal et al., 1985; Denton et al., 1999; Philp et al., 2003; Negri et al., 2006; Rao et al., 2009). Concentrations of these elements were also found to be substantially higher than for other sponges from Darwin Harbour (*Paratetilla* sp., *Iotrochota* sp. and *Halichondria phakellioides*, Padovan unpublished data). Arsenic, copper, lead, manganese, silicon and sulphur levels in *S. vagabunda* were in the range reported for other sponge species (Patel et al., 1985; Verdenal et al., 1985; Araújo et al., 1999, 2003; Denton et al., 1999; Rao et al., 2009). Phosphorus was present at 703–1,760 ppm in sponge tissue (no published values could be found for comparisons).

Element concentrations in sponges collected from the Larrakeyah reference site, between diffusers and at diffusers were not significantly different from each other, but they were significantly different to sponges from the other reference sites. Element concentrations in sponges from the three reference sites were also significantly different from each other. This suggests that differences in sponge metal content may be related to regional differences in metal bioavailability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◀ **Fig. 5** XFM elemental maps of *S. vagabunda* tissue (sample AS 8206) collected from the Larrakeyah diffuser. Panels show distribution of chromium (**A**), manganese (**B**), nickel (**C**), iron (**D**), zinc (**E**) and a close-up of the outlined area of the zinc map (**F**). Concentration scales are in ppm mass except for iron which is % mass

in dissolved and/or particulate phases. For example, sediment grainsize and mineralogical content varies across Darwin Harbour (Munksgaard & Parry, 2002; Fortune, 2006) and this will influence the binding of metals accumulated within the sediment. Consequently, it is plausible that the bioavailability of metals in suspended particles derived from such sediments may vary between sites with different sediment characteristics and may have a direct bearing on sponge metal content in resident *S. vagabunda*. In this study, there were significant differences between sediment metal levels at the Larrakeyah diffusers and East Point, and to a lesser degree Channel Island. Sponges can integrate chemicals from both particulate matter (Reiswig, 1971) and dissolved phases (Olesen & Weeks, 1994; Hansen et al., 1995). In this study, the very high BCF calculated using either filtered seawater or sediment metal concentrations showed that *S. vagabunda* may take up metals from either or both sources, except for iron which only had a high BCF relative to seawater. This suggests that *S. vagabunda* has a highly effective and active uptake mechanism, not necessarily correlated to contaminant sources.

Cadmium and nickel were largely responsible for the differences between Channel Island and diffuser sponges, whereas zinc and arsenic drove the differences between East Point and diffuser sponges. So, although sponges at the diffuser were consistently different from the other sites, the key metals were different depending on the sites compared, which means that we cannot attribute metals in the sewage effluent as the cause of the observed differences. It is possible that some intra-site variation in sponge metal composition is likely to reflect differences in sponge sizes and ages. Seasonal differences may also be a factor since seasonal variations in the concentration of trace elements have been reported (Patel et al., 1985).

The concentrations of cadmium, cobalt, iron, nickel and zinc in *S. vagabunda* were higher than levels reported in most other sponge species. Only a few sponges with naturally high cadmium concentrations have been reported in the literature (Patel et al., 1985; Bargagli et al., 1996) with the Antarctic sponge *Tedania charcoti* having up to 15,000 ppm (Capon et al., 1993). Even at polluted sites, cadmium levels in other sponge species only ranged between 0.15 and 27.9 ppm (Philp, 1999; Perez et al., 2005; Rao et al., 2009). *Spheciospongia vesparia* was reported to have 0.94–14.24 ppm cadmium concentration (Philp, 1999), which is lower than for *S. vagabunda*. Cobalt concentrations have received relatively little attention, with published values ranging from below detection limits to 50 ppm (Bowen & Sutton, 1951; Patel et al., 1985; Rao et al., 2006, 2009). High iron content has been reported in *Spongia agaricina*, *Ircinia fasciculata*, *Cacospongia scalaris* and *Spongia officinalis* (Verdenal et al., 1985; Vacelet et al., 1988; Araújo et al., 1999, 2003) and has been attributed to incorporation of iron into spongin fibres. *Suberites carnosus* also had high iron content but in this species, the iron was due to incorporation of sediment particles (Araújo et al., 1999). High zinc concentrations have been measured in *Hymeniacidon perlevis*, *Cliona celata* (Araújo et al., 1999; Aguiar et al., 2010) and *Spirastrella cuspidifera* (Patel et al., 1985). Naturally high nickel concentrations occur in *Suberites carnosus* and *Cliona viridis* and like *S. vagabunda*, these sponges also contain high concentrations of zinc and iron (Araújo et al., 1999, 2003). The reasons for cadmium and zinc accumulation in sponges are not clear, with suggestions including conferring antibacterial properties and defence against fouling organisms and grazers (Capon et al., 1993). Therefore, like other sponge species, the high metal levels in *S. vagabunda* may have a role in defence, but also precludes these species as biomonitors of metal contamination, unless there is an understanding of the mechanism of metal uptake.

Metal distribution maps in *S. vagabunda* showed that domains of highly concentrated iron, nickel and zinc occurred in seams or (semi-) circular clusters. In contrast, chromium and manganese were highly dispersed, mainly in small areas a few microns in size. These areas may represent small inclusions of sediment grains embedded in sponge tissue near water channels as elevated chromium and manganese concentrations are commonly associated with aluminium- and iron-oxide rich fine grained sediment in Darwin Harbour (Munksgaard & Parry, 2002).

The clusters of iron, nickel and zinc ‘hot spots’ in the XFM maps appear to be similar to zooxanthellae

distributions found in other sponges (Rützler, 1997). Individual metal 'hot spots' in the XFM images are approximately 10 μm in diameter, which correlates with the size for individual zooxanthellae (Sará & Liaci, 1964; Rützler, 1997; Garson et al., 1998). In sponges, these algal cells can group into clumps from 6 to 20 cells (Rützler, 1997; Garson et al., 1998). In *S. vagabunda* XFM maps, abundant clusters of metal hot spots occur with an aggregate size up to approximately 200 μm which may represent clusters of zooxanthellae. Bowen & Sutton (1951) first suggested that high nickel concentrations in *D. crawshayi* may be due to microflora. Zooxanthellae separated from coral colonies had higher concentration of metals compared with coral soft tissue or skeletal components (Reichelt-Brushett & McOrist, 2003; Shah, 2008) and recently, zooxanthellae were associated with the nickel accumulating sponge *Cliona viridis* (Esteves et al., 2010). Zooxanthellae have been observed in *S. vagabunda* from New Caledonia (Kelly-Borges & Vacelet, 1998) and in this study which suggests that the high metal concentrations discovered in this study may be associated with these dinoflagellates. However, the mechanisms and significance of metal sequestering in *S. vagabunda* in Darwin Harbour are currently unknown.

Our data support the hypothesis that metal levels in seawater were elevated at the sewage outfall site compared with the unimpacted sites. Metals were also elevated in sediment but not above sediment quality guidelines. Despite the fact that water and sediment metals were elevated at the impacted site, metal levels were elevated in all *S. vagabunda* samples tested, regardless of impact. Upon closer assessment there were differences in sponge metal levels between sites but these differences could not be attributed to the metals associated with sewage effluent. While our data do show that *S. vagabunda* can accumulate metals, we reject the hypothesis that sponges collected from impacted sites had higher metal levels than sponges collected from reference sites. We offer an alternative hypothesis that differences in sponge metal content may be related to regional differences in metal bioavailability. Iron, nickel and zinc sequestration in *S. vagabunda* was clustered, which supports our hypotheses of microflora-mediated metal bioaccumulation. In contrast, chromium and manganese were highly dispersed which points to an association with aluminium- and iron-oxide rich fine grained sediment typical for Darwin Harbour.

While the similarity in metal levels in sponges at both impact and reference sites appears to rule out the use of *S. vagabunda* as an effective biomonitor, the discovery presents an opportunity to investigate the distribution and mechanism of metal accumulation in marine sponges. Research is now focused on coupling metal microanalysis to sponge and symbiont ultrastructure, separation of sponge and symbionts for chemical analyses, and molecular microbial community identification, to further investigate the nature and mechanism of metal accumulation in *S. vagabunda*.

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References

Aguiar, P., J. Medeiros, A. Costa, M. Humanes & M. Barreto, 2010. Distribution of nickel in the tissue of *Cliona viridis* from the coast of São Miguel (Azores). VIII World Sponge Conference, Girona, Spain.

Anderson, M. J., R. N. Gorley & K. R. Clarke, 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. PRIMER-E Ltd, Plymouth, UK.

ANZECC, 2000. Australian and New Zealand Guidelines for Fresh and Marine Water Quality. ANZECC, Australia.

Araújo, M., A. Cruz, M. Humanes, M. Lopes, J. A. L. da Silva & J. J. R. da Silva, 1999. Elemental composition of Demospongiae from the eastern Atlantic coastal waters. Chemical Speciation and Bioavailability 11: 25–36.

Araújo, M., A. Conceição, T. Barbosa, M. Lopes & M. Humanes, 2003. Elemental composition of marine sponges from the Berlengas natural park, western Portuguese coast. X-Ray Spectrometry 32: 428–433.

Australian Synchrotron, 2010. Australian Synchrotron Technical Information (XFM). Australian Synchrotron [available on internet at http://www.synchrotron.org.au/index.php/aussyncbeamlines/x-ray-fluorescence-microprobe-x-ray-microspectroscopy/technical-information].

Bargagli, R., L. Nelli, S. Ancora & S. Focardi, 1996. Elevated cadmium accumulation in marine organisms from Terra Nova Bay (Antarctica). Polar Biology 16: 513–520.

Becerro, M., X. Turon & M. J. Uriz, 1997. Multiple functions for secondary metabolites in encrusting marine invertebrates. Journal of Chemical Ecology 23: 1527–1547.

Bowen, V. & D. Sutton, 1951. Comparative studies of mineral constituents of marine sponges I. The genera Dysidea, Chondrilla, Terpios. Journal of Marine Research X: 153–167.

Capon, R., K. Elsbury, M. Butler, C. Lu, J. Hooper, J. Rostas, K. O'Brien, L. Mudge & A. Sim, 1993. Extraordinary levels of cadmium and zinc in a marine sponge, *Tedania charcoti* Topsent: inorganic chemical defense agents. Cellular and Molecular Life Sciences 49: 263–264.

Cebrian, E., M. Uriz & X. Turon, 2007. Sponges as biomonitors of heavy metals in spatial and temporal surveys in northwestern Mediterranean: multispecies comparison. Environmental Toxicology and Chemistry 26: 2430–2439.

Cox, G. & A. Larkum, 1983. A diatom apparently living in symbiosis with a sponge. Bulletin of Marine Science 33: 943–945.

CSIRO, 2010. Quantitative Imaging Using Dynamic Analysis. CSIRO [available on internet at http://nmp.csiro.au/dynamic.html].

Denton, G., L. Concepcion, H. Wood, V. Eflin & G. Pangelinan, 1999. Heavy Metals, PCBs and PAHs in Marine Organisms from Four Harbor Locations on Guam. Technical Report No. 87. Water and Environmental Research Institute of the Western Pacific University of Guam.

Dettrick, D. & K. Schlusser, 2006. A Report on Larrakeyah Sewage Outfall, Darwin Harbour, August 2006. Northern Territory Government: 1–17.

Esteves, A., S. Chaves, J. Xavier, R. Tenreiro & M. Humanes, 2010. A new *Symbiodinium* sp. lineage in Atlanto-Mediterranean *Cliona viridis*. VIII World Sponge Conference, Girona, Spain.

Flowers, A., M. Garson, R. Webb, E. Dumdei & R. Charan, 1998. Cellular origin of chlorinated diketopiperazines in the dictyoceratid sponge *Dysidea herbacea* (Keller). Cell and Tissue Research 292: 597–607.

Fortune, J., 2006. The Grainsize and Heavy Metal Content of Sediment in Darwin Harbour. Report No. 14/2006D. Aquatic Health Unit (Northern Territory Government) and the Environment Protection Agency.

Garson, M., J. Thompson, R. M. Larsen, C. H. Battershill, P. T. Murphy & P. R. Bergquist, 1992. Terpenes in sponge cell membranes: cell separation and membrane fractionation studies with the tropical marine sponge *Amphimedon* sp. Lipids 27: 378–388.

Garson, M., A. Flowers, R. Webb, R. Charan & E. J. McCaffrey, 1998. A sponge/dinoflagellate association in the haplosclerid sponge *Haliclona* sp.: cellular origin of cytotoxic alkaloids by Percoll density gradient fractionation. Cell and Tissue Research 293: 365–373.

Hansen, I., J. Weeks & M. Depledge, 1995. Accumulation of copper, zinc, cadmium and chromium by the marine sponge *Halichondria panicea* Pallas and the implications for biomonitoring. Marine Pollution Bulletin 31: 133–138.

Hentschel, U., J. Hopke, M. Horn, A. Friedrich, M. Wagner, J. Hacker & B. Moore, 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. Applied and Environmental Microbiology 68: 4431–4440.

Holmes, B. & H. Blanch, 2007. Genus-specific associations of marine sponges with group I crenarchaeotes. Marine Biology 150: 759–772.

Kelly-Borges, M. & J. Vacelet, 1998. Sponges of the New Caledonian Lagoon: class Demospongiae, order Hadromerida. In Levi, C. (ed.), Sponges of the New Caledonian Lagoon. Orstom Editions, Paris: 87–93.

Long, E., D. MacDonald, S. Smith & F. Calder, 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. Environmental Management 19: 81–97.

Moir, C. M., 1995. The effects of sewage discharge on the water quality of Darwin Harbour and Buffalo Creek. Northern Territory University Honours thesis.

Munksgaard, N. & D. Parry, 2001. Trace metals, arsenic and lead isotopes in dissolved and particulate phases of North Australian coastal and estuarine seawater. Marine Chemistry 75: 165–184.

Munksgaard, N. & D. Parry, 2002. Metals, arsenic and lead isotopes in near-pristine estuarine and marine coastal sediments from northern Australia. Marine & Freshwater Research 53: 719–729.

Negri, A., K. Burns, S. Boyle, D. Brinkman & N. Webster, 2006. Contamination in sediments, bivalves and sponges of McMurdo Sound, Antarctica. Environmental Pollution 143: 456–467.

Olesen, T. & J. Weeks, 1994. Accumulation of Cd by the marine sponge *Halichondria panicea* Pallas: effects upon filtration rate and its relevance for biomonitoring. Bulletin of Environmental Contamination and Toxicology 52: 722–728.

Padovan, A., 2002. Darwin Harbour Water Quality Monitoring 2001/02 Report. Report No. 23/2002. Water Monitoring Section, Natural Resource Management Division, Conservation and Natural Resources Group: 1–16.

Padovan, A., 2003. Darwin Harbour water and sediment quality. Proceedings of the Darwin Harbour Public Presentations, February 2003, Darwin, NT, Australia: 5–18.

Patel, B., M. Balani & S. Patel, 1985. Sponge 'sentinel' of heavy metals. The Science of the Total Environment 41: 145–152.

Perez, T., D. Longet, T. Schembri, P. Rebouillon & J. Vacelet, 2005. Effects of 12 years' operation of a sewage treatment plant on trace metal occurrence within a Mediterranean commercial sponge (*Spongia officinalis*, Demospongiae). Marine Pollution Bulletin 50: 301–309.

Philp, R., 1999. Cadmium content of the marine sponge *Microciona prolifera*, other sponges, water and sediment from the eastern Florida panhandle: possible effects on *Microciona* cell aggregation and potential roles of low pH and low salinity. Comparative Biochemistry and Physiology Part C 124: 41–49.

Philp, R., F. Leung & C. Bradley, 2003. A comparison of the metal content of some benthic species from coastal waters of the Florida Panhandle using high-resolution inductively coupled plasma mass spectrometry (ICP-MS) analysis. Archives of Environmental Contamination and Toxicology 44: 218–223.

Power and Water Corporation, 2006. Wastewater Treatment, Reuse and Discharge Report 2006. Power and Water Corporation.

Preston, C., K. Wu, T. Molinski & E. DeLong, 1996. A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. Proceedings of the

National Academy of Sciences of the United States of America 93: 6241–6246.

Quinn, G. & M. Keough, 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press, Cambridge, UK.

Rao, J., P. Kavitha, N. Reddy & T. Rao, 2006. *Petrosia testudinaria* as a biomarker for metal contamination at Gulf of Mannar, southeast coast of India. Chemosphere 6: 634–638.

Rao, J. V., K. Srikanth, R. Pallela & T. G. Rao, 2009. The use of marine sponge, *Haliclona tenuiramosa* as bioindicator to monitor heavy metal pollution in the coasts of Gulf of Mannar, India. Environmental Monitoring and Assessment 156: 451–459.

Reichelt-Brushett, A. J. & G. McOrist, 2003. Trace metals in the living and nonliving components of scleractinian corals. Marine Pollution Bulletin 46: 1573–1582.

Reiswig, H., 1971. Particle feeding in natural populations of three marine demosponges. Biological Bulletin 141: 568–591.

Ridley, S. O., 1884. Spongiida. In Report on the Zoological Collections made in the Indo-Pacific Ocean During the Voyage of H.M.S. 'Alert', 1881-2. British Museum of Natural History, London: 366–482, 339–343, 582–630, 353–354.

Rützler, K., 1990. Associations between Caribbean sponges and photosynthetic organisms. In Rützler, K. (ed.), New Perspectives in Sponge Biology. Smithsonian Institution Press, Washington, DC: 455–466.

Rützler, K., 1997. The role of psammobiontic sponges in the reef community. Proceedings of the 8th International Coral Reef Symposium, Vol. 2: 1393–1397.

Ryan, C. G., 2000. Quantitative trace element imaging using PIXE and the nuclear microprobe. International Journal of Imaging Systems and Technology 11: 219–230.

Ryan, C. G., R. Kirkham, R. M. Hough, G. Moorhead, D. P. Siddons, M. D. de Jonge, D. J. Paterson, G. De Geronimo, D. L. Howard & J. S. Cleverley, 2010. Elemental X-ray imaging using the Maia detector array: the benefits and challenges of large solid-angle. Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment 619: 37–43.

Sará, M. & L. Liaci, 1964. Symbiotic association between zooxanthellae and two marine sponges of the genus Cliona. Nature 203: 321.

Shah, S., 2008. Study of heavy metal accumulation in scleractinian corals of Viti Levu, Fiji Islands. University of the South Pacific Masters thesis.

Skinner, L., S. Townsend & J. Fortune, 2009. The Impact of Urban Land-Use on Total Pollutant Loads Entering Darwin Harbour. Report 06/2008D. Aquatic Health Unit, Department of Natural Resources, Environment, the Arts and Sport, Northern Territory Government: 1–66.

Sutcliffe, P. R., J. N. A. Hooper & C. R. Pitcher, 2010. The most common sponges on the Great Barrer Reef seabed, Australia, include species new to science (Phylum Porifera). Zootaxa 2616: 1–30.

Turon, X., M. Becerro & M. Uriz, 2000. Distribution of brominated compounds within the sponge *Aplysina aerophoba*: coupling of X-ray microanalysis with cryofixation techniques. Cell and Tissue Research 301: 311–322.

Vacelet, J. & C. Donadey, 1977. Electron microscope study of the association between some sponges and bacteria. Journal of Experimental Marine Biology and Ecology 30: 301–314.

Vacelet, J., B. Verdenal & G. Perinet, 1988. The iron mineralization of *Spongia officinalis* L. (Porifera, Dictyoceratida) and its relationships with the collagen skeleton. Biology of the Cell 62: 189–198.

Verdenal, B., C. Diana, A. Arnoux & J. Vacelet, 1985. Pollutant levels in Mediterranean commercial sponges. In Rützler, K. (ed.), Proceedings of the 3rd International Sponge Conference. Smithsonian Institution Press, Washington, DC: 516–524.

Welch, M., J. Schult & A. J. Padovan, 2008. Effects of Urban Stormwater on Heavy Metal and Nutrient Concentrations in Mangrove Sediments of Darwin Harbour. Aquatic Health Unit, Department of Natural Resources, Environment, the Arts and Sport, Northern Territory Government Report 08/2008D: 1–37.

Wilkinson, C. R., 1980. Cyanobacteria symbiotic in marine sponges. In Schwemmler, W. & H. E. A. Schenk (eds), Endocytobiology. Walter de Gruyter, Berlin: 553–563.

Woods, B. B., 1998. Heavy metals in Darwin Harbour sediments and the use of lead isotope ratios for the determination of anthropogenic sources of lead. Northern Territory University Honours thesis.

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SPONGE RESEARCH DEVELOPMENTS

Does concentrating chemical defenses within specific regions of marine sponges result in enhanced protection from predators?

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Abstract Chemical defenses are an effective mode of predator deterrence across benthic marine organisms, but their production may come with associated costs to the organism as limited resources are diverted away from primary processes like growth and reproduction. Organisms concentrating ecologically relevant levels of these defenses in tissues most at risk to predator attack may alleviate this cost while deterring predators. We addressed this hypothesis by investigating the deterrence of chemical extracts from the inner and outer regions of the sponges *Aplysina fulva*, *Ircinia felix*, and *I. campana* from a temperate hard-bottom reef in the South Atlantic Bight. Assays were conducted using natural fish assemblages and sea urchins. Although, *A. fulva* and *I. felix* have higher concentrations of defensive metabolites in the outer and inner regions, respectively, extracts from these regions did not display enhanced deterrency against fish or mobile invertebrate predators. Likewise, extracts from both regions of the sponge *Ircinia campana*, which has a uniform distribution of defensive chemicals throughout, did not differ in their ability to deter either group of predators. Since chemical defenses were effective deterrents at lower concentrations, secondary metabolite allocation patterns observed among these sponges are likely not driven by predation pressure from generalist fish and mobile invertebrate predators on these reefs. Alternatively, these patterns may be driven by other ecological stressors, another suite of predators, or may be more effective at deterring predators when combined with structural defenses.

Keywords Chemical defense · Temperate sponges · South Atlantic Bight · Feeding assays

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Introduction

Secondary metabolite production is a common method of defense against consumers in sessile terrestrial and marine organisms (Rhoades, 1979; Paul, 1992; Pawlik et al., 1995). While the effectiveness of these chemicals against predators is well-documented, their production may come with considerable metabolic cost to the prey (McKey, 1974, 1979; Rhoades, 1979). Organisms able to maintain defenses at the lowest, but still effective concentration or organisms that sequester these defenses in

particular regions of the body may thus be at a selective advantage. In plants, the Optimal Defense Theory (ODT) hypothesizes that the cost of chemical defenses can be reduced by concentrating defensive compounds in regions of the body with the highest fitness value or those that are most at risk to consumers (Rhoades, 1979). In recent years, the ODT has been tested more and more frequently on marine algae and soft-bodied invertebrates such as sponges (Hay, 1996) because, like plants, these marine organisms coexist with numerous species of potential consumers and lack active escape mechanisms.

Consistent with the ODT, some sponges show higher concentrations of deterrent compounds in the outer regions of the sponge, where the risk of predator attack may be greatest. For instance, brominated compounds are found in spherulous cells common to the ectosomal region of sponges in the genus *Aplysina* (Thompson et al., 1983; Turon et al., 2000), and toxic guanidine alkaloids accumulate in spherulous cells in *Crambe crambe*, leading to increased toxicity toward the periphery (Becerro et al., 1997). Likewise, *Rhopaloeides odorabile* has higher levels of diterpenes in the outer 2 cm (Thompson et al., 1987) of the sponge body and *Negombata magnifica* concentrates the metabolite latrunculin B toward its periphery (Gillor et al., 2000). While increased concentrations of these metabolites in the outer region imply a defensive role against fish and large mobile invertebrate predators, elevated levels of deterrent compounds toward the interior of other sponge species and high variation in chemical defense allocation within some genera imply that there may be multiple, complex forces driving the selection of chemical defense production and allocation within sponges (Freeman & Gleason, 2010; Sacristán-Soriano et al., 2011). In addition, since investigations on the effectiveness of allocating defensive chemistry to the outer region of a sponge have produced conflicting results, our understanding of the ecological significance of surface allocation on predator–prey interactions is lacking. For example, Furrow et al. (2003) reported that the Antarctic sponge *Latrunculia apicalis* sequesters defensive compounds in the outer 2 mm of its tissues and that chemical extracts from this region are significantly more deterrent against sea stars than extracts from the inner region. In contrast, while the sponge *Chondrilla nucula*, six species of Red Sea sponges, and *Melophlus sarasinorum* sequester defensive compounds toward their periphery, chemical extracts from this outer region do not display an enhanced ability to deter predators when compared to extracts from within the sponge (Swearingen & Pawlik, 1998; Burns et al., 2003; Rohde & Schupp, 2011).

One method to address the disparity across studies is to combine quantification of chemical defenses with tests of their ability to deter relevant consumers (as in Furrow et al., 2003; but not in Swearingen & Pawlik, 1998; Burns et al., 2003). Using this methodology, Schupp et al. (1999) confirmed that increased concentrations of defensive chemicals in regions of a sponge most vulnerable to attack by fishes made them deterrent. In contrast, Becerro et al. (1998) found that concentrations of major metabolites were higher in the tips than the base of the sponge *Cacospongia* sp., but extracts from the tips were consumed by fish just as readily as extracts from the base. Contrasting results from studies such as those described above highlight the need for further work to fully understand the relationship between allocation of chemical defenses within sponges and predator deterrence.

We have recently reported the presence of three distinct chemical defense allocation patterns in three sponge species that are common on temperate reefs of the North Atlantic Ocean off the coast of Georgia, USA (Freeman & Gleason, 2010). We used liquid chromatography-mass spectrometry (LC-MS) to identify a suite of brominated tyrosine derivatives isolated from the sponge *Aplysina fulva* (Pallas, 1766) and gas chromatography-mass spectrometry (GC-MS) to identify furanosesterterpene tetronic acids (FTAs) from the sponges *Ircinia felix* (Duchassaing & Michelotti, 1864), and *Ircinia campana* (Lamarck, 1814). Finally, we used high performance liquid chromatography (HPLC) to quantify the natural concentrations of these metabolites within the inner and outer regions of these sponges with the assumption that tissues toward the periphery are more vulnerable to consumption by fish and macroinvertebrate predators (Freeman & Gleason, 2010). While the ability of both brominated tyrosine derivatives and FTAs to deter predators has been reported previously (Ebel et al., 1997; Pawlik et al., 2002; Thoms et al., 2004), allocation patterns we

documented in these three sponge species were not consistent with predictions arising from the ODT. Specifically, higher concentrations of brominated tyrosine derivatives were measured in the outer regions of the branching sponge, *A. fulva*, as might be expected by the ODT, but levels of FTAs were greater in the inner regions of the amorphous massive sponge *I. felix* and were equal in inner and outer regions of the vase sponge *I. campana*. Thus, the study reported here was conducted to determine whether the higher levels of chemical defense observed within some of these sponges translates into enhanced predator deterrence. Based on the documented allocation patterns, we predicted that the higher levels of chemical defenses in the outer tissue regions of *A. fulva* and the inner regions of *I. felix* would result in enhanced deterrence, while the similar concentrations found in both regions of *I. campana* would deter predators equally.

Methods

Artificial food preparation

To determine if higher concentrations of defensive compounds within certain regions of sponges correspond with increased protection of those areas from predators, we compared the palatability of extracts from the outer 2 mm and the corresponding inner regions (hereafter referred to as outer and inner regions as in Freeman & Gleason, 2010) of a sponge to fish and urchin predators. Methods for the extraction of crude organic extracts from *A. fulva*, *I. felix* and *I. campana* and artificial food preparation followed those described by Becerro et al. (2003) and Ruzicka & Gleason (2008). We did not have enough sponge tissue to test the palatability of *I. felix* extracts against generalist reef fish, so only *A. fulva* and *I. campana* were used in this assay. All three species were used in feeding assays with the sea urchin *Arbacia punctulata*.

Sponges were collected from J-Y Reef (31°36.056N, 80°47.431W), a hard-bottom area in the South Atlantic Bight (SAB) characterized by sandstone and scallop shell ridges (Freeman et al., 2007; Freeman & Gleason, 2010). We collected a total of 24 individuals of *A. fulva*, 20 individuals of *I. felix*, and 22 individuals of *I. campana* from this site (Freeman & Gleason, 2010). Each of these individuals were separated into inner and outer regions and then utilized for HPLC quantification of metabolite concentrations, nutritional quality, and structural component analyses as reported in Freeman & Gleason (2010) and for feeding assays as discussed below. Discrepancies between the total number of sponges collected and those used in feeding assays were due to the fact that there was sometimes not enough tissue on the smaller samples for all analyses. In all cases though, at least 14 individuals of each sponge species were used to make food cubes for feeding assays. Chemical extracts from multiple sponges were never combined thus allowing for adequate replication in all feeding assays.

For fish feeding assays, frozen sponge samples from single individuals were thawed and 5 ml of the outer and inner regions were measured by displacement of water. These samples were freeze dried and extracted three times in 10 ml of DCM:MeOH (Freeman & Gleason, 2010). The three extracts obtained from a single sponge were combined, filtered, and evaporated to dryness. The dried crude extract was weighed and then reconstituted in 0.50 ml of MeOH and sonicated to ensure complete dissolution. Artificial food was made in 150 ml batches from a mixture of 7.5 g powdered squid mantle, 2.975 g Type I Carrageenan, 0.525 g agar, and 150 ml distilled water. This food mixture resulted in artificial food approximating the average nutritional quality present in sponges (Chanas & Pawlik, 1995). Once heated, approximately 4.5 ml of food mixture was added to each 27 mm diameter × 58 mm height vial containing 0.50 ml of MeOH and dissolved sponge extract. The mixture was homogenized by stirring rapidly and then was allowed to cool. Once the artificial food cube had hardened, it was cut into 1 ml cubes for feeding assays. Control food cubes were prepared the same way, but with crude extract omitted. To ensure that selection of food cubes by predators was not influenced by color, food coloring was added to both control and treatment mixtures before heating until both appeared similar in color by visual comparison. For the urchin feeding assays, frozen sponge samples from all three species were thawed and processed in the same manner as in the fish trials, but 7.5 g of powdered algal disks were substituted for powdered squid mantle and only 3 ml of tissue from the outer

and inner regions of each replicate and 0.30 ml of MeOH were used. In both fish and urchin feeding assays, the deterrence of chemical defenses was tested at natural concentrations. This was accomplished by incorporating extracts from 5 and 3 ml of sponge tissue into 5 and 3 ml food cubes for the fish and urchin assays, respectively. Thus, chemicals extracted from the sponge were dissolved and incorporated into artificial foods that were at a similar volumetric concentration.

Feeding assays

Fish feeding assays carried out with extracts from *A. fulva* and *I. campana* were conducted in the field at J-Y Reef (Freeman & Gleason, 2010) in one dive per species. The assays began by releasing several control food cubes to stimulate feeding activity of natural assemblages of generalist reef fish including black seabass (*Centropristus striata*), spottail pinfish (*Diplodus holbrooki*), pinfish (*Lagodon rhomboides*), and the gray triggerfish (*Balistes capriscus*). Inner, outer, and control food cubes were then offered one or two at a time. Once the released food cubes had been consumed or rejected (typically <5–10 s), additional food cubes were offered. Since each food cube represents extract obtained from a single sponge, a total of 21 food cubes of each type (inner, outer, and control) were released for *I. campana* and 14 of each type were released for *A. fulva*. Food cubes were released haphazardly so that not more than four of the same types of cube were released in a row and the fish could not habituate to a pattern of cube release or become accustomed to one type of food. A food cube was considered unpalatable if it was rejected by fishes two or more times or if it sank to the bottom uneaten. In these assays, the dominant consumers of food cubes were the black seabass (*Centropristus striata*) and spottail pinfish (*Diplodus holbrooki*). These fish species were also the dominant consumers for chemical assays conducted by Ruzicka & Gleason (2009) off the Georgia coast, consuming 80% of the 570 food cubes fed to fish.

Feeding assays were also conducted for *A. fulva*, *I. felix*, and *I. campana* in the wet lab at Georgia Southern University using the urchin *Arbacia punctulata* as the predator. This is a prevalent echinoderm species and the most abundant urchin species at J-Y Reef and is a common invertebrate grazer on hard-bottom reefs off the coast of Georgia (Freeman & Gleason, pers. obs.). While we have not observed *A. punctulata* feeding on sponges at J-Y Reef, other investigators have reported that this species, as well as others in this genus are omnivorous scavengers preying on a wide variety of algae and benthic animals, including coral polyps and sponges (reviewed in Ridder & Lawrence, 1982). In addition, although we did not use strictly spongivorous predators in either set of feeding assays, the use of generalist predators in assays to determine the deterrency of sponge chemical defenses is widespread in the literature (Waddell & Pawlik, 2000a, b; Burns et al., 2003) and the utility of using generalist predators has been well-outlined by Pawlik et al. (1995) and Ruzicka & Gleason (2009).

The experimental design for the urchin assays followed a method similar to that employed by Hay et al. (1994) using fiberglass window screening to hold the artificial food in place. Before adding artificial food to the sponge crude extract, a small ($\sim 7 \times 7$ mm) piece of fiberglass window screening (with 1×2 mm openings) was added to the vial containing the extract to act as webbing for attachment of the food cube. Approximately, 2.7 ml of artificial food was then added to the vial and stirred vigorously until the extract was thoroughly mixed within the food. Before allowing the artificial food to harden, the small piece of window screening was pushed to the bottom of the vial in the middle of the food cube. Once the food had hardened, the food cube was removed from the vial, blotted dry, and weighed.

For each replicate, urchins were given a choice of cubes containing compounds from inner and outer sponge tissues or a control cube containing no sponge extract. To do this, we attached pre-weighed food cubes to a large ($\sim 140 \times 130$ mm) piece of window screening by sewing the small piece of screen embedded in the food cube to the larger screen base with monofilament line. To keep food cubes equidistant from each other, we used an acrylic template containing three holes oriented in a triangle to position the cubes on the large screen base. This triangular arrangement ensured that when an urchin was placed in the center, its tube feet would be in contact with all three food types.

In preparation for the feeding assay, the large screen base containing all three food cubes was attached to the bottom of a feeding arena using

monofilament line. These arenas were 3.1 l Glad© plastic containers that had holes drilled in the sides, bottom, and lid to allow water flow. Stainless steel hex nuts were cable tied to the sides for negative buoyancy. The lids prevented the urchins from escaping during the assay period and also allowed us to conduct 20 assays at once by stacking arenas within the aquarium. In order to prevent positional effects, the orientation of the window screen within the feeding arena was adjusted haphazardly among replicates.

Arbacia punctulata were collected from J-Y Reef and transported to Georgia Southern University in aerated coolers. Urchins were held in 75.7 l aquaria containing filtered, artificial seawater at 30–32 ppt. Urchins were maintained on a diet of Wardley® algal disks until 48 h before their assay. All urchins used in the assay had a test diameter between 4 and 5.5 cm. An assay began when an urchin was placed in the center of the three food types, with as little stress due to exposure to air and handling as possible, and the arena was covered and placed on the bottom of the aquarium. A complete set of assays for a sponge species was completed in 1 day because these arenas could be stacked. Preliminary assays found that urchins allowed to feed for more than 8 h could consume all of at least one type of food, so all assays were stopped at 8 h. We found that urchins typically moved around on the window screen, trying at least two types of food, but usually fed extensively only on one. Urchins that did not feed at all ($\sim$10% of runs) provided no information on the relative deterrence of extracts and were not included in the statistical analysis. Urchins were only used once in these assays. At the completion of the assay, urchins were removed from the feeding arenas. Individual food cubes were removed from the window screening, blotted dry, and weighed to determine how much of each cube had been consumed.

Tests assessing weight loss and degradation of food cubes resulting from the 8 h exposure to water were conducted simultaneously with the urchin feeding assay by placing food cubes in feeding arenas lacking *A. punctulata*. In all instances, weight loss in these cubes was below 2% and there was no obvious degradation of secondary compounds due to water exposure. The integrity of the chemical defenses in artificial food cubes used in both fish and urchin assays was confirmed by re-extraction of leftover cubes and analysis by thin layer chromatography (TLC) and HPLC. In all instances, the compounds extracted from the sponges were present in the artificial food and had not undergone obvious degradation during the experimental procedure.

To test for significant differences in consumption of different food cube types by fish (expressed as % of cubes consumed), we carried out multiple 2×2 contingency tables with χ^2 analysis. For each species, the following comparisons were made: inner vs. control, outer vs. control, and inner vs. outer. For the urchin feeding assays, we analyzed square root arcsine transformed data (expressed as % of weight loss of each food type) with a repeated measures ANOVA. A repeated measures analysis was used because all three food cubes were exposed to one urchin simultaneously and were therefore not independent.

Results

Fish that consumed experimental food cubes were primarily the black seabass (*Centropristus striata*) and spottail pinfish (*Diplodus holbrooki*), with occasional consumption by the gray triggerfish (*Balistes capriscus*) and pinfish (*Lagodon rhomboides*). In both *A. fulva* and *I. campana*, 100% of the control food cubes were consumed while treatment cubes were consumed less frequently (*A. fulva*: Pearson's test: $X^2 = 6.222$, $P < 0.05$ and $X^2 = 10.435$, $P < 0.01$ for inner vs. control and outer vs. control, respectively; *I. campana*: Pearson's test: $X^2 = 4.909$, $P < 0.05$ and $X^2 = 4.190$, $P < 0.05$ for inner vs. control and outer vs. control, respectively) (Fig. 1). The frequency at which the two types of treatment food cubes were consumed, however, was not significantly different in either sponge species (Pearson's test: $X^2 = 0.735$, $P > 0.30$ and $X^2 = 0.094$, $P > 0.70$ for *A. fulva* and *I. campana*, respectively) (Fig. 1).

In laboratory feeding assays with the urchin, *A. punctulata*, control food cubes lost more weight than both treatment cubes in all sponge species, but this trend was significant in *A. fulva* ($F_{2,32} = 13.96$, $P < 0.001$ for inner vs. control and $F_{2,32} = 8.246$, $P < 0.01$ for outer vs. control) and not significant for either of the *Ircinia* spp. ($P > 0.05$). There was no significant difference ($P > 0.05$) in the percent change in weight between the two treatment cubes in any of the sponge species (Fig. 2).

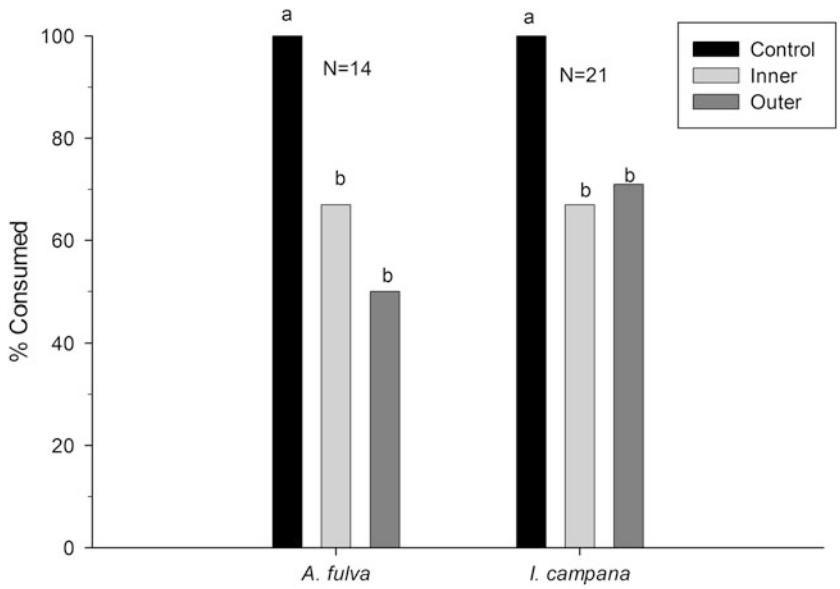

Fig. 1 Consumption of food cubes by a natural assemblage of temperate reef fish. Cubes containing crude extract from both the inner and outer regions of these sponges as well as control cubes possessing no extract were offered. Percentages are based on the number of cubes consumed out of the number indicated at the top of the bars. Data were analyzed using 2 × 2 contingency tables. Letters a and b indicate significant differences between treatment foods (outer vs. inner) or between a treatment food and control

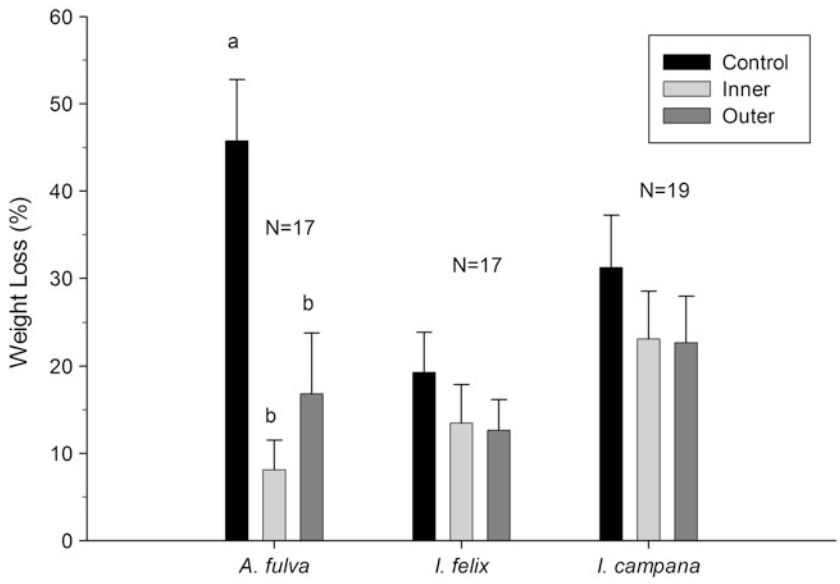

Fig. 2 Percent weight loss (±SE) due to feeding by the urchin *Arbacia punctulata* on food cubes created from chemical extracts of three sponge species. Extracts were obtained from inner and outer tissue regions of each sponge. Controls represent food cubes containing no chemical extract. Data were analyzed using a repeated measures ANOVA followed by a least squares comparison. Letters a and b indicate significant differences between treatment foods (outer vs. inner) or between a treatment food and control

Discussion

There is a paucity of experimental evidence supporting the contention that allocating chemical defenses to regions of the body most vulnerable to attack is ecologically beneficial to marine sponges (Becerro et al., 1997, 1998; Schupp et al., 1999; Burns et al., 2003; Furrow et al., 2003). Although such a strategy might confer protection at lower metabolic cost to the sponge, the concentrations of defensive metabolites throughout the sponge body are rarely measured (Swearingen & Pawlik, 1998; Burns et al., 2003), or the ecological benefit of increased concentrations of chemical defenses is not experimentally verified (Freeman & Gleason, 2010). In addition, investigations approaching this topic on both fronts have produced conflicting results, thus making generalization difficult (Becerro et al., 1997, 1998; Schupp et al., 1999; Furrow et al., 2003). In an initial survey of the defensive compounds in the sponges *A. fulva*, *I. felix*, and *I. campana* (Nuñez et al., 2008; Freeman & Gleason, 2010), we confirmed the presence of

secondary metabolites that are known to deter predators (Ebel et al., 1997; Waddell & Pawlik, 2000a, b; Pawlik et al., 2002; Thoms et al., 2004) and showed that the concentrations of these compounds differ between the inner and outer tissue regions in two of these species, *A. fulva* and *I. felix*, (Freeman & Gleason, 2010). Interestingly, the distribution of chemical defenses within these three species was not consistent with predictions of the ODT that costly defenses should be concentrated in regions of highest fitness value or regions that are most likely to encounter predators (McKey, 1974, 1979; Rhoades, 1979). Feeding assays conducted here provide support for this conclusion by showing that extracts obtained from sponge regions possessing higher concentrations of secondary metabolites (i.e., inner tissue regions of *I. felix* and outer regions of *A. fulva*) did not display enhanced deterrency to generalist temperate reef fish and one species of sea urchin. Thus, we conclude that differences in secondary metabolite allocation patterns observed among *A. fulva*, *I. felix*, and *I. campana* on temperate reefs of the southeastern US may not be driven by predation pressure from fish and urchins and that these known deterrent compounds may be allocated to different regions of these sponges to mediate other interactions besides predation.

The feeding trials conducted here do corroborate previous findings that extracts from these sponge species deter predators because treatment food cubes were consumed less often than controls (Pawlik et al., 1995, 2002; Ebel et al., 1997; Thoms et al., 2004, 2006). The lack of evidence for a positive relationship between compound concentrations and predator deterrence may be attributable to at least three factors. First, defensive compounds may serve multiple functions and higher concentrations of these compounds may actually represent an adaptation to defend against competitors, reduce fouling, or inhibit microbial infection (Uriz et al., 1992; Becerro et al., 1997; Kubanek et al., 2002). Second, because multiple factors typically play a role in prey deterrence, testing chemical defenses in isolation may not sufficiently address the suite of defenses, such as structural components or variability in nutritional quality, encountered by predators (Hay et al., 1994; Hay, 1996). The three aspiculate species investigated here show within individual heterogeneity in the levels of structural components (i.e., spongin fibers) and nutritional quality (protein and carbohydrate) so this hypothesis warrants further investigation (Freeman & Gleason, 2010). Finally, if a threshold concentration for predator deterrence exists and the lower metabolite concentration (from the inner region of *A. fulva* and the outer region of *I. felix*) is above the threshold, then higher concentrations in adjacent regions would not confer increased deterrence. This hypothesis might also be relevant here because metabolite concentrations in the inner and outer regions of these two sponges differed by only 0.06% (*I. felix*) and approximately 4% of sponge dry weight (*A. fulva*) (Freeman & Gleason, 2010).

It should also be pointed out that different suites of chemical defenses are not necessarily functionally equivalent when it comes to deterring predators (Hay et al., 1988; Pennings & Paul, 1992). The FTAs and brominated tyrosine derivatives identified here are known to be deterrent compounds, but it is likely that they differ in their effectiveness against specific predators. This is supported in the current study, where FTA extracts from *I. campana* were only deterrent to fish predators whereas brominated tyrosine derivative extracts from *A. fulva* were deterrent to both fish and urchins. It is also possible that chemical variability within these sponges may be driven by other predators or even that the full suite of possible predators of these three species at J-Y Reef has yet to be adequately enumerated. For instance, a recent study by Sacristán-Soriano et al. (2011) suggests that higher levels of one compound in the outer region of the sponge *Aplysina aerophoba* may defend this sponge from generalist predators, while higher concentrations of other metabolites toward the interior of the sponge may act to defend the sponge from specialist predators. Thus, extending our methods to other predators or testing the effects of specific compounds against various predators may have yielded different results. Future assays should test the ability of individual compounds, at natural concentrations, to deter an array of potential predators including generalist and specialist fish and invertebrate predators and small, cryptic invertebrates (Hay et al., 1988). In addition, since chemical defenses may act synergistically to deter predators in some sponges (Hill et al., 2005) and sponges display some ability to deter predators using both chemical and physical defenses (Ruzicka & Gleason, 2009), future assays should include, at the very least,

the interactive effects of chemical and structural defenses to more accurately identify the effectiveness of variation in anti-predatory defenses within sponges.

In conclusion, while we have documented previously significant within-sponge variation in the distribution of defensive chemicals in two out of three temperate sponge species of the North Atlantic (Freeman & Gleason, 2010), this variation is not consistent with allocation models like the ODT that predict at risk regions should be more heavily defended from predators. Rather, results reported here suggest increased concentrations of chemical defenses do little to provide enhanced protection from damage by large biting or mobile invertebrate generalist predators. However, the possibility that concentrating chemical defenses within certain regions of these sponges is due to predation pressure from consumers not investigated here must be acknowledged. For instance, specialist spongivores like angelfish (*Holacanthus* sp. and *Pomacanthus* sp.), trunkfish (*Acanthostracion* sp.), and filefish (*Cantherhines macrocerus*) are present at J-Y and surrounding reefs and may exert strong selective pressure for differential within-sponge allocation of chemical defenses. In addition, other ecological stresses including competition with other reef organisms, risks of bacterial infection, or potential for biofouling might select for the allocation of defenses to certain regions or throughout sponge bodies. Given that we are just beginning to understand how sponges use secondary metabolites to mediate biotic interactions (Becerro, 2008), future research should extend beyond predator–prey interactions and address the other potential roles these compounds play in sponge defense and, ultimately, sponge community structure (Ruzicka & Gleason, 2009).

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References

Becerro, M. A., 2008. Quantitative trends in sponge ecology research. Marine Ecology 29: 167–177.

Becerro, M. A., M. J. Uriz & X. Turon, 1997. Chemically-mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera, Poecilosclerida). Hydrobiologia 356: 77–89.

Becerro, M. A., V. J. Paul & J. Starmer, 1998. Intracolonial variation in chemical defenses of the sponge *Cacospongia* sp. and its consequences on generalist fish predators and the specialist nudibranch predator *Glossodoris pallida*. Marine Ecology Progress Series 168: 187–196.

Becerro, M. A., R. W. Thacker, X. Turon, M. J. Uriz & V. J. Paul, 2003. Biogeography of sponge chemical ecology: comparisons of tropical and temperate defenses. Oecologia 135: 91–101.

Burns, E., I. Ifrach, S. Carmeli, J. R. Pawlik & M. Ilan, 2003. Comparison of anti-predatory defenses of Red Sea and Caribbean sponges. I. Chemical defense. Marine Ecology Progress Series 252: 105–114.

Chanas, B. & J. R. Pawlik, 1995. Defenses of Caribbean sponges against predatory reef fish. II. Spicules, tissue toughness, and nutritional quality. Marine Ecology Progress Series 127: 195–211.

Ebel, R., M. Brenzinger, A. Kunze, H. J. Gross & P. Proksch, 1997. Wound activation of protoxins in marine sponge *Aplysina aerophoba*. Journal of Chemical Ecology 23: 1451–1462.

Freeman, C. J. & D. F. Gleason, 2010. Chemical defenses, nutritional quality, and structural components in three sponge species: *Ircinia felix*, *I. campana*, and *Aplysina fulva*. Marine Biology 157: 1083–1093.

Freeman, C. J., D. F. Gleason, R. Ruzicka, R. W. M. van Soest, A. W. Harvey & G. McFall, 2007. A biogeographic comparison of sponge fauna from Gray's Reef National Marine Sanctuary and other hard-bottom reefs of coastal Georgia, USA. In Hajdu, E. & G. Muricy (eds), Porifera Research: Biodiversity, Innovation, and Sustainability. Museu Nacional, Rio de Janeiro: 319–325.

Furrow, F. B., C. D. Amsler, J. B. McClintock & B. J. Baker, 2003. Surface sequestration of chemical feeding deterrents in the Antarctic sponge *Latrunculia apicalis* as an optimal defense against sea star spongivory. Marine Biology 143: 443–449.

Gillor, O., S. Carmeli, Y. Rahamim, Z. Fishelson & M. Ilan, 2000. Immunolocalization of the toxin Latrunculin B within the Red Sea sponge *Negombata magnifica* (Demospongiae, Latrunculiidae). Marine Biotechnology 2: 213–223.

Hay, M. E., 1996. Marine chemical ecology: what's known and what's next. Journal of Experimental Marine Biology and Ecology 200: 103–134.

Hay, M. E., P. E. Renaud & W. Fenical, 1988. Large mobile versus small sedentary herbivores and their resistance to seaweed chemical defenses. Oecologia 75: 246–252.

Hay, M. E., Q. E. Kappel & W. Fenical, 1994. Synergisms in plant defenses against herbivores: interactions of chemistry, calcification, and plant quality. Ecology 75: 1714–1726.

Hill, M. S., N. A. Lopez & K. A. Young, 2005. Anti-predator defenses in western North Atlantic sponges with evidence of enhanced defense through interactions between spicules and chemicals. Marine Ecology Progress Series 291: 93–102.

Kubanek, J., K. E. Whalen, S. Engel, S. R. Kelly, T. P. Henkel, W. Fenical & J. R. Pawlik, 2002. Multiple defensive roles for triterpene glycosides from two Caribbean sponges. Oecologia 131: 125–136.

McKey, D., 1974. Adaptive patterns in alkaloid physiology. The American Naturalist 108: 305–320.

McKey, D., 1979. The distribution of secondary compounds within plants. In Rosenthal, G. A. & D. H. Janzen (eds), Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York: 55–133.

Nuñez, C. V., E. V. R. de Almeida, A. C. Granato, S. O. Marques, K. O. Santos, F. R. Pereira, M. L. Macedo, A. G. Ferreira, E. Hajdu, U. S. Pinheiro, G. Muricy, S. Peixinho, C. J. Freeman, D. F. Gleason & R. G. S. Berlinck, 2008. Chemical variability within the marine sponge *Aplysina fulva*. Biochemical Systematics and Ecology 36: 283–296.

Paul, V. J., 1992. Chemical defenses of benthic marine invertebrates. In Paul, V. J. (ed.), Ecological Roles of Marine Natural Products. Comstock Publishing, Ithaca, NY: 164–188.

Pawlik, J. R., B. Chanas, R. J. Toonen & W. Fenical, 1995. Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. Marine Ecology Progress Series 127: 183–194.

Pawlik, J. R., G. McFall & S. Zea, 2002. Does the odor from sponges of the genus *Ircinia* protect them from fish predators? Journal of Chemical Ecology 28: 1103–1115.

Pennings, S. C. & V. J. Paul, 1992. Effect of plant toughness, calcification, and chemistry on herbivory by *Dolabella auricularia*. Ecology 73: 1606–1619.

Rhoades, D. F., 1979. Evolution of plant chemical defense against herbivores. In Rosenthal, G. A. & D. H. Janzen (eds), Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York: 3–54.

Ridder, C. D. & J. M. Lawrence, 1982. Food and feeding mechanisms: Echinoidea. In Jangoux, M. & J. M. Lawrence (eds), Echinoderm Nutrition. A.A. Balkema, Rotterdam: 57–116.

Rohde, S. & P. J. Schupp, 2011. Allocation of chemical and structural defenses in the sponge *Melophlus sarasinorum*. Journal of Experimental Marine Biology and Ecology 399: 76–83.

Ruzicka, R. & D. F. Gleason, 2008. Latitudinal variation in spongivorous fishes and the effectiveness of sponge chemical defenses. Oecologia 154: 785–794.

Ruzicka, R. & D. F. Gleason, 2009. Sponge community structure and anti-predator defenses on temperate reefs of the South Atlantic Bight. Journal of Experimental Marine Biology and Ecology 380: 36–46.

Sacristán-Soriano, O., B. Banaigs, E. O. Casamayor & M. A. Becerro, 2011. Exploring the links between natural products and bacterial assemblages in the sponge *Aplysina aerophoba*. Applied and Environmental Microbiology 77: 862–870.

Schupp, P., C. Eder, V. J. Paul & P. Proksch, 1999. Distribution of secondary metabolites in the sponge *Oceanapia* sp. and its ecological implications. Marine Biology 135: 573–580.

Swearingen, D. C. & J. R. Pawlik, 1998. Variability in the chemical defense of the sponge *Chondrilla nucula* against predatory reef fishes. Marine Biology 131: 619–627.

Thompson, J. E., K. D. Barrow & D. J. Faulkner, 1983. Localization of two brominated metabolites, Aerothionin and Homoaerothionin, in spherulous cells of the marine sponge *Aplysina fistularis*. Acta Zoologica (Stockholm) 64: 199–210.

Thompson, J. E., P. T. Murphy, P. R. Bergquist & E. A. Evans, 1987. Environmentally induced variation in diterpene composition of the marine sponge *Rhopaloeides odorabile*. Biochemical Systematics and Ecology 15: 595–606.

Thoms, C., M. Wolff, K. Padmakumar, R. Ebel & P. Proksch, 2004. Chemical defense of Mediterranean sponges *Aplysina cavernicola* and *Aplysina aerophoba*. Z Naturforsch 59: 113–122.

Thoms, C., R. Ebel & P. Proksch, 2006. Activated chemical defense in *Aplysina* sponges revisited. Journal of Chemical Ecology 32: 97–123.

Turon, X., M. A. Becerro & M. J. Uriz, 2000. Distribution of brominated compounds within the sponge *Aplysina aerophoba*: coupling of X-ray microanalysis with cryofixation techniques. Cell and Tissue Research 301: 311–322.

Uriz, M. J., D. Martin & D. Rosell, 1992. Relationships of biological and taxonomic characteristics to chemically mediated bioactivity in Mediterranean littoral sponges. Marine Biology 113: 287–297.

Waddell, B. & J. R. Pawlik, 2000a. Defenses of Caribbean sponges against invertebrate predators. I. Assays with hermit crabs. Marine Ecology Progress Series 195: 125–132.

Waddell, B. & J. R. Pawlik, 2000b. Defenses of Caribbean sponges against invertebrate predators. II. Assays with sea stars. Marine Ecology Progress Series 195: 133–144.

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SPONGE RESEARCH DEVELOPMENTS

Temporal variations in growth and reproduction of *Tedania anhelans* and *Chondrosia reniformis* in the North Adriatic Sea

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Abstract Most works concerning growth and reproduction of Mediterranean sponges have been performed in the oligotrophic western Mediterranean while little is known about sponge dynamics in the North-western Adriatic Sea, a basin characterized by low winter temperature and eutrophy. In order to deepen our understanding of sponges in the North Adriatic Sea and verify how its peculiar trophic and physical conditions affect sponge life cycles, temporal trend of sponge cover (%) and reproductive timing of *Chondrosia reniformis* and *Tedania* (*Tedania*) *anhelans* were studied over a 1-year period looking for a possible relation with variations of temperature or food availability. In *C. reniformis,* although little variations of sponge cover were evidenced around the year, the number of individuals and their size increase during spring. Asexual reproduction, via drop-like propagules, mainly occurs in spring and summer, while sexual reproduction is characterized by a maximum number of oocytes in August. *T. anhelans* progressively grows from spring to summer and develops propagules on its surface that reach their maximum size in July. In autumn, the sponge undergoes a process of progressive shrinkage and almost disappears in winter when temperature reaches 7–8°C. Larvae occur during summer. In the North Adriatic Sea sponges have larger sizes, higher density and a wider period of oocytes production compared with the same species from the Mediterranean Sea, suggesting these differences could be due to high food availability characterizing the eutrophic Adriatic basin. On the contrary, the sharp water temperature variations and the very low winter temperature, 5–6°C lower than what has been reported for the Mediterranean Sea, regulate temporal variations in abundance and cause the disappearance of thermophile species during winter.

Keywords *Tedania anhelans* · *Chondrosia reniformis* · Porifera · Sponge cover · Reproduction · North Adriatic Sea

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Introduction

Due to their filter-feeding activity on particulate organic matter, sponges play a key role in coastal food

chains, directly regulating primary production and indirectly secondary production (Gili & Coma, 1998). In fact, it has been demonstrated that sponges, besides feeding on nano- and picoplankton (Reiswig, 1971; Ribes et al., 1999) , can intake large amounts of DOC and DOM (Yahel et al., 2003; de Goeij & van Duyl, 2007; Maldonado et al., 2010). Moreover, siliceous sponges play a role in the production and recycling of biogenic silica, and due to their ability to retain Si they can be considered as Si sinks (Maldonado et al., 2005).

Sponges establish a wide range of interactions with other organisms competing for space, providing a colonisable substrate for other animals and photosynthetic organisms, producing a large amount of bioactive compounds, or giving rise to bioerosive processes (Rützler, 1985, 2004; Cerrano et al., 2006; Wulff, 2006). Sponges, and in particular, demosponges are generally known as slow growing, long-living animals characterized by low adult mortality, and only smaller individuals show higher growth rates (Sarà, 1970; Ayling, 1983; Garrabou & Zabala, 2001). Temporal patterns of abundance are recognized to be of great importance in the understanding of the structure of benthic communities in temperate shallow waters. There are only few studies concerning the growth dynamics of demosponges, especially for the Mediterranean basin. De Caralt et al. (2008) studied the monthly growth rates of *Corticium candelabrum* at 10-12 m depth in the North-western Mediterranean Sea highlighting seasonal variations of the growth rates, with the highest values recorded in summer. Also the encrusting sponge *Crambe crambe* from the shallow waters of Blanes (Spain, western Mediterranean Sea) shows a seasonal growth pattern, with size increases occurring from May to October, in coincidence with the rise of seawater temperature (Turon et al., 1998). Dynamics of the same species from the Medes Islands were studied for 14 years (Teixidó et al., 2009) in deeper waters (between 12 and 16 m). Smaller individuals exhibited elevated growth rates, but high mortality, while large, old sponges grew slowly, but survived for the entire considered period.

A long-term study on Mediterranean sponges was carried out for 6 years in Portofino (Italy, Ligurian sea) at a depth ranging from 12 to 26 m (Pansini & Pronzato, 1985). The 13 monitored species showed long life spans, but no clear seasonal growth pattern, albeit several individuals underwent fragmentation or merging.

In environments characterized by wide annual temperature variations, sponge species may show evident seasonal trends driven by water temperature and food supply. For example, the biomass of *Halichondria panicea* in the Baltic Sea varied considerably with season: during winter the values were low, started increasing in spring when temperature rose and reached a peak in summer (Barthel, 1988). Similarly, volume and growth rates of *Haliclona oculata* from the Netherlands coasts showed a seasonal increase in accordance with higher temperatures and more abundantly available suspended particulate matter (Koopmans & Wijffels, 2008).

Studies concerning ecology of Mediterranean demosponges were mainly conducted in the western basin. In the Adriatic Sea, the sponges are mainly known from a taxonomic point of view, and few papers deal with growth dynamics or reproduction of the species (e.g., Scalera Liaci et al., 1971, 1973; Corriero et al., 1984, 1996, 1998; Nonnis Marzano et al., 2000, 2003a, b). The Adriatic Sea is an area characterized by peculiar trophic and hydrologic conditions (Artegiani et al., 1997a, b). In particular, the North Adriatic Sea is the shallowest area of the basin with an average depth of 35 m. Nutrient-rich freshwater inputs discharged by the Po river and the other minor affluents (Artegiani & Azzolini, 1981) strongly influence the productivity of the basin that is almost 2-fold higher than what has been reported for the western Mediterranean Sea (Giordani et al., 2002). During winter, water temperature is 5–6°C lower compared to the minimal values reported for the Mediterranean Sea (http://www.mareografico.it). Since the sharp temperature shift between summer and winter and the food availability high all year round, we hypothesized that in the North Adriatic Sea sponges could show different dynamics of growth or reproduction respect to the western Mediterranean, where sponges are present all year round and show limited period of fertility. In order to test our hypothesis, temporal trend of abundance, evaluated as sponge cover of the substratum and reproductive timing of two of the commonest sponge species in the North Adriatic Sea—*Chondrosia reniformis* (Nardo, 1847) and *Tedania* (*Tedania*) *anhelans* (Lieberkühn, 1859)—were studied over a 1 year period looking for a possible relation with variations of temperature or food availability.

Materials and methods

Sampling site and studied species

The study area was the "Scoglio del Trave", a natural rocky barrier rising from the coast of the Conero Promontory (Ancona, Italy) and reaching a maximum depth of 8 m (Fig. 1). The Italian coasts of the northern Adriatic Sea are generally sandy, and the Conero Promontory represents one of the rare zones characterized by hard bottoms. These, together with scattered substrates of anthropic origin, break the continuity of the Adriatic sandy coast and represent important stepping stones for species dispersal. This zone is characterized by shallow waters, a high sedimentation rate and a wide annual range of temperature (from 7°C in winter to 27°C in summer).

Monthly and daily air and superficial sea temperature trends of Conero Promontory were downloaded from the National Tidegauge Network website (http://www.mareografico.it). Data of solar irradiance in the area have been reported by Byun & Pinardi (2007). For estimating of the food availability in the Conero area, we used the chlorophyll concentrations obtained during an ARPAM coastal monitoring campaign from January to November 2009 in the station WGS84 (43°34′53.6″N–13°34′17″E) with the Idronaut Ocean Seven plus Probe (Cicero & Di Girolamo, 2001).

Fig. 1 Map of the study area in the North Adriatic Sea. The *inset* shows the Conero Promontory and the sampling site (Scoglio del Trave)

Variations of sponge cover

In order to estimate the sponge cover (%) of each sponge species, from April 2008 to November 2009, monthly samplings were conducted by scuba diving at Scoglio del Trave at a depth ranging between 5 and 7 m. Nine square areas of 0.5 × 0.5 m were marked on the rocky vertical walls where individuals of *C. reniformis* and *T. anhelans* were located. Even if the two sponges have been monitored considering a reduced total surface (4.5 m^2), the sampling site, a vertical cliff with homogeneous physical characteristics (depth range, light exposition, inclination, hydrodynamics), shows a homogeneous benthic assemblage and the replicates realistically represent the species composition of the rocky wall. Every month, each marked area was photographed with an underwater digital camera. Later, images were analyzed with the Image J software to estimate the number of individuals and their cover. For each frame, values of sponge cover were expressed as percentage of substratum covered by a sponge species, and then, the average of the nine replicates ±SD were calculated. Obviously, the sponge cover did not consider the vertical development of the two species that is especially evident in spring and summer in *T. anhelans*. The monthly average of the number of individuals in each frame ±SD was also determined.

In order to show the seasonal variations of the sponge cover, the dataset of spring (sponge cover values from March to May), summer (values from June to August), autumn (values from September to November), and winter (from December to February) was averaged and the SD was calculated.

We hypothesized that the sponge cover differed significantly over time and that these differences were in accordance with the seasonal cycle. The correlations with trends of temperature, chlorophyll, and irradiance were therefore tested. To test our hypothesis, non-transformed data of sponge cover of the two monitored sponge species, *T. anhelans* and *C. reniformis*, were tested by means of Kruskal–Wallis (Sokal & Rohlf, 1981). The non-parametric procedure was applied because data, checked with Shapiro–

Wilk's test, were not normally distributed. For statistical analysis, we grouped sponge cover under four different levels, according to the fixed factor season: spring (from March to May), summer (from June to August), autumn (from September to November), and winter (from December to February). Analyses were performed using PAST for Windows version 1.91 (Hammer et al., 2001).

Reproductive biology

Presence, density and diameter of the reproductive elements (oocytes, sperm cysts, embryos and larvae) were determined from January 2009 to November 2009 during the monthly underwater samplings. Ten randomly chosen pieces of sponges (collected from 10 different individuals of *C. reniformis* and *T. anhelans* living outside the frames) were taken with a cutter and immediately fixed in 2.5% glutaraldehyde (buffered in filtered seawater 7.8 pH). Three hours later, samples were washed in filtered seawaters and then dehydrated in a graded ethanol series. After the passage in ethanol 70°, pieces of *T. anhelans* were immersed for 2 h in hydrofluoric acid 5% to dissolve siliceous spicules. Successively, sponges were included in a cold-curing resin (Technovit 8100) and finally mounted on plastic supports. The sections obtained by microtome Histo-Line MRS3500 were colored with Toluidine blue, and then analyzed under a compound microscope.

Since, during previous surveys *T. anhelans* and *C. reniformis* were observed to produce larvae and oocytes, respectively, starting from spring, in this period we collected 30 pieces of each species instead of just ten to intensify the observations in the most critical reproductive moments. When present, propagules of *T. anhelans* were also collected to compare their structure with that of the massive portions of the sponge.

Density of gametes, embryos, and larvae were obtained by modifying of the formula used by Elvin (1976):

$$d = N(t/D + t)/V$$

where d is the density of reproductive elements per cube millimeter of sponge tissue, N indicates the number of reproductive elements in each histological section, t is the thickness of the section (8 μm in our case), D is the diameter of the reproductive elements, and V is the volume of the histological section, the latter calculated for each slice. The area of the sections was determined by taking pictures of each slice under a stereomicroscope and then processing the images with ImageJ software. Their volume was calculated by multiplying the thickness of the section (8 μm) for the area.

Average density values ±SD of oocytes, embryos, larvae, and spermatic cysts, as well as diameter measurements (in μm) of these reproductive elements, were calculated monthly for the hermaphroditic viviparous sponge *T. anhelans*, while only average densities of oocytes and spermatic cysts were determined for the gonocoric oviparous sponge *C. reniformis.*

Results

Tedania anhelans

Tedania anhelans from the Conero Promontory evidenced strong monthly variations (Fig. 2a): growth started in early spring (6.5% ± 9.27 SD and 8.3% ± 10.24 SD cover in April 2008 and 2009 respectively), when the sea temperature was about 13°C. Sponge size increased progressively during late spring and summer reaching the maximum cover in July (12.08% ± 13.18 SD and 9.38% ± 11.92 in July 2008 and 2009 respectively; $T = 25$°C) and started decreasing at the end of summer. In autumn, *T. anhelans* went through a progressive shrinkage (1.28% ± 2.63 SD and 4.44 ± 7.47 SD in September 2008 and 2009 respectively; $T = 20$–24°C), and it almost disappeared in winter (minimum value was 0.47% ± 1.95 SD in December 2008; $T = 7$–8°C).

The average seasonal variations of sponge cover have been showed in Fig. 2b. The highest value was observed in summer (7.79% ± 10.28 SD), while the minimum was recorded in winter (1.31% ± 2.73 SD). Statistical analysis shows significant variations in the sponge cover of *T. anhelans* among all the considered seasons (Kruskal–Wallis, $H = 92.32$; $P < 0.0001$). Summer values were significantly different from those of autumn and winter but not from those observed in spring, while there were significant differences between autumn and winter values.

Variations of the sponge cover were correlated with temperature and irradiance values (respectively with $r = 0.48$, $N = 21$ and $r = 0.63$, $N = 12$, $P < 0.05$),

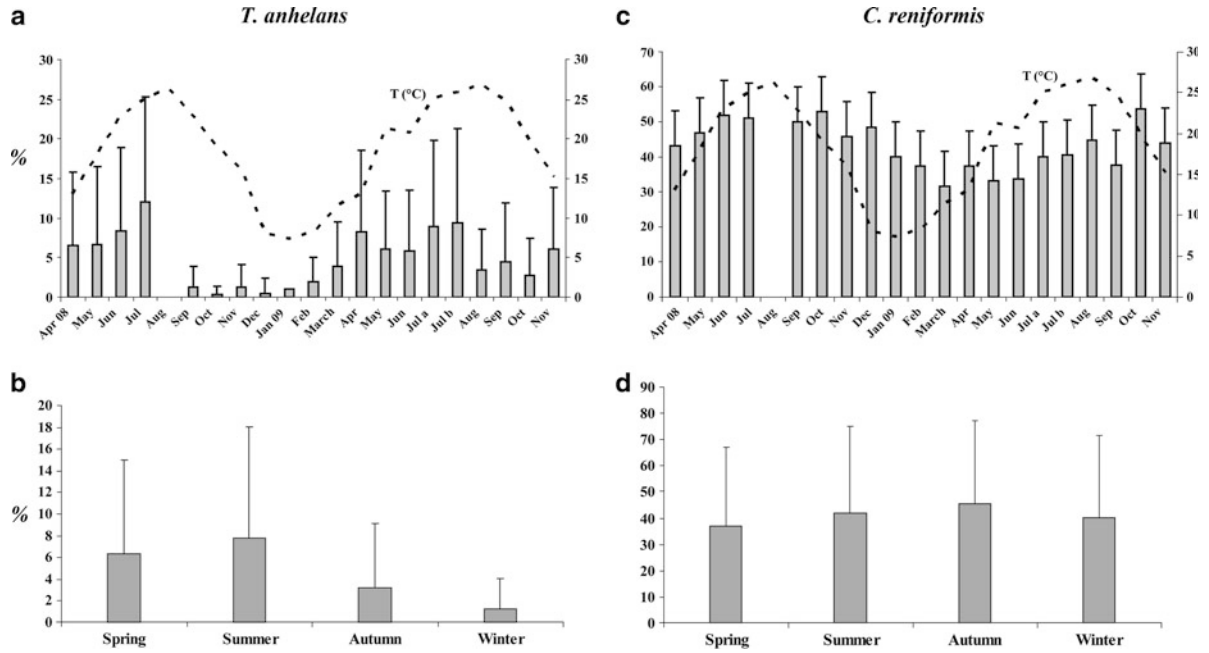

Fig. 2 Sponge cover (%) from April 2008 to November 2009. Gray bars represent monthly and seasonal variations of sponge cover ± SD of *Tedania anhelans* (**a**, **b**) and *Chondrosia reniformis* (**c**, **d**). The dotted line is the water temperature trend in the considered period. It was not possible to collect samples in August for adverse meteo-marine conditions

but not with those of chlorophyll ($r = 0.29$, $N = 12$, $P > 0.05$).

In *T. anhelans*, the average number of individuals m^{-2} varied in the study period (Fig. 3a, b), with the highest values observed in spring (23.3 ± 36.5 SD in April 2008, with a maximum of 94 individuals in one of the replicates) and minimal values in winter (3.9 ± 5.6 SD in December 2008). The trend of the number of individuals was correlated with that of the sponge cover ($r = 0.49$, $N = 21$, $P < 0.05$), but not with that of temperature, irradiance, and chlorophyll (with $r = 0.03$, $r = 0.26$, $r = 0.2$ respectively; $N = 21$, $P > 0.05$). From June to August, the number of individuals decreased while the sponge surface increased. From September to November, the number of sponges continued to increase, while in winter, the sponge almost disappeared (December–February).

Photographic analysis showed deep modifications in the morphology of the sponge during the considered period, with phases of growth during spring and summer and others of shrinkage occurring in autumn and winter. The sponge was able to reproduce asexually via propagule formations: in early spring, *T. anhelans* was encrusting and covered a small surface. In late spring, it developed thin propagules on its surface that grew and branched in early summer reaching its maximum size in July (up to 12 cm length). In autumn, propagules disappeared, and the sponge started shrinking.

Concerning reproductive biology, variations in density of the reproductive elements are showed in Fig. 4a. No differences were noticed between density of the reproductive elements in the pieces cut from the basal part of the sponge and from propagules. Oocytes were present starting from January and reached the highest density in August (8.54 oocytes mm^{-3}), while embryos appeared in April and reached the highest density in June (18.49 embryos mm^{-3} ± 14.77 SD), and gradually decreased until October. We found sperm cysts only in June (4.83 cysts mm^{-3} ± 0.22 SD), but probably the spermatogenesis occurred from April since embryos were found starting from this month. Production of larvae was observed from May to October, and their density always remained low (highest value in July, 1.18 larvae mm^{-3} ± 0.13 SD).

Concerning measurements of the reproductive elements, oocytes of *T. anhelans* ranged from 37 to 43 μm (average 42.38 ± 12.86 SD), embryos from 25 to 250 μm (average 93.70 ± 45.27 SD), and spermatic cysts from 27.5 to 120 μm (average

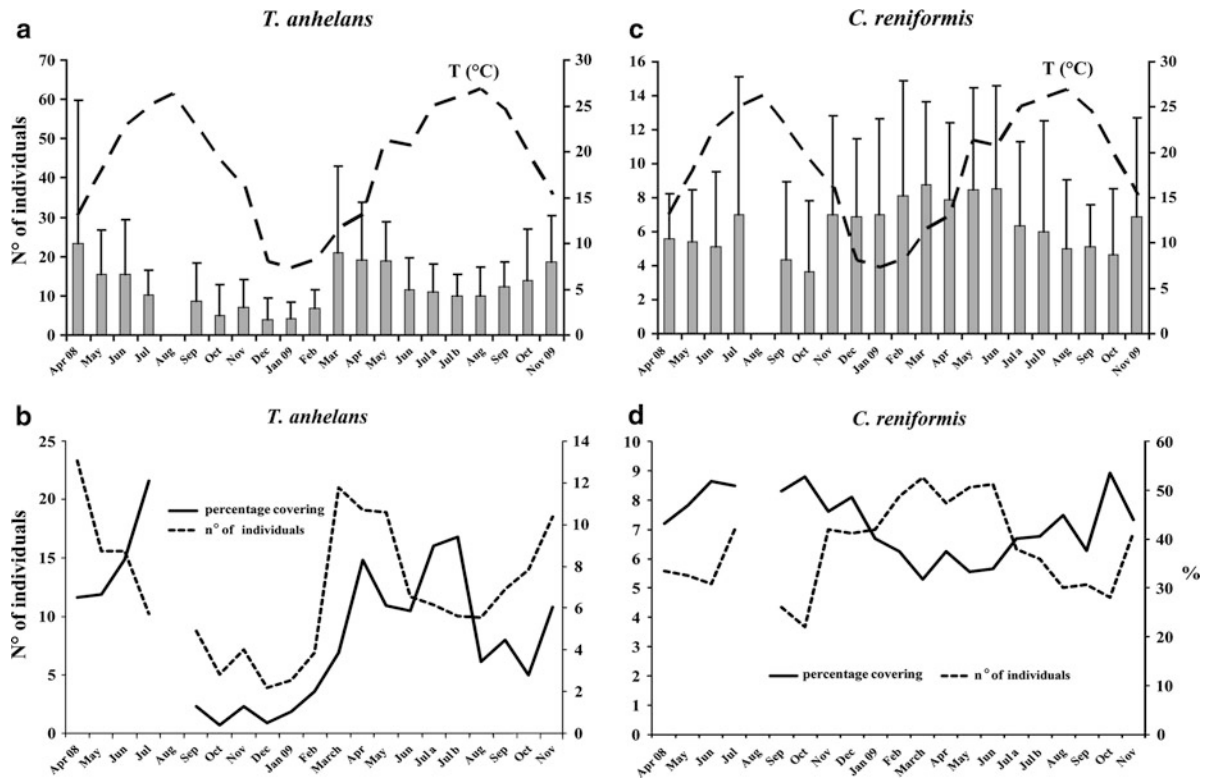

Fig. 3 Trend of the number of individuals ± SD of *T. anhelans* in relation to temperature (**a**) and sponge cover (%) (**b**). Trend of the number of individuals ± SD of *C. reniformis* in relation to temperature (**c**) and sponge cover (%) (**d**)

76.62 ± 87.32 SD). Larvae ranged from 48.5 to 920 μm (average 512.54 ± 152.13 SD).

Chondrosia reniformis

Chondrosia reniformis is the most common and largest sponge of the studied area. Several giant individuals larger than one square meter were observed in the sampling area. The monthly (Fig. 2c) and seasonal (Fig. 2d) variations of the sponge cover of *C. reniformis* from April 2008 to November 2009 showed little change during the study period.

Statistical analysis did not show any significant variation in the sponge cover in the considered seasons (Kruskal–Wallis, $H = 12.83$; $P < 0.01$). The highest value of sponge cover was recorded in autumn (average 42.04% ± 32.96 SD) while the minimum was observed in spring (average 36.87% ± 29.94 SD).

The trend of the sponge cover was correlated neither with temperature nor with irradiance or chlorophyll concentration (with $r = 0.21$, $N = 21$; $r = 0.29$, $N = 12$; $r = 0.44$, $N = 12$; $P > 0.05$, respectively).

The monthly average number of individuals of *C. reniformis* showed only slight variations from April 2008 to November 2009 increasing from autumn to spring, decreasing in summer and reaching the highest value in May 2009 (8.44 individuals ± 6.02 SD) (Fig. 3c, d).

The trend of the number of individuals resulted inversely correlated with both the sponge cover ($r = 0.75$, $N = 21$, $P < 0.01$) and the water temperature variations ($r = 0.44$, $N = 21$, $P < 0.05$), but was not correlated with either irradiance or chlorophyll ($r = 0.22$ and $r = 0.01$ respectively; $N = 21$, $P > 0.05$).

Asexual reproduction via drop-like propagules occurred all the year round, but during summer, the phenomenon was particularly evident. The formation of dripping portions (*creeping* for Bonasoro et al., 2001; Parma et al., 2007) could have originated from different areas of one specimen. Most of these propagules showed living epibionts (hydroid or

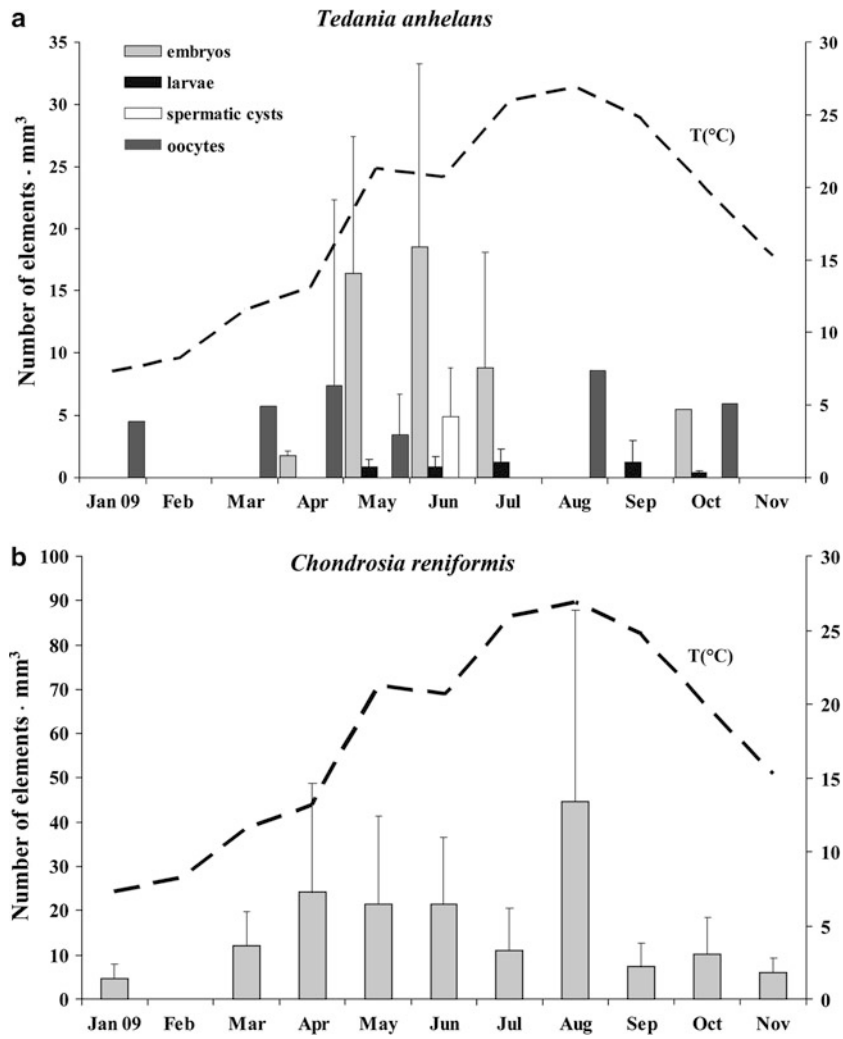

Fig. 4 Density of the reproductive elements ± SD of *T. anhelans* (**a**) and *C. reniformis* (**b**)

zoanthid colonies, serpulids, ascidians, and other sponge species) or parts of dead animals (i.e., shells of bivalves) attached to the distal portions.

Concerning the reproductive biology of *C. reniformis*, oocytes were recorded throughout the entire period of observation with the maximum density observed in August (44.62 oocytes mm^{-3} ± 43.36 SD) and the minimal in January (4.73 oocytes mm^{-3} ± 3.33 SD) (Fig. 4b). Diameter of oocytes varied from 20 to 75 μm (average 32.60 μm ± 8.57 SD) with 85.9% (2057 of the 2396 measures) ranging from 20 to 40 μm. Sperm cysts were never found during the study period even if their spawning was observed underwater in July 2009.

Discussion

This study provides new information about the life cycle of *T. anhelans* and *C. reniformis* from the Italian coasts of the North Adriatic Sea. The sponge life cycle includes four principal stages: (i) recruitment, (ii) growth, (iii) fusion and fragmentation, and (iv) mortality of individuals (Teixidó et al., 2009). In *T. anhelans*, an increase in the number of individuals is observed in spring, from March to May. The first larvae were found from May, suggesting that from March to April the increase is only due to the fragmentation of the genets, while in May the increase also depends on larval settlement with the formation of

new individuals. From June to September, the number of individuals decreases, probably due to both fusion of individuals originating from the same genet and death of some newly recruited sponges. From April to July, the sponge reaches its largest size, both increasing the covered surface and branching vertically. From September to November, breaking of propagules is observed together with the decrease in the sponge cover probably due to fragmentation and surface shrinkage. During this phase, the number of individuals still continues to increase, due to both fragmentation and also quite likely to the recruitment of propagules. In winter, the sponge almost disappears (December–February). The life cycle of this species reflects that of the majority of the benthic filter feeders living in the study area characterized by growth and reproduction periods in summer and dormancy in winter (Di Camillo et al., 2010).

On the contrary, *C. reniformis* is present and abundant all the year round. However, even if the sponge does not show important seasonal variations, it slightly increases in size from April to December and shrinks from January to March. Inversely, the number of individuals is low during summer and suddenly increases from autumn to spring. Spawning was observed in July and since sponge larvae generally have low dispersal (Maldonado, 2006), the most plausible hypothesis is that larvae settle in summer. However, the newly recruited sponges are probably not big enough to be detectable before autumn. The settlement of propagules asexually generated in summer and fragmentation of large individuals could occur in autumn, contributing to an increase in the number of individuals in this period.

In *C. reniformis,* creeping and detachment of the propagules precede the fragmentation of the individuals. In particular, the phenomenon occurs from spring to early summer as already described by Parma et al. (2007). The creeping rate is positively correlated with temperature, and the formation of propagules is particularly evident in the large individuals from Conero Promontory with respect to the small sponges of Paraggi (Ligurian Sea). This fact is probably due to the difference in size of the individuals between the two populations since large individuals may creep from several points. Parma et al. (2007) suggested that creeping was a possible response to gravity and our study supports this hypothesis, thanks to the weight of the epibionts attached on the propagules promoting collagen stretching and the detachment of sponge pieces. In areas of high sedimentation, like Conero Promontory, the tips of the dripping portions can act as small sacs containing sand and even in this case the weight of the sediments enhances propagule formation. Since food is particularly abundant in this eutrophic area, the strong reduction of *T. anhelans* in winter and size variations and sexual or asexual reproduction of *T. anhelans* and *C. reniformis* are not driven by food availability but by other factors such as temperature variations.

In fact the Conero area is subjected to sharp water temperature variations occurring during the year (7–27°C vs. 13–25°C temperature ranges registered in the Adriatic and western Mediterranean basins, respectively) and to the very low winter temperature, 5–6°C lower than what has been generally reported for the Mediterranean Sea (Fig. 5c). These factors could cause the disappearance of thermophile species during the cold season. The trend of *T. anhelans* results correlated with both temperature and irradiance. In the Mediterranean Sea, the trends of solar irradiance and water temperature have shifted (Bavestrello & Arillo, 1992), while in the North Adriatic Sea, the trends of these two environmental factors are strongly correlated ($r = 0.86$, $N = 19$) and having a synergic effect on *T. anhelans* growth.

Most of the studies concerning growth of Mediterranean sponges state that demosponges are long-living animals that do not show seasonal cycles. However, the monitored sponges proved to be very dynamic organisms that were able to undergo fragmentation, contraction, or merging and showed seasonal growth trends, with peaks generally occurring during spring or summer (Sarà, 1970; Ayling, 1983; Pansini & Pronzato, 1985; Turon et al., 1998; Garrabou & Zabala, 2001; De Caralt et al., 2008; Teixidó et al., 2009; Cardone et al., 2010).

So far, *T. anhelans* from the North Adriatic Sea is the only known Mediterranean demosponge that is able to almost disappear during a whole season (winter). In the same area, another sponge, *Oscarella* sp., shows the same seasonal trend (unpublished). This species grows during summer forming enormous individuals over 50-cm long and then undergoes a strong reduction in winter. Short phases of disappearance were reported for the demospongiae *Halichondria panicea* from the brackish Lake of Lesina (Apulia, Southern Italy) (Nonnis Marzano et al.,

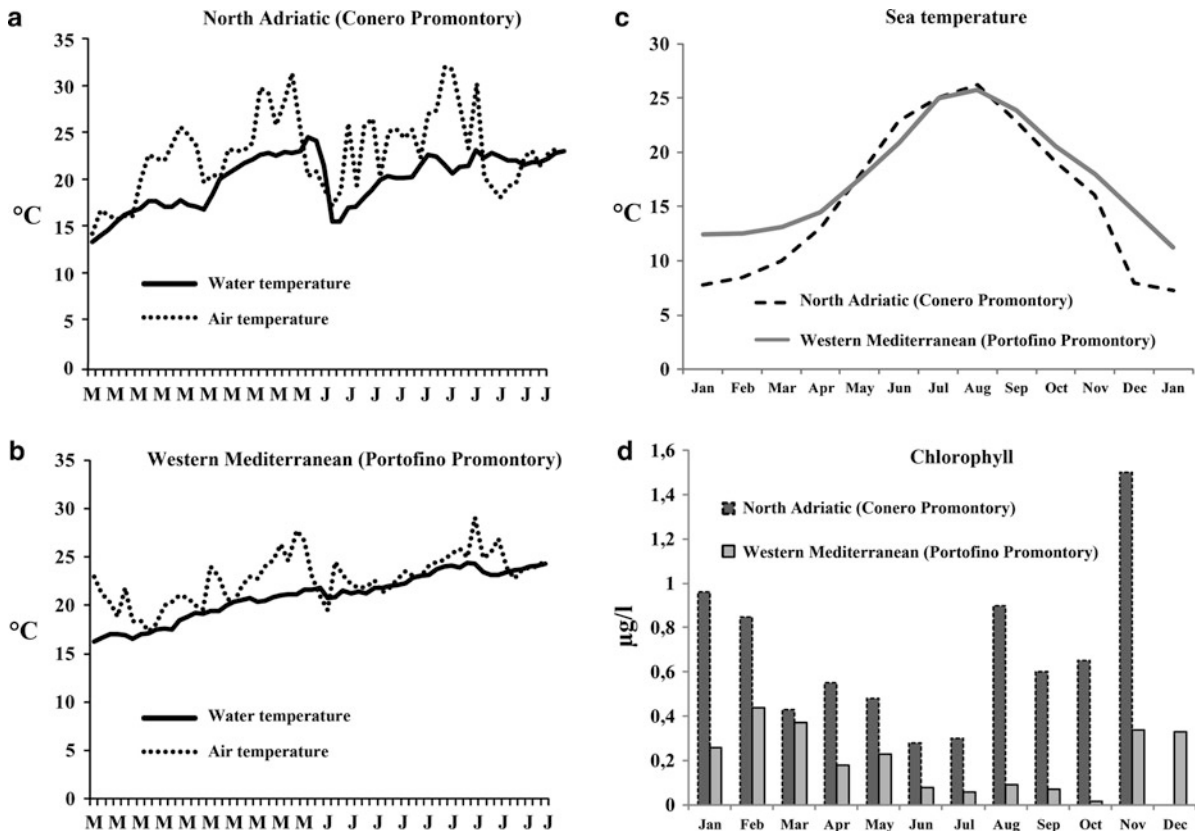

Fig. 5 Comparison of the trends of water and air temperature and chlorophyll in the North Adriatic Sea (Conero Promontory) with those of a locality of the western Mediterranean basin (Portofino Promontory). **a**–**b** Daily variations of air and water temperature in the Conero Promontory (**a**) and Portofino Promontory (**b**) from May to June 2009; **c** Trends of monthly average sea temperature (°C) from January 2008 to January 2009; **d** Monthly variations of chlorophyll levels (µg l^{-1}) from January to December 2009

2003a, b). The sponge was characterized by high fluctuations of density and size and underwent two mortality episodes (May and September 2001) in about 2 years of study. The life cycle was not related to the trend of the hydrologic parameters (temperature, oxygen, and salinity), suggesting that the disappearance of this species was probably due to food paucity or disease.

Concerning reproduction, demosponges do not usually show a seasonal trend of abundance, and sexual and asexual reproductions are generally observed only in some periods of the year in relation to temperature variations. The comparison of periods of production of oocytes, sperm cysts, embryos, and larvae in some Mediterranean sponges is showed in Table 1. According to Lévi (1956) and Mercurio et al. (2007) in the Mediterranean Sea, many sponge species present oocytes in spring or summer, probably as a consequence of a gradual increase in temperature (Maldonado & Riesgo, 2009), even if in *Axinella damicornis* the oocytes are present only during the cold season (from autumn to early spring). The only sponges whose oogenesis has been recorded continuously throughout the year are *C. reniformis* and *T. anhelans* from the North Adriatic Sea (present work), *Spongia officinalis* var. *adriatica* from the Ionian sea (Baldacconi et al., 2006), *Corticium candelabrum* from the Western Mediterranean Sea (Riesgo et al., 2007; Maldonado & Riesgo, 2008; Riesgo & Maldonado, 2008) and *H. panicea* from the Lake of Lesina, although marked differences from year to year may occur in this species (Nonnis Marzano et al., 2003a). In all the records presented, the period of presence of sperm cysts is shorter and generally slightly

shifted compared to the production of oocytes. Table 1 shows that the periods of presence of oocytes, embryos, and larvae in *T. anhelans* and *C. reniformis* vary in the same species from different localities.

T. anhelans from the Conero Promontory continuously produced oocytes during the study period, while embryos were observed from April to October and larvae from May to October. Spermatic cysts were found only in June, but since embryos were present from April to October, it is possible to hypothesize that fertilization occurred before and the duration of spermatogenesis was longer. The same species studied along the Ionian coasts of Apulia in 1994 (Nonnis Marzano et al., 2000) exhibited a reduced period of presence of oocytes (from February to August) and embryos and larvae (from June to October), while spermatic cysts were observed from April to September. During the second year of samplings (1995), female gametes were never observed in the collected sponges, and the duration of production of embryos and larvae (from May to August 1995) and spermatic cysts (from May to July 1995) was shorter. *T. anhelans* from Apulia was collected in a peculiar, semi-enclosed environment—*La Strea*—where the maximum depth is 2.5 m and the great variability in the sponge life cycle could be due to sharp variations in physical parameters.

As already observed in *T. anhelans*, also in *C. reniformis* from the Conero Promontory the production of oocytes was continuous during the year. Both in *C. reniformis* from Conero Promontory and from Spain (Riesgo & Maldonado, 2008), the highest value of oocyte density was observed in August suggesting that high temperatures favor oocyte production in this species. Spermatic cysts were never observed in histological sections, but we witnessed a spawning event involving several individuals during July 2009, with a temperature of 25.9°C. *C. reniformis* from other localities produces oocytes from May to August along Apulian coasts (Scalera Liaci et al., 1971) and only for 3 months (from June to August) along the Mediterranean coasts of Spain (Riesgo & Maldonado, 2008). In both places, spermatic cysts have been observed only in July and August.

Temperature is one of the main factors regulating oogenesis and spermatogenesis in sponges (Kinne, 1970; Sarà & Vacelet, 1973) even if there are several cases of gametogenesis unrelated to this parameter (Sarà & Relini Orsi, 1975; Corriero et al., 1996; Riesgo & Maldonado, 2008). As highlighted by Riesgo & Maldonado (2008), the relationship between temperature and gametogenesis is not reducible to a simple generic pattern. Intrinsic physiological processes also regulate gamete production. The comparison of daily variations of sea and air temperatures of Conero Promontory and western Mediterranean Sea (Portofino Promontory) highlighted that in the study area water temperature varies more quickly in response to air temperature with respect to western Mediterranean (Fig. 5a, b). Due to these sharp temperature variations, periods of spermatic cyst production could undergo slight variations from one year to another, and the continuous presence of oocytes could grant a higher probability of fertilization.

The comparison of the reproductive effort in *T. anhelans* and *C. reniformis* of the study area and *T. anhelans* from the Ionian sea (Nonnis Marzano et al., 2000) is showed in Table 2. Both species from the North Adriatic Sea involve a higher percentage of tissue in the production of oocytes compared to *T. anhelans* from Apulia; values relative to embryos and larvae and spermatic cysts are inferior but similar to those of Apulian sponges.

Table 3 shows the comparison of density of reproductive elements in *T. anhelans* and *C. reniformis* from the studied area, *T. anhelans* from the Ionian Sea (Nonnis Marzano et al., 2000), *C. reniformis* from the western Mediterranean (Riesgo & Maldonado, 2008), and *Tedania* (*Tedania*) *ignis* (Duchassaing & Michelotti, 1864) from the Caribbean (Maldonado & Young, 1996). Even if Authors used different thickness of histological sections, and some data are referred to embryos and larvae (or oocytes and embryos) considered together, it is possible to observe that density of oocytes, embryos, and larvae are similar in *T. anhelans* from the North Adriatic and Ionian seas and in *T. ignis* from the Caribbean. On the contrary, the density of spermatic cysts is higher in samples from Apulia. The density of oocytes in *C. reniformis* from the Western Mediterranean is much higher than values obtained for samples from the North Adriatic Sea.

The North Adriatic Sea is characterized by high productivity at several trophic levels from phytoplankton to fish (Gilmartin & Revelante 1980; Fonda Umani, 1996; Bernardi Aubry et al., 2006), and the continuous oogenesis together with the large amount of tissue involved in oocyte production could be due to high food availability in this area that drives sponges

Table 1 Comparison of reproductive periods of some Mediterranean sponges

References	Locality	Species	J	F	M	A	M	J	J	A	S	O	N	D
Present work	Conero Promontory (North Adriatic Sea)	*Tedania anhelans*												
Nonnis Marzano et al. (2000)	Apulia (Ionian Sea)	*Tedania anhelans*												
Present work	Conero Promontory (North Adriatic Sea)	*Chondrosia reniformis*												
Riesgo & Maldonado (2008)	Blanes, Tossa del Mar (Western Mediterranean Sea)	*Chondrosia reniformis*												
Scalera Liaci et al. (1971)	Apulia (South Adriatic Sea)	*Chondrosia reniformis*												
Scalera Liaci et al. (1971)	Apulia (South Adriatic Sea)	*Chondrilla nucula*												
Riesgo & Maldonado (2008)	Blanes, Tossa del Mar (Western Mediterranean Sea)	*Axinella damicornis*												
Riesgo et al. (2007), Maldonado & Riesgo (2008), Riesgo & Maldonado (2008)	Blanes, Tossa del Mar (Western Mediterranean Sea)	*Corticium candelabrum*												
Riesgo et al. (2008), Riesgo & Maldonado (2009)	Blanes, Tossa del Mar (Western Mediterranean Sea)	*Raspaciona aculeata*												
Baldacconi et al. (2006)	Apulia (Ionian Sea)	*Spongia officinalis* var. *adriatica*												
Mariani et al. (2000)	Blanes (Western Mediterranean, Sea)	*Cliona viridis*												
Corriero et al. (1998)	Apulia (Ionian Sea)	*Mycale contarenii*												
Riesgo et al. (2008), Maldonado & Riesgo (2009)	Western Mediterranean Sea	*Petrosia ficiformis*												
Scalera Liaci et al. (1973)	Apulia (Southern Adriatic and Ionian Sea)	*Petrosia ficiformis*												
Rodriguez Perez-Porro et al. (2010)	Western Mediterranean Sea	*Hemimycale columella*												
Rodriguez Perez-Porro et al. (2010)	Western Mediterranean Sea	*Crella elegans*												

– – – Oocytes, – · – · sperm cysts, ——— larvae

Table 2 Comparison of reproductive effort (expressed as percentage of tissue occupied by reproductive elements) of *T. anhelans* and *C. reniformis*

	Oocytes	Embryos	Larvae	Spermatic cysts
T. anhelans				
Ionian Sea				
Nonnis Marzano et al. (2000)	0.004% ± 0.002 SD	3.8% ± 2.9 SD (embryos and larvae)		0.8% ± 0.7 SD
North Adriatic Sea				
Present study	0.029% ± 0.047 SD	1.23% ± 1.43 SD (embryos and larvae: 2.46% ± 3.39 SD)	5.89% ± 4.66 SD	0.74% ± 3.36 SD
C. reniformis				
North Adriatic Sea				
Present study	0.045% ± 0.06 SD			

Table 3 Comparison of density of reproductive elements (highest average values)

	Oocytes	Embryos	Larvae	Cysts
T. anhelans				
Ionian Sea				
Nonnis Marzano et al. (2000)	0.2 mm^{-2} ± 0.1 SD	0.5 mm^{-2} ± 0.4 SD (embryos and larvae)		6.3 mm^{-2} ± 5.1 SD
North Adriatic Sea				
Present study	8.54 mm^{-3}	18.49 mm^{-3} ± 14.77 SD	1.18 mm^{-3} ± 0.13 SD	4.83 mm^{-3} ± 0.22 SD
	0.35 mm^{-2}	0.55 mm^{-2} ± 0.49 SD	0.33 mm^{-2} ± 0.17 SD	0.31 mm^{-2} ± 0.22 SD
T. ignis				
The Caribbean				
Obtained from Maldonado & Young (1996)	0.212 ± 0.054 mm^{-2} (oocytes and embryos)			
C. reniformis				
North Adriatic Sea				
Present study	44.62 mm^{-3} ± 43.36 SD			
	1.29 mm^{-2} ± 1.16 SD			
Blanes, Tossa del Mar (Western Mediterranean Sea)				
Riesgo & Maldonado (2008)	7.2 mm^{-2} ± 3.3 SD			

to invest their energy surplus in oocytes production. Since spermatogenesis in *T. anhelans* and *C. reniformis* is shorter than oogenesis, many oocytes can not be fertilized; it is possible that some young oocytes are used to nourish mature oocytes, zygotes, and embryos (Sarà, 1955; Maldonado et al., 2005).

In the study area of Conero Promontory, *T. anhelans* and *C. reniformis* as well as other benthic animals (Di Camillo et al., 2010; Betti et al., 2011) reach densities or sizes perceptibly bigger than the same species observed in other Mediterranean sites. *C. reniformis* from the study area, with its over one-meter surface, is probably one of the largest sponges of the Mediterranean Sea. *T. anhelans* generally forms small encrusting or cushion specimens (Corriero et al., 2007), and giant morphs have been observed only in the North Adriatic Sea (Venice Lagoon, Corriero et al., 2007; Tegnue, Bertolino et al., 2008).

The high incidence of large and very abundant species in the North Adriatic Sea is mainly due to the high concentrations of nutrients discharged by rivers (Artegiani & Azzolini, 1981; Artegiani et al., 1997a, b; Zavatarelli et al., 2000). In this area, food is never a limiting factor since productivity rates are high and comparable to those of the North Sea (Gilmartin & Revelante, 1983). The chlorophyll concentration

(Fig. 5d) is always higher than that reported for the Mediterranean Sea even during summer, which is generally considered an oligotrophic period (Coma et al., 2000). Sponges can feed on a wide spectrum of food sources (DOC, bacteria, cyanobacteria, phytoplankton, and zooplankton), but the most important resource for these animals is phytoplankton (Ribes et al., 1999, Reiswig, 1971, Pile et al., 1996). In particular, nano- and pico-plankton are abundant especially in summer time in the North Adriatic Sea, and they could represent an important food supply during this period (Gilmartin & Revelante, 1980; Fonda Umani, 1996; Bernardi Aubry et al., 2006; Zuschin & Stachowitshc, 2009) that is generally considered oligotrophic for the Mediterranean Sea (Coma et al., 2000).

C. reniformis is able to incorporate foreign materials, selecting sand grains of crystalline quartz and amorphous siliceous opaline spicules (Bavestrello et al., 1998a, b) and the collagenous production in *C. reniformis* may be enhanced by quartz dissolution (Bavestrello et al., 2003; Cerrano et al., 2007). This lead us to consider that, due to the high sedimentation rates characterizing the Conero Promontory, the sponge growth could be enhanced by the incorporation of large amounts of sediments.

References

Artegiani, A. & R. Azzolini, 1981. Influence of the Po floods on the western Adriatic coastal water up to Ancona and beyond. Rapport de la Commission Internazionale pour l'exporation scientifique de la Mer Méditerranée 27(6): 115–119.

Artegiani, A., D. Bregant, E. Paschini, N. Pinardi, F. Raicich & A. Russo, 1997a. The Adriatic Sea general circulation. Part I: Air–sea interactions and water mass structure. Journal of Physical Oceanography 27(8): 1492–1514.

Artegiani, A., D. Bregant, E. Paschini, N. Pinardi, F. Raicich & A. Russo, 1997b. The Adriatic Sea general circulation. Part II: Baroclinic circulation structure. Journal of Physical Oceanography 27(8): 1515–1532.

Ayling, A. L., 1983. Growth and regeneration rates in thinly encrusting Demospongiae from temperate waters. The Biological Bullettin 165: 343–352.

Baldacconi, R., F. Cardone, C. Nonnis Marzano & G. Corriero, 2006. Riproduzione sessuale in una popolazione naturale e in frammenti reimpiantati di *Spongia officinalis* var. *adriatica* (Porifera, Demospongiae). Biologia Marina Mediterranea 13(1): 797–800.

Barthel, D., 1988. On the ecophysiology of the sponge Halichondria panicea in Kiel Bight. II. Biomass, production, energy budget and integration in enviromental processes. Marine Ecology Progress Series 43: 87–93.

Bavestrello, G. & A. Arillo, 1992. Irradiance, temperature and circannual cycle of *Eudendrium glomeratum* Picard (Hydrozoa, Cnidaria). Bollettino di Zoologia 59(1): 45–48.

Bavestrello, G., A. Arillo, B. Calcinai, C. Cerrano, S. Lanza, R. Cattaneo-Vietti, E. Gaino & M. Sarà, 1998a. Siliceous particles in corporation in *Chondrosia reniformis* (Porifera, Demospongiae). Italian Journal of Zoology 65: 343–348.

Bavestrello, G., A. Arillo, B. Calcinai, C. Cerrano, S. Lanza, R. Cattaneo-Vietti, E. Gaino & M. Sarà, 1998b. Body polarity and mineral selectivity in the demosponge *Chondrosia reniformis*. Biological Bullettin 195(2): 120–125.

Bavestrello, G., U. Benatti, R. Cattaneo-Vietti, C. Cerrano & M. Giovine, 2003. Sponge cell reactivity to various forms of silica. Microscopy Research and Technique 62: 327–335.

Bernardi Aubry, F., F. Acri, M. Bastianini, A. Pugnetti & G. Socal, 2006. Picophytoplankton contribution to phytoplankton community structure in the Gulf of Venice (NW Adriatic Sea). Hinternational Review of Hydrobiology 91(1): 51–70.

Bertolino, M., B. Calcinai & C. Cerrano, 2008. Poriferi. In Mandragora (ed.), Poriferi. Meraviglie sommerse delle Tenue. Guida alla scoperta degli organismi marini: 85–135.

Betti, F., M. Bo, C. G. Di Camillo & G. Bavestrello, 2011. Life history of *Cornularia cornucopiae* (Anthozoa: Octocorallia) on the Conero Promontory (North Adriatic Sea). Marine Ecology. doi:10.1111/j.1439-0485.2011.00456.x.

Bonasoro, F., I. C. Wilkie, G. Bavestrello, C. Cerrano & M. D. Bavestrello, 2001. Dynamic structure of the mesohyl in the sponge *Chondrosia reniformis* (Porifera, Demospongiae). Zoomorphology 121: 109–121.

Byun, D.-S. & N. Pinardi, 2007. Comparison of marine insolation estimating methods in the Adriatic Sea. Ocean Science Journal 42(4): 211–222.

Cardone, F., E. Gaino & G. Corriero, 2010. The budding process in *Tethya citrina* Sara & Melone (Porifera, Demospongiae) and the incidence of post-buds in sponge population maintenance. Journal of Experimental Marine Biology and Ecology 389: 93–100.

Cerrano, C., B. Calcinai, S. Pinca & G. Bavestrello, 2006. Reef sponges as hosts of biodiversity: cases from North Sulawesi. In Suzuki, Y., T. Nakamori, M. Hidaka, H. Kayanne, B. E. Casareto, K. Nadao, H. Yamano & M. Tsuchiya (eds), Proceedings Xth Coral Reef Symposium. Japanese. Coral Reef Society, Okinawa: 208–213.

Cerrano, C., B. Calcinai, C. G. Di Camillo, L. Valisano & G. Bavestrello, 2007. How and why do sponges incorporate foreign material? Strategies in Porifera. Porifera Research: Biodiversity Innovation and Sustainability: 239–246.

Cicero, A.M., & I. Di Girolamo, 2001. Programma di monitoraggio per il controllo dell'ambiente marino-costiero (triennio 2001–2003). Metodologie analitiche di riferimento, Ministero dell'Ambiente e della Tutela del Territorio Servizio Difesa Mare, Lo Studio Editoriale s.r.l., Roma: 1–12.

Coma, R., M. Ribes, J.-M. Gili & M. Zabala, 2000. Seasonally in coastal benthic ecosystems. Trends in Ecology & Evolution 15: 448–453.

Corriero, G., M. Pansini & M. Sarà, 1984. Sui poriferi dell'insenatura della Strea a Porto Cesareo (Lecce). Thalassia Salentina 14: 3–10.

Corriero, G., M. Sarà & P. Vaccaio, 1996. Sexual and asexual reproduction in two species of Tethya (Porifera, Demospongiae) from a Mediterranean coastal lagoon. Marine Biology 126: 175–181.

Corriero, G., L. Scalera Liaci, C. Nonnis Marzano & E. Gaino, 1998. Reproductive strategies of *Mycale contarenii* (Porifera: Demospongiae). Marine Biology 131: 319–327.

Corriero, G., C. Longo, M. Mercurio, A. Marchini & A. Occhipinti-Ambrogi, 2007. Porifera and Bryozoa on artificial hard bottoms in the Venice Lagoon: spatial distribution and temporal changes in the northern basin. Italian Journal of Zoology 74: 21–29.

De Caralt, S., M. J. Uriz & R. H. Wijffels, 2008. Grazing, differential size-class dynamics and survival of the Mediterranean sponge *Corticium cadelabrum*. Marine Ecology Progress Series 360: 97–106.

de Goeij, J. M. & F. C. van Duyl, 2007. Coral cavities are sinks of dissolved organic carbon (DOC). Limnology and Oceanography 52: 2608–2617.

Di Camillo, G. C., M. Bo, I. Bartolucci, F. Betti, B. Calcinai, C. Cerrano, M. Coppari, M. Martinelli, S. Puce & G. Bavestrello, 2010. The benthic assemblage of Conero Promontory: a model for the study of seasonal cycles in the North Adriatic Sea. Biologia Marina Mediterranea 17: 112–115.

Elvin, D. W., 1976. Seasonal growth and reproduction of an intertidal sponge, *Haliclona permollis* (Bowerbank). Biological Bulletin 151: 108–125.

Fonda Umani, S., 1996. Pelagic biomass and production in the Adriatic Sea. In Palomera, J. & P. Rubies (eds), The European Anchovy and Its Environment. Scientia Marina 60 (Suppl): 67–77.

Garrabou, J. & M. Zabala, 2001. Growth dynamics in four Mediterranean Demosponges. Estuarine, coastal and shelf science 52: 293–303.

Gili, J. M. & R. Coma, 1998. Benthic suspension feeders: their paramount role in littoral marine food webs. Trends in Ecolology & Evolution 13: 316–321.

Gilmartin, M. & N. Revelante, 1980. Nutrient input and the summer nanoplankton bloom in the northern Adriatic Sea. Marine Ecology Progress Series 1: 169–180.

Gilmartin, M. & N. Revelante, 1983. The phytoplankton of the Adriatic Sea: standing crop and primary production. Thalassia Jugoslavica 19: 173–188.

Giordani, P., W. Helder, E. Koning, S. Miserocchi, R. Danovaro & A. Malaguti, 2002. Gradients of benthic-pelagic coupling and carbon budgets in the Adriatic and Northern Ionian Sea. Journal of Marine Systems 33–34: 365–387.

Hammer, Ø., D. A. T. Harper & P. D. Ryan, 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Paleontologia Electronica 4: 1–9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Kinne, O., 1970. Temperature-invertebrates. In Kinne, O. (ed.), Marine Ecology: A Comprehensive Integrated Treatise of Life in Oceans and Coastal Waters. Wiley-Interscience, London, UK: 407–514.

Koopmans, M. & R. H. Wijffels, 2008. Seasonal growth rate of the sponge *Haliclona oculata* (Demospongiae: Haplosclerida). Marine Biotechnology 10: 502–510.

Lévi, C., 1956. Étude des Halisarca de Roscoff. Archives de Zoologie Expérimentale et Générale 93: 1–181.

Maldonado, M., 2006. The ecology of the sponge larva. Canadian Journal of Zoology 84: 175–194.

Maldonado, M. & A. Riesgo, 2008. Reproductive output in a Mediterranean population of the homosclerophorid *Corticium candelabrum* (Porifera, Demospongiae), with notes on the ultrastructure and behavior of the larva. Marine Ecology 29: 298–316.

Maldonado, M. & A. Riesgo, 2009. Gametogenesis, embryogenesis, and larval features of the oviparous sponge *Petrosia ficiformis*. Marine Biology 156: 2181–2197.

Maldonado, M. & C. M. Young, 1996. Effects of physical factors on larval behavior, settlement and recruitment of four tropical demosponges. Marine Ecoogy Progress Series 138: 169–180.

Maldonado, M., N. Cortadellas, M. I. Trillas & K. Rützler, 2005. Endosymbiotic yeast maternally transmitted in a marine sponge. Biological Bulletin 209: 94–106.

Maldonado, M., X. Zhang, X. Cao, L. Xue, H. Cao & W. Zhang, 2010. Selective feeding by sponges on pathogenic microbes: a reassessment of potential for abatement of microbial pollution. Marine Ecology Progress Series 403: 75–89.

Mariani, S., M.-J. Uriz & X. Turon, 2000. Larval bloom of the oviparous sponge *Cliona viridis*: coupling of larval abundance and adult distribution. Marine Biology 137: 783–790.

Mercurio, M., G. Corriero & E. Gaino, 2007. A 3-year study of sexual reproduction in *Geodia cydonium* (Jamenson 1811) (Porifera, Demospongiae) from a semi-enclosed Mediterranean bay. Marine Biology 151: 1491–1500.

Nonnis Marzano, C., M. Mercurio & L. Scalera Liaci, 2000. Il ciclo sessuale di *Tedania anhelans* Lieberkühn (Porifera, Demospongiae) nell'insenatura della strea di Porto Cesareo. Biologia Marina Mediterranea 7: 703–706.

Nonnis Marzano, C., G. Corriero & L. Scalera Liaci, 2003a. Distribution and life cycle of *Halichondria panicea* (Porifera, Demospongiae) in the Lake of Lesina. Biologia Marina Mediterranea 10: 159–167.

Nonnis Marzano, C., L. Scalera Liaci, A. Fianchini, F. Gravina, M. Mercurio & G. Corriero, 2003b. Distribution, persistence and change in the macrobenthos of the lagoon of Lesina (Apulia, Southern Adriatic Sea). Oceanologica Acta 26: 57–66.

Pansini, M. & R. Pronzato, 1985. Observations on the dynamics of a Mediterranean sponge community. In Rützler, K. (ed.), New Perspectives in Sponge Biology: 3d International Sponge Conference, 1985. Smithsonian Institution Press, Washington, D. C.: 404–415.

Parma, L., D. Fassini, G. Bavestrello, I. C. Wilkie, F. Bonasoro & D. C. Carnevali, 2007. Ecology and physiology of mesohyl creep in *Chondrosia reniformis*. Porifera Research: Biodiversity, Innovation and Sustainability: 503–508.

Pile, A. J., M. R. Petterson & J. D. Witman, 1996. In situ grazing on plankton <10 μm by the boreal sponge *Mycale lingua*. Marine Ecology Progres Series 141: 95–102.

Reiswig, H. M., 1971. Particle feeding in natural populations of three marine demosponges. Biological Bullettin 141: 568–591.

Ribes, M., R. Coma & J.-M. Gili, 1999. Natural diet and grazing rate of the temperate sponge *Dysidea avara* (Demospongiae, Dendroceratida) throughout an annual cycle. Marine Ecology Progress Series 176: 179–190.

Riesgo, A. & M. Maldonado, 2008. Differences in reproductive timing among sponges sharing habitat and thermal regime. Invertebrate Biology 127: 357–367.

Riesgo, A. & M. Maldonado, 2009. Ultrastructure of oogenesis in two oviparous demosponges: *Axinella damicornis* and *Raspaciona aculeata*. Tissue & Cell 41: 51–56.

Riesgo, A., M. Maldonado & M. Durfort, 2007. Dynamics of gametogenesis, embryogenesis, and larval release in a Mediterranean homosclerophorid demosponge. Marine and Freshwater Research 58: 398–417.

Riesgo, A., M. Maldonado & M. Durfort, 2008. Occurence of somatic cells within the spermatic cysts of demosponges: a discussion of their role. Tissue & Cell 40: 387–396.

Rodriguez Perez-Porro, A., J. Gonzales & M. J. Uriz, 2010. Life cycle and larval ultrastructure of two Mediterranean Poecilosclerida sharing habitat. Book of abstracts, VIII World Sponge Conference, Girona (Spain), 20–24 September 2010: p. 118.

Rützler, K., 1985. Associations between Caribbean sponges and photosynthetic organisms. In Rützler, K. (ed.), New Perspectives in Sponge Biology: 3d International Sponge Conference, 1985. Smithsonian Institution Press, Washington, D. C.: 455–466.

Rützler, K., 2004. Sponges on coral reefs: a community shaped by competitive cooperation. Bollettino dei Musei e degli Istituti Biologici dell'Università di Genova 68: 85–148.

Sarà, M., 1955. La nutrizione dell'ovocita in Calcispongiae Omoceli. Annuario dell'Istituto e Museo di Zoologia dell'Università di Napoli 7: 1–30.

Sarà, M., 1970. Competition and cooperation in sponge populations. Symposia of the Zoological Society of London 25: 273–284.

Sarà, M. & J. Vacelet, 1973. Ecologie des Démosponges. In P. P. Grassé (ed.), Traité de Zoologie. Spongiaires (Masson et Cie: Paris) 3: 462–576

Sarà, M. & L. Relini Orsi, 1975. Sex differentiation in *Sycon* (Porifera, Calcispongiae). Pubblicazioni della Stazione Zoologica di Napoli 39: 618–634.

Scalera Liaci, L., M. Sciscioli & A. Matarrese, 1971. La riproduzione sessuale di alcuni Tetractinomorpha (Porifera). Proceeding of the Società Peloritana di Scienze Fisiche Matematiche e Naturali 17 (3–4).

Scalera Liaci, L., M. Sciscioli & A. Matarrese, 1973. Raffronto tra il comportamento sessuale di alcune ceractinomorpha. Estratto da: Rivista di Biologia 66(1–2): 135–162.

Sokal, R. R. & F. J. Rohlf, 1981. Biometry: The principle and practice of statistics in biological research. Freeman W. H. & Company, New York.

Teixidó, N., M. C. Pineda & J. Garrabou, 2009. Decadal demographic trends of a long-lived temperate encrusting sponge. Marine Ecology Progress Series 375: 113–124.

Turon, X., I. Tarjuelo & M. J. Uriz, 1998. Growth dynamics and mortality of the encrusting sponge *Crambe crambe* (Poecilosclerida) in contrasting habitats: correlation with population structure and investment in defence. Functional Ecology 12: 631–639.

World Porifera Database – http://www.marinespecies.org/porifera/porifera.php?p=taxdetails&id=134112.

Wulff, J. L., 2006. Rapid diversity and abundance decline in a Caribbean coral reef sponges community. Biological Conservation 127: 167–176.

Yahel, G., J. H. D. Sharp, M. C. Haese & A. Genin, 2003. In situ feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei*: Bulk DOC is the major source for carbon. Limnology and Oceanography 48: 141–149.

Zavatarelli, M., J. W. Baretta, J. C. Baretta-Bekker & N. Pinardi, 2000. The dynamics of the Adriatic Sea Ecosystem. An idealized model study. Deep-Sea Research 47: 937–970.

Zuschin, M. & M. Stachowitshc, 2009. Epifauna-dominated benthic shelf assemblages: lessons from the modern Adriatic Sea. Palaios 24: 211–221.

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SPONGE RESEARCH DEVELOPMENTS

Reproductive traits explain contrasting ecological features in sponges: the sympatric poecilosclerids *Hemimycale columella* and *Crella elegans* as examples

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Abstract Our study focused on the Mediterranean species *Hemimycale columella* and *Crella elegans*, which have overlapping ecological distributions but contrasting population densities and resilience. We formulated the hypothesis that differential reproductive traits were the main cause underlying these ecological differences in the study area. The issues addressed were whether recruits compete for the substrate, either because their respective reproductive cycles overlap or/and larval performance differs between species, and whether a contrasting investment in reproduction contributed to explain their diverse densities. Both species were simultaneous hermaphrodites and incubated their larvae. The reproductive period was notably shorter in *C. elegans* than in *H. columella*, while investment in reproductive tissue was higher in individuals of *H. columella* than in *C. elegans*. In contrast, *C. elegans* larvae harbored larger amounts of lipids and yolk inclusions than *H. columella* larvae. Moreover, the former contained amazing collagen masses densely packed among their inner cells. *H. columella* is likely to produce a higher number of poorer equipped larvae while *C. elegans* seems to produce a lower number of more resistant, better fitted with energy reserves, and thus potentially more successful larvae. These reproductive patterns agree with a higher and stable density of *C. elegans* in the study site compared with a more variable abundance of *H. columella.*

Keywords Reproductive cycle · Embryo ultrastructure · Larva ultrastructure · Parenchymella · Demosponges · Reproductive investment · *Hemimycale columella* · *Crella elegans* · Mediterranean Sea

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Introduction

Sponges are morphologically plastic animals that do not possess gonads or reproductive tracts. Gametes are usually found widespread throughout the entire sponge body (Simpson, 1984), although larvae can be found in brood chambers (e.g., Whalan et al., 2005) and, in some cases, oocytes form clusters (Riesgo et al., 2007). Several reproductive modes have been reported in sponges (gonochorism, hermaphroditism, oviparism, viviparism), as well as different types of larvae (amphiblastula and calciblastula for calcareous sponges, parenchymella, coeloblastula, and dispherula

for demosponges, cinctoblastula for homoscleromorphs, and trichimella for hexactinellids) (Leys & Ereskovsky, 2006). Many studies have focused on sponge reproduction (as reviewed by Fell, 1974; Reiswig, 1983; Simpson, 1984; Boury-Esnault & Jamieson, 1999) but, because of sponge phenotypic plasticity and genotypic differences, new features are continuously discovered as more species are investigated (e.g., Riesgo & Maldonado, 2009).

The order Poecilosclerida is one of the most diverse of the class Demospongiae for the number of genera and species it contains (Ereskovsky, 2010). Accordingly, species would be expected to contain extensive variation in reproductive traits. However, although previous studies indicated that most families in Poecilosclerida are viviparous, simultaneous hermaphrodites, show a yearly reproductive cycle (Reiswig, 1973; De Vos et al., 1991; Ilan, 1995; Ereskovsky, 2000; Ilan et al., 2004; Leys & Ereskovsky, 2006; Riesgo & Maldonado, 2009), and release parenchymella larvae (Ereskovsky, 2010), when studied in detail, original traits have been reported for some species. These include the particular spermatozoa features in *Crambe crambe* (Schmidt, 1862) (Riesgo & Maldonado, 2009) and the fertilization system of the carnivorous sponges within the family Cladorhizidae (Vacelet & Boury-Esnault, 1995; Riesgo et al., 2007). Other specific reproductive traits can be anticipated when studying new poecilosclerid species, in particular when the species belong to families that have not been previously studied.

The study of reproduction patterns may reveal biological characters with diagnostic value for taxonomic issues. Reproduction in the family Crellidae (Poecilosclerida) is still poorly known, although it harbors genera with widespread representatives in several oceans (Hooper & Van Soest, 2002). On the other hand, the taxonomic position of the genus *Hemimycale* remains unclear. This genus was moved from Halichondrida (Lévi, 1973) to Poecilosclerida (Hooper & Van Soest, 2002) mainly on the basis of the surface pore-sieves that the type species share with most Hymedesmiidae, and Crellidae. However, the genus does not have decisive diagnostic characters as it has one type of monaxon megasclere and lacks any of the typical microscleres of Poecilosclerida. Biological, as well as genetic characters, of the type species, *Hemimycale columella*, would be very useful to establish its phylogenetic position.

Reproductive features also contribute, in the medium-and long-term, to population success of sessile organisms such as sponges (e.g., Uriz et al., 1998). Reproductive investment, fertilization success, sperm and larval dispersal, and recruitment contribute to the permanence of populations in a given area, since they determine their genetic diversity and, thus, their capacity to survive environmental changes and perturbations (Blanquer & Uriz, 2010; Guardiola et al., this issue). Moreover, some reproductive traits may help to explain how species competing for limited resources (i.e., substrate and food) manage to coexist in the same habitat. There are several descriptors of reproductive characters, which can provide information about potential dispersal and expected success of a given species. Among these, resource investment in reproductive tissue and larval performance for settlement and survival are particularly relevant.

We have selected for study two poecilosclerid species, which share growth habit (encrusting), external morphology (inhalant orifices clustered in small ringed areas) and habitat (sublittoral rocky, semi-sciaphilous bottoms), and attempted to understand why they show differential densities in the study area. We pose the hypothesis that several reproduction traits were key factors underlying the ecological patterns observed. To test this hypothesis, we formulated and tried to answer the following questions: (1) Does their reproductive cycle overlap in such a way that new recruits may compete for the substrate? (2) Does a contrasting investment in reproduction contribute to understanding their differential densities? and (3) Do their larvae show particular characteristics that could indicate different recruitment success? To gain insight into the reproductive aspects that may help to answer these questions, we compare the timing of gamete, embryo, and larval production, and investment in reproductive tissue of both species. We also examine the ultrastructure of gametes, embryos and larvae of both species searching for cues such as reserve inclusions or collagen, which could predict larval performance and colonization success.

Materials and methods

Species studied and sampling site

Hemimycale columella and *Crella elegans* are Atlanto-Mediterranean, thick encrusting pale orange to pink

sponges with a slippery smooth surface covered by densely distributed roundish pore-sieves. They are particularly abundant in the sublittoral rocky-bottom assemblages of the western Mediterranean. The species share habitat at the study locality: rocky walls and overhangs along the Tossa de Mar sublittoral (Girona, Spain, North-western Mediterranean, (41°43′13″N, 2°56′25″E)), at a depth of ca. 18 m.

Sponge survey, sample collection, and treatment

To survey the reproductive cycle of both poecilosclerid species we tagged five large individuals of *H. columella* and *C. elegans* with two-component, water-resistant mastic (IVEGOR, S.A.). Two of the tagged *C. elegans* disappeared at the end of the reproductive season of 2008, and thus only three individuals were surveyed during 2009. All individuals were sampled monthly from September 2008 to October 2009 by SCUBA diving. Samples consisted of ca. 1 × 1 cm tissue pieces, which were taken from the basal sponge zone where reproductive elements were expected to accumulate (Ereskovsky, 2010).

The samples for light microscopy were fixed in 10% formaldehyde in seawater for 5 h and then changed to 4% formaldehyde in seawater. These samples were desilicified with 5% hydrofluoric acid for 4 h, rinsed in distilled water, dehydrated in an ethanol series (70, 96, 100%) (30 min each) and then rinsed first in a solution of 1:1 toluene/ethanol (30 min), and then in pure toluene (15 min). The samples were embedded in paraffin and sectioned in 5-µm-thick sections with an Autocut 2040 Reichert-Jung microtome. Sections were then dewaxed with xylene, stained with Harris hematoxylin, mounted in DPX, and observed under a Zeiss Axioplan II compound microscope connected to a Spot Cooled Color digital camera. To count and measure the reproductive elements we took three pictures (10×) of two non-serial sections per individual each month. The two sections were separated by a minimum of 240 µm to avoid measuring the same reproductive elements. The total analyzed area represents ca. 6 mm^2 of sponge tissue. Image analysis was performed with the free access software Fiji (http://pacific.mpi-cbg.de/). We estimated the number, area, and the largest diameter of each reproductive element in every digitized, histological image. We computed the percent area of each sponge section and the average density (number of reproductive elements per unit area ± SD) occupied by reproductive structures, as well as the average size of each reproductive element (whether gamete, embryo, or larva).

For transmission electron microscopy (TEM) samples ca. 2 mm^3 in size were fixed in 1% OsO_4 and 2% glutaraldehyde in 0.45 M sodium acetate buffer (pH 6.4) with 10% sucrose (1:3) (Leys & Reiswig, 1998) for 12 h at 4°C. Samples were repeatedly rinsed in the same buffer, dehydrated in an ethanol series and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a TEM (JEOL 1010), implemented with a Bioscan system (Gatan) for image digitalization (Microscopy Unit of the Scientific and Technical Services of the Universitat de Barcelona).

Results

Hemimycale columella

Reproductive cycle

Hemimycale columella is a simultaneous hermaphroditic, viviparous sponge. Spermatic cysts and oocytes coexisted in all the specimens studied. Gametogenesis likely started with the increase of seawater temperature in midsummer (Fig. 1), although oocytes were only observed in August of 2009 (1.54 ± 3.8% sponge tissue) and spermatic cysts at different stages of development from August (0.24 ± 0.08%) to September (0.9 ± 0.4%) during 2009 (Fig. 1). Embryo development lasted for 2 months. Several embryo stages, pre-larvae and mature larvae were simultaneously found in the same individuals from September to the end of October 2008. Larval release took place mainly during October, in accordance with a sharp decrease of seawater temperature (Fig. 1), although occasional larvae were also released at the beginning of November.

Reproductive investment

The average sponge tissue occupied by embryos pre-larvae, and larvae increased from September 2008 (15 ± 15.9%, 3.7 ± 4.6%, and 3.1 ± 4.2% of the sponge section, respectively) to mid-October, 2008 (36.8 ± 24.20%, 9 ± 20.1%, and 13.28 ± 29.7%,

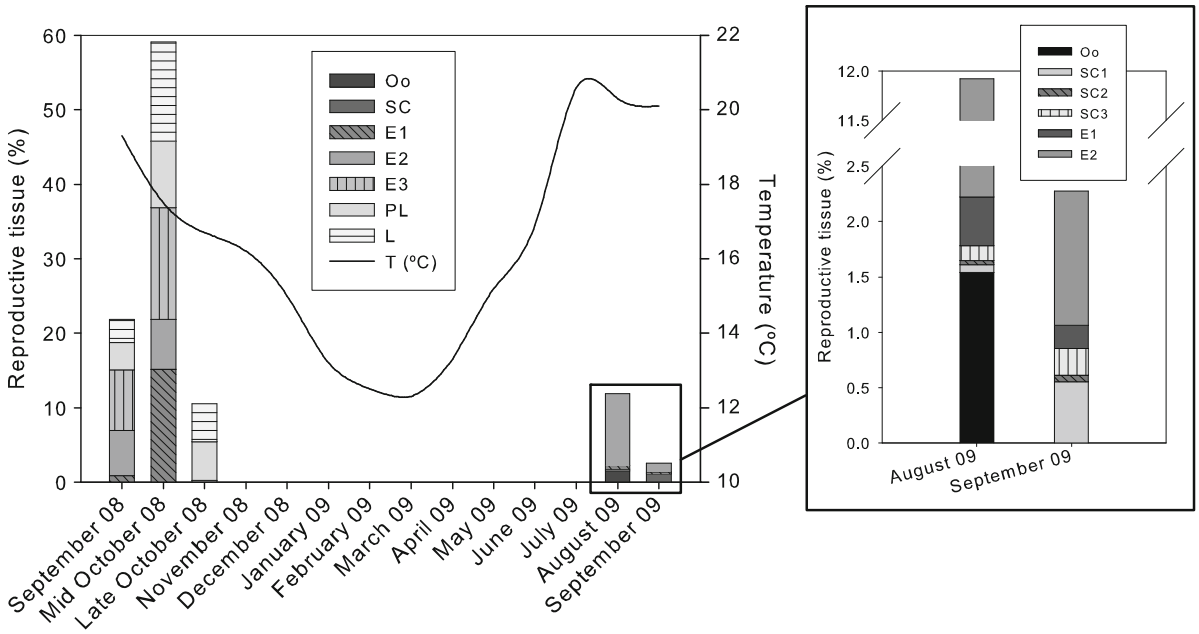


Fig. 1 *Hemimycale columella*. *Stacked bars* representing the relative abundance of the different reproductive elements with respect to sponge tissue (measured in % of area occupied from histological sections) in the target individuals over the two study years. Reproductive elements: oocytes (Oo), three different stages of spermatic cysts development (SC1, SC2, and SC3, from early to late development stages), three different stages of embryo development (E1, E2, and E3, from early to late development stages), pre-larvae (PL), and larvae (L). *Right insert* is an enlargement of the *stacked bars* from August–September 2009

respectively). Then, during October 2008, the number of embryos, pre-larvae, and larvae decreased to 0.3 ± 0.5%, 5.2 ± 6.5%, and 5.1 ± 6.8% values, respectively, at the end of the month. Altogether, the reproductive stages occupied ca. 60% of the sponge tissue at the peak of the reproductive period (mid-October).

Gamete and embryo densities varied notably among the individuals surveyed. Oocyte density was 4.04 ± 24.3 oocytes per mm^2 of sponge section) (Fig. 2). Spermatocysts had an average density of 0.67 ± 4 cysts per mm^2 in August of 2009, and increased to 0.79 ± 4.75 after 1 month (Fig. 2). The average density of embryos at E2 and E3 stages decreased from September of 2008 (0.33 ± 1.04 and 0.43 ± 1.28 embryos per mm^2, E2 and E3 stages, respectively) to mid-October of 2008 (0.1 ± 0.6 and 0.17 ± 0.5 embryos per mm^2, E2 and E3 stages, respectively) while the density of early embryos (E1) increased (from 0.03 ± 0.2 to 0.23 ± 1.2 E1 per mm^2) in the same period. Pre-larvae kept very similar values of average density during September, mid-October, and late October, 2008 (0.16 ± 0.54, 0.17 ± 0.55, and 0.17 ± 0.58 pre-larvae per mm^2, respectively) (Fig. 2). Mature larvae were first recorded in September-2008 at an average density of 0.07 ± 0.2 larvae per mm^2. Larval density increased from September to mid-October (2008) when it reached 0.17 ± 1 larvae per mm^2, and than remained constant (0.17 ± 0.7 larvae per mm^2) until the end of the month (Fig. 2).

Reproductive elements

Oocytes, 26.3 ± 4.5 (mean ± SD) μm in diameter, with 8.5–10 μm diameter nucleolate nucleus, were surrounded by numerous nurse cells (not shown). They were spread throughout the sponge mesohyl but mainly accumulated at the sponge basal zone (Fig. 3a).

Spermatic cysts, surrounded by a follicular layer of pinacocyte-like cells, were spherical to ovoid in shape at all stages of development and were spread throughout the sponge mesohyl. Cells at the development stages of spermacysts were pseudo-cubic to round in shape. Spermatogenesis was synchronous within the

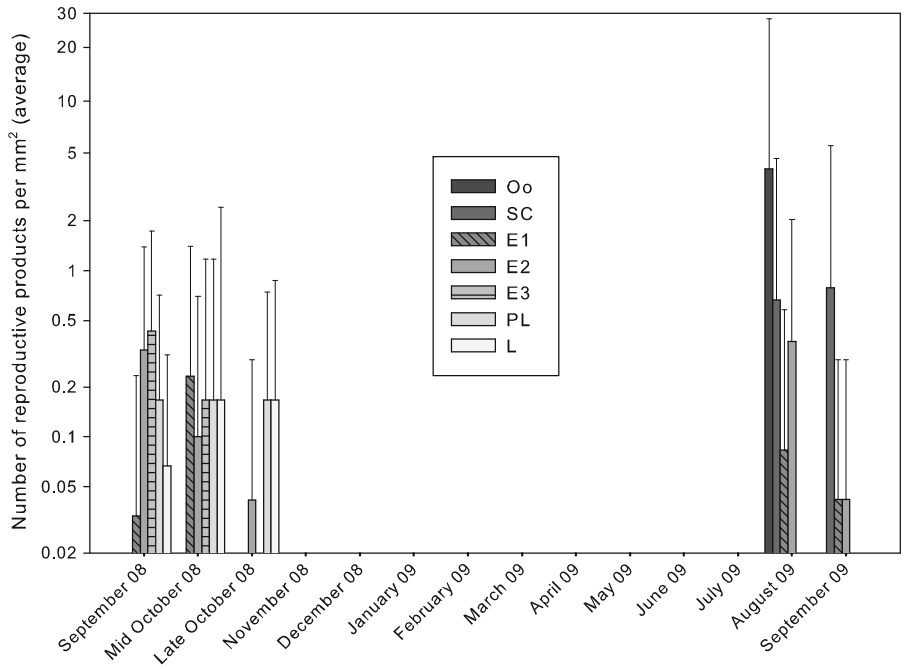


Fig. 2 *Hemimycale columella.* Density (±SD) of reproductive products in the target individuals over the two study years: oocytes (Oo), spermatic cysts (SC), three different stages of embryo development (E1, E2, E3, from early to late development stages), pre-larvae (PL), and larvae (L)

cysts (Fig. 4). Three different development stages co-occurred in the same sponge individuals (Figs. 1, 4). Cyst size slightly decreased during the maturation process. The first development stage (SC1) (56.9 ± 16.9 μm), showed densely packed, globulous spermatogonia-like cells without apparent flagella, which were strongly stained by Harris hematoxylin. Spermatic cysts at a mid-stage of development (SC2) measured 52 ± 6.1 μm of diameter (Fig. 4a, b). Cells were more loosely packed in comparison with SC1, and the presence of flagella that could be glimpsed though the light microscope was confirmed through TEM (Fig. 4a). Cysts at the end of spermatogenesis (SC3) measured 42.1 ± 18.8 μm in diameter and had an oval nucleus with highly condensed chromatine (Fig. 4d). A follicular layer of interlaced pinacocyte-like cells was conspicuous (Fig. 4). The cytoplasm of spermatids surrounded the basal part of the flagellum forming a tunnel-like structure (Fig. 4c). Small, clear to electrons vesicles accumulated at the base of the cell flagella (Fig. 4c, d). Cytoplasmic bridges between spermatids were observed at this stage for the first time during the maturation process (Fig. 4c). Cell flagella in SC3 concentrated at several zones of the spermatic cyst in particular at the peripheral of the cyst, always surrounding the cells (Fig. 4b, c). Mitochondria were present in the spermatids (Fig. 4d). No acrosome vesicles could be differentiated.

Embryos at several stages of development (E1, E2, and E3) coexisted in the same individual, from September to mid-October of 2008 (Figs. 1, 3b, c), while only E2 stages were found at the end of October of 2008, (Fig. 2). In 2009, only the E2 and E3 stages were observed from August to September (Fig. 2), while E1 stage was not found. Early embryos (E1) were round to oval in cross-section, with an average diameter of 259.4 ± 89.9 μm (Fig. 3b). They contained numerous yolk inclusions and were already surrounded by a follicular layer (Fig. 3b). Cleavage was already perceived at the second stage (E2), when the embryos measured 257.4 ± 73.6 μm (Figs. 3c, 5a). Cleavage was clearer in E3 embryos (276.8 ± 92.5 μm in diameters). Blastomeres of two sizes, 79.3 ± 29 and 19.1 ± 11.3 μm in diameter likely corresponding to macromeres and micromeres, could be differentiated (Fig. 3c). Through the light microscope, embryonic cells appeared filled with numerous yolk inclusions (bright purple stained with

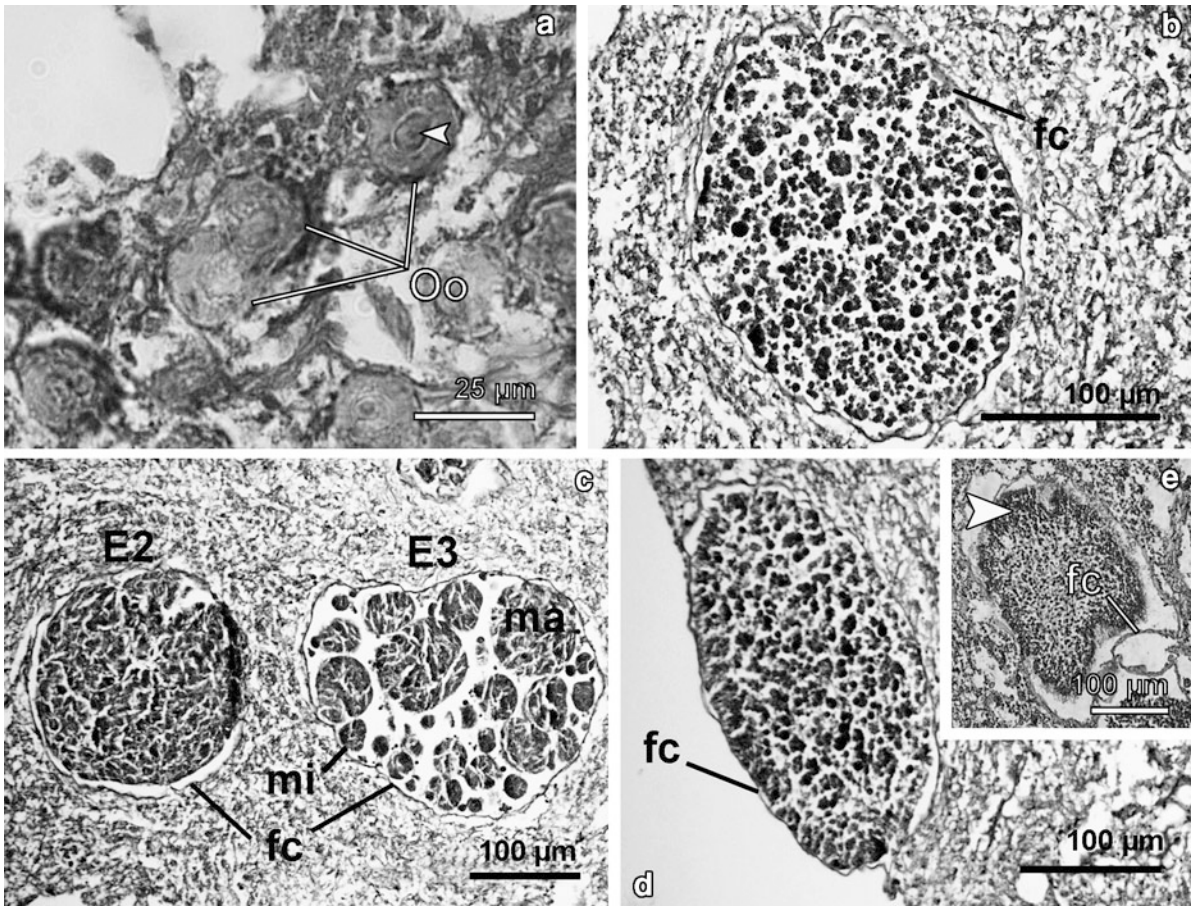


Fig. 3 *Hemimycale columella*. Several reproductive products through the light microscope. **a** Nucleolate (*arrowhead*) oocytes (Oo) distributed throughout the mesohyl. **b** Embryo at an early stage of development surrounded by follicular cells (fc). **c** Two embryos (E2 and E3) in different stages of development, surrounded by follicular cells (fc). See putative macromere (ma) and micromere (mi) cells in E3. **d** Pre-larva surrounded by follicular cells (fc). See the already elongated cells at the pre-larva periphery. **e** Ciliated larva with an completely formed ciliated epithelium (*arrow head*), surrounded by follicular cells (fc)

Harris hematoxylin), which were particularly abundant in early embryos (E1) (Fig. 5a), and in micromeres of later stages of embryo development (Figs. 3c, 5b, 6c). Later embryo developing stages were considered as pre-larvae stages (PL) (Fig. 3d). Pre-larvae reached the largest size (495.6 ± 51.1 µm in diameter) of all embryo stages and were present in the target specimens from September to the end of October of 2008 at the same average density (Fig. 2). They were roundish non-ciliated stereoblastulae, with evenly distributed small cells. After this, two types of cells differentiated: globular-irregular cells in the larval inner zone and elongated cells at the larval periphery (Fig. 3d). Electron microscopy images showed a progressive decrease of the number and size of yolk inclusions from embryo to pre-larvae (Fig. 6a, d). Cells become thinner and elongated and, flatten at the pre-larva periphery during this process (Fig. 6a,d).

Mature larvae were typical parenchymellae, more elongated in shape but similar in size to embryos (339.5 ± 65.6 µm in diameter). Free larvae belonged to type IIa, described in Mariani et al. (2005), which is typical of the order Poecilosclerida. Larvae were conical at release with a naked posterior zone that was alternatively retracted and protruded during larval swimming, and changed to a more ovoid shape later on. The anterior wider zone was evenly covered by relatively short cilia. In the parental tissue, through light microscopy, an empty space between the larva and the surrounding follicular layer could be observed

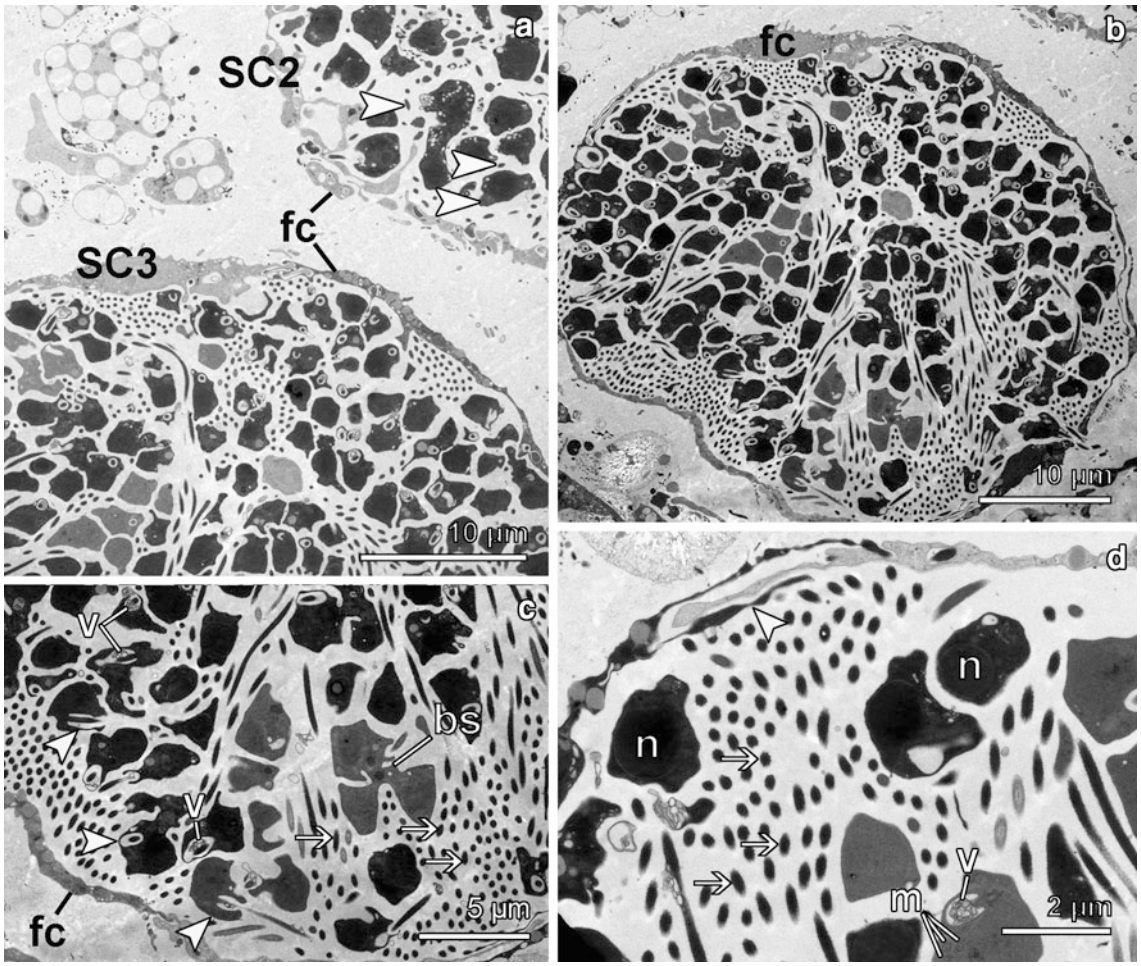


Fig. 4 *Hemimycale columella* SEM images of spermatic cysts. **a** Mid- and late-developmental stage spermatic cysts (SC2 and SC3) both surrounded by follicular cells. Flagella (*arrowheads*) in SC2. **b** Late spermatic cyst (SC3) with the typical rounded shape, surrounded by follicular cells (fc). Note flagella concentration, mainly in the cyst peripheral zone. **c** Detail of a late spermatic cyst (SC3). Note spermatid cytoplasma surrounding the basal zone of flagella (*arrowheads*) and the presence of bridges between spermatids (bs), and the periflagellar vesicles (v). **d** Detail of the follicular layer surrounding a late spermatic cyst (SC3). Note the interlaced pinacocyte-like cells (*arrowhead*), the spermatid nucleus with highly condensed chromatine (n), cross-sections of flagella (*arrows*), the periflagellar vesicles (v) around the flagella, and the mitochondria in the spermatid cytoplasm (m)

(Figs. 3c, 6d). Follicular cells around larvae were more elongated than those in previous stages of embryo development (Figs. 5, 6d). Larvae were clearly distinguishable from embryos by the presence of columnar peripheral ciliated cells, forming a pseudostratified monolayer epithelium, which surrounds the densely arranged inner cells (Fig. 3e). Through the electron microscope, the differences between these two cell types became more conspicuous. The cells forming the peripheral layer were irregularly elongated, practically free from yolk and lipid inclusions, but contained mitochondria and vesicles. Their nuclei were at a basal position (Fig. 6d). Inner cells were more rounded, with a nucleolate nucleus and many pseudopodia, and had more lipid droplets and yolk inclusions (Fig. 6e). In both cases, yolk inclusions decreased in number compared with embryos. In the inner larval zone, sclerocytes with huge amounts of mitochondria, secreting spicules and collencytes with pseudopodia, and secreting collagen were relatively abundant

(Fig. 6e, f). Purported symbiotic bacteria presumably transmitted from maternal tissue were also present (Fig. 6d–f).

Crella elegans

Reproductive cycle

Crella elegans was also simultaneously hermaphroditic and viviparous. Gametogenesis started earlier than in *H. columella* (Fig. 7). Spermatic cysts at different stages of development were found throughout the sponge mesohyl from May to June (Fig. 7). A few oocyte-like cells were found from May to August (not shown). The low number of female gametes found in *C. elegans* may be an artifact resulting from inappropriate sampling of the sponge tissue (i.e., missing the basal part were oocytes accumulate, Ereskovsky, 2010). Spermatic cysts at different stages of development were present from May to June (Fig. 7). As in *H. columella,* the beginning of gametogenesis in *C. elegans* was coincident with the midsummer increase of sea temperature (Fig. 7). Two different stages of embryo development (E1 and E2) were found coexisting in the same individuals in August. Larvae developed completely during September–October of both studied seasons. Larval release occurred during September and October in both study years, in accordance with the autumn sea temperature decrease (Fig. 7), as we reported for *H. columella* (see above). The size of the adults decreased considerably after larval release to the extent that some of them disappeared completely (e.g., two of the five target specimens after the first study year).

Fig. 6 *Hemimycale columella* TEM images of pre-larvae and larvae through TEM. **a** Pre-larva surrounded by follicular cells with macromeres (ma) and micromeres. **b** Micromere of a pre-larva with a nucleolate nucleus (nu), and yolk inclusions (y) with lipid droplets (*arrowheads*). **c** Follicular interlaced (*arrowhead*) cells (fc) surrounding a pre-larva. The external cells are probably involved in collagen secretion (cl), which form an envelop that surrounds the follicular layer. **d** Peripheral zone of a ciliated larvae surrounded by thin follicular cells (fc). Note the cross-sectioned cilia (*arrowheads*). A collagen envelop (cl) around the follicular layer is also shown. **e** Detail of the inner part of a larva, showing archaeocyte-like, nucleolate (nu) cells filled with yolk inclusions (y). Collencytes secreting collagen (cl) are also conspicuous. Some bacteria-like microorganisms are throughout the larval mesohyl (*arrowheads*). *g* Detail of bacteria-like microorganism inside the larva (*arrowheads*). **f** A mitochondria-full (*arrowheads*) larval sclerocyte secreting the axial filament of a spicule (sp). Yolk (y) and lipid (li) material is visible in an adjacent cell. Collagen fibrils (cl) throughout the larval mesohyl

Reproductive investment

Spermatic cysts at different stages of development accounted for 1.6 ± 2% sponge tissue in May and

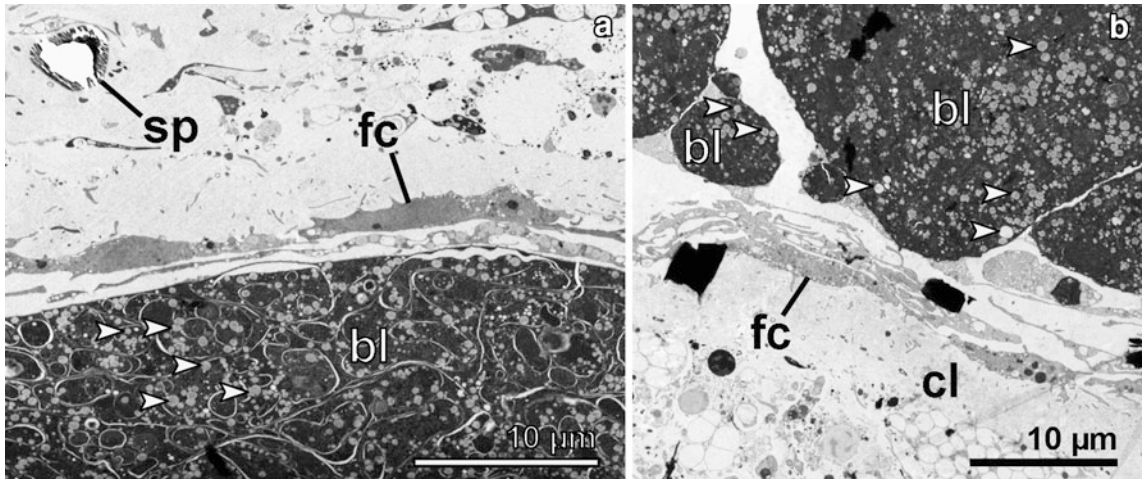
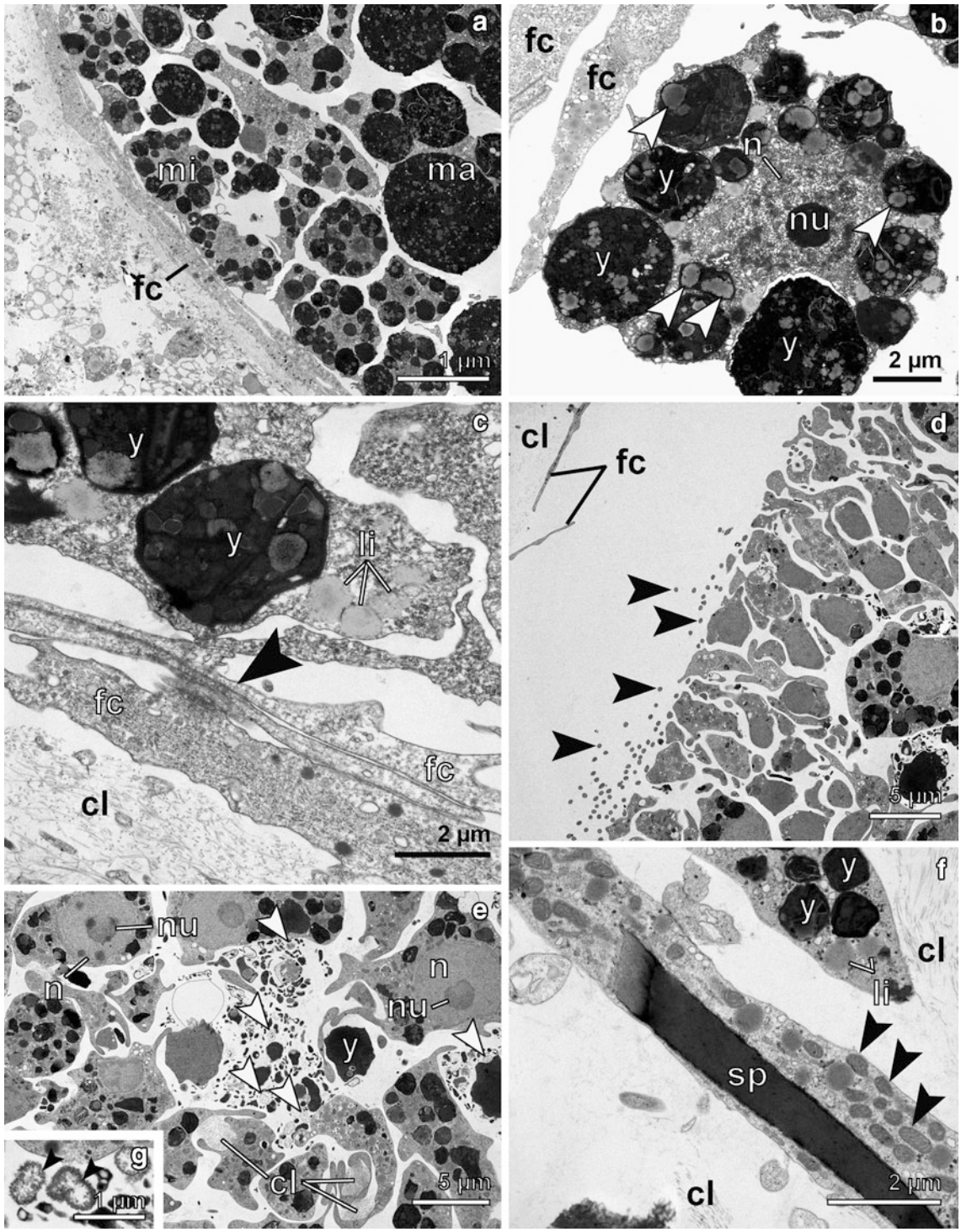


Fig. 5 *Hemimycale columella* TEM images of embryos. **a** Embryo at a mid-stage of development (E2) surrounded by follicular cells (fc), showing its blastomeres (bl) filled by numerous yolk inclusions (darker to the electrons) and lipid droplets (*arrowheads*). Note the presence of a sectioned spicule in the maternal tissue (sp). **b** Embryo at a late stage of development (E3) surrounded by follicular cells (fc) and a thin layer of collagen (cl). Different in size blastomeres (bl) filled with yolk inclusions and lipid droplets (*arrowheads*) coexisted in the same embryo



36.2 ± 11.1% in June (Fig. 7). Early spermatic cysts (SC1) showed mean densities of 1.6 ± 2.8 and 3.8 ± 4.5 spermatocysts per mm^2 of sponge section in May and June, respectively (Fig. 8). In June, spermatic cysts SC2 and SC3 showed mean densities of 14.16 ± 10 and 18.22 ± 11.8 spermatocysts per

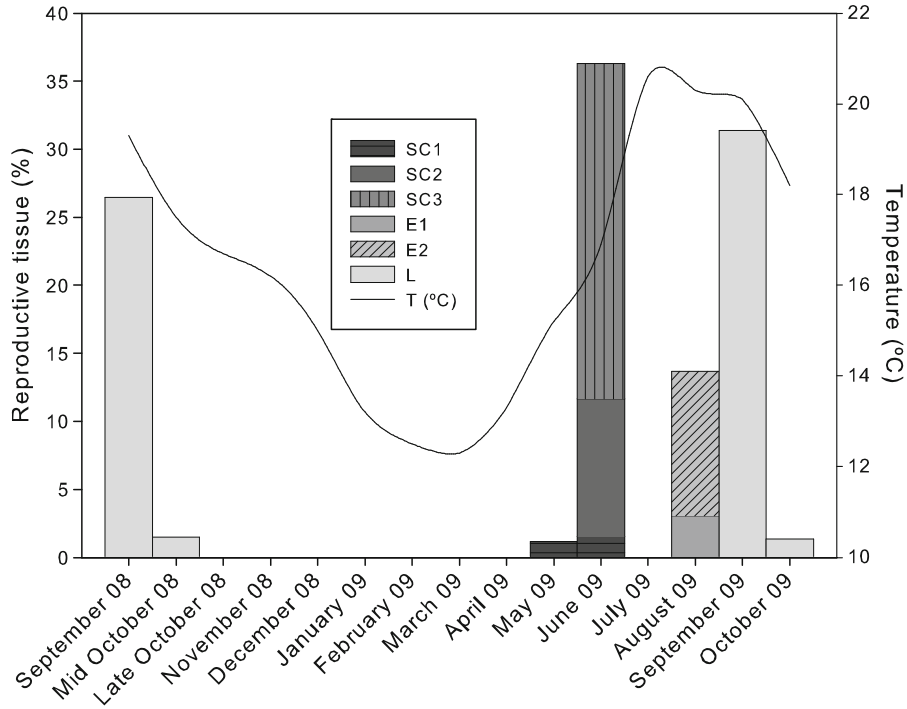


Fig. 7 *Crella elegans*. *Staked bars* representing the relative abundance of the different reproductive elements with respect to the sponge tissue (measured in % area occupied, from histological sections) in the target specimens over the two study years. Reproductive elements: three developmental stages of spermatic cysts (SC1, SC2, SC3 from early to late developmental stages), two embryo developmental stages (E1, E2, from early to late stages), and larvae (L)

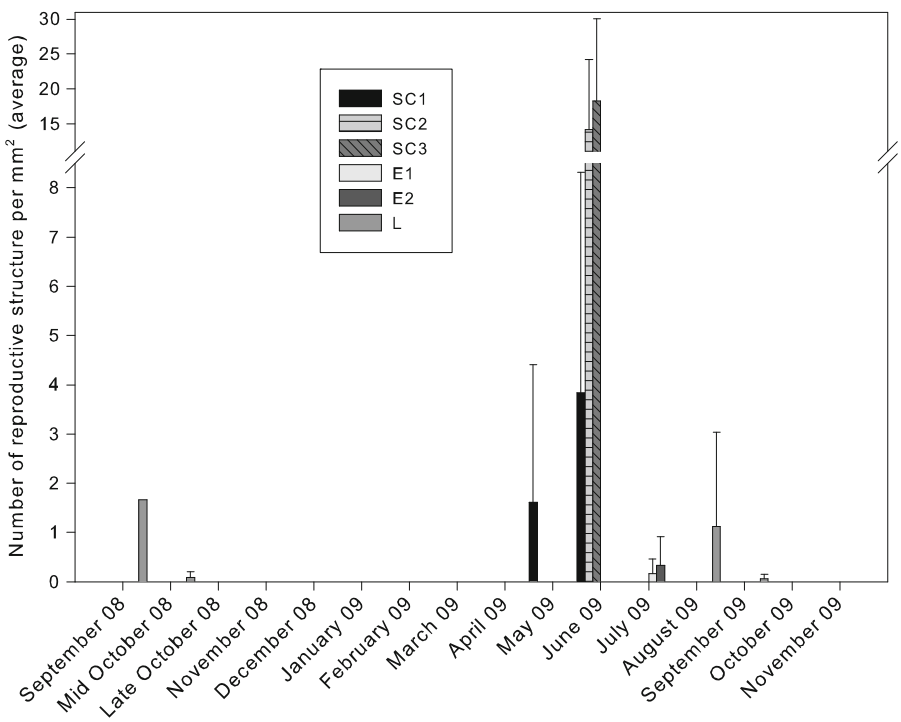


Fig. 8 *Crella elegans*. Density (±SD) of reproductive products in the target individuals over the two study years: three development stages of spermatic cysts (SC1, SC2, SC3 from early to late stage), two embryo development stages (E1, E2), and larvae (L)

mm^2, respectively (Fig. 8). Oocyte-like cells occupied up to 0.016 ± 0.003% of sponge tissue in May, 0.006 ± 0.002% in June, 0.004% in July and 0.003% in August (not shown). Embryos E1 and E2 occupied up to 3 ± 5.2 and 10.6 ± 18.4% of sponge tissue, respectively (Fig. 7), giving average densities of 0.17 ± 0.3 and 0.33 ± 0.6 per mm^2 of sponge section, respectively. Larvae occupied a similar percentage of the sponge tissue in both years: 26.45% (2008) versus 31.4 ± 44.4% in September (2009), and 1.5 ± 2.1% (2008) and 1.4 ± 2.4% (2009) in October. They showed average densities of 1.6 ± 0.1 and 1.1 ± 1.9 larvae per mm^2 in 2008 and 2009, respectively.

Reproductive elements

Oocyte-like cells were oval to round with a brightly stained, 7 ± 2.3 μm in diameter nucleus (not shown). Three developmental stages of spermatocysts, all of them rounded or oval in cross-section, were observed only through the light microscope. Early spermatic cysts (SC1), 40.51 ± 10.92 μm in diameter, were observed in May and June 2009 (Fig. 8) and were uniform in size, densely packed, rounded cells, without apparent flagella (Fig. 9a). Spermatic cysts at a middle stage of development (SC2), 49.31 ± 6.39 μm in diameter, appeared in June (Fig. 8). They were formed by a mass of small cells difficult to differentiate through light microscopy (Fig. 9a). The largest spermatic cysts (SC3), 65.57 ± 2.48 μm in size, were observed at the end of the spermatogenesis process (Fig. 8). Spermatid heads were intensely stained, and disarranged cell flagella could be intuited in light microscope sections (Fig. 9a). Cells in each spermatocyst developed synchronously (Fig. 9a). In contrast to from *H. columella,*

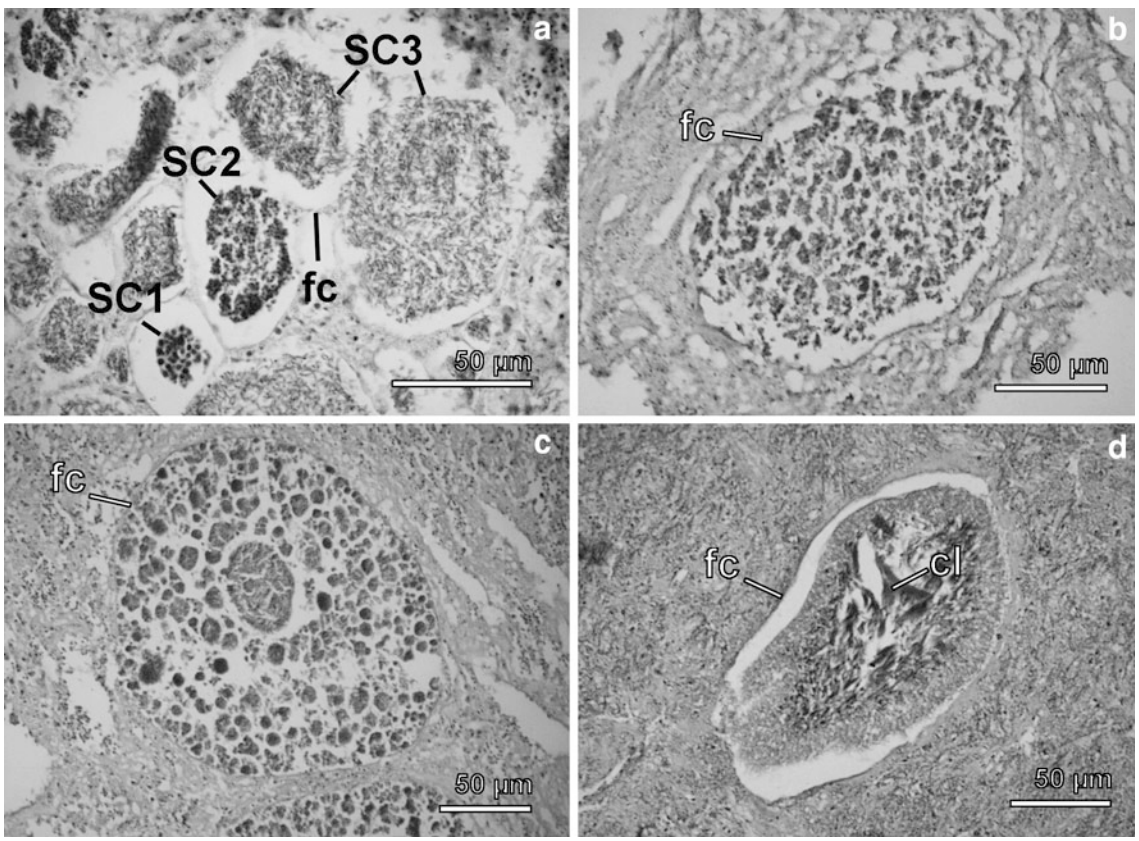


Fig. 9 *Crella elegans* images of reproductive products through light microscope. **a** Various spermatic cysts at three stages of development (SC1, SC2, and SC3) surrounded by follicular cells (fc). **b** Early embryo (E1) surrounded by follicular cells. **c** Late embryo (E2) surrounded by follicular cells. Cleavage is noticed. **d** Mature larva showing a relatively thick peripheral ciliated layer and high density of collagen (cl) at the inner, upper, and mid zones

spermatocysts slightly increased in size as development proceeded (Fig. 9a).

Embryos of *Crella elegans* were recorded only in August 2009. Two different stages of embryo development (E1, E2) were observed (Fig. 8). Both stages were oval, similar in shape and size, and were surrounded by follicular cells. E1 measured 247 ± 84.3 μm in diameter (Fig. 9b). E2 measured 296.6 ± 13.3 μm in diameter and showed cleavage furrows with micromeres and macromeres distinguishable for the first time (Fig. 9c). Through the electron microscope, most cells in E1 appeared to contain a great number of yolk and lipid inclusions, with only a few cells with few inclusions (Fig. 10a). The amount of yolk decreased considerably in the E2 cells (Fig. 10b, c). All blastomeres were nucleolate (Fig. 10c). A thin collagen layer surrounded the follicular cells at both stages of embryo development (Fig. 10a, b).

The typical parenchymella larvae were egg-shaped and measured 248.4 ± 22 μm in length (Fig. 9d). An empty space between the larva and the sponge mesohyl was conspicuous through the light (Fig. 9d) and electron microscopes (Fig. 11a). As in *H. columella*, larvae of *C. elegans* were distinguished from embryos by the presence of a monolayered pseudostratified epithelium formed by elongated ciliated cells. TEM images confirmed the presence of follicular cells surrounding the larva (Fig. 11a). Peripheral ciliated cells, dense to electrons at their proximal zone, also contained small clear to electrons vesicles, and the basal apparatus of cilia surrounded by mitochondria (Fig. 11e). The structure of the basal apparatus of cilia was unclear in most of the images but in some cases a basal foot and a laminar rootlet, typical of poecilosclerid larval cells (Woollacott & Pinto, 1995) could be shown (Fig. 11e). The medium part of these cells harbor huge amounts of lipid droplets, while the cell nuclei are at the basal zone (Fig. 11a). Larval inner cells showed a nucleolate nucleus and were irregular to elongate in shape with cytoplasmic pseudopodia indicating ameboid behavior. Compared with the peripheral ciliated cells (Fig. 11a), the inner cells were smaller in size with less lipid droplets and a higher number of yolk inclusions (Fig. 11b). The dense collagen bundles, which appeared below the basal part of the ciliated cells, penetrated into the inner cell mass giving strength to the larva (Fig. 11b). Pseudospherical nanobodies with an unknown function were observed between the collagen fibrils (Fig. 11c). No sclerocyte secreting spicules were found (Fig. 11b). As in *H.*

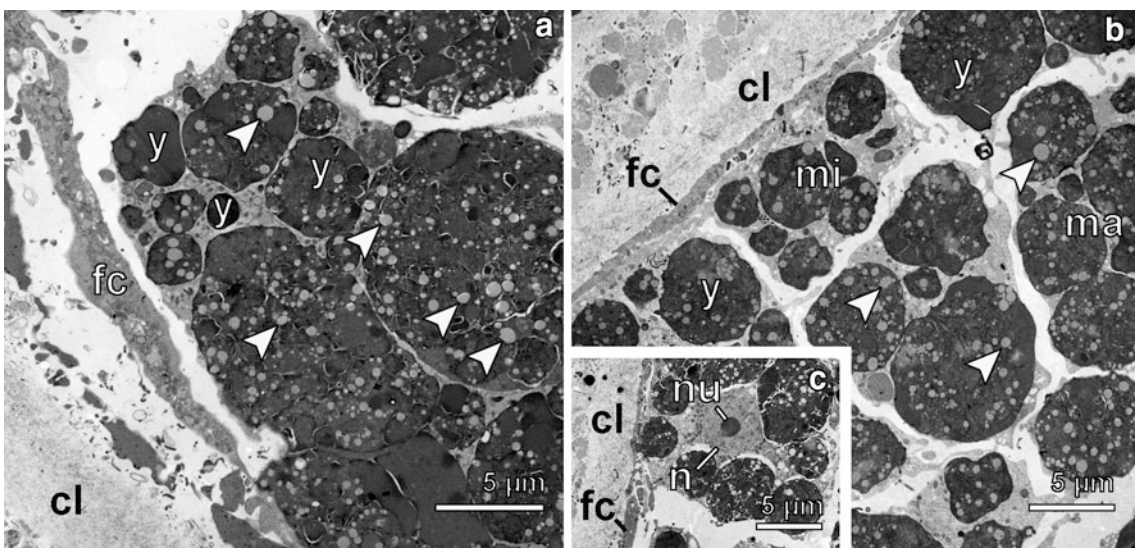


Fig. 10 *Crella elegans* TEM images of embryo cleavage. **a** Early embryo at the beginning of cleavage surrounded by follicular cells (fc) and a collagen layer (cl). Yolk inclusions (y) including lipid droplets (*arrowheads*) are abundant. **b** Later embryo with micromeres (mi) and macromeres (ma) differentiated. The follicular (fc) and collagen (cl) layers are also conspicuous. **c** Same embryo as in (**b**) showing a micromere with a nucleolate nucleus

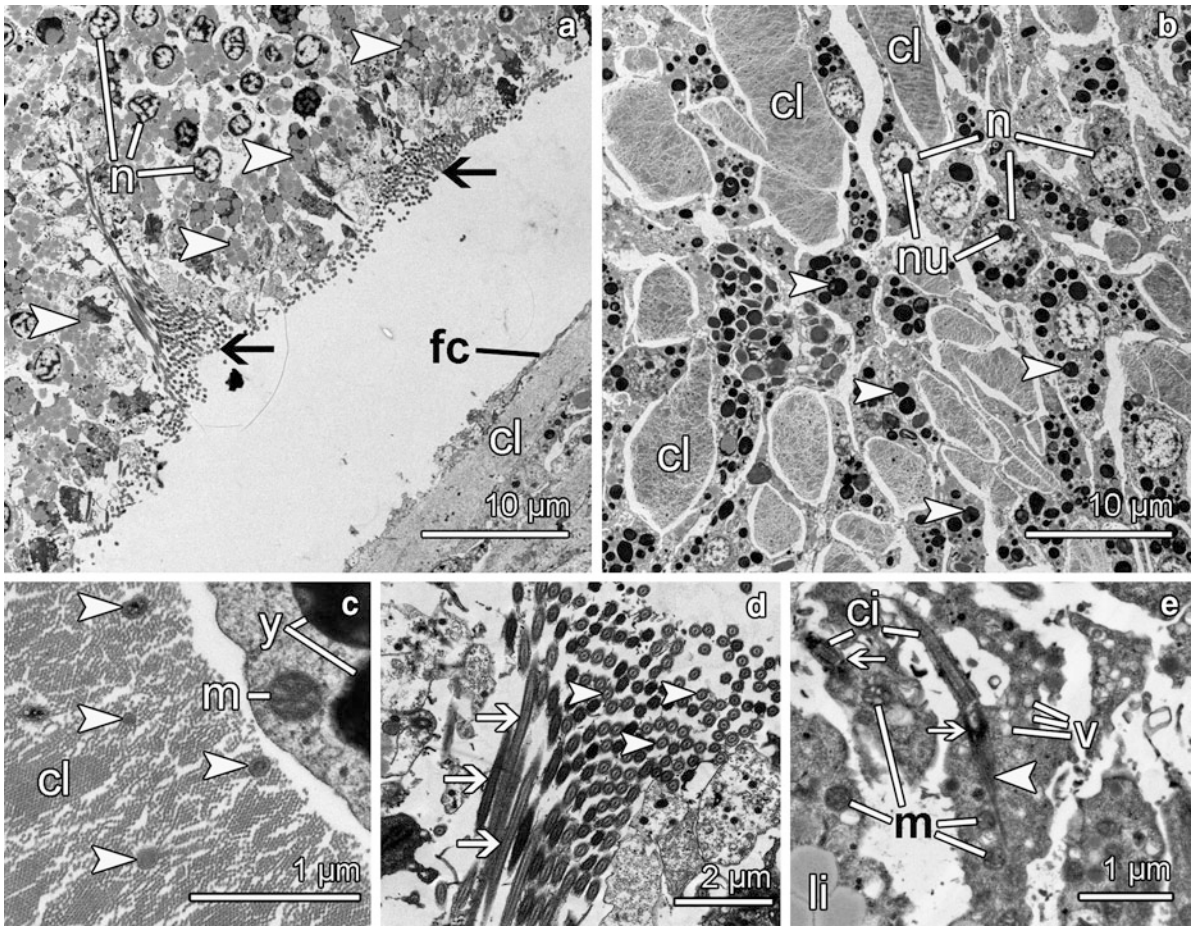


Fig. 11 *Crella elegans* TEM images of larval details. **a** Pseudostratified monolayered epithelium showing ciliated cells with their nuclei (n) at a basal position, and large amounts of lipids surrounding the nuclei and at the mid zone of the cells (*arrowheads*). Note the high density of cross-sectioned cilia (*arrows*). Follicular cells and a thin layer of collagen surround the larva. **b** Inner cells with nucleolate (nu) nuclei (n) and abundant yolk (*arrowheads*). Collagen masses (cl) are densely packed among the cells. **c** Close view of a cross-sectioned collagen mass showing membrane-bounded, spherical nanobodies (*arrowheads*), which might correspond to an unknown microorganism. Yolk inclusions (y) and one mitochondria are visible in an adjacent inner cell. **(d)** Cross-sections (*arrowheads*) and longitudinal sections (*arrows*) of densely arranged cilia. **(e)** Detail of the basal foot (*arrows*) and the ciliary rootlet (*arrowhead*) surrounded by mitochondria (mi) of a ciliated (ci) cell. Many clear to the electrons vesicles (v) accumulate in this proximal zone of the cells

columella, yolk seemed to diminish from embryos to larvae (Figs. 10, 11). No bacteria were observed in the larvae (Fig. 11).

Discussion

Hemimycale columella and *Crella elegans* overlap in their ecological distributions, but their population densities differ at the study site, suggesting contrasting long-term ecological strategies for the two species. We have found species-specific reproductive traits for each species, which may help to understand their different ecological successes. All the sampled individuals of *H. columella* and *C. elegans* were simultaneous hermaphrodites and incubated their larvae, which is typical of most incubating poecilosclerid sponges (Ereskovsky, 2010). They have a relatively short reproductive cycle, as already reported for some incubating demosponges inhabiting seasonal seas (Scalera-Liaci & Sciscioli, 1970; Lepore et al., 2000; Riesgo & Maldonado, 2008), although exceptions do exist for example in oviparous Mediterranean sponges (e.g., Piscitelli et al., 2011). In the analyzed specimens, spermatogenesis lasted for

2 months in both species but did not overlap occurring earlier in *C. elegans* (May–June) than in *H. columella* (August–September). Oogenesis appeared to be a faster process in *H. columella* so we only found oocytes in August while oocyte-like cells were present in *C. elegans* from May to August. Oocyte-like cells in *C. elegans* were smaller than those generally reported for poecilosclerid sponges (Corriero et al., 1998; Riesgo & Maldonado, 2008), and were more similar to the oocytes of the cladorhizid *Asbestopluma occidentalis* (Lambe, 1893) (Riesgo et al., 2007). Embryogenesis was also shorter in *H. columella* and embryo development was asynchronous (September–October of 2008 or August–September of 2009). *C. elegans* produced fewer embryos but embryo development was synchronous (in August) and larvae were incubated in the parental tissue for a longer period (September–October). Larval release was gradual in both species, although it co-occurred with embryogenesis in *H. columella*. Asynchrony in larval release is a common strategy reported for incubating sponge species inhabiting both tropical and subtropical areas (Ilan, 1995; Whalan et al., 2007) including seasonal seas (Baldacconi et al., 2007) and has been considered a strategy to increase the chances of finding favorable settling conditions (Ilan & Loya, 1990; de Caralt et al., 2007).

In terms of reproductive investment (percentage of tissue occupied by reproductive elements), and despite its variation among the target individuals, we found differences between the studied specimens of *H. columella* and *C. elegans*. *H. columella* had a highest average of 59.08% ± 50.72 (embryos and larvae) in mid-October 2008. *C. elegans* had two periods of maximal reproductive investment in June 2009 (36.29% ± 11.1 of tissue occupied by spermatocysts) and September 2009 (31.4% ± 44.3 of tissue occupied by larvae).

Thus, both species differ in the length of their reproductive periods, which was almost twice as long in *C. elegans* than in *H. columella.* The reproductive process took ca. 5 months in the studied individuals of *C. elegans* and only 3 months in those of *H. columella*. The period of gamete formation was similar (ca. 2 months in both species). However, embryo development and larva maturation took considerably longer in *C. elegans* than in *H. columella.* Although the period of larval release may overlap in both species, larvae of *C. elegans* were more able to survive at release under adverse conditions for settlement, than *H. columella* larvae (see discussion on larval ultrastructure below). As the timing of the reproductive events was similar in all specimens surveyed of each species, we may extrapolate some of these traits to the respective populations at the study site.

We found large differences in the larval ultrastructure of the two species. *C. elegans* larva took longer in complete its formation and acquired more reserves from the parental sponge. It accumulated more lipids in their ciliated peripheral cells and many more yolk inclusions in their inner cells than *H. columella* larva. Moreover, the formed had substantially larger amounts of collagen, which was densely packed between the proximal zone of the ciliated cells and the inner larval cells. *C. elegans* species produced fewer but, according to their ultrastructure characteristics, potentially more competent larvae. Larval peripheral cells were monociliated in both species. However, cilia density appeared to be much higher in *C. elegans* larvae than in *H. columella* larvae. We infer this difference in density from a more crowded arrangement of the ciliated cells in cross-sections observed in the TEM images, which were additionally more elongated in *C. elegans*.

Data on reproductive investment derived from the percentage of sponge tissue that was involved in reproductive functions should be interpreted with care because of the low number of individuals of each species analyzed, and the intra-species variation found despite the consistent larval ultrastructure characteristics and length of the incubation period. Thus, *H. columella* seems to release more sexual propagules than *C. elegans* per sponge tissue unit. However, the latter invests noticeably more energy in its larvae, inferred from the larger amounts of lipids and yolk inclusions they contain. *C. elegans* larvae may rely on their reserves for longer and thus, may resist delayed settlement during unfavorable sea conditions. Moreover, the higher cilia density per square mm of surface in *C. elegans* larvae, possibly because of a thither arrangement of its ciliated cells, would indicate a better swimming capability. Similarly, the extraordinarily compact collagen masses of *C. elegans* larvae, which are absent from *H. columella*, would give mechanical resistance (i.e., the joining between the ciliated layer and the inner cells) to its larvae. Differences in ultrastructure features between the larvae of *Cacospongia mollior* (Schmidt, 1862) and *Scopalina lophyropoda* (Schmidt, 1862) were clearly

correlated with their respective success for survival in laboratory experiments (Uriz et al., 2008). Thus, we predict that larvae of *C. elegans* will perform better, in swimming ability and survival under adverse conditions for settlement, than the larva of *H. columella.*

Previous studies showed that larvae of *Scopalina lophyropoda* contained only lipids as energy source, which was correlated with passive behavior in laboratory experiments and phylopatric dispersal in natural conditions (Uriz et al., 2008). This larval behavior resulted in a patchy distribution of *S. lophyropoda* populations. In contrast, the larva of *C. mollior,* Schmidt showed rapid photonegative responses due to a fast energy source (glycogen). Larval reinforcement by thick cuneiform collagen bundles, and diverse energy sources suggested good potential for *C. mollior* larvae to travel long distances in a healthy state, which was also confirmed experimentally in the laboratory. The differential larval ultrastructure and behaviors agreed with the contrasting distribution of the species, which were patch for *S. lophyropoda* and widespread in *C. mollior* (Uriz et al., 2008). Our results confirm that larval ultrastructure may also provide useful information on larval behavior, potential dispersal, and recruitment success, which in the long term might be translated into species abundances, of *C. elegans* and *H. columella*.

Gametogenesis and brooding embryos and larvae caused obvious disturbance to the aquiferous system of both species, because of the high percentage of sponge tissue occupied by those reproductive elements. Although the disrupted tissue was higher in the monitored individuals of *H. columella* than in those of *C. elegans*, both species decreased drastically in size at the end of the reproductive period and many specimens of *H. columella* and, to a losser extent, of *C. elegans* disappeared completely (authors current research). As reported for other sponge species (Rosell, 1993), both *C. elegans* and *H. columella* underwent a resting phase that varied in length in autumn–winter to recover from the tissue disruption that occurred during the reproductive season.

To summarize, even though the studied species are very similar morphologically and coexist in the study zone, they had different reproductive features, which result in contrasting ecological traits. While *H. columella* produced a higher number of larvae, which incubated for a shorter period of time, *C. elegans* produced better performing larvae potentially more resistant to mechanical disturbance due to collagen reinforcement, and better swimmers because of higher cilia density, and with longer energetic reserves. These larval ultrastructure patterns allow us to predict a higher recruitment success for *C. elegans* larvae, which agrees with the more dense and stable populations of *C. elegans* occurring at the study site (authors, pers. obs.).

As more sponge larval types are investigated from morphological and functional standpoints, the crucial link between ultrastructure, behavior, and ecological traits will become more firmly established and understood, in particular in species with small larvae difficult to track, making behavioral at sea studies impracticable.

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References

Baldacconi, R., C. Nonnis-Marzano, E. Gaino & G. Corriero, 2007. Sexual reproduction, larval development and release in *Spongia officinalis* L. (Porifera, Demospongiae) from the Apulian coast. Marine Biology 152(4): 969–979.

Blanquer, A. & M. J. Uriz, 2010. Population genetics at three spatial scales of a rare sponge living in fragmented habitats. BMC Evolutionary Biology 10: 13

Boury-Esnault, N. & B. Jamieson, 1999. Porifera. In Adiyodi, K. G. & R. G. Adiyodi (eds), Reproductive Biology of Invertebrates IX Progress in Male Gamete Ultrastructure and Phylogeny. Wiley, Chichester: 1–20.

Corriero, G., L. S. Liaci, C. N. Marzano & E. Gaino, 1998. Reproductive strategies of *Mycale contarenii* (Porifera : Demospongiae). Marine Biology 131(2): 319–327.

de Caralt, S., M. J. Uriz, A. V. Ereskovsky & R. H. Wijffels, 2007. Embryo development of *Corticium candelabrum* (Demospongiae : Homosclerophorida). Invertebrate Biology 126(3): 211–219.

De Vos, L., K. Rützler, N. Boury-Esnault, C. Donadey & J. Vacelet, 1991. Atlas of sponge morphology. Smithsonian Institution Press, Washington and London: 117.

Ereskovsky, A. V., 2000. Reproduction cycles and strategies of the cold-water sponges *Halisarca dujardini* (Demospongiae, Halisarcida), *Myxilla incrustans* and *Iophon piceus* (Demospongiae, Poecilosclerida) from the White Sea. Biological Bulletin 198(1): 77–87.

Ereskovsky, A. V., 2010. The Comparative Embryology of Sponges. Springer, Dordrecht, London.

Fell, P. E., 1974. Porifera. In Giese, A. & J. Pearse (eds), Reproduction of Marine Invertebrates: Acoelomate and Pseudocoelomate Metazoans. Academis Press, New York: 51–132.

Guardiola, M., J. Frotscher & M. J. Uriz, 2011. Genetic structure and temporal differentiation of cohorts of a recently introduced sponge (*Paraleucilla magna*) to the western Mediterranean. Hydrobiologia (in press).

Hooper, J. N. A. & R. W. M. Van Soest (eds), 2002. Systema Porifera: A Guide to the Classification of Sponges. Kluwer Academis/Plenum Publishers, New York.

Ilan, M., 1995. Reproductive Biology, Taxonomy, and Aspects of Chemical Ecology of Latrunculiidae (Porifera). Biological Bulletin 188: 306–312.

Ilan, M. & Y. Loya, 1990. Sexual reproduction and settlement of the coral reef sponge *Chalinula* sp. from the Red Sea. Marine Biology 105(1): 25–31.

Ilan, M., J. Gugel & R. W. M. Van Soest, 2004. Taxonomy, reproduction and ecology of new and known Red Sea sponges. Sarsia 89(6): 388–410.

Lepore, E., M. Sciscioli, L. Scalera-Liaci, G. Santarelli & E. Gaino, 2000. Sexual reproduction of *Cinachyra tarentina* (Porifera, Demospongiae). Italian Journal of Zoology 67: 153–158.

Lévi, C., 1973. Systématique de la classe des Demospongiaria (Démosponges). In Grassé, P. P., P. Brien, C. Lévi, M. Sarà, O. Tuzet & J. Vacelet (eds), Traité de Zoologie: Anatomie, systématique, biologie Spongiaires. Masson et Cie, Paris.

Leys, S. P. & A. V. Ereskovsky, 2006. Embryogenesis and larval differentiation in sponges. Canadian Journal of Zoology 84: 262–287.

Leys, S. P. & H. M. Reiswig, 1998. Transport pathways in the neotropical sponge *Aplysina*. Biological Bulletin 195: 30–42.

Mariani, S., M. J. Uriz & X. Turon, 2005. The dynamics of sponge larvae assemblages from northwestern Mediterranean nearshore bottoms. Journal of Plankton Research 27(3): 249–262.

Piscitelli, M., G. Corriero, E. Gaino & M.-J. Uriz, 2011. Reproductive cycles of the sympatric excavating sponges Cliona celata and Cliona viridis in the Mediterranean Sea. Invertebrate Biology 130(1): 1–10.

Reiswig, H. M., 1973. Population-dynamics of three Jamaican demospongiae. Bulletin of Marine Science 23(2): 191–226.

Reiswig, H. M., 1983. Porifera. In Adiyodi, K. G. & R. G. Adiyodi (eds), Reproductive Biology of Invertebrates II Spermatogenesis and Sperm function. Wiley, Chichester: 1–21.

Riesgo, A. & M. Maldonado, 2008. Differences in reproductive timing among sponges sharing habitat and thermal regime. Invertebrate Biology 127(4): 357–367.

Riesgo, A. & M. Maldonado, 2009. An unexpectedly sophisticated, V-shaped spermatozoon in Demospongiae (Porifera): reproductive and evolutionary implications. Biological Journal of the Linnean Society 97: 413–426.

Riesgo, A., C. Taylor & S. P. Leys, 2007. Reproduction in a carnivorous sponge: the significance of the absence of an aquiferous system to the sponge body plan. Evolution & Development 9(6): 618–631.

Rosell, D., 1993. Effects of reproduction in *Cliona viridis* (Hadromerida) on zooxanthellae. Scientia Marina 57: 405–413.

Scalera-Liaci, L. & M. Sciscioli, 1970. Il ciclo sessuale di *Erylus discophorus* (Schmidt) (Porifera: Tetractinellida). Rivista di Biologia 63: 255–270.

Simpson, T. L., 1984. Gamete, embryo, larval development. In Simpson, T. L. (ed.), The cell biology of sponge. Springer, Berlin: 341–413.

Uriz, M. J., M. Maldonado, X. Turon & R. Martí, 1998. How do reproductive output, larval behaviour, and recruitment contribute to adult spatial patterns in Mediterranean encrusting sponges? Marine Ecology Progress Series 167: 137–148.

Uriz, M. J., X. Turon & S. Mariani, 2008. Ultrastructure and dispersal potential of sponge larvae: tufted *versus* evenly ciliated parenchymellae. Marine Ecology 29: 280–297.

Vacelet, J. & N. Boury-Esnault, 1995. Carnivorous sponges. Nature 373(6512): 333–335.

Whalan, S., M. S. Johnson, E. Harvey & C. Battershill, 2005. Mode of reproduction, recruitment, and genetic subdivision in the brooding sponge *Haliclona* sp. Marine Biology 146(3): 425–433.

Whalan, S., C. Battershill & R. de Nys, 2007. Sexual reproduction of the brooding sponge *Rhopaloeides odorabile*. Coral Reefs 26(3): 655–663.

Woollacott, R. M. & R. L. Pinto, 1995. Flagellar basal apparatus and its utility in phylogenetic analyses of the Porifera. Journal of Morphology 226: 247–265.

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SPONGE RESEARCH DEVELOPMENTS

Epibiont–basibiont interactions: examination of ecological factors that influence specialization in a two-sponge association between *Geodia vosmaeri* (Sollas, 1886) and *Amphimedon erina* (de Laubenfels, 1936)

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Abstract The objective of this study was to determine what ecological benefits *Geodia vosmaeri* (*Gv*) and *Amphimedon erina* (*Ae*) gain from their symbiosis. The prevailing, though untested, hypotheses are that *Ae* protects *Gv* from predators through chemical defenses and that *Gv* provides *Ae* access to substrata. Data from our experiments support these hypotheses. During field surveys, *Ae* was never found growing without *Gv* in this habitat. *Ae* was the only epibiont on 81% of the *Gv* surveyed. Field feeding assays using chemical extracts indicated that *Ae* is less palatable than *Gv*. Laboratory feeding assays using sponge tissue demonstrated that spongivorous sea stars avoided contact with *Ae* tissue and frequently accepted *Gv* tissue for consumption. In caging experiments, predator exclusion had no effect on *Gv* tissue loss. *Amphimedon erina* may benefit from the vertical substrata represented by *Gv* colonies because *Ae* grown in a vertical orientation experienced less tissue loss compared to *Ae* grown horizontally. Taken together, our results provide empirical support for the hypothesis that *Gv* is afforded chemical protection from predators through *Ae* and some evidence that *Ae* benefits from growing on *Gn* substrata.

Keywords Symbiosis · Sponge · Facilitation · *Geodia* · *Amphimedon*

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Introduction

Marine sponges interact with a multitude of organisms in a variety of ecological contexts (reviewed in Wulff, 2005, 2006a). Sponges are consumed by vertebrate and invertebrate predators (Meylan, 1988; Wulff, 1995), they compete with sessile invertebrates for space (Hill, 1998; Engel & Pawlik, 2005a, b), they are important in benthic:pelagic coupling (e.g., Southwell et al., 2008), and they form symbiotic interactions with phototrophic organisms (e.g., Weisz et al., 2010). Sponge ecologists have focused on negative (e.g., competition and predation; Pawlik et al., 1995; Hill, 1998; Waddell & Pawlik, 2000a) and positive (e.g., mutualism, epizoism, and facilitation; Rützler, 1970; Hill, 1996; Wulff, 1997a, 2008a) ecological interactions. While negative

ecological interactions play important roles in structuring communities, we have only a limited understanding of the importance of facilitation in sponge communities (Rützler, 1970; Sarà, 1970), especially in tropical ecosystems (e.g., Wulff, 2008a, b).

For example, branching sponges appear able to increase their resistance to predators and storm damage by adhering to branches of other sponge species (Wulff, 1997a). Tolerating overgrowth may represent a strategy that some sponges employ to persist in habitats with intense predation pressures (Wilcox et al., 2002; Wulff, 2008a) or to reduce competition for space (Rützler, 1970; Wulff, 2005). In some sponge–sponge epizoic associations, one sponge completely overgrows another sponge. Overgrowth associations between sponges have been observed in the Adriatic, Pacific, and Caribbean (Rützler, 1970; Wilcox et al., 2002; Ávila et al., 2007). Sponge overgrowth is intriguing for a variety of reasons, most notably because the epibiont represents a barrier to water flow for the basibiont sponge and presumably restricts access to particulate food. How basibiont sponges maintain water circulation is not known (Rützler, 1970; Wilcox et al., 2002). Interestingly, in many cases, the overgrown sponge does not show any negative effects from the association and thus competition does not seem to be the driving ecological force (Wilcox et al., 2002; Ávila et al., 2007).

An unusual two-sponge symbiosis from the Florida Keys provides a model system to study the ecological and evolutionary consequences of positive interactions in marine systems (Wilcox et al., 2002). In this overgrowth association, the external surface of the basibiont *Geodia vosmaeri* (*Gv*) is covered by the epibiont *Amphimedon erina* (*Ae*). There has been some taxonomic confusion about the sponges in this association. In the previous study, the basibiont sponge was considered *G. gibberosa* (Engel & Pawlik, 2000; Wilcox et al., 2002). Recent findings indicate that the basibiont is in the *G. neptuni* group, and should more accurately be referred to as *G. vosmaeri*, which has taxonomic precedence (Silva, 2002; P. Cardenas, pers. comm.). In many cases, *Ae* covers >90% of the available outer surface of *Gv*, which seems unsustainable for a filter-feeding organism. The two-sponge associations between *Ae* and *Gv* appear restricted to shallow sea grass beds and hard-bottom communities close to shore. The symbiotic interaction commonly occurs at several sites, occurs in many size classes, and appears stable through time (Wilcox et al., 2002).

The overgrowth of *Gv* by *Ae* has been suggested to be an associational defense (e.g., Wilcox et al., 2002), but this hypothesis has not been tested directly. Chemical extracts of *Ae* are deterrent to the non-spongivorous fish *Thalassoma bifasciatum* (Pawlik et al., 1995). Assays using *Ae* tissue also support the idea that *Ae* is chemically defended against starfish and parrotfish (Wulff, 1995, 1997b, 2008a). In contrast, chemical extracts of *Geodia* sponges (e.g., *Gv* = *G. neptuni*, *G. gibberosa*) do not deter sea stars and *T. bifasciatum* (Pawlik et al., 1995, Waddell & Pawlik, 2000b). Several studies also document the palatability of *Gv* = *G. neptuni*, *G. gibberosa* tissue to sponge predators (Dunlap & Pawlik, 1996; Wulff, 1997b, Waddell & Pawlik, 2000b). These assays suggest that *Ae* provides chemical defense to *Gv* (Wilcox et al., 2002).

The benefits to *Ae* from the two-sponge association are less clear than those presumed for *Gv*. The dominant hypothesis is that sponge epibionts gain access to stable substrata in habitats where such substrata is limited (e.g., Rützler, 1970; Wulff, 2008a). Epizoism may represent a mechanism to avoid competition for space or deal with fluxes in sedimentation (Rützler, 1970; Engel & Pawlik, 2000; Wilcox et al., 2002). Sedimentation may influence the two-sponge association discussed here as preliminary evidence suggests that *Ae* can tolerate moderate coverage by sediment but not deep burial (Wulff, 2008a). While feeding assays have been performed with *Gv* and *Ae* (e.g., Pawlik et al., 1995), additional field work is necessary to fully understand the nature of the relationship between these two sponges in natural contexts. Our aim was to test the hypotheses that *Ae* confers chemical defensive traits to *Gv*, and that *Gv* provides *Ae* with preferred substrata. We conducted field surveys, manipulative field experiments, and feeding preference studies to identify the benefits that *Gv* and *Ae* accrue in their symbiotic interaction.

Methods

Two-sponge associations involving *Gv* and *Ae* were studied at the Mote Marine Tropical Research Laboratory on Summerland Key in the Florida Keys, FL, USA (24°39′41.69″N, 81°27′16.53″W).

Field surveys

We surveyed two-sponge associations in June 2009 at the north end of Summerland Key, in Niles Channel. Six 50 m transects were run parallel to shore between 0.5 and 1 m depth. Each transect was separated by approximately 5 m. *Geodia vosmaeri* colonies within 0.5 m of either side of the transect line were counted. We placed *Gv* into one of three categories: *Ae* present, *Ae* not present but other sponge epibionts present, or no sponge epibionts present. When *Ae* was present, we estimated its coverage of *Gv* surface as 25, 50, 75, or 100% coverage. We divided percentage estimates into "high" (75 and 100%) and "low" (25 and 50%) categories for statistical analysis. We also recorded whether *Gv* appeared to have been recently damaged by predators. A chi-square test for goodness-of-fit was used to determine whether *Ae* coverage was related to the proportion of damaged colonies.

Feeding assays

To test the hypothesis that these sponges are capable of chemical defense, we conducted feeding assays with naturally occurring predators in July 2003. We used artificial foods containing organic extracts obtained from *Ae* and *Gv* that were involved in two-sponge associations. The use of organic extracts controls for differences in spicule type, spicule orientation, and nutritional differences between sponges.

To assess the efficacy of chemical defenses of each sponge, we used the screen-gel assay of Hay et al. (1994). 40 cm^3 samples of *Ae* and *Gv* were collected from three different two-sponge associations in Long Key Bight, Long Key, FL. Excised pieces were frozen on dry ice and stored at −80°C. Crude organic extracts were prepared from *Ae* and *Gv* pieces following the protocol of Hill et al. (2005). Crude organics were extracted at 4°C using two 24 h methanol extractions followed by a single 24 h 1:1 methanol:dichloromethane extraction. Extraction solutions were combined and rotary-evaporated at <40°C. The resulting residue was lyophilized and stored at −80°C. Artificial food consisted of lyophilized, powdered squid mantle suspended in a 1.5% agar matrix (0.05 g squid ml^{-1} agar solution; Chanas & Pawlik, 1995). We liquefied the squid:agar mixture in a microwave, allowed the solution to cool, and then added crude extract that had been re-suspended in a small volume of methanol. Control artificial foods were made by adding an equal volume of methanol to the liquefied squid:agar mixture. The final volume of the squid:agar mixture equaled the volume of sponge from which the extracts were derived, therefore, the concentration of organics in the food mixture was approximately the same as in the sponge tissue. The solutions were mixed well and then poured into molds. As the solutions cooled, they solidified and attached to a layer of window screening underneath the molds. Once solidified, the window screening was cut to form 1 cm^2 × 2 mm food squares.

Amphimedon erina extract, *Gv* extract, and control food squares from each association were attached to fishing line at 5 cm intervals in random order. Six squares from each treatment group (*Ae*, *Gv*, and control) were placed on a single line. Thus, there were two lines with 18 squares per line. Although it would have been best to randomly arrange food squares from each of the three sponges across lines, there was no easy way to mark squares as coming from any particular sponge. Therefore, each line represented a nested treatment (replicates nested by sponge of origin). Lines were placed on the bottom of a shallow mangrove channel just east of Long Key Lake, Long Key, and retrieved after 24 h. The number of exposed screening squares was counted. We used the non-parametric Mack-Skillings statistic (Mack & Skillings, 1980) to determine if the amount of food removed was independent of the two-sponge association from which extracts were obtained because the data could not be normalized. We used the Kruskal–Wallis statistic to test for extract effects and the Dwass–Steel–Critchlow–Fligner multiple comparisons test to specify significance among treatment pairs (Hollander & Wolfe, 1999).

We conducted sea star choice assays to determine if any of the sponge epibionts observed on *Gv* elicited avoidance behavior from sea star predators. Choice assays were conducted in July 2009. We conducted our assays with *Echinaster sentus* collected on the south side of Summerland Key, FL. *Echinaster sentus* is a very common sea star in this habitat. Approximately 80 sea stars were collected within a 100 m^2 area where two-sponge associations are also found. Sea stars were held for 48 h without food before beginning the feeding assays. Three colonies of each sponge species (*Gv, Ae, Tedania klausi, Biemna caribea,* and *Haliclona implexiformis*) were collected

from the same location as *E. sentus*. For *Gv*, choanoderm tissue and cortex tissue were separated and tested independently. Experiments were conducted at night when the sea stars were most active. In every assay, one arm of *E. sentus* was placed on a piece (~ 1 cm^3) of sponge. Each sea star was used to assay a single piece of sponge. If the sea star moved away from the sponge, we recorded a rejection. If the starfish moved onto the sponge and remained there, we recorded an acceptance. These behaviors typically occurred within 30 s of contact with the sponge. Every sponge type was presented to at least 10 starfish and the frequency of acceptance was calculated for each sponge type.

Predator exclusion experiment

A two-factor caging experiment was employed to determine the effect of predator exclusion and *Ae* removal on the survival of *Gv*. The caging experiment ran for 12 weeks between June and August 2009. We chose 20 two-sponge associations of similar size and similar *Ae* surface coverage. Two-sponge associations were divided into two treatments: *Ae* was removed from *Gv* with a soft brush or *Ae* was left on *Gv*. Removal of *Ae* left the cortex of *Gv* intact. A vertical metal rod attached to a concrete-filled PVC anchor was inserted into each *Gv*. Sponges from both *Ae* treatments were then randomly positioned on the PVC anchor. Half of the brushed *Gv* ($n = 5$) and half of the intact associations ($n = 5$) were caged with 1 cm^2 plastic mesh. Sponges and cages were cleaned of sediment weekly. Any evidence of damage was recorded and photographed.

To determine the effect of *Ae* removal and predator exclusion, we measured sponge volume at the onset of the experiment and after 12 weeks. We measured the volume of each sponge using water displacement. We placed the sponges in a container with a known volume of water and used a valve to drain the container back to the original volume. The drained water was then measured in a graduated cylinder. One replicate was not used in statistical analysis because it experienced a much larger increase in volume than was physically possible. A two-way ANOVA was used to determine whether the *Ae* treatment or the caging treatment affected changes in sponge volume. A magnitude of effects estimate was calculated for the *Ae* treatment following Graham & Edwards (2001). Degrees of freedom and sum of squares for the caging treatment and the interaction term were pooled with the error to eliminate negative variance components. Plaster-of-paris clods were used to determine whether cages affected water flow (Hill, 1998). Five clods were placed inside and outside cages and collected after 24 h. Clods were dried to a constant weight and analyzed using a student's *t* test.

Cortex thickness in *Gv* and *Ae* growth experiments

We collected plugs of tissue from randomly selected two-sponge associations in Niles Channel in June 2009. Samples were taken from 15 sponges in regions of *Gv* where the cortex was exposed to the external environment. Another 20 samples were taken from regions where *Gv* cortex was covered with *Ae*. Tissue samples were preserved in 70% ethanol and sections were analyzed in ImageJ. The distance between the dermal layer at the outer edge of *Gv* to the sterraster-armor layer (sensu de Laubenfels, 1936) was measured at least five times for each sponge. Average distances for covered *Gv* and exposed *Gv* were compared using a *t* test.

We manipulated the orientation of *Ae* to test the hypothesis that sediment load restricts the distribution of *Ae* to the vertical sides of *Gv*. *Ae* tissue is thicker on the sides of *Gv* than on the top (see Wilcox et al., 2002, Fig. 1). This experiment lasted for 7 weeks in June and July 2008. Two-sponge associations were cut to create slices of *Ae* that were still attached to a very small portion of *Gv*. *Amphimedon erina* slices were attached to terracotta tiles using cable ties. Tiles were then fixed in horizontal and vertical orientations and placed in Niles Channel. Aerial photos of each sponge were taken at the beginning of the experiment and again 7 weeks later. ImageJ was used to calculate the surface area of each sponge, and changes in surface area were compared using a *t* test.

At the end of the experiment, we carefully collected sediment that had accumulated on the sponges by slowly removing the tiles from the water and washing sediment into collection bags. Excess water was removed from the bags once all material had settled. The sediment was transferred to scintillation vials, and 3.7% formaldehyde was added to prevent degradation of organic material. The material was dried to constant weight at 60°C and then placed in a muffle furnace at 450°C for 4 h. Ash-free dry weight (AFDW) and the weight of the inorganic fraction were determined from

these measurements. Dry weight and AFDW were compared for horizontal *Ae* and vertical *Ae* using a *t* test.

Results

The epibionts found on *Gv* do not appear to be a random sample of the sponges in the community. We found *Gv* more frequently associated with *Ae* (81%) than with all other sponge epibionts combined (16%, Table 1). Over 60% of the *Gv* had high rates of *Ae* coverage, which we defined as any situation where ≥50% of *Gv*'s surface area was occluded (Table 2). When *Ae* was present on *Gv*, we rarely found any other sponge species interacting with that *Gv* individual. *Ae* was only found on *Gv* in this habitat. When *Ae* was absent, we often found multi-species sponge assemblages of *T. klausi, B. caribea*, and *H. implexiformis* on *Gv*. These assemblages covered less *Gv* surface than *Ae*.

Only five *Gv* individuals were observed without epibionts and three showed signs of damage (Table 1). *Geodia vosmaeri* was damaged much less frequently when found with epibionts. The identity of the epibiont, however, did not seem to affect the proportion of colonies showing signs of damage (Table 1).

Table 1 Survey data of the relative abundance of *Gv* and its associated epibionts from Niles Chanel, Summerland Key, FL

Gv epibiont	Number observed	Colonies damaged (*n*)	Density (m^{-2})
Ae	144	17.4% (25)	0.48
Other sponges	29	10.3% (3)	0.10
Without epibionts	5	60.0% (3)	0.02

All two-sponge associations occurring in an area of 300 m^2 were examined. The 'other sponges' include *T. klausi*, *H. implexiformis*, and *B. caribea*

Table 2 Relative abundance of *Gv* with different *Ae* coverage

Ae coverage (%)	Coverage category	Count (*n*)	Colonies damaged (*n*)	Density (m^{-2})
100	High	40	5% (2)	0.13
75	High	48	13% (6)	0.16
50	Low	29	31% (9)	0.10
25	Low	27	30% (8)	0.09

Colonies damaged indicates the proportion of damaged *Gv* with different levels of *Ae* coverage

Geodia vosmaeri with *Ae* and *Gv* with other sponge epibionts were found damaged in similar proportions. There was a negative relationship between the level of *Ae* coverage and the amount of *Gv* damage (Table 2). A positive relationship was observed between the level of *Ae* coverage and the density of *Gv* in the community (Table 2). Two-sponge associations with "high" *Ae* coverage were damaged significantly less frequently than would be expected based upon their abundance in the population (Table 2; $\chi^2 = 10.8$; $df = 1$; $P < 0.01$).

In the field feeding assay, the amount of artificial food removed was independent of the two-sponge association used to make the extract (Mack-Skillings MS = 0.76, $k = 3$, $P < 0.02$). Therefore, data from all three associations were pooled. Extract type significantly affected how much artificial food was removed from each food square ($H = 9.1$, $k = 3$, $P < 0.02$). Consumers removed significantly less artificial food that contained *Ae*-extract and there was no significant difference between the *Gv* treatment and the control (Fig. 1A).

Echinaster sentus was observed to initiate feeding behavior on several of the sponges used in our feeding assays (Fig. 1B). *Geodia vosmaeri* was accepted at a high rate, but we found large differences between the feeding responses to *Gv* tissue types. *Geodia vosmaeri* choanoderm was accepted 75% of the time whereas *Gv* cortex was accepted only 10% of the time (Table 3). Only two epibionts, *Ae* and *T. klausi*, were completely effective at deterring *E. sentus*. *Haliclona implexiformis* and *B. caribea* were accepted at rates that were similar to *Gv* cortex (Table 3).

Many sponges in all treatments lost volume during the predator exclusion experiment (Fig. 2). *Geodia vosmaeri* without *Ae* lost, on average, 20% of their initial volume regardless of whether they were exposed or caged. *Geodia vosmaeri* with *Ae* lost, on average, 5% of their initial volume. However, neither caging, the presence of *Ae*, nor the interaction had a significant effect on sponge volume (Table 4). *Ae* removal only explained 11% of the variance in *Gv* volume (Table 4). Clod card analysis indicated that there was no significant effect of the cages on water motion near the sponges ($t_8 = 1.52$, $P = 0.17$), which is further evidenced by the fact that caged and uncaged sponges had virtually the same change in volume (Fig. 2). The greatest variability within treatments was seen in *Gv* without *Ae* that were exposed to predators.

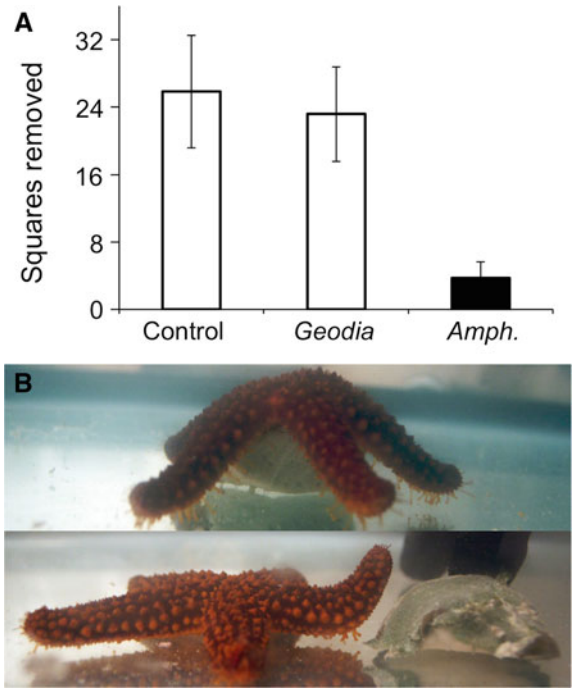

Fig. 1 **A** The number of food squares removed containing one of three chemical extracts: *Ae, Gv*, or no extract. Significantly fewer *Ae* food squares were consumed than *Gn* or control food squares. Means were calculated for 12 food squares per source. *Error bars* represent ±1 standard error. *Bars* of a *different color* are significantly different from each other. **B** The sea star *E. sentus* feeding on *Gv* choanoderm and rejecting *Ae*. Following contact with *Ae*, *E. sentus* raised one arm and moved in the opposite direction from *Ae*

Table 3 Results of feeding assays with the sea star *E. sentus*

Potential food item	Percent accepted
Gv choanoderm	75
Gv cortex	10
Ae	0
Tedania klausi	0
Haliclona implexiformis	14
Biemna caribea	10

The sterraster-armor layer of the cortex of *Gv* was significantly thicker when in areas that were covered by *Ae* (Fig. 3; $t_{32} = 6.73$, $P < 0.001$). *Amphimedon erina* growing in a horizontal orientation lost significantly more surface area than *Ae* growing vertically (Fig. 4B; $t_7 = 2.41$, $P < 0.05$). Significantly more sediment accumulated on horizontal *Ae* compared to

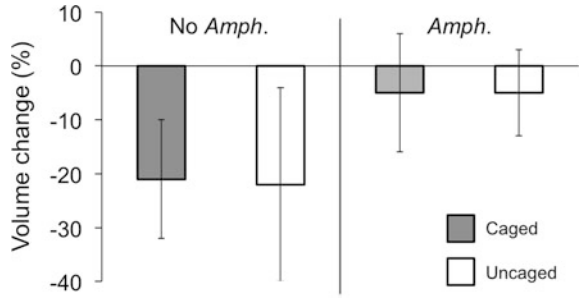

Fig. 2 Volume change during the predator exclusion experiment. The two-factor design consisted of *Ae* removal ("No *Amph*.," "*Amph*.") and a caging treatment (*gray bars* caged; *white bars* uncaged). Five sponges were used in each *Ae* + caging treatment combination except for No *Amph* + uncaged where four sponges were used in the analysis (see text). Neither the main effects nor the interaction were significant. *Error bars* represent ±1 standard error

vertical *Ae* (Fig. 4B; $t_7 = 2.56$, $P < 0.05$). The accumulation of organic matter (AFDW) was significantly greater for horizontal *Ae* (Fig. 4B; $t_7 = -3.38$, $P < 0.05$).

Discussion

The interaction between *Ae* and *Gv* is an intriguing association due to its specificity, uniqueness, and abundance. Our evidence indicates that *Ae* protects *Gv* from potential predators. We also present the first data indicating benefits that *Ae* accrues by growing on *Gv*. Taken together, these findings raise important questions about how facilitative ecological interactions affect community structure, how ecological interactions can vary spatially and temporally (e.g., Palumbi, 1985), and how sponges can influence and expand each other's realized niche (e.g., Wulff, 2008a).

The shallow water and mangrove habitats in the Florida Keys can be stressful locations (Pawlik et al., 2007). Conditions favorable for solitary *Ae* growth appear not to exist in the sediment-laden flats in the Florida Keys (this study; Wilcox et al., 2002). *Amphimedon erina* may have found a strategy and basibiont partner (*Gv*) to deal with some of the physical stressors in these habitats. Sediment burial can cause discoloration and death for *Ae* (Wulff, 2008a). By growing on the vertical surfaces of *Gv*, *Ae* avoids any costs that high sediment loads impart (e.g., investment in mucus to clear particles). Our finding

Table 4 ANOVA results for the caging experiment

Source	Df	SS	MS	*F* ratio	*P* value	ω^2
Amph	1	0.1264	0.1264	1.984	0.1793	11.1
Cage	1	0.0002	0.0002	0.0024	0.9619	0
Amph * Cage	1	0.0000	0.0000	0.0002	0.9882	0
Residual	15	0.9552	0.0637			88.9

Amphimedon erina removal and caging did not affect changes in *Gv* volume. Df and SS for Cage, Amph * Cage, and residual were pooled to eliminate negative variance components. Magnitude of effects (ω^2) were calculated for Amph and error as a percentage of the total variance components in the model

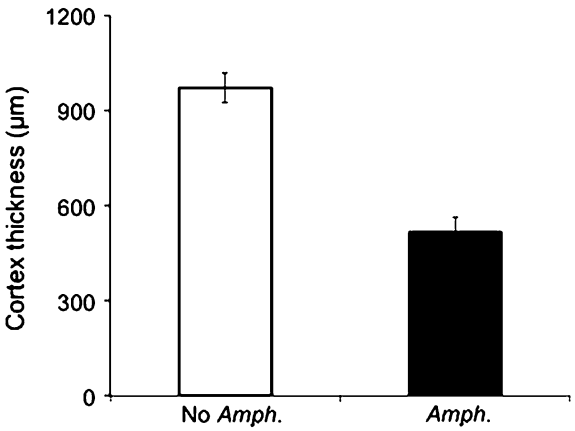

Fig. 3 Cortex thickness of exposed *Gv* tissue and *Gv* tissue underneath *Ae*. The cortex of Gv is significantly thinner when covered by *Ae* (see text). *Columns* indicate means ±1 standard error. *Bars* of a *different color* are statistically significant from one another

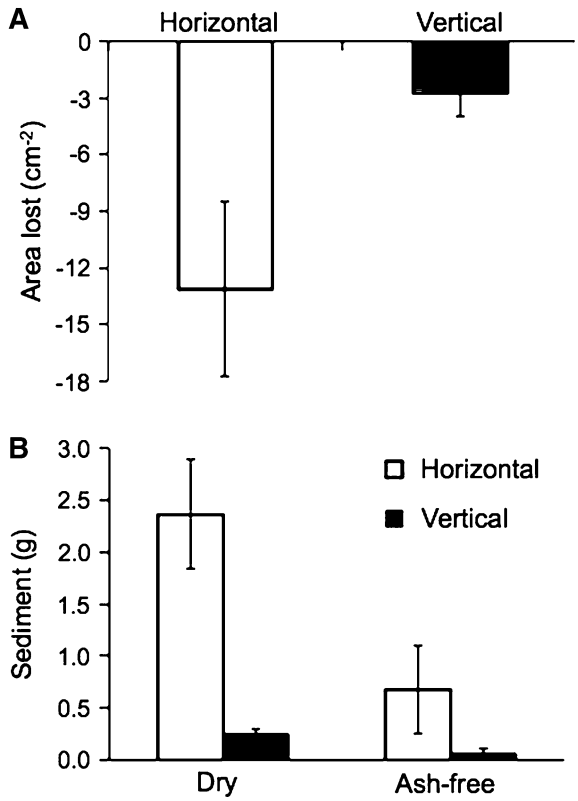

Fig. 4 **A** Area lost over a 40-day period by *Ae* in horizontal and vertical orientations. **B** Dry weight and ash-free dry weight of sediment that accumulated on *Ae* over a 40 day period. In **A** and **B**, vertical *Ae* (*black bars*) was significantly different from horizontal *Ae*. *Bars* indicate treatment means and error bars represent ±1 standard error

that *Ae* suffers elevated loss of surface area when growing horizontally (Fig. 4A) indicates that there are abiotic stressors for *Ae* in this habitat. The energetic cost may be in the form of sponge-derived mucus, as we observed greater AFDW on horizontal *Ae* (Fig. 4B), but this is confounded by the higher organic material present in detritus. *Amphimedon erina* may be using a similar strategy to the rope sponges observed by Wulff (1997a) that increase survival and tolerance of abiotic stressors by adhering to heterospecific sponges.

While *Ae* clearly provides chemical defenses, we also propose that *Gv* has morphological defenses that provide a first line of attack against potential predators. Only 10% of the *Gv* cortex offered to *E. sentus* were accepted, which contrasted with the 75% acceptance rate for *Gv* choanosome (Table 3). The cortex of *Gv* appears to be an effective but potentially costly defense against the weak-jawed vertebrate predators found on coral reefs and in associated habitats (see also Hill & Hill, 2002). The cortex may be less effective against strong-jawed predators such as hawksbill turtles (Meylan, 1988) and sea stars that evert their stomachs (e.g., Waddell & Pawlik, 2000b). In several instances, we exposed choanoderm of *Gv* individuals by cutting off one side of the two-sponge

association. We found that these sponges were rapidly consumed by a variety of organisms not normally known to consume sponges (e.g., pinfish, *Lagodon rhomboides*, Supplementary Fig. 1A). Known spongivores will also attack *Geodia* at higher than normal rates if choanoderm is exposed. As is seen in Supplementary Fig. 1B, two angelfish (*Pomacanthus paru* and *Holacanthus ciliaris*) were observed feeding on *G. gibberosa* sponge that had just been damaged by a hawksbill turtle. Furthermore, studies suggest that siliceous spicules can be defensive (e.g., Hill & Hill, 2002; Burns & Ilan, 2003; Hill et al., 2005), and that spicules can act in synergy with secondary metabolites to enhance defenses in certain sponge species (e.g., Hill et al., 2005; Jones et al., 2005). Nonetheless, our feeding assay results agreed with other studies using *Ae*, *Gv* (=*G. neptuni*), or *T. klausi* (Wulff, 1995, 2006b; Waddell & Pawlik, 2000b). Together, these findings indicate that reef and mangrove populations of *Gv* are preferred by, and incapable of defense against, *E. sentus*.

The results from our feeding trials with *Ae* and *T. klausi* raise an important question about partner fidelity: Why isn't *T. klausi* a more common epibiont of *Gv*? In our choice assay, *T. klausi* and *Ae* were equally deterrent to *E. sentus* (Table 3), yet 81% of *Gv* have *Ae* as an epibiont (Table 1). Thus, additional features of the *Gv*:*Ae* association are likely responsible for the specificity including the possibility of tight morphologic complementation that permits greater movement of water for *Gv*. The exclusivity of this association may also be driven by chemical signaling between the two sponges. Engel & Pawlik (2000) found that *G. gibberosa* promoted overgrowth of several sponges, and they proposed that the two-sponge association described here might involve active recruitment of *Ae* by the basibiont sponge. We observed small ($\sim$1 cm^2), circular *Ae* "recruits" on several of our *Ae* removal replicates in the predator exclusion experiment. These "recruits" may be of larval origin, and if so, would indicate a specialization in habitat recruitment that might represent an advantage of *Ae* over other potential epibionts for *Gv*.

Amphimedon erina removal had no statistical effect on *Gv* biomass in the ANOVA, and explained only 11% of the variation in changes in *Gv* volume (Table 4). Measuring changes in volume presented practical challenges as has been seen in other field experiments with sponges (Ruzicka & Gleason, 2009). The removal of *Ae* was stressful for caged and uncaged *Gv* given that both lost volume during the experiment. We believe that for significant effects to be detectable, greater replication and longer experiments are required.

The tightly coupled nature of this association is also reflected in the plasticity in the sterraster-armor layer of *Gv* (Fig. 3; see also, Fig. 12 in Cardenas, 2010). Cardenas (2010) proposed that silica may be a limiting resource for shallow-water sponges of the Geodiidae. If this hypothesis is true, the plasticity in the sterraster-armor layer of *Gv* might be explained by the fact that obtaining silica is costly in shallow tropical waters. The presence of *Ae* provides a mechanism by which *Gv* can reduce energy expenditure by decreasing the production of silica-rich sterrasters. If *Gv* only produces a thick sterraster layer when epibionts such as *Ae* are absent, this plasticity points toward a role for the sterraster layer in defense against potential predators.

Facilitative interactions have the potential to be extremely important in shaping the structure of ecological communities (Callaway, 2007), yet they remain under-studied. Epibiosis in sponges provide a model system to explore factors that are important in shaping facilitative associations (Wulff, 2005, 2008a, b). In the two-sponge association between *Ae* and *Gv*, we have a clear example of the reciprocal effects that organisms can have on one another. We've identified some of the costs and benefits that appear important in this symbiosis but temporal or environmental changes can alter interspecific interactions (Palumbi, 1985). Much additional work needs to be done to fully understand this interaction, but we argue that the *Gv*:*Ae* association provides opportunities to test important hypotheses on the ecological significance of facilitation.

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References

Ávila, E., J. L. Carballo, & J. A. Cruz-Barraza, 2007. Symbiotic relationships between sponges and other organisms from the Sea of Cortes (Mexican Pacific coast): same problems, same solutions. Porifera Research: Biodiversity, Innovation and Sustainability: 147–156.

Burns, E. & M. Ilan, 2003. Comparison of anti-predatory defenses of Red Sea and Caribbean sponges. II. Physical defense. Marine Ecology Progress Series 252: 115–123.

Callaway, R. M., 2007. Positive interactions and interdependence in plant communities. Springer, Dordrecht, The Netherlands.

Cardenas, P., 2010. Phylogeny, taxonomy and evolution of the Astrophorida (Porifera, Demospongiae). Ph.D. Dissertation. University of Bergen (http://bora.uib.no/handle/1956/4020).

Chanas, B. & J. R. Pawlik, 1995. Defenses of Caribbean sponges against predatory reef fish. II. Spicules, tissue toughness, and nutritional quality. Marine Ecology Progress Series 127: 195–211.

de Laubenfels, M. W., 1936. A discussion of the sponge fauna of the Dry Tortugas in particular and the West Indies in General, with material for a revision of the families and orders of the Porifera. Papers from Tortugas Laboratory, 30: 1–225, pls 1–22.

Dunlap, M. & J. R. Pawlik, 1996. Video-monitored predation by Caribbean reef fishes on an array of mangrove and reef sponges. Marine Biology 126: 117–123.

Engel, S. & J. R. Pawlik, 2000. Allelopathic activities of sponge extracts. Marine Ecology Progress Series 207: 273–281.

Engel, S. & J. R. Pawlik, 2005a. Interactions among Florida sponges. I. Reef habitats. Marine Ecology Progress Series 303: 133–144.

Engel, S. & J. R. Pawlik, 2005b. Interactions among Florida sponges. II. Mangrove habitats. Marine Ecology Progress Series 303: 145–152.

Graham, M. H. & M. S. Edwards, 2001. Statistical significance versus fit: estimating the importance of individual factors in ecological analysis of variance. Oikos 93: 505–513.

Hay, M. E., Q. E. Kappel & W. Fenical, 1994. Synergisms in plant defenses against herbivores—interactions of chemistry, calcification, and plant-quality. Ecology 75: 1714–1726.

Hill, M. S., 1996. Symbiotic zooxanthellae enhance boring and growth rates of the tropical sponge *Anthosigmella varians* forma *varians*. Marine Biology 125: 649–654.

Hill, M. S., 1998. Spongivory on Caribbean reefs releases corals from competition with sponges. Oecologia 117: 143–150.

Hill, M. S. & A. L. Hill, 2002. Morphological plasticity in the tropical sponge *Anthosigmella varians*: responses to predators and wave energy. The Biological Bulletin 202: 86–95.

Hill, M. S., N. A. Lopez & K. A. Young, 2005. Anti-predator defenses in western North Atlantic sponges with evidence of enhanced defense through interactions between spicules and chemicals. Marine Ecology Progress Series 291: 93–102.

Hollander, M. & D. A. Wolfe, 1999. Nonparametric statistical methods. Wiley-Interscience, Inc., New York.

Jones, A., J. Blum & J. Pawlik, 2005. Testing for defensive synergy in Caribbean sponges: bad taste or glass spicules? Journal of Experimental Marine Biology and Ecology 322: 67–81.

Mack, G. A. & J. H. Skillings, 1980. A Friedman-type rank test for main effects in a two-factor. ANOVA Journal of the American Statistical Association 75: 947–951.

Meylan, A., 1988. Spongivory in hawksbill turtles: a diet of glass. Science 239: 393–395.

Palumbi, S., 1985. Spatial variation in an alga-sponge commensalism and the evolution of ecological interactions. The American Naturalist 126: 267–274.

Pawlik, J. R., B. Chanas, R. J. Toonen & W. Fenical, 1995. Defenses of Caribbean sponges against predatory reef fish: I. Chemical deterrency. Marine Ecology Progress Series 127: 183–194.

Pawlik, J. R., S. E. McMurray & T. P. Henkel, 2007. Abiotic factors control sponge ecology in Florida mangroves. Marine Ecology Progress Series 339: 93–98.

Rützler, K., 1970. Spatial competition among Porifera: solution by epizoism. Oecologia 5: 85–95.

Ruzicka, R. & D. F. Gleason, 2009. Sponge community structure and anti-predator defenses on temperate reefs of the South Atlantic Bight. Journal of Experimental Marine Biology and Ecology 380: 36–46.

Sarà, M., 1970. Competition and cooperation in sponge populations. Symposium of the Zoological Society of London 25: 273–284.

Silva, C. M. M., 2002. Revisão das espécies de Geodia Lamarck, 1815 (Porifera, Astrophorida, Geodiidae) do Atlântico Ocidental e Pacífico Oriental. Ph.D. Dissertation. Universidade de São Paulo, São Paulo.

Sollas, W. J., 1886. Preliminary account of the tetractinellid sponges dredged by H.N.S. Challenger, 1872–1876. Part. I. The Choristida. Scientific Proceedings of the Royal Dublin Society. (new ser.) 5: 177–199.

Southwell, M. W., J. B. Weisz, C. S. Martens & N. Lindquist, 2008. In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. Limnology and Oceanography 53: 986–996.

Waddell, B. & J. R. Pawlik, 2000a. Defenses of Caribbean sponges against invertebrate predators. I. Assays with hermit crabs. Marine Ecology Progress Series 195: 125–132.

Waddell, B. & J. R. Pawlik, 2000b. Defenses of Caribbean sponges against invertebrate predators: II. Assays with sea stars. Marine Ecology Progress Series 195: 133–144.

Weisz, J. B., A. J. Massaro, B. D. Ramsby & M. S. Hill, 2010. Zooxanthellar symbionts shape host sponge trophic status through translocation of carbon. Biological Bulletin 219: 189–197.

Wilcox, T. P., M. Hill & K. DeMeo, 2002. Observations on a new two-sponge symbiosis from the Florida Keys. Coral Reefs 21: 198–204.

Wulff, J. L., 1995. Sponge-feeding by the Caribbean starfish *Oreaster reticulatus*. Marine Biology 123: 313–325.

Wulff, J. L., 1997a. Mutualisms among species of coral reef sponges. Ecology 78: 146–159.

Wulff, J. L., 1997b. Parrotfish predation on cryptic sponges of Caribbean coral reefs. Marine Biology 129: 41–52.

Wulff, J. L., 2005. Trade-offs in resistance to competitors and predators, and their effects on the diversity of tropical marine sponges. The Journal of Animal Ecology 74: 313–321.

Wulff, J. L., 2006a. Ecological interactions of marine sponges. Canadian Journal of Zoology 84: 146–166.

Wulff, J. L., 2006b. Sponge systematics by starfish: predators distinguish cryptic sympatric species of Caribbean fire sponges, *Tedania ignis* and *Tedania klausi* n. sp. (Demospongiae, Poecilosclerida). Biological Bulletin 211: 83–94.

Wulff, J. L., 2008a. Collaboration among sponge species increases sponge diversity and abundance in a seagrass meadow. Marine Ecology 29: 193–204.

Wulff, J. L., 2008b. Life-history differences among coral reef sponges promote mutualism or exploitation of mutualism by influencing partner fidelity feedback. The American Naturalist 171: 597–609.

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SPONGE RESEARCH DEVELOPMENTS

Deep sequencing reveals diversity and community structure of complex microbiota in five Mediterranean sponges

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Abstract Marine sponges harbor dense microbial communities of exceptionally high diversity. Despite the complexity of sponge microbiota, microbial communities in different sponges seem to be remarkably similar. In this study, we used a subset of a previously established 454 amplicon pyrosequencing dataset (Schmitt and Taylor, unpublished data). Five Mediterranean sponges were chosen including the model sponge *Aplysina aerophoba* to determine the extent of uniformity by defining (i) the core microbial community, consisting of bacteria found in all sponges, (ii) the variable microbial community, consisting of bacteria found in 2–4 sponges, and (iii) the species-specific community, consisting of bacteria found in only one sponge. Using the enormous sequencing depth of pyrosequencing the diversity in each of the five sponges was extended to up to 15 different bacterial phyla per sponge with Proteobacteria and Chloroflexi being most diverse in each of the five sponges. Similarity comparison of bacteria on phylum and phylotype level revealed most similar communities in *A. aerophoba* and *A. cavernicola* and the most dissimilar community in *Pseudocorticium jarrei*. A surprising minimal core bacterial community was found when distribution of 97% operational taxonomic units (OTUs) was analyzed. Core, variable, and species-specific communities were comprised of 2, 26, and 72% of all OTUs, respectively. This indicates that each sponge contains a large set of unique bacteria and shares only few bacteria with other sponges. However, host species-specific bacteria are probably still closely related to each other explaining the observed similarity among bacterial communities in sponges.

Keywords 454 amplicon pyrosequencing · Bacterial symbiont · Core microbiota · Microbial diversity · Sponge · Vertical transmission

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Guest editors: M. Maldonado, X. Turon, M. A. Becerro & M. J. Uriz / Ancient animals, new challenges: developments in sponge research

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Introduction

The recent advent of next-generation sequencing technologies (Metzger, 2010) has led to exciting advances in the study of microorganisms in natural

environments. Among the most significant has been the identification of a microbial "rare biosphere" (Sogin et al., 2006), which comprises a phylogenetically diverse set of organisms that are present only at very low abundances. An initial, 454 pyrosequencing-based survey of deep sea samples indicated that the resident bacterial communities were up to two orders of magnitude more complex than previously reported for any other environment (Sogin et al., 2006). While acknowledging the limitations of this technique, e.g., sequencing artifacts that led to inflation of diversity (Quince et al., 2009; Huse et al., 2010; Kunin et al., 2010) subsequent studies on plants and humans (Turnbaugh et al., 2009; Redford et al., 2010), soil (Roesch et al., 2007; Chou et al., 2010), and other marine habitats (Galand et al., 2009; Gilbert et al., 2009; Andersson et al., 2010) supported these initial conclusions.

Few habitats are better suited to exploration using next-generation sequencing approaches than the highly diverse microbial communities of marine sponges. Indeed, 454 pyrosequencing of 16S rRNA gene amplicons has already yielded much new information about microbes in Great Barrier Reef (Webster et al., 2010) and Red Sea (Lee et al., 2010) sponges. In the former study, more than 250,000 16S rRNA gene sequences were obtained from the Great Barrier Reef sponges *Ianthella basta*, *Ircinia ramosa*, and *Rhopaloeides odorabile* (Webster et al., 2010). A broad taxonomic range of bacteria were detected, including common sponge associates such as Acidobacteria, Actinobacteria, Chloroflexi, Proteobacteria, and Poribacteria as well as a number of taxa recorded from sponges for the first time (e.g., Deferribacteres, Tenericutes, candidate phylum WS3). Invariably, the "new" taxa were at low abundance, demonstrating the utility of pyrosequencing for uncovering the rare microbial biosphere of marine sponges. It is clear that this new technology offers much to the study of marine sponge microbiology.

In a previous, large-scale 454 amplicon pyrosequencing study (Schmitt and Taylor, unpublished data) we analyzed the diversity and community structure of the total sponge microbiota (obtained from 32 sponge species) as well as the global biogeography of sponge symbionts. Here we used a subset of the previous dataset to investigate in detail the diversity of bacteria associated with each of five Mediterranean sponges (*Aplysina aerophoba*, *Aplysina cavernicola*, *Ircinia variabilis*, *Petrosia ficiformis*, and *Pseudocorticium jarrei*). These sequencing data are also used to delineate the bacterial community into core, variable, and (host) species-specific subsets.

Materials and methods

Amplicon 454 pyrosequencing data were previously generated using a set of 32 sponge species (GenBank accession number SRP003545; Schmitt and Taylor, unpublished data). Briefly, high-quality DNA was extracted from three individuals of each species and a ca. 145 bp fragment of the 16S rRNA gene, including the hypervariable V3 region, was amplified using the modified primer pair 338f and 533r ($338f_{deg}$: ACW CCT ACG GGW GGC WGC AG, $533r_{deg}$: TKA CCG CRG CTG CTG GCA C). Equal amounts of PCR products were pooled from all three individuals and amplicon libraries were sequenced with a 454 Life Sciences FLX pyrosequencer (University of Otago, Dunedin, New Zealand).

For this study, tag sequence data from the five Mediterranean sponges *Aplysina aerophoba*, *Aplysina cavernicola*, *Ircinia variabilis*, *Petrosia ficiformis*, and *Pseudocorticium jarrei* (collection: *A. aerophoba*: Croatia, 45°05′N, 13°38′E, all other sponges: France, 43°12′N, 5°21′E, all at a depth of 15 m) were extracted from the whole dataset and analyzed in greater detail. To avoid overestimating the true diversity due to erroneous tag reads as a result of sequencing errors and formation of homopolymers and chimeras (Reeder & Knight, 2009), the approach of Kunin et al. (2010) was followed: the tag sequences were end-trimmed based on quality scores at a stringency of 0.2% per base error probability using LUCY (Chou & Holmes, 2001). Unique sequences were identified with Mothur 1.9.0 (Schloss et al., 2009), aligned against a SILVA alignment (available at http://www.mothur.org/wiki/Alignment_database) using a kmer search and a Needleman algorithm, and then grouped into 97% OTUs based on uncorrected pairwise distance matrices with the furthest neighbor algorithm. A representative sequence (defined as implemented in Mothur) of each OTU was used for the taxonomic assignment using customized perl scripts similar to the approach used by Sogin et al. (2006) and Webster et al. (2010). For each tag

sequence, a BLAST search (Altschul et al., 1990) was performed against a manually modified SILVA version 98 database. Pairwise global alignments were performed between each of the 10 best hits against the tag sequence using a Smith–Waterman algorithm. The most similar sequence to the tag sequence (or multiple sequences if within a range of 0.1% sequence divergence) was then used for assignment according to the RDP taxonomy implemented in the SILVA database. For assignment at phylum, class, order, family, and genus level, sequence similarity thresholds of 75, 80, 85, 90, and 95% were applied. In cases where the taxonomy of the most similar sequences was inconsistent, a majority rule was applied, and the tag sequence was only assigned if at least 60% of all reference sequences shared the same taxonomic annotation at the respective taxonomic level. All previously published, sponge-derived sequences in the SILVA reference database were labeled as such and it was noted when a tag sequence was assigned to a sponge-derived sequence. Based on the taxonomic assignment as well as on the presence/absence of each OTU in the sponges, Bray-Curtis similarities were calculated using the program PRIMER-6 (PRIMER-E, UK) and visualized as heatmaps using JColorGrid (Joachimiak et al., 2006). Bray-Curtis similarities were also used for unweighted pair-group average cluster analysis with PRIMER-6.

Results

Phylogenetic affiliation of OTUs

Overall, 831, 908, 709, 595, and 255 high-quality tag sequences and 133, 119, 111, 142, and 44 different OTUs were obtained from the sponges *A. aerophoba*, *A. cavernicola*, *I. variabilis*, *P. ficiformis*, and *P. jarrei*, respectively (Fig. 1). These OTUs were affiliated with 15 described bacterial phyla, 5 candidate phyla, and an unclassified bacterial lineage that was previously termed SAUL (sponge-associated unidentified lineage, Schmitt and Taylor, unpublished data) (Fig. 1, Table S1). *A. aerophoba* showed the highest phylum level diversity with 15 different bacterial phyla whereas *P. jarrei* revealed the lowest diversity with 8 bacterial phyla. The remaining three sponges all contained OTUs affiliated with 13 or 14 bacterial phyla. Acidobacteria, Actinobacteria, Chloroflexi, Nitrospira, Proteobacteria, and Spirochaetes as well as the candidate phylum Poribacteria were present in all five investigated sponges. In contrast, Chlamydiae, Lentisphaerae, and Planctomycetes, as well as the candidate phyla TM6 and TM7, were only found in one of the five sponge species. Most diverse in this dataset were the Chloroflexi and Proteobacteria with up to 53 and 45 different OTUs per host species, respectively. Acidobacteria, Actinobacteria, and Poribacteria were represented by an average of 11, 6, and 9.4 OTUs. All other phyla were less diverse with 7 or less different OTUs.

Bacterial community similarity among the five sponges

At phylum level, analysis of Bray-Curtis similarity values showed a high similarity (>75%) for bacterial communities present in *A. aerophoba*, *A. cavernicola*, *P. ficiformis*, and *I. variabilis* with the two *Aplysina* sponges having most similar bacterial communities (Fig. 2A). The bacterial community in *P. jarrei* was more dissimilar to the other sponges with a Bray-Curtis value of 60%. At phylotype level, the same similarity pattern was obtained with both *Aplysina* sponges having most similar bacterial communities and *P. jarrei* having the most dissimilar bacterial community, though with overall lower similarity values (Fig. 2B).

Distribution of OTUs within the five sponges

The bacterial communities of the 5 sponges were divided into the following three categories: (i) core community, represented by OTUs that are present in all 5 sponge species; (ii) variable community, represented by OTUs that are present in 2–4 sponge species; (iii) species-specific community, represented by OTUs that are present in a single sponge species (Fig. 3). The core community consisted of 2% of all OTUs and contained representatives of five different bacterial phyla. The variable community comprised 26% of all OTUs and included 11 different bacterial phyla and the SAUL lineage. The species-specific community was biggest with 72% of all OTUs and represented 18 bacterial phyla and the SAUL lineage. Core, variable, and species-specific OTUs were further divided into "Plus-OTUs" and "Minus-OTUs" depending on whether the respective tag sequences were assigned to a previously

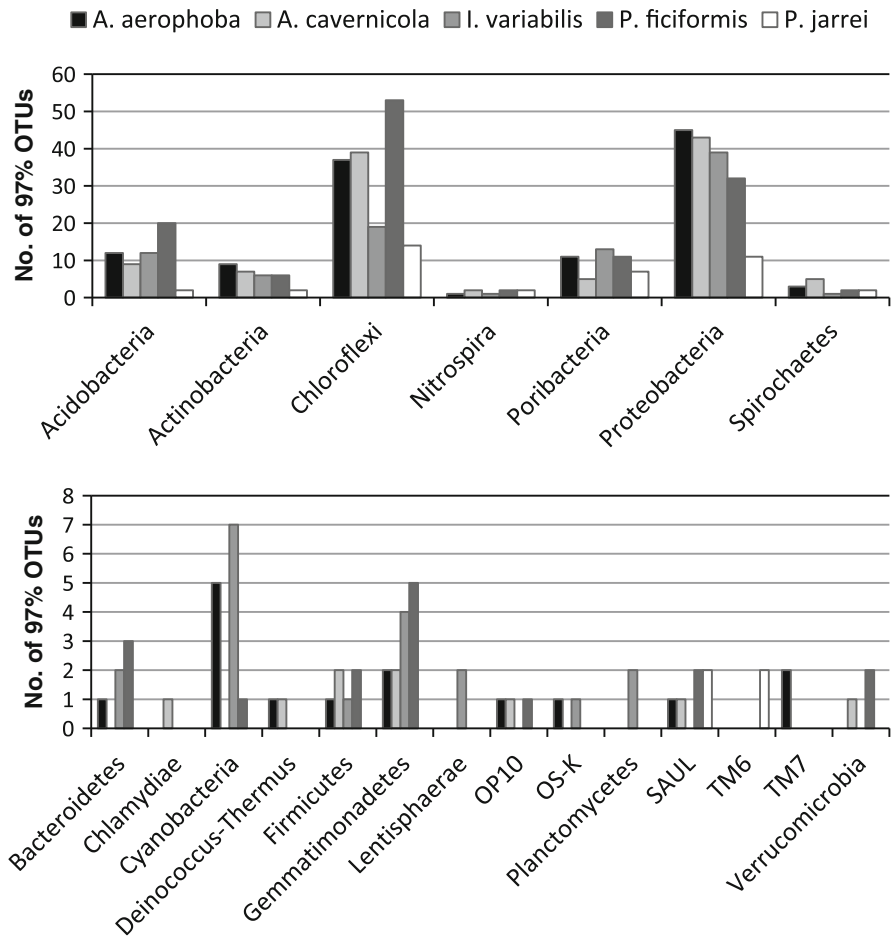

Fig. 1 Bacterial phyla found in the five sponges. Poribacteria, OP10, OS-K, TM6, and TM7 are candidate phyla. SAUL (sponge-associated unclassified lineage) is a bacterial lineage that could not be assigned to any known bacterial phylum. The number of different OTUs per phylum is illustrated

sponge-derived 16S rRNA gene sequence (Plus-OTU) or to a non-sponge-derived 16S rRNA gene sequence (Minus-OTU) during the taxonomic assignment. 89% of core OTUs were Plus-OTUs. The variable community comprised 75% Plus-OTUs whereas the species-specific community contained 69% Plus-OTUs.

Discussion

Taxonomic richness of sponge microbiota

Aplysina aerophoba is a model sponge for the investigation of sponge-microbe associations. Studies on the morphological diversity of bacteria associated with *A. aerophoba* reach back to 1970s (Vacelet, 1975) and were later complemented by cultivation approaches (Hentschel et al., 2001; Pimentel-Elardo et al., 2003) and molecular studies on microbial diversity (Hentschel et al., 2002; Fieseler et al., 2004). Subsequently, different aspects of the association of *A. aerophoba* with diverse microbes were analyzed in greater detail such as the stability of the association (Friedrich et al., 2001), physiology of microbial symbionts (Bayer et al., 2008; Schlappy et al., 2010), disturbance of the association during sponge disease (Webster et al., 2008), and secondary metabolites (Ahn et al., 2003; Siegl & Hentschel, 2010).

In this study, a 454 amplicon pyrosequencing dataset obtained from *A. aerophoba* was re-analyzed together with tag sequence data from four other Mediterranean sponges (Fig. 1). From the 15 bacterial phyla found in *A. aerophoba*, 10 were discovered previously from this sponge (Hentschel et al., 2002; Fieseler et al., 2004), four were not known from this species until now while members of the Firmicutes were only detected in *A. aerophoba* by cultivation

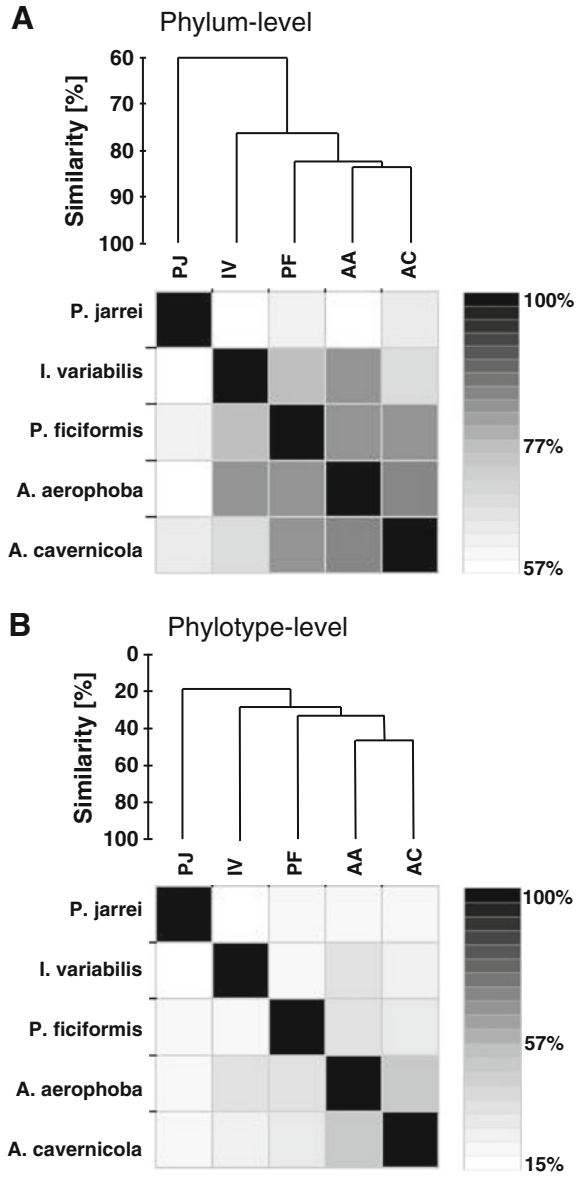

Fig. 2 Phylum level (**A**) and phylotype level (**B**) similarity of bacterial communities among the five sponges. Heat map illustrating Bray-Curtis similarity values and cluster analysis of assigned OTUs on phylum level (**A**) as well as presence/absence of each OTU in sponges (**B**) are shown. Sponges are abbreviated in the order as shown on the left side

approaches (Hentschel et al., 2001; Pabel et al., 2003). The SAUL lineage was found in several other sponges before (Taylor et al., 2007; Kamke et al., 2010) but was not detected in *A. aerophoba* so far. The phylogenetic affiliation of the SAUL lineage is still unclear although previous phylogenetic analysis placed it into the Planctomycetes–Verrucomicrobia–Chlamydiae (PVC) superphylum (Wagner & Horn, 2006). Among the four newly found phyla are Deinococcus-Thermus and the candidate phylum TM7 which were also previously found in other sponges albeit always at low diversity (Schirmer et al., 2005; Thiel et al., 2007; Schmitt et al., 2008; Webster et al., 2010). In addition, two new candidate phyla, OS-K and OP10, were detected. These data show the great potential of amplicon pyrosequencing to discover less abundant members of bacterial communities that may be missed with conventional molecular methods, and therefore to extend our knowledge of bacterial diversity.

Similar to the results for *A. aerophoba*, some hitherto undetected phyla were also found within the other four Mediterranean sponges (Fig. 1). The microbial diversity within *A. cavernicola* was also studied before by molecular methods although not as extensively as in *A. aerophoba* (Friedrich et al., 1999; Thoms et al., 2003; Lafi et al., 2009). In this study, 13 different bacterial phyla were detected in *A. cavernicola* including two candidate phyla (Poribacteria, OP10), as well as the unclassified SAUL lineage. Particularly interesting was the finding of a Chlamydiae-affiliated OTU as this phylum has only been reported once from sponges investigated by conventional molecular methods (Zhu et al., 2008) but was found in a recent 454 amplicon pyrosequencing study in two more sponges (Webster et al., 2010). This might indicate that members of the Chlamydiae could actually be widespread among sponges but likely belong to the rare biosphere in many sponges and were therefore overlooked in most previous studies. Only few 16S rRNA gene sequence data are available for *I. variabilis*, either from cultivated bacteria (De Rosa et al., 2003) or from cyanobacteria-specific studies (Usher et al., 2004). Here, members of 14 different bacterial phyla were found including two candidate phyla (Poribacteria, OS-K) as well as Planctomycetes and Lentisphaerae. Although the latter two phyla have been found before in sponges (Taylor et al., 2007; Webster et al., 2010) they do not seem to be very diverse in these hosts. The respective 16S rRNA gene sequences recovered in this study do not match with other sponge-derived but with environmental 16S rRNA gene sequences and might therefore represent contaminants from seawater. The association of *P. ficiformis* with

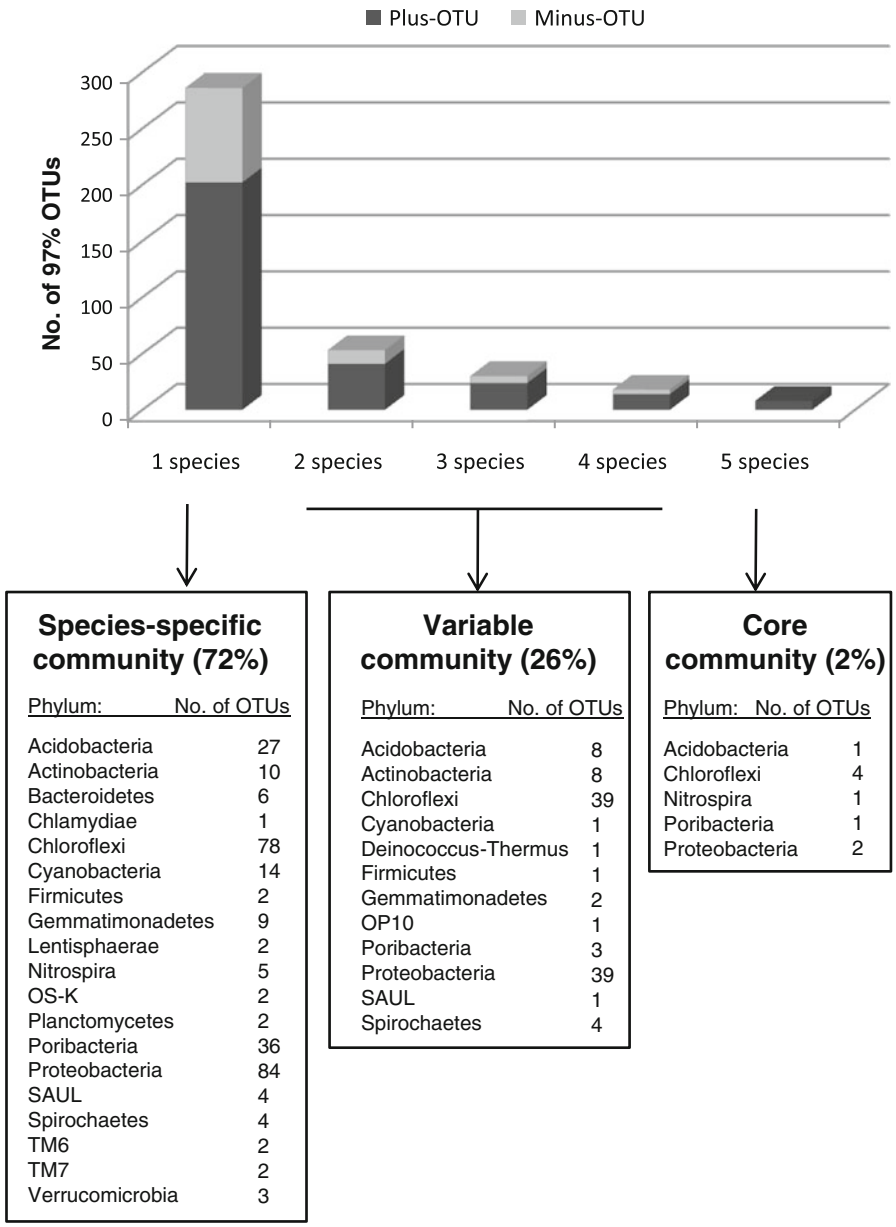

Fig. 3 Number of OTUs that comprise species-specific, variable, and core bacterial communities defined as presence of OTUs in 1 sponge, 2–4 sponges, and all 5 sponges, respectively. Phylogenetic composition and number of different OTUs per phylum is given for each of the three communities. Species-specific, variable and core OTUs are further divided into Plus- or Minus-OTUs depending on whether a previously sponge-derived 16S rRNA gene sequence was used for taxonomic assignment or a non-sponge-derived sequence

microbes has long been studied (Vacelet & Donadey, 1977; Wilkinson & Vacelet, 1979), however, many of the 16S rRNA gene sequences from this sponge available in public databases such as GenBank (http://www.ncbi.nlm.nih.gov) were derived from cultivation-based studies (Chelossi et al., 2007; Muscholl-Silberhorn et al., 2008). In this study, *P. ficiformis* contained members of 13 different bacterial phyla including two candidate phyla (Poribacteria, OP10) and the SAUL lineage. Two OTUs found in *P. ficiformis* were affiliated with the phylum Verrucomicrobia, a phylum that is only irregularly found in sponges. Interestingly, these two OTUs were identified as Plus-OTUs meaning their most similar sequences in the database were also retrieved from sponges. This also might indicate a closer relationship than previously thought. Finally, the microbiology of *P. jarrei*, a representative of the group Homoscleromorpha, is completely unknown so far. Here, we found eight different bacterial phyla including the candidate phyla Poribacteria and TM6, as well as the SAUL lineage.

Similarity among Mediterranean sponge-associated bacteria

The four sponges *A. aerophoba*, *A. cavernicola*, *I. variabilis*, and *P. ficiformis* exhibit more than 75% overlap between their microbial communities at phylum level (Fig. 2A), with 8 phyla being present in all four of these sponges (Fig. 1). With the exception of Firmicutes, all of these phyla are known to contain sponge-specific clusters and represent the typical bacterial profile of bacteriosponges (Hentschel et al., 2002; Taylor et al., 2007). Most diverse in all sponges are the phyla Proteobacteria and Chloroflexi, which is in agreement with previous data obtained from 16S rRNA gene clone libraries (Mohamed et al., 2008; Lee et al., 2009; Kamke et al., 2010). The similarity pattern on phylum level where both *Aplysina* sponges have most similar bacterial communities followed by *P. ficiformis* and *I. variabilis* was supported by the comparison of bacterial communities on phylotype level (Fig. 2B). The bacterial community in *P. jarrei* seems to differ more on both phylum level, with only 60% similarity, and phylotype level, with 20% similarity (Fig. 2). This might indicate a different bacterial profile in *P. jarrei*, but more likely reflects the much lower number of analyzed OTUs due to fewer 454 reads. It is therefore conceivable that the more abundant bacteria were detected in *P. jarrei* whereas the rare bacteria are underrepresented in the dataset which then has an effect on the comparison analysis.

Community structure of complex sponge microbiota

Core (presence in all sponges), variable (presence in 2–4 sponges), and species-specific (presence in only one sponge) communities were defined by determining the presence of each OTU in every sponge species (Fig. 3). The core community was rather small with only 2% of all OTUs found in all sponges. In stark contrast, the species-specific community comprised almost three quarters of all OTUs. Core, variable, and species-specific communities are phylogenetically diverse and not dominated by a single phylum (Fig. 3). Assuming that 97% sequence similarity is an approximate threshold for bacterial species, then these data suggest that sponges do not share a lot of their bacterial species. Instead each sponge species contains a large set of unique bacterial species. This seemingly contrasts with current understanding of microbiota in sponges that are believed to be highly similar (Hentschel et al., 2002; Olson & McCarthy, 2005; Hill et al., 2006; Taylor et al., 2007; Lee et al., 2009; Anderson et al., 2010). The hypothesis of a uniform bacterial community in sponges is mainly based on the finding of sponge-specific clusters, e.g., clusters of only sponge-derived 16S rRNA gene sequences from different sponge species and/or locations (Hentschel et al., 2002). However, these clusters are not limited by a sequence similarity threshold and, in fact, within-cluster similarity can be as low as 77% (Hentschel et al., 2002). Because of the short length of our tag sequences (ca. 145 bp) we could not determine sponge-specific clusters in our dataset. Instead, we used a similar designation into Plus- and Minus-OTUs depending on whether the respective 16S rRNA gene sequence was assigned to a sponge-derived (Plus-OTU) or a non-sponge-derived (Minus-OTU) 16S rRNA gene sequence in the database. Interestingly, we found a high percentage of Plus-OTUs ranging from 69% in the species-specific community to 89% in the core community. We therefore propose that different sponges might contain different bacterial species. But these species are still more closely related to each other than to, e.g., seawater bacteria as indicated by the high percentage of Plus-OTUs in our dataset and generally by sponge-specific clusters.

Symbiont transmission model revisited

In a previous study we proposed a symbiont transmission model including a combination of both vertical and horizontal transmission to maintain complex microbial consortia in sponges (Schmitt et al., 2008). Here, we provide an update including the current literature on the mechanism of formation of this specific association (Fig. 4). There are now several microscopic and molecular studies showing that bacterial symbionts in sponges are vertically transmitted through reproductive stages (Usher et al., 2001; Ereskovsky et al., 2005; Oren et al., 2005; Enticknap et al., 2006; DeCaralt et al., 2007; Maldonado, 2007; Schmitt et al., 2007a, b; Sharp et al., 2007; Steger et al., 2008; Lee et al., 2009; Webster et al., 2010). The alternative mechanism of horizontal or environmental transmission, e.g., uptake of symbionts from seawater, is much more difficult to

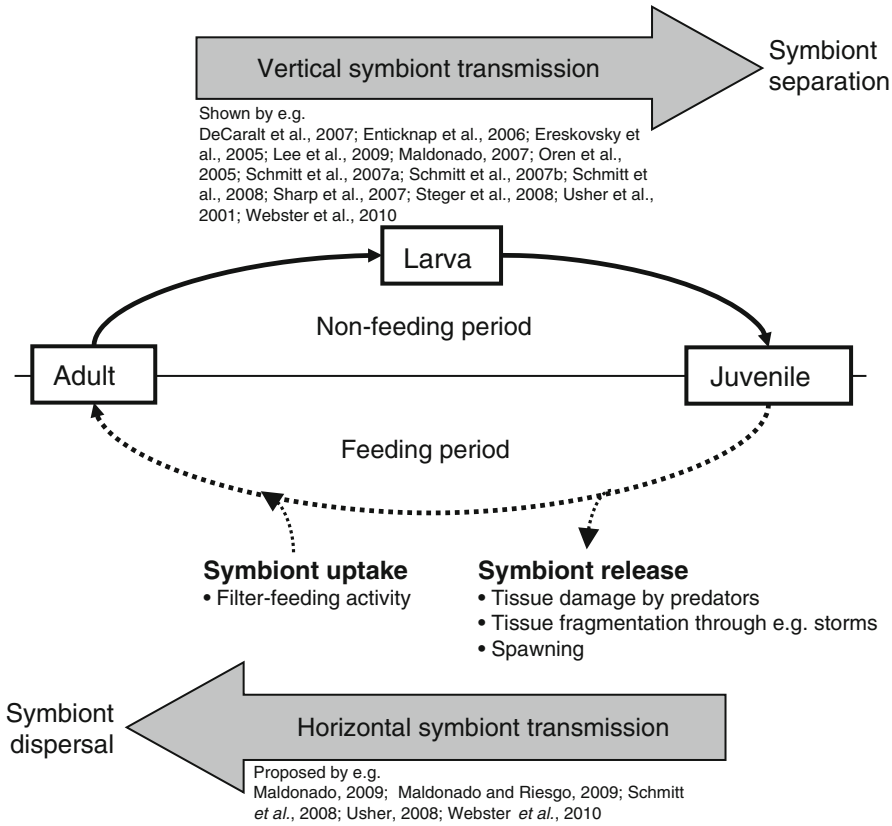

Fig. 4 Update of the previously proposed microbial symbiont transmission model in sponge hosts hypothesizing a combination of vertical and horizontal transmission to maintain complex sponge-microbe associations. Figure amended, with permission, from Schmitt et al. (2008)

prove and so far there is only indirect evidence for it. For *P. ficiformis*, a species that was also used in this study, the gametes do not contain any bacterial cells and it was therefore concluded that bacteria must be acquired from the environment by juveniles of each new generation (Maldonado & Riesgo, 2009). A similar argument was used for cyanobacterial cells in *A. aerophoba* and *Chondrilla australiensis* (Usher, 2008; Maldonado, 2009). Both species contain cyanobacteria in the mesohyl of adult sponges but these bacteria could not be detected at all in oocytes of *A. aerophoba* (Maldonado, 2009) and only in some gametes of *C. australiensis* (Usher, 2008). Strictly vertically transmitted bacterial symbionts co-speciate with their hosts which results in congruent phylogenies (Zientz et al., 2004). Such a co-speciation signal was not found in a comprehensive phylogenetic study of vertically transmitted symbionts in sponges and it was assumed that additional horizontal symbiont transmission might obscure a co-speciation pattern (Schmitt et al., 2008). Finally, a recent 454 amplicon pyrosequencing study detected sponge-specific microbes in seawater albeit at very low abundances (Webster et al., 2010). The authors speculated that sponge-specific microbes might in fact be present in seawater as part of the rare seawater biosphere which might serve as a seed bank for colonization of sponges. In this case, sponges might harvest these microbial lineages from seawater in addition to vertical transmission of symbionts. Altogether, these new results corroborate the theory that horizontal/environmental transmission of sponge symbionts occurs and that a combination of horizontal and environmental transmission maintains complex sponge microbes associations.

Conclusions

This study complements a previous large-scale study on whole sponge microbiome diversity and global sponge symbiont biogeography (Schmitt and Taylor, unpublished data) on a small-scale level including microbial diversity and community structure on a sponge species level. For this study the tag dataset from five Mediterranean sponges was chosen

including the model sponge *Aplysina aerophoba*. Deep sequencing of these sponges revealed a previously unknown diversity including several phyla so far not known from these sponges. The sponge microbiome consists of very few core bacteria found in all five sponges and a majority of host species-specific bacteria found in only one sponge. However, these host species-specific bacteria are probably still closely related to each other explaining the observed similarity among bacterial communities in sponges. These important new results allow us to better assess and understand one of the most diverse and possibly most ancient symbiotic systems.

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References

Ahn, Y. B., S. K. Rhee, D. E. Fennell, L. J. Kerkhof, U. Hentschel & M. M. Haggblom, 2003. Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge *Aplysina aerophoba*. Applied and Environmental Microbiology 69: 4159–4166.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers & D. J. Lipman, 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

Anderson, S. A., P. T. Northcote & M. J. Page, 2010. Spatial and temporal variability of the bacterial community in different chemotypes of the New Zealand marine sponge *Mycale hentscheli*. FEMS Microbiology Ecology 72: 328–342.

Andersson, A. F., L. Riemann & S. Bertilsson, 2010. Pyrosequencing reveals contrasting seasonal dynamics of taxa within Baltic Sea bacterioplankton communities. The ISME Journal 4: 171–181.

Bayer, K., S. Schmitt & U. Hentschel, 2008. Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge *Aplysina aerophoba*. Environmental Microbiology 10: 2942–2955.

Chelossi, E., R. Pantile, R. Pronzato, M. Milanese & U. Hentschel, 2007. Bacteria with antimicrobial properties isolated from the Mediterranean sponges *Chondrilla nucula* and *Petrosia ficiformis*. Aquatic Microbial Ecology 49: 157–163.

Chou, H. H. & M. H. Holmes, 2001. DNA sequence quality trimming and vector removal. Bioinformatics 17: 1093–1104.

Chou, H. Y., N. Fierer, C. L. Lauber, J. G. Caporaso, R. Knight & P. Grogan, 2010. Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes. Environmental Microbiology 12: 2998–3006.

De Rosa, S., M. Mitova & G. Tommonaro, 2003. Marine bacteria associated with sponge as source of cyclic peptides. Biomolecular Engineering 20: 311–316.

DeCaralt, S., M. J. Uriz & R. H. Wijffels, 2007. Vertical transmission and successive location of symbiotic bacteria during embryo development and larva formation in *Corticium candelabrum* (Porifera: Demospongiae). Journal of the Marine Biological Association of the United Kingdom 87: 1693–1699.

Enticknap, J. J., M. Kelly, O. Peraud & R. T. Hill, 2006. Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. Applied and Environmental Microbiology 72: 3724–3732.

Ereskovsky, A. V., E. Gonobobleva & A. Vishnyakov, 2005. Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). Marine Biology 146: 869–875.

Fieseler, L., M. Horn, M. Wagner & U. Hentschel, 2004. Discovery of the novel candidate phylum "Poribacteria" in marine sponges. Applied and Environmental Microbiology 70: 3724–3732.

Friedrich, A. B., H. Merkert, T. Fendert, J. Hacker, P. Proksch & U. Hentschel, 1999. Microbial diversity in the marine sponge *Aplysina cavernicola* (formerly *Verongia cavernicola*) analyzed by fluorescence in situ hybridization (FISH). Marine Biology 134: 461–470.

Friedrich, A. B., I. Fischer, P. Proksch, J. Hacker & U. Hentschel, 2001. Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. FEMS Microbiology Ecology 38: 105–113.

Galand, P. E., E. O. Casamayor, D. L. Kirchman & C. Lovejoy, 2009. Ecology of the rare microbial biosphere of the Arctic Ocean. Proceedings of the National Academy of Sciences of the United States of America 106: 22427–22432.

Gilbert, J. A., D. Field, P. Swift, L. Newbold, A. Oliver, T. Smyth, P. J. Somerfield, S. Huse & I. Joint, 2009. The seasonal structure of microbial communities in the Western English Channel. Environmental Microbiology 11: 3132–3139.

Hentschel, U., M. Schmid, M. Wagner, L. Fieseler, C. Gernert & J. Hacker, 2001. Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. FEMS Microbiology Ecology 35: 305–312.

Hentschel, U., J. Hopke, M. Horn, A. B. Friedrich, M. Wagner, J. Hacker & B. S. Moore, 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. Applied and Environmental Microbiology 68: 4431–4440.

Hill, M., A. Hill, N. Lopez & O. Harriott, 2006. Sponge-specific bacterial symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida). Marine Biology 148: 1221–1230.

Huse, S., D. B. M. Welch, H. G. Morrison & M. L. Sogin, 2010. Ironing out the wrinkles in the rare biosphere

through improved OTU clustering. Environmental Microbiology 12: 1889–1898.
Joachimiak, M. P., J. L. Weisman & B. C. H. May, 2006. JColorGrid: software for the visualization of biological measurement. BMC Bioinformatics 27: 7–225.
Kamke, J., M. W. Taylor & S. Schmitt, 2010. Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. The ISME Journal 4: 498–508.
Kunin, V., A. Engelbrektson, H. Ochman & P. Hugenholtz, 2010. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. Environmental Microbiology 12: 118–123.
Lafi, F. F., J. A. Fuerst, L. Fieseler, C. Engels, W. W. L. Goh & U. Hentschel, 2009. Widespread distribution of Poribacteria in Demospongiae. Applied and Environmental Microbiology 75: 5695–5699.
Lee, O. O., P. Y. Chui, Y. H. Wong, J. R. Pawlik & P. Y. Qian, 2009. Evidence for vertical transmission of bacterial symbionts from adult to embryo in the Caribbean sponge *Svenzea zeai*. Applied and Environmental Microbiology 75: 6147–6156.
Lee, O. O., Y. Wang, J. Yang, F. F. Lafi, A. Al-Suwailem & P.-Y. Qian, 2010. Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. The ISME Journal, epub ahead of print, doi:10.1038/ismej.2010.165.
Maldonado, M., 2007. Intergenerational transmission of symbiotic bacteria in oviparous and viviparous demosponges, with emphasis on intracytoplasmically-compartmented bacterial types. Journal of the Marine Biological Association of the United Kingdom 87: 1701–1713.
Maldonado, M., 2009. Embryonic development of verongid demosponges supports the independent acquisition of spongin skeletons as an alternative to the siliceous skeleton of sponges. Biological Journal of the Linnean Society 97: 427–447.
Maldonado, M. & A. Riesgo, 2009. Gametogenesis, embryogenesis, and larval features of the oviparous sponge *Petrosia ficiformis* (Haplosclerida, Demospongiae). Marine Biology 156: 2181–2197.
Metzger, M. L., 2010. Sequencing technologies—the next generation. Nature Reviews Genetics 11: 31–46.
Mohamed, N. M., V. Rao, M. T. Hamann, M. Kelly & R. T. Hill, 2008. Monitoring bacterial diversity of the marine sponge *Ircinia strobilina* upon transfer into aquaculture. Applied and Environmental Microbiology 74: 4133–4143.
Muscholl-Silberhorn, A., V. Thiel & J. F. Imhoff, 2008. Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean sea. Microbial Ecology 55: 94–106.
Olson, J. B. & P. J. McCarthy, 2005. Associated bacterial communities of two deep-water sponges. Aquatic Microbial Ecology 39: 47–55.
Oren, M., L. Steindler & M. Ilan, 2005. Transmission, plasticity and the molecular identification of cyanobacterial symbionts in the Red Sea sponge *Diacarnus erythraenus*. Marine Biology 148: 35–41.
Pabel, C. T., J. Vater, C. Wilde, P. Franke, J. Hofemeister, B. Adler, G. Bringmann, J. Hacker & U. Hentschel, 2003. Antimicrobial activities and matrix-assisted laser desorption/ionization mass spectrometry of Bacillus isolates from the marine sponge *Aplysina aerophoba*. Marine Biotechnology 5: 424–434.
Pimentel-Elardo, S., M. Wehrl, A. B. Friedrich, P. R. Jensen & U. Hentschel, 2003. Isolation of planctomycetes from *Aplysina* sponges. Aquatic Microbial Ecology 33: 239–245.
Quince, C., A. Lanzen, T. P. Curtis, R. J. Davenport, N. Hall, I. M. Head, L. F. Read & W. T. Sloan, 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods 6: 639–641.
Redford, A. J., R. M. Bowers, R. Knight, Y. Linhart & N. Fierer, 2010. The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environmental Microbiology 12: 2885–2893.
Reeder, J. & R. Knight, 2009. The 'rare biosphere': a reality check. Nature Methods 6: 636–637.
Roesch, L. F., R. R. Fulthorpe, A. Riva, G. Casella, A. K. M. Hadwin, A. D. Kent, S. H. Daroub, F. A. O. Camargo, W. G. Farmerie & E. W. Triplett, 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME Journal 1: 283–290.
Schirmer, A., R. Gadkari, C. D. Reeves, F. Ibrahim, E. F. DeLong & C. R. Hutchinson, 2005. Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge *Discodermia dissoluta*. Applied and Environmental Microbiology 71: 4840–4849.
Schlappy, M. L., S. I. Schottner, G. Lavik, M. M. M. Kuypers, D. de Beer & F. Hoffmann, 2010. Evidence of nitrification and denitrification in high and low microbial abundance sponges. Marine Biology 157: 593–602.
Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn & C. F. Weber, 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75: 7537–7541.
Schmitt, S., M. Wehrl, N. Lindquist, J. B. Weisz & U. Hentschel, 2007a. Morphological and molecular analyses of microorganisms in Caribbean reef adult sponges and in corresponding reproductive material. In Porifera Research: Biodiversity, Innovation & Sustainability. Rio de Janeiro Museu Nacional, Buzios, Brazil: 561–568.
Schmitt, S., J. B. Weisz, N. Lindquist & U. Hentschel, 2007b. Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. Applied and Environmental Microbiology 73: 2067–2078.
Schmitt, S., H. Angermeier, R. Schiller, N. Lindquist & U. Hentschel, 2008. Molecular microbial diversity survey of sponge reproductive stages and mechanistic insights into vertical transmission of microbial symbionts. Applied and Environmental Microbiology 74: 7694–7708.
Sharp, K. H., B. Eam, D. J. Faulkner & M. G. Haygood, 2007. Vertical transmission of diverse microbes in the tropical sponge *Corticium* sp. Applied and Environmental Microbiology 73: 622–629.

Siegl, A. & U. Hentschel, 2010. PKS and NRPS gene clusters from microbial symbiont cells of marine sponges by whole genome amplification. Environmental Microbiology Reports 2: 507–513.

Sogin, M. L., H. G. Morrison, J. A. Huber, D. Mark Welch, S. M. Huse, P. R. Neal, J. M. Arrieta & G. J. Herndl, 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences of the United States of America 103: 12115–12120.

Steger, D., P. Ettinger-Epstein, S. Whalan, U. Hentschel, R. de Nys, M. Wagner & M. W. Taylor, 2008. Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. Environmental Microbiology 10: 1087–1094.

Taylor, M. W., R. Radax, D. Steger & M. Wagner, 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiology and Molecular Biology Reviews 71: 295–347.

Thiel, V., S. Leininger, R. Schmaljohann, F. Brummer & J. F. Imhoff, 2007. Sponge-specific bacterial associations of the Mediterranean sponge *Chondrilla nucula* (Demospongiae, Tetractinomorpha). Microbial Ecology 54: 101–111.

Thoms, C., M. Horn, M. Wagner, U. Hentschel & P. Proksch, 2003. Monitoring microbial diversity and natural product profiles of the sponge *Aplysina cavernicola* following transplantation. Marine Biology 142: 685–692.

Turnbaugh, P. J., M. Hamady, T. Yatsunenko, B. L. Cantarel, A. Duncan, R. E. Ley, M. L. Sogin, W. J. Jones, B. A. Roe, J. P. Affourtit, M. Egholm, B. Henrissat, A. C. Heath, R. Knight & J. I. Gordon, 2009. A core gut microbiome in obese and lean twins. Nature 457: 480–487.

Usher, K. M., 2008. The ecology and phylogeny of cyanobacterial symbionts in sponges. Marine Ecology and Evolutionary Perspective 29: 178–192.

Usher, K. M., J. Kuo, J. Fromont & D. C. Sutton, 2001. Vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). Hydrobiologia 461: 15–23.

Usher, K. M., J. Fromont, D. C. Sutton & S. Toze, 2004. The biogeography and phylogeny of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean. Microbial Ecology 48: 167–177.

Vacelet, J., 1975. Etude en microscopie electronique de l'association entre bacteries et spongiaires du genre Verongia (Dictyoceratida). Journal De Microscopie 23: 271–288.

Vacelet, J. & C. Donadey, 1977. Electron-microscope study of association between some sponges and bacteria. Journal of Experimental Marine Biology and Ecology 30: 301–314.

Wagner, M. & M. Horn, 2006. The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. Current Opinion in Biotechnology 17: 241–249.

Webster, N. S., J. R. Xavier, M. Freckelton, C. A. Motti & R. Cobb, 2008. Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. Environmental Microbiology 10: 3366–3376.

Webster, N. S., M. W. Taylor, F. Behnam, S. Luecker, T. Rattei, S. Whalan, M. Horn & M. Wagner, 2010. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environmental Microbiology 12: 2070–2082.

Wilkinson, C. R. & J. Vacelet, 1979. Transplantation of marine sponges to different conditions of light and current. Journal of Experimental Marine Biology and Ecology 37: 91–104.

Zhu, P., Q. Z. Li & G. Y. Wang, 2008. Unique microbial signatures of the alien Hawaiian marine sponge *Suberites zeteki*. Microbial Ecology 55: 406–414.

Zientz, E., T. Dandekar & R. Gross, 2004. Metabolic interdependence of obligate intracellular bacteria and their insect hosts. Microbiology and Molecular Biology Reviews 68: 745–770.