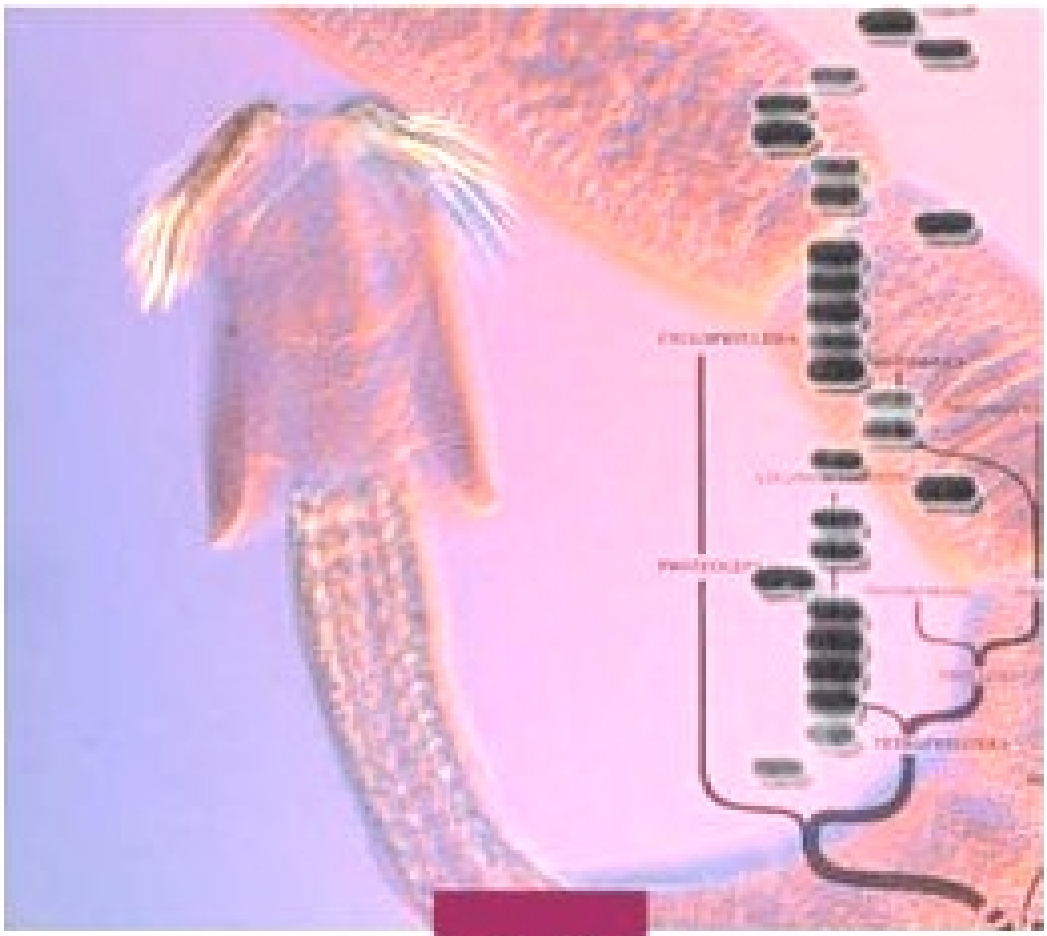
ADVANCES IN PARASITOLOGY



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Preface

The volume opens with a review by Tomoyoshi Nozaki, Vahab Ali and Masaharu Tokoro, of Gunma University, the Institute of Infectious Diseases, and Kanazawa University in Japan, of sulfur-containing amino acids in parasitic protozoa. These compounds are involved in many metabolic processes, including the biosynthesis of proteins, polyamines and glutathione. Genome analysis of the relevant enzymes in a range of parasitic protozoa (species of *Cryptosporidium*, *Entamoeba*, *Plasmodium*, *Trichomonas* and *Trypanosoma*) has revealed wide differences between the parasites and, perhaps more importantly, between them and their mammalian hosts. Some of these enzymes and pathways are present in the parasites but absent from their hosts. This diversity should enable selected parasitic protozoa to serve as models in studies aimed at further elucidating the biological significance of the metabolism of these compounds. The sulfur-containing amino acids of the parasites also represent interesting potential targets for future chemotherapeutic interventions.

Mark Taylor of the Liverpool School of Tropical Medicine, UK, Claudio Bandi of the University of Milan, Italy, and Achim Hoerauf of the University of Bonn, Germany, review the presence and importance of the symbiotic, intracellular, bacterium *Wolbachia* in filariae of medical importance. The bacterium is essential for parasite fertility and survival and this is leading to promising results with the use of antibiotics as chemotherapeutic agents against lymphatic filariasis and onchocerciasis. The authors also discuss the role of *Wolbachia* in inflammatory-mediated pathogenesis resulting from these filarial infections and in adverse reactions to classical microfilaricidal drugs.

The next two reviews are closely linked; both are concerned with recent developments in molecular systematics. The first is a

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contribution from Matthew Nolan and Tom Cribb, The University of Queensland, Brisbane, Australia who examine in detail the use of ribosomal DNA for the identification of species of Digenea. They pay particular attention to the internal transcribed spacer of ribosomal DNA (ITS rDNA) as this has been much used in recent taxonomic studies. Indeed they consider 63 studies that have reported partial or complete ITS rDNA sequences. The authors consider the wider implications of ITS sequencing and how best to assess and use the molecular information in association with morphological or biological traits. They conclude by highlighting what they believe to be the characteristics of an effective taxonomic study to explore trematode species boundaries. The second review takes a much broader look at the application of molecular systematics to the parasitic Platyhelminthes (Cestoda, Digenea and Monogenea). Peter Olson, The Natural History Museum, London, UK and Vasyl Tkach, University of North Dakota, USA review the many advances that have been made through molecular studies in the understanding of the systematics, evolution, taxonomy and species identification in these groups. It is interesting to see how different questions have been tackled in each of the three groups and the current state of play in unravelling the respective phylogenies. Future directions are considered, including the future value of mitochondrial and nuclear genomics in systematic studies.

The coccidian *Eimeria* is one of the most important parasites of poultry and in the final chapter Martin Shirley, Adrian Smith and Fiona Tomley of the Institute of Animal Health, Compton, UK, review the biology of avian species and particularly recent work on their control by vaccination. Sequencing of the nuclear genome of one species (*Eimeria tenella*) is providing new information on life cycles and on protective immune responses. While the use of live vaccines provides the most successful current vaccine strategy, recombinant expressed antigens, maternal immunization and DNA vaccines are promising new lines of research.

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Sulfur-Containing Amino Acid Metabolism in Parasitic Protozoa

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ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
ACCS	1-aminocyclopropane-1-carboxylic acid synthase
AdoHcy	*S*-adenosylhomocysteine
AdoMet	*S*-adenosylmethionine
AdoMetDC	*S*-adenosylmethionine decarboxylase
AT	aminotransferases
CAT	cysteine aminotransferase
CBL	cystathionine β-lyase
CBS	cystathionine β-synthase
cdMS	cobalamin-dependent methionine synthase
CDO	cysteine dioxygenase
CGL	cystathionine γ-lyase

CGS	cystathionine γ-synthase
ciMS	cobalamin-independent methionine synthase
CoAS	coenzyme A synthetic pathway
CS	cysteine synthase
CSDC	cysteine-sulfinate decarboxylase
CTADO	cysteamine dioxygenase
DcAdoMet	decarboxylated *S*-adenosylmethionine
DFMO	DL-α-difluoromethylornithine
DHFR	dihydrofolate reductase
DS	direct sulfhydrylation
eIF-5A	eukaryotic translation initiation factor 5A
GDH	glycerate dehydrogenase
GGS	γ-glutamylcysteine synthase
GK	glycerate kinase
GS	glutathione synthase
HAT	human African trypanosomiasis
HD	hypotaurine dehydrogenase
HETA	5′-deoxy-5′-(hydroxyethyl)thioadenosine
HP	hydroxypyruvate
ISC	iron–sulfur cluster
KMTB	α-ketomethiobutyrate
MAOEA	5′-deoxy-5′-[(2-aminooxyethyl)methylamino] adenosine
MAT	methionine adenosyltransferase
MDL 73811	[(*Z*)-4-amino-2-butenyl]methylamino)-5′-deoxyadenosine
MGBG	methylglyoxal-bis(guanylhydrazone)
MGL	methionine γ-lyase
MHZEA	5′-deoxy-5′-[(2-hydrazinoethyl)-methylamino] adenosine
MT	methyltransferases
MTA	methylthioadenosine
MTAN	5′-methylthioadenosine nucleosidase
MTAP	methylthioadenosine phosphorylase
MTR	5-methylthioribose
MTRK	5-methylthioribose kinase
NCBI	National Center of Biotechnology Information
NIF	nitrogen fixation
OAS	*O*-acetylserine

ODC	ornithine decarboxylase
PGDH	phosphoglycerate dehydrogenase
PHP	3-phosphoglycerate
Pi	inorganic phosphate
PK	pyruvate kinase
PLP	pyridoxal 5′-phosphate
PPi	inorganic pyrophosphate
PPPi	tripolyphosphate
PSAT	phosphoserine aminotransferase
PSP	phosphoserine phosphatase
SAHH	*S*-adenosylhomocysteine hydrolase
SAT	serine *O*-acetyltransferase
SD	serine dehydratase
SHMT	(GHMT) serine (glycine) hydroxymethyl transferase
SPAT	(AGAT) serine pyruvate (alanine glyoxylate) aminotransferase
SS	spermidine synthase or spermine synthase
TEAT	thioethanolamine *S*-acetyltransferase
THF	tetrahydrofolate
THP	tetrahydropteroyltriglutamate
TIGR	the Institute of Genomic Research
TS	thymidylate synthase

ABSTRACT

Sulfur-containing amino acids play indispensable roles in a wide variety of biological activities including protein synthesis, methylation, and biosynthesis of polyamines and glutathione. Biosynthesis and catabolism of these amino acids need to be carefully regulated to achieve the requirement of the above-mentioned activities and also to eliminate toxicity attributable to the amino acids. Genome-wide analyses of enzymes involved in the metabolic pathways of sulfur-containing amino acids, including transsulfuration, sulfur assimilatory *de novo* cysteine biosynthesis, methionine cycle, and degradation, using genome databases available from a variety of parasitic protozoa, reveal remarkable diversity between protozoan parasites and

their mammalian hosts. Thus, the sulfur-containing amino acid metabolic pathways are a rational target for the development of novel chemotherapeutic and prophylactic agents against diseases caused by protozoan parasites. These pathways also demonstrate notable heterogeneity among parasites, suggesting that the metabolism of sulfur-containing amino acids reflects the diversity of parasitism among parasite species, and probably influences their biology and pathophysiology such as virulence competence and stress defense.

1. INTRODUCTION

Two major sulfur-containing amino acids, methionine and cysteine, are metabolized by a variety of reactions involving at least two dozen intermediates and products. These sulfur-containing amino acids and some of the intermediates are essential for survival of virtually all living organisms from bacteria to higher eukaryotes. Despite the fact that these molecules are indispensable for all organisms, there are remarkable differences in both biosynthesis and catabolism of these sulfur-containing amino acids between parasitic protozoa and their mammalian hosts. Owing to rapid expansion of available genome databases of parasitic protozoa, genome-wide searches of genes involved in metabolism of sulfur-containing amino acids have become possible to comprehensively understand distribution of individual enzymes and whole metabolic pathways. Such comparative genomics should enable us to address important biological, biochemical, and evolutionary questions related to these essential metabolic pathways. In this review, we attempt to summarize current knowledge on heterogeneity in the metabolism of sulfur-containing amino acids among representative protozoan parasites, with emphasis on major differences between parasites and their mammalian hosts. Comprehensive understanding of differences in metabolism between parasites and mammalian hosts should help us to identify and exploit unique targets to develop novel chemotherapeutic and prophylactic agents.

2. BIOLOGICAL IMPORTANCE OF SULFUR-CONTAINING AMINO ACIDS AND THEIR METABOLIC PATHWAYS

2.1. General Features and Functions of Sulfur

Sulfur is the fourth most abundant element after oxygen, silicon, and hydrogen in the Earth's crust (Beinert, 2000). In all living organisms, sulfur is the third most abundant element after carbon and nitrogen. Sulfur constitutes only 0.1% of dry matter of plants while carbon and nitrogen are present at 45% and 1.5%, respectively (Leustek *et al.*, 2000). Sulfur is often involved in catalytic and electrochemical reactions rather than constituting structural biomolecules. The sulfur atom is unique due to its extraordinary properties. Sulfur is light enough for its nuclear charge to be overcome and also to enable mobilization or immobilization of its electrons, compared to selenium and tellurium. In addition, the sulfur atom is large enough to populate orbital 3d, in contrast to the oxygen atom. Sulfur can occur in formal valencies from 2^- to 6^+; thus it can donate or accept electrons to reach the neon or argon configurations, respectively. The fact that reduced sulfur in cysteine (thiol, –SH) is strongly nucleophilic, makes it react easily with electrophilic compounds. On the contrary, two cysteine thiol groups are oxidized to form a stable covalent disulfide bond in macromolecules, e.g. proteins. This disulfide bond can be reduced to restore two thiol moieties in cysteine. This reversibility serves a regulatory role that determines the structure and activity of proteins. These physical and chemical properties, unique to sulfur, led to the intriguing hypothesis that iron–sulfur proteins represent one of the first catalysts in nature (Beinert *et al.*, 1997; Cody *et al.*, 2000).

2.2. Functions of Sulfur-Containing Amino Acids

2.2.1. General Functions of Sulfur-Containing Amino Acids

Cysteine and methionine are incorporated into proteins. Cysteine plays an important role in stability, structure, catalytic activity, and

regulation of numerous proteins due to the unique properties of sulfur and thiol. These versatile functions exploit unique aspects of sulfur chemistry as described above. The high nucleophilicity of thiols facilitates the role of cysteine as an active-site covalent catalyst. In addition, oxidized derivatives of cysteine, e.g. cystine, form disulfide bonds which stabilize the tertiary structure of proteins. Due to its high reactivity, cysteine is also involved in synthesis of glutathione, which plays an important role in protection from oxidative stress. Glutathione, an enzymatically synthesized tripeptide composed of glutamate, cysteine, and glycine, and its derivatives have a critical function as redox buffers detoxifying noxious oxygen species including hydrogen peroxide. The high nucleophilicity of the cysteine residue of glutathione allows this compound to scavenge and detoxify electrophiles. The easy formation and low reactivity of sulfur-free radicals permit glutathione to capture and detoxify the more reactive free radicals of oxygen and carbon (Meister and Anderson, 1983; Doelman and Bast, 1990). Glutathione also plays a role in detoxification of xenobiotics and herbicides (Rea *et al.*, 1998) and functions as a stress signal and a trigger for development (Sanchez-Fernandez *et al.*, 1997). Glutathione also serves as a store of cysteine and reduced sulfur, and as a signal for the regulation of sulfur assimilation (Noctor *et al.*, 1998). Insufficient availability of glutathione leads to reduced cell survival *in vitro* and also hemolytic anemia and neurological abnormalities *in vivo* (Grimble and Grimble, 1998; Dringen *et al.*, 1999; Wullner *et al.*, 1999; Ceccon *et al.*, 2000). Two molecules of glutathione are conjugated with one molecule of spermidine to form trypanothione (N^1,N^8-bis(glutathionyl)spermidine), a glutathione derivative that was initially thought to be present only in kinetoplastids (Fairlamb *et al.*, 1985). However, a related glutathionylspermidine was later demonstrated in other organisms including *Escherichia coli* (Bollinger *et al.*, 1995; Smith *et al.*, 1995). Trypanothione metabolism and its physiological functions such as the detoxification of peroxide and the maintenance of intracellular redox balance have been reviewed by Fairlamb and Cerami (1992), Walker, J. and Barrett (1997), Flohe *et al.* (1999), Rahlfs *et al.* (2002), Steenkamp (2002), Krauth-Siegel and Inhoff (2003), Muller, S. *et al.* (2003) and Turrens (2004). Polyamine metabolism, described below, is also a part of

trypanothione biosynthesis. Details of polyamine synthesis in protozoan parasites are given in previous reviews (Yarlett and Bacchi, 1994; Marton and Pegg, 1995; Muller, S. *et al.*, 2001; Bacchi and Yarlett, 2002; Heby *et al.*, 2003; Kaiser *et al.*, 2003).

Cysteine also serves as a precursor of important biomolecules including coenzyme A, cysteamine, and taurine. Coenzyme A and acyl carrier protein, which contains 4-phosphopantothenic acid, mediate various acyl transfer reactions involving thioesters of the cysteamine residue of these molecules; the poor resonance stability of thioesters serves to maintain the high transfer potential of the acyl group (Stipanuk, 2004). Taurine has important physiological roles in detoxification, bile acid formation, membrane stabilization, and neurotransmission (Stipanuk, 2004). Cysteine is also further utilized for the production of ubiquitous iron–sulfur clusters, which play various important roles in electron transfer, redox regulation, nitrogen fixation, and sensing for regulatory processes (Beinert *et al.*, 1997), and are probably present in all living organisms.

Methionine is also an important constituent of proteins. Although methionine is not involved in interpeptide bond formation like cysteine, it is also vulnerable to oxidation (forming methionine sulfoxide). Thus, oxidation and reduction of methionine in a protein are involved in the regulation of enzymatic activities (Sun *et al.*, 1999). In addition, methionine plays various roles through its activated intermediate *S*-adenosylmethionine (AdoMet) (see Section 2.2.2.).

2.2.2. Functions of AdoMet

In all organisms, most of the intracellular methionine is converted into AdoMet (Figure 1), a sulfonium compound which serves in transfer reactions as donor of a methyl moiety to various molecules including bases of nucleic acids, arginine, histidine and lysine residues of proteins, and phosphatidylethanolamine (Stipanuk, 1986). In fact, AdoMet serves as the methyl donor in essentially all known biological methylation reactions, with the notable exception of those involved in methylation of homocysteine (Stipanuk, 2004). AdoMet is also utilized for the formation of polyamines including spermidine

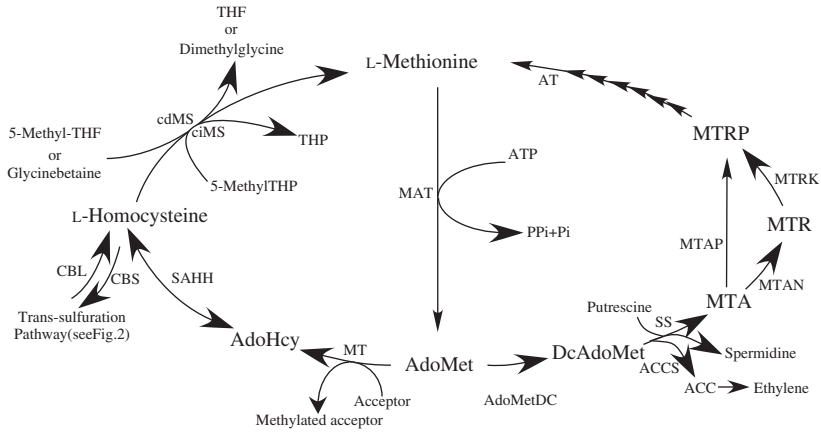

Figure 1 A general scheme of methionine activation and methionine regeneration. The central portion of the scheme represents methionine activation. The left and right halves represents methionine recycling and the MTA cycle, respectively. The abbreviations of substrates and products are: ACC, 1-aminocyclopropane-1-carboxylic acid; AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; DcAdoMet, decarboxylated AdoMet; MTA, 5′-methylthioadenosine; MTR, 5′-methylthioribose; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; THF, tetrahydrofolate; and THP, tetrahydropteroyltriglutamate. The abbreviations of enzyme names are: ACCS, 1-aminocyclopropane-1-carboxylic acid synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14); AdoMetDC, *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50); AT, various aminotransferases (e.g. glutamine/tyrosine/aspartate transaminase, EC 2.6.1.X); CBL, cystathionine-β-lyase (β-cystathionase, EC 4.4.1.8); CBS, cystathionine β-synthase (EC 4.2.1.22); cdMS, cobalamin-dependent methionine synthase (5-methyltetrahydrofolate-homocysteine *S*-methyltransferase, EC 2.1.1.13, or betaine-homocysteine *S*-methyltransferase, EC 2.1.1.5); ciMS, cobalamin-independent methionine synthase (5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase, EC 2.1.1.14); MAT, methionine adenosyltransferase (*S*-adenosyl-L-methionine synthetase, EC 2.5.1.6); MT, various methyltransferases (EC 2.1.1.X); MTAN, 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (EC 3.2.2.9); MTAP, 5′-methylthioadenosine phosphorylase (EC 2.4.2.28); MTRK, *S*-methyl-5-thioribose kinase (EC 2.7.1.100); SAHH, adenosylhomocysteinase (*S*-adenosyl-L-homocysteine hydrolase, EC 3.3.1.1); and SS, spermidine synthase (EC 2.5.1.16) or spermine synthase (EC 2.5.1.22).

and spermine via decarboxylated AdoMet (DcAdoMet), which is produced from AdoMet by AdoMet decarboxylase (AdoMetDC, EC 4.1.1.50; Figure 1). Polyamines are also involved in the control of various biological processes including regulation of transcription and translation of many genes, modulation of enzyme activities, regulation of ion channels, and the formation of hypusine, and an essential post-translational modification of eukaryotic translation initiation factor 5A (eIF-5A) (Pegg, 1988; Marton and Pegg, 1995; Bachrach *et al.*, 2001; Casero and Woster, 2001; Hillary and Pegg, 2003). Elevated polyamine levels were demonstrated in rapidly growing cells (Tabor and Tabor, 1984; Pegg, 1988). It was also shown that biosynthesis of large quantities of polyamines from methionine, together with cysteine and phospholipids, is essential immediately before DNA replication (Marton and Pegg, 1995). Ethylene, a plant hormone, which plays an important role in shoot and root growth, leaf abscission, fruit ripening, flower senescence, healing and defense against wounding, pathogen attack, and environmental stress (Parsons, B.L. and Mattoo, 1991), is also synthesized from AdoMet via 1-aminocyclopropane-1-carbocylic acid (ACC). ACC is produced from AdoMet by ACC synthase (EC 4.4.1.14) (Fu, P.C. *et al.*, 1979; Yu *et al.*, 1979). In prokaryotes, it was also shown that AdoMet is utilized for transfer of the aminocarboxyl moiety to the uridilyl residue (Nishimura *et al.*, 1974).

2.3. Significance of Sulfur-Containing Amino Acids in Parasitic Protozoa

2.3.1. Significance of Methionine in Parasitic Protozoa

As in other organisms, sulfur-containing amino acids and their metabolic intermediates play indispensable roles in all parasitic protozoa. The significance of methionine for cell proliferation has been appreciated in parasites by analogy with prokaryotic and other eukaryotic organisms. It was previously shown that *Plasmodium falciparum* apparently requires extracellular methionine in addition to that obtained by proteolysis of the hemoglobin of red blood cells to sustain

growth (Sherman, 1979). It was also shown that extracellular methionine was required for the growth of *Trypanosoma brucei* procyclic (Brun and Schonenberger, 1979) and bloodstream (Duszenko *et al.*, 1992) forms and also for promastigotes of *Leishmania donovani* and *L. braziliensis* (Steiger and Steiger, 1977) in chemically semidefined media.

2.3.2. *Requirement of Extracellular Cysteine for Anaerobic or Microaerophilic Protozoa*

It has been demonstrated that extra cysteine in the culture milieu is required for growth, attachment, and survival against oxidative stress of *Entamoeba histolytica* and *Giardia duodenalis* (Gillin and Diamond, 1980a, b, 1981a, b; also see Section 4.3). It should be noted that the requirement of L-cysteine and other reducing compounds, i.e. D-cysteine, L-cystine, ascorbic acid, dithiothreitol, and β-mercaptoethanol, for growth and antioxidative activity varies between these two parasitic species. For instance, L-cysteine, D-cysteine, or ascorbic acid, but neither dithiothreitol nor β-mercaptoethanol, supported the growth and defence against hydrogen peroxide of *E. histolytica*, while all of these compounds protected *G. duodenalis* from oxidative killing, but only L-cysteine supported growth of *G. duodenalis* trophozoites (Gillin and Diamond, 1981b). The observed differences in the requirement of extracellular reducing agents between these parasites suggest that cysteine is involved in distinct biological processes in these parasites, and retrospectively agreed well with current understanding of the presence or absence of the *de novo* cysteine biosynthetic pathway in *E. histolytica* and *G. duodenalis*, respectively (see Section 4.2).

2.3.3. *Significance of Cysteine as an Antioxidant in* Entamoeba histolytica, Giardia duodenalis *and* Trichomonas vaginalis

It has previously been shown that, when parasites were cultured in a glutathione-depleted medium, cysteine was the major thiol molecule

in *Entamoeba histolytica* (Fahey *et al.*, 1984) and *Giardia duodenalis* (Gillin and Diamond, 1981a, b; Brown *et al.*, 1993). Cysteine was present largely in a thiol form, i.e. reduced but not oxidized (Fahey *et al.*, 1984). It has been assumed that cysteine plays a major role in maintaining redox balance of thiol compounds in microaerophiles. Although *Entamoeba* is known as an anaerobic or microaerophilic protozoon, it consumes oxygen (Takeuchi *et al.*, 1979) and thus probably produces toxic oxygen derivatives, similar to aerophilic organisms. Although these protozoa possess superoxide dismutase (Bruchhaus and Tannich, 1994; Mehlotra, 1996) for detoxification of the reactive oxygen intermediates, they lack catalase, peroxidase, and enzymes for glutathione biosynthesis, reduction, and regeneration (Weinbach and Diamond, 1974; Fahey *et al.*, 1984; Mehlotra, 1996). Instead, these microaerophilic protozoan parasites possess alternative mechanisms including cysteine (Mehlotra, 1996), NADPH:flavin oxidoreductase (Eh34) (Bruchhaus *et al.*, 1998) and NAD(P)H peroxidase (only *Giardia*). Recent genome-wide survey also revealed that *E. histolytica* possesses rubrethrin, an uncommon reductant distributed mainly in anaerobic prokaryotes (Loftus *et al.*, 2005).

2.3.4. *Physiological Importance of Cysteine in* Trypanosoma *and* Leishmania

The significance of cysteine in *Trypanosoma* and *Leishmania* may be partially attributable to the production of trypanothione, which is involved in the detoxification of reactive oxygen species in these organisms (Muller, S. *et al.*, 2003). The amount of trypanothione, glutathione covalently linked to spermidine, varies among species, e.g. *Trypanosoma brucei*, *T. cruzi*, *Leishmania* spp., and *Crithidia fasciculata*, a monoxenous parasite of arthropods, and also among developmental stages (Fairlamb, 1989; Ariyanayagam and Fairlamb, 2001; Ariyanayagam *et al.*, 2003). Trypanothione and its metabolism were recently reported in *Entamoeba histolytica* by Ondarza *et al.* (1999), but this is still controversial (Ariyanayagam and Fairlamb, 1999; Tamayo *et al.*, 2005). The essentiality of cysteine for growth of bloodstream forms of *T. brucei* was demonstrated using a cysteine-free

minimum essential medium (Duszenko *et al.*, 1992). L-Cysteine alone or a mixture of L-cystine and reducing agents (monothioglycerol or 2-mercaptoethanol), but none of the reducing agents, or D-cysteine alone supported cell growth. Since cystine is not incorporated by bloodstream-form trypanosomes (Duszenko *et al.*, 1985), L-cysteine was proposed to be an essential growth factor (Duszenko *et al.*, 1992).

3. METABOLISM OF SULFUR-CONTAINING AMINO ACIDS

3.1. An Overview of Metabolism of Sulfur-Containing Amino Acids

Animals including humans and other non-ruminants have a dietary requirement for methionine as they are incapable of *de novo* production of cysteine. They take methionine from the diet and ultimately oxidize its sulfur to inorganic sulfate. Plants, bacteria, and fungi play an indispensable role in nature in completing the sulfur cycle by reductive assimilation of inorganic sulfate to cysteine and methionine, and thus providing the source of methionine necessary for animals. Plants in particular are the most important producers of sulfur-containing amino acids, assimilating an estimated 4.6×10^{11} kg of inorganic sulfur per annum (Anderson, 1978).

Aspects of methionine metabolism discussed in this review include methionine activation, methionine recycling, and transsulfuration leading to cysteine production in fungi and mammals. Two major pathways are currently known for methionine recycling: a relatively common recycling pathway utilizing homocysteine methyltransferase (methionine synthase) as a final enzyme and the so-called methionine thioadenosine cycle. Cysteine metabolism discussed in this review includes sulfur assimilatory *de novo* cysteine synthesis, transsulfuration in the direction of cysteine to homocysteine found mainly in bacteria and plants, and cysteine degradation.

3.2. Methionine Metabolism

3.2.1. A General Scheme of Methionine Activation

Methionine is activated by the transfer of an adenosyl group of ATP to methionine in a reaction catalyzed by methionine adenosyltransferase (MAT, EC 2.5.1.6; Figure 1) to produce AdoMet. Two sequential enzymatic activities have been shown for all MAT enzymes described to date. The first activity catalyzes the formation of AdoMet and tripolyphosphate (PPPi), while the second activity catalyzes hydrolysis of PPPi to give rise to pyro- and orthophosphate, in a reaction strongly induced by AdoMet (Chiang and Cantoni, 1977; Mato *et al.*, 1990; Kotb and Geller, 1993). MAT genes often exist as multiple isoenzymes in many organisms including humans (Kotb and Kredich, 1985), *Escherichia coli* (Takusagawa *et al.*, 1996) and rats (Mingorance *et al.*, 1997). Two MAT isoenzymes showing different tissue distributions and subunit structures have been described in mammals. MAT I/MAT III (Alvarez *et al.*, 1993) are expressed only in the liver, while MAT II is expressed in all tissues (De La Rosa *et al.*, 1995). MAT I and MAT III are composed of two and four identical catalytic α_1 subunits, respectively (Alvarez *et al.*, 1993). MAT II is a heterotetrameric enzyme consisting of two catalytic subunits (α_1/α_2) and a β regulatory subunit (LeGros *et al.*, 2000).

AdoMet is involved primarily in the transfer of several possible acceptors of the *S*-methyl group including glycine (forming sarcosine), guanidoacetate (forming creatine), phosphatidylethanolamine (forming phosphatidylcholine), purine and pyrimidine bases of tRNA, and various xenobiotics bearing hydroxyl, amino, or sulfhydryl groups (Cantoni, 1975). It is estimated that about 95% of the AdoMet formed is consumed in the methyl transfer reactions (Mudd and Poole, 1975). AdoMet also serves as a substrate in the production of DcAdoMet, in a reaction catalyzed by AdoMetDC (Figure 1), which is further utilized for the formation of spermidine from putrescine and of spermine from spermidine. In these reactions, DcAdoMet serves as a donor of aminopropyl groups with concomitant formation of methylthioadenosine (MTA) as a by-product. MTA is further recycled back to methionine in the so-called methionine

thioadenosine cycle involving 9–10 steps (see Section 3.2.2(c), Schwartz and Shapiro, 1954; Trackman and Abeles, 1983; Furfine and Abeles, 1988; Myers *et al.*, 1993; Wray and Abeles, 1993). AdoMet has also been indicated as a potential source of 5′-deoxyadenosyl radicals in a large number of reductive metabolic processes (Fontecave *et al.*, 2001). While AdoMet synthesis is present in a wide variety of cells, its transport has also been demonstrated in a few organisms including *Saccharomyces cerevisiae* (Murphy and Spence, 1972), *Pneumocystis carinii* (Merali *et al.*, 2000), *Rickettsia prowazekii* (Tucker *et al.*, 2003), and rats (liver mitochondria) (Horne *et al.*, 1997).

3.2.2. A General Scheme of Two Pathways for Methionine Recycling

(*a*) *S-adenosylhomocysteine hydrolase* (*SAHH*) *is involved in both the salvaging of methionine and reverse transsulfuration.* Methylation using AdoMet as a methyl donor yields *S*-adenosylhomocysteine (AdoHcy) as a by-product, which is further hydrolyzed to regenerate adenosine with concomitant production of homocysteine. Homocysteine then enters one of two pathways: regeneration of methionine by homocysteine methyltransferase (see Section 3.2.2(b)) or via the reverse transsulfuration (or transsulfuration) pathway (Section 3.3). In the reverse transsulfuration pathway, AdoMet is ultimately converted into cysteine via cystathionine. Transsulfuration was first described in mammalian liver (Vigneaud, 1952). In the 1960s transsulfuration in the opposite direction was found in bacteria (Kaplan and Flavin, 1966) and fungi (Nagai and Flavin, 1967), in the latter occurring in both directions. In this review, the terminology of 'forward' and 'reverse' transsulfuration indicates reactions leading from cysteine to methionine (via homocysteine) and from methionine to cysteine, respectively, according to Soda (1987) and Steegborn *et al.* (1999) (see also the detailed description in Section 3.3.1).

AdoMet is first demethylated by specific or non-specific methyltransferase to form AdoHcy. AdoHcy is reversibly hydrolyzed to adenosine and homocysteine in a reaction catalyzed by AdoHcy hydrolase (SAHH, EC 3.3.1.1; Figure 1), which plays a crucial role by

removing AdoHcy to regulate the overall methylation capacity of the cell, often expressed as the methylation index (the ratio of AdoMet to AdoHcy). It is important to mention that the equilibrium constant of the reaction by SAHH favors AdoHcy synthesis, and AdoHcy is a strong competitive inhibitor of the AdoMet-dependent methylases (Hoffman *et al.*, 1981). Thus, further utilization or degradation of AdoHcy and homocysteine to maintain their low concentrations is critical for various methylation reactions to occur efficiently in the cell (Walker, R.D. and Duerre, 1975).

(*b*) *Homocysteine methyltransferase* (*methionine synthase*) *is involved in the final step of the methionine production from homocysteine*. Synthesis *de novo* of methionine from cysteine or homocysteine (also known as the forward transsulfuration pathway, Figure 2) has been reported in some bacteria, fungi and plants (reviewed by Thomas and Surdin-Kerjan, 1997; Ravanel *et al.*, 1998; Sekowska *et al.*, 2000; also see Section 3.2.2(a)). Although mammals do not possess a complete forward transsulfuration pathway, they utilize part of this methionine biosynthetic pathway for the recycling of methionine. Since the forward transsulfuration pathway, which forms methionine from cysteine, is energetically expensive, mammals dispense of most of the enzymes involved in this pathway except for vitamin B_{12} (cobalamin)-dependent homocysteine methyltransferase catalyzing the last step of methionine production (Figure 1). This final step is mainly catalyzed by 5′-methyltetrahydrofolate-homocysteine *S*-methyltransferase (EC 2.1.1.13, also called methionine synthase), which utilizes N^5-methyltetrahydrofolate as the major methyl donor *in vivo* under normal nutritional conditions (Finkelstein *et al.*, 1971; Finkelstein, 1974; Finkelstein and Martin, 1986). In mammals, this final step of methionine recycling is also catalyzed in part by betaine-homocysteine methyltransferase (EC 2.1.1.5) using betaine as a methyl donor. This enzyme is distributed in only a few organs including liver, where its expression is induced by high protein diet or high methionine intake, suggesting that it is involved in decomposition of sulfur-containing amino acids via homocysteine (Finkelstein and Martin, 1986). In bacteria (Whitfield *et al.*, 1970), yeasts (Burton and Sakami, 1971), and fungi (Burton and Metzenberg, 1975), which are incapable of producing vitamin B_{12}, this reaction is also catalyzed by the vitamin

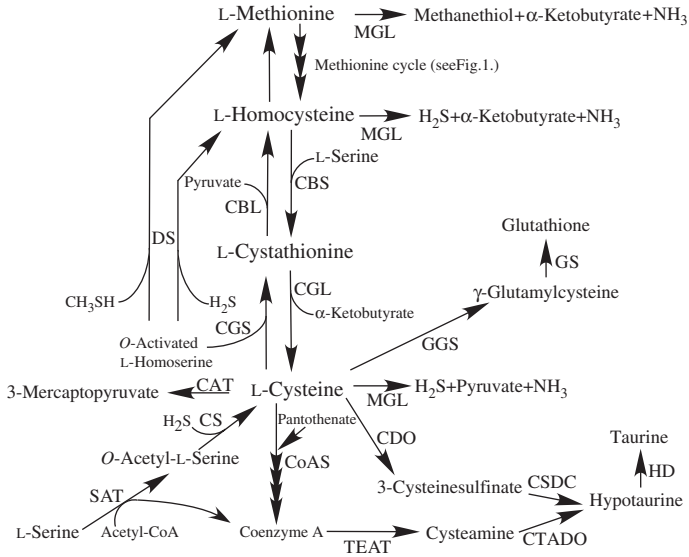

Figure 2 A general scheme of transsulfuration and sulfur assimilatory *de novo* cysteine biosynthetic, and sulfur-containing amino acid degradation pathways. The abbreviations used are: CAT, cysteine aminotransferase (EC 2.6.1.3); CBL, cystathionine β-lyase (β-cystathionase, EC 4.4.1.8); CBS, cystathionine β-synthase (serine sulfhydrase, EC 4.2.1.22); CDO, cysteine dioxygenase (EC 1.13.11.20); CGL, cystathionine γ-lyase (homoserine deaminase, EC 4.4.1.1); CGS, cystathionine γ-synthase (*O*-succinylhomoserine (thiol)-lyase, EC 2.5.1.48); CoAS, coenzyme A synthetic pathway; CS, cysteine synthase (*O*-acetyl-L-serine sulfhydrylase, EC 2.5.1.47); CSDC, cysteine-sulfinate decarboxylase (EC 4.1.1.29); CTADO, cysteamine dioxygenase (EC 1.13.11.19); DS, direct sulfhydrylation (*O*-acetyl-L-homoserine sulfhydrylase, methionine synthase, *O*-acetylhomoserine (thiol)-lyase, EC 2.5.1.49); GGS, γ-glutamylcysteine synthase (EC 6.3.2.2); GS, glutathione synthase (EC 6.3.2.3); HD, hypotaurine dehydrogenase (EC 1.8.1.3); MGL, methionine γ-lyase (L-methioninase, EC 4.4.1.11); SAT, serine *O*-acetyltransferase (EC 2.3.1.30); and TEAT, thioethanolamine *S*-acetyltransferase (EC 2.3.1.11).

B_{12}-independent enzyme known as 5′-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase (EC 2.1.1.14).

(*c*) *MTA cycle is involved in the salvaging of methionine*. There is a second route, referred as the MTA cycle or methionine salvage pathway, for methionine recycling. This pathway is present in many organisms including yeasts (Marchitto and Ferro, 1985), protozoa

(Ghoda *et al.*, 1988; Riscoe *et al.*, 1988), rats (Wray and Abeles, 1995) and plants (Van der Straeten *et al.*, 1990). These organisms regenerate methionine from MTA in seven or eight reactions (Myers *et al.*, 1993; Heilbronn *et al.*, 1999; Figure 1). Although this pathway has been studied in a number of organisms, the complete pathway has been fully delineated only in *Klebsiella pneumoniae* by Wray and Abeles (1995), where a series of unusual enzymes involved in the formation of methionine from MTA through α-ketomethiobutyrate (KMTB), which is a final key intermediate leading to methionine, was discovered. The MTA cycle shares its first half with the polyamine biosynthetic pathway. AdoMet is produced through decarboxylation of AdoMet by AdoMetDC as described above. The aminopropyl moiety of methylthiopropylamine is subsequently transferred to putrescine to form spermidine (spermidine synthase, EC 2.5.1.16) or to spermidine to form spermine (spermine synthethase, EC 2.5.1.22). In plants the aminopropyl moiety is transferred from methylthiopropylamine to ACC to produce ethylene by ACC synthase. These reactions concomitantly form MTA. Subsequent paths of the MTA metabolism, leading to the production of methionine, differ among organisms (Figure 1). In mammals, MTA is hydrolyzed by MTA phosphorylase (MTAP, EC 2.4.2.28) (Pegg and Williams-Ashman, 1969) to form methylthioribose-1-phosphate and adenine. In contrast, in prokaryotes MTA is hydrolyzed to 5′-methylthioribose (MTR) and adenine by 5′-methylthioadenosine nucleosidase (MTAN, EC 3.2.2.9, also called AdoHcy nucleosidase) (Duerre, 1962; Ferro *et al.*, 1976), followed by further phosphorylation of MTR by MTR kinase (MTRK, EC 2.7.1.100, Ferro *et al.*, 1978) at the expense of ATP to produce MTR 1-phosphate. Subsequently, MTR 1-phosphate is converted into methionine via a diketo intermediate and KMTB to complete the methionine cycle. The final step of the MTA recycling pathway appears to be catalyzed by various aminotransferases (AT, EC 2.6.1.X). Although glutamine was initially implicated as an amino donor for this reaction (Cooper, J.L. and Meister, 1972), various amino acids including aspartate, tryptophan, phenylalanine, tyrosine, and glutamate have been reported as amino donors for this reaction (Hall *et al.*, 1993; Berger *et al.*, 1996, 1998; Heilbronn *et al.*, 1999).

3.2.3. *Ubiquity and Significance of Methionine Activation in Parasitic Protozoa*

(*a*) *Genome-wide survey of genes involved in sulfur-containing amino acid metabolisms*. In order to evaluate the distributions of enzymes involved in sulfur-containing amino acid metabolism among protozoan parasites and to understand how ubiquitous they are, we conducted a wide-scale search of all the known enzymes involved in methionine activation and regeneration (see Sections 3.2.3–4), transsulfuration (Section 3.3.4), sulfur assimilatory cysteine biosynthesis (Section 4.2), degradation of sulfur-containing amino acids (Section 5.2), and serine metabolism (Section 6.2) using public genome databases available at the Institute of Genomic Research (TIGR, http://www.tigr.org/tdb/e2k1/tca1/, http://www.tigr.org/tdb/e2k1/pfa1/, http://www.tigr.org/tdb/e2k1/tvg/), the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/projects/E_histolytica/, http://www.sanger.ac.uk/projects/L_major/, http://www.sanger.ac.uk/projects/T_brucei/), National Center of Biotechnology Information (NCBI, http://www.ncbi.nih.gov/) and GiardiaDB (http://gmod.mbl.edu/perl/site/giardia?page=intro). Only representative species were used for the survey to ensure maximal coverage of putative genes. We chose three anaerobic and amitochondrial protozoa (*Entamoeba histolytica*, *Giardia duodenalis* and *Trichomonas vaginalis*), two apicomplexan parasites (*Plasmodium falciparum* and *Cryptosporidium parvum*) and three trypanosomatids (*Trypanosoma cruzi*, *T. brucei* and *Leishmania major*). The genome drafts were complete and regularly updated for *P. falciparum* (Gardner *et al.*, 2002), *C. parvum* (Abrahamsen *et al.*, 2004), and *E. histolytica* (Loftus *et al.*, 2005). The *G. duodenalis* database contains approximately 11-fold genome sequence data. The genome databases of *T. brucei* (complete or close to completion for 8 of 11 chromosomes (I–VIII) and 4–9-fold coverage for chromosomes IX–XI; approximately 13 000 protein-coding genes were predicted in the ~25.5 Mb haploid genome), *T. cruzi* (19-fold coverage; http://www.genedb.org/genedb/tcruzi/index.jsp) and *L. major* (approximately 8300 genes over 36 chromosomes were predicted in the ~33.6 Mb haploid genome; http://www.genedb.org/genedb/leish/index.jsp) are close to the final stage and sufficient for gene-finding purposes. Owing to the unexpectedly

large size of the *T. vaginalis* genome (160–180 Mb) (J. M. Carlton, personal communication), the coverage of the *T. vaginalis* database cannot be predicted.

We identified putative homologs by blastp and tblastn search against protein and nucleotide databases of the genome database of these parasites using protein sequences from *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana* and *Homo sapiens*. We designated homologs when proteins revealed $>30\%$ overall amino acid identity and the signature motifs, domains and residues were conserved. To differentiate pyridoxal 5′-phosphate (PLP)-dependent enzymes, protozoan sequences as well as homologs from other organisms were subjected to phylogenetic analyses by the neighbor joining method using Clustal W. If a sequence formed a statistically significant (supported by high i.e., $>60\%$, bootstrap proportions) relationship with homologs possessing known enzymological characteristics, the protein was assumed to possess a homologous enzyme function. If no homolog was present in the organisms listed above (e.g. methionine γ-lyase), other prokaryotic organisms and protists (e.g. archaeal *Methanosarcina* species and *E. histolytica*) were used for both blast search and phylogenetic analyses.

(*b*) *Ubiquity of methionine activation in parasitic protozoa*. With one exception, all the protozoan parasites included in the review (*E. histolytica*, *T. vaginalis*, *P. falciparum*, *C. parvum*, *T. brucei*, *T. cruzi* and *L. major*) possess genes encoding MAT and SAHH; the exception, *G. duodenalis*, possesses MAT but lacks SAHH (Table 1). This conservation indicates that activation of methionine by MAT and regulation of methylation capacity through SAHH are widespread among parasitic protozoa as in other prokaryotic and eukaryotic organisms (Cantoni, 1975).

(*c*) *MAT in parasitic protozoa.* MAT activity has been previously demonstrated in many protozoan parasites. Two MAT isoenzymes in bloodstream forms of *Trypanosoma brucei* with different kinetic constants were shown to be insensitive to allosteric inhibition by AdoMet (Yarlett and Bacchi, 1988a; Yarlett *et al.*, 1993), in contrast to mammalian MAT (Lu, 2000). The insensitivity of trypanosomal MAT to AdoMet is responsible in part for the well-studied trypanocidal effects of DL-α-difluoromethylornithine (DFMO) (Bacchi and Yarlett,

1993), which is used therapeutically in *T. b. rhodesiense* infections (Sjoerdsma and Schechter, 1984; Van Nieuwenhove *et al.*, 1985; Bacchi *et al.*, 1987). It is well accepted that DFMO is a specific inhibitor of ornithine decarboxylase (ODC), the first enzyme of polyamine biosynthesis (Figure 1), and causes accumulation of polyamine precursors including AdoMet and DcAdoMet in trypanosomes (Yarlett *et al.*, 1993). Since these trypanosomes are capable of utilizing exogenous polyamines very poorly, and also lack the interconversion pathway between spermine and spermidine, they rely on *de novo* synthesis of polyamines (Fairlamb and LeQuesne, 1997), indicating that the trypanocidal effect of DFMO is attributable to the inhibition of polyamine and trypanothione biosynthesis (Fairlamb and Henderson, 1987; Fairlamb *et al.*, 1987; Bitonti *et al.*, 1988). In addition to perturbation of polyamine and trypanothione synthesis, the abnormally high transmethylation activity, which is caused by the unregulated synthesis of AdoMet due to AdoMet-insensitive MAT, has also been reported to be responsible for the trypanocidal effect of DFMO (Bacchi and Yarlett, 1993; Bacchi *et al.*, 1993).

[(*Z*)-4-Amino-2-butenyl]methylamino-5′-deoxyadenosine (MDL 73811), an enzyme-activated irreversible inhibitor of AdoMetDC, was also found to be very effective against *T. b. brucei* infections in mice and rats. The fact that bloodstream trypanosomes disappeared before polyamine depletion was detected strongly suggested that the antitrypanosomal effect of the drug was mainly attributable to an increase in the level of AdoMet and not polyamine depletion (Byers *et al.*, 1991). Cloning and characterization of a recombinant MAT from *Leishmania infantum* (Reguera *et al.*, 1999, 2002) and *L. donovani* (Perez-Pertejo *et al.*, 2003) suggested that *Leishmania* MAT was also partially insensitive to allosteric inhibition by AdoMet. MAT activity was also demonstrated in *Trichomonas vaginalis* and *T. foetus* (Thong *et al.*, 1987a). Although it was shown that *T. vaginalis* ODC was sensitive to inhibition by DFMO (Yarlett *et al.*, 1992), the methylation index was not changed by DFMO treatment in *T. vaginalis* (Yarlett and Bacchi, 1988b), probably due to the absence of polyamine biosynthesis from *Trichomonas*.

Recombinant *Cryptosporidium parvum* MAT was shown to be competitively inhibited by a methionine analog, cycloleucine;

Table 1 Enzymes involved in the methionine cycle, transsulfuration, sulfur assimilatory cysteine biosynthesis and degradation of sulfur-containing amino acids in parasitic protozoa[a]

Organisms	Enzymes															
	Methionine cycle								Transsulfuration				Sulfur assimilation		Degradation	
	MAT	SAHH	Ado Met DC	MTAN	MTAP	Asp AT	cdMS	ciMS	Forward CGS	Forward CBL	Reverse CBS	Reverse CGL	SAT	CS	MGL	CDO
E. histolytica	+	+	–	+	–	+[b]	–	–	–	–	–	–	+	+	+	–
G. duodenalis	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
T. vaginalis	+	+	–	+	+	+	–	+	–	–	–	–	–	+	+	–
P. falciparum	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–
C. parvum	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
T. brucei	+	+	+	–	+	+	+	+	–	–	+	+[c]	–	–	–	–
T. cruzi	+	+	+	+	+	+	–	–	–	–	+	+[c]	+	+	–	–
L. major	+	+	+	–	+	+	–	–	–	–	+	+[c]	–	–	–	–

Accession numbers of putative homologs (either protein or nucleotide) are as follows: *E. histolytica*, MAT (2390555.c000316748), SAHH (2390555.c000227129), MTAN (2390555.c000506367), AspAT (2390555.c000505226), SAT (AB023954), CS1 (AB000266), CS2 (AB006900), MGL1 (AB094499), MGL2 (AB094500); *G. duodenalis*, MAT (AACB01000070), MTAP (AY132352), AspAT

(AF326991); *T. vaginalis*, MAT (38080.m00046, 39085.m00036, 56641.m00042,38383.m00037, 50773.m00002, 39081.m00025), SAHH (43465.m00075), MTAN (38081.m00042), MTAP (61898.m00072, 55953.m00025), AspAT (43456.m00085, 43271.m00035, 40895.m00018, 40895.m00017), ciMS (53871.m00008, 39399.m00130, 49217.m00002), CS (39878.m00035), MGL1 (AJ000486), MGL2 (AJ000487); *P. falciparum*, MAT (AL929357), SAHH (2272.m00210), AdoMetDC (AF094833), AspAT (1400.m02475); *C. parvum*, MAT (AB119646), SAHH (AAEE01000015); *T. brucei*, MAT (Tb06.30P15.650), SAHH (Tb11.01.1350), AdoMetDC (Tb06.26G9.750), MTAP (Tb07.21H15.360), AspAT (AF326988, AF326989, AF326990, AF326991), cdMS (AL929604), ciMS (Tb08.26A17.600), CBS (Tb11.02.5400), CGL (Tb09.211.3330); *T. cruzi*, MAT (5924.m00005), SAHH (8730.m00020, 6080.m00005), AdoMetDC (AAC26796, AAC33263), MTAN [1047053469207(4486-5142)], MTAP (AY144609), AspAT (AY762327, 6015.m00007), CBS (AF296842, AF296843, AF296844, AF296845, AF296846, 7625.m00036, 5907.m00001, 7626.m00011, 7652.m00014), CGL (8458.m00025), SAT (AF296848, 4865.m00004), CS (7472.m00002, 5442.m00005); and *L. major*, MAT (LmjF30.3520), SAHH (LmjF36.3910), AdoMetDC (LmjF30.3110, LmjF30.3120), MTAP (AL157415), AspAT (AAG18601), CBS (LmjF17.0250), CGL [LMFP1295(87875-89392)].

[a]See the legend of Figure 3 for abbreviations used in this table; + and – symbols denote presence or absence of the gene.

[b]This putative *E. histolytica* AspAT is denoted AlaAT in the genome database.

[c]These putative CGL showed only marginal kinship with CGL from other organisms in a phylogenetic reconstruction using CGL, CGS, CBL and MGL belonging to α-type PLP-dependent enzymes and thus may be a functional homolog of other members of the family.

however, allosteric inhibition by AdoMet was not investigated (Slapeta *et al.*, 2003). Putative MAT genes from other *Cryptosporidium* spp. (*C. hominis*, *C. meleagiridis* and *C. parvum* of a ferret genotype) (personal observation) and *E. histolytica* (Loftus *et al.*, 2005) have also been identified. Interestingly, these MAT genes form a monophyletic clade with MAT from *P. falciparum*, while other protozoa, i.e. *T. vaginalis, T. brucei*, *T. cruzi* and *L. infantum*, form a statistically well separated monophyletic clade, together with plants. Therefore, protozoan MAT genes seem to be derived from at least two distinct ancestral organisms. Molecular modeling of MAT from *P. falciparum* based on the X-ray crystal structure of *Escherichia coli* MAT revealed that the subunit structure of *P. falciparum* MAT is probably a dimer, and differs from that of *Escherichia coli* MAT, which is a tetramer (Chiang *et al.*, 1999). Since *Plasmodium* and human MAT showed comparable sensitivity toward cycloleucine (K_i values of 17 and 10 μM, respectively), cycloleucine or its closely related inhibitors could not be considered as lead compounds to selectively inhibit parasite MAT. A remarkable increase in the polyamine content of erythrocytes, which contain only a trace amount of polyamines and lack active polyamine biosynthesis, following infection with malaria parasites, together with the presence of ODC and AdoMetDC genes (see below) and their activities, indicate that active polyamine anabolism is present in proliferating malaria parasites (Assaraf *et al.*, 1984). Thus, it is still worth investigating whether *Plasmodium* MAT is insensitive to allosteric feedback inhibition as is trypanosomal MAT, and to consider further methionine activation and polyamine metabolism as drug targets for malaria. A unique AdoMetDC–ODC bifunctional enzyme from *Plasmodium* is discussed in Section 3.2.5(a) Since polyamine and trypanothione biosynthesis and their functions as antioxidative stress mechanisms are beyond the scope of this review, the following excellent reviews should be consulted for details (Walker, J. and Barrett, 1997; Flohe *et al.*, 1999; Rahlfs *et al.*, 2002; Steenkamp, 2002; Krauth-Siegel *et al.*, 2003; Muller, S. *et al.*, 2003; Turrens, 2004).

(*d*) *SAHH from parasitic protozoa as a drug target*. Since SAHH is the sole enzyme responsible for the removal of AdoHcy from the cell, inhibition of SAHH causes substantial expansion of the intracellular

pool of AdoHcy as well as significant diminution of the AdoMet/AdoHcy ratio, leading to inhibition of transmethylation reactions (Whaun *et al.*, 1986; Henderson *et al.*, 1992). Various adenosine analogs have been used as potent inhibitors of SAHH. Some of these compounds have previously been tested as antimalaria and anti-leishmaniasis drugs. A carbocyclic analog of adenosine, Neoplanocin A, and its derivatives showed growth inhibitory effects against the intraerythrocytic stages of *Plasmodium falciparum* (Whaun *et al.*, 1986; Nakanishi *et al.*, 2001; Shuto *et al.*, 2002). Since SAHH activity was detected in neither *P. berghei* nor *P. falciparum* isolated from infected erythrocytes, the growth inhibitory effects of Neoplanocin A have been attributed to inhibition of the host SAHH (Bitonti *et al.*, 1990a). However, the SAHH gene and its transcript were demonstrated in *P. falciparum* by Creedon *et al.* (1994) (see also Section 7.2). Furthermore, a series of related adenosine derivatives also showed antimalarial effects, reinforcing the premise that *Plasmodium* SAHH provides a practical target for antimalarial drugs (Kitade *et al.*, 2000; Nakanishi *et al.*, 2001; Kojima *et al.*, 2002; Kitade *et al.*, 2003). However, further modifications that increase the chemotherapeutic index are required to enable these adenosine analogs to be considered as possible candidates for antimalarial and anticryptosporidiosis drugs.

The growth of *Leishmania donovani* promastigotes was also inhibited by micromolar concentrations of 3-deazaaristeromycine and 9-(*trans*-2′-, *trans*-3′-dihydroxycyclopentanyl) adenine, both of which are well-known inhibitors of mammalian SAHH (Henderson *et al.*, 1992). SAHH activity was also detected in crude homogenates of *Trichomonas vaginalis*, *T. foetus* and *Trichomitus batrachorum* (Thong *et al.*, 1985). Multicopy genes of *T. vaginalis* SAHH (Bagnara *et al.*, 1996) and characterization of the recombinant SAHH, which was inhibited by arabinosyl adenine (Minotto *et al.*, 1998), have also been reported. However, the effects of SAHH inhibition on the growth of *Trichomonas* spp. have not been studied.

(*e*) *AdoMet transporter in* Trypanosoma. AdoMet synthesis is energetically expensive as it consumes one molecule of ATP. Consequently, mammals often scavenge extracellular AdoMet by incorporation with specific AdoMet transporters. It has previously

been demonstrated that uptake of AdoMet was three times faster than AdoMet synthesis (Goldberg *et al.*, 1998). The fact that the serum concentration of AdoMet (70 nM; Stramentinoli, 1987) is higher than that of methionine (26 nM; Blom *et al.*, 1989) also argues for the premise that cellular uptake of AdoMet, but not methionine, is biologically reasonable.

In *Trypanosoma*, the AdoMet level is not regulated by the product inhibition of MAT, but probably by transporter activities (Goldberg *et al.*, 1997, 1998). Putative transporters for AdoMet and an AdoMet analog, sinefungin, were demonstrated in *Leishmania mexicana*, *L. braziliensis* (Avila and Polegre, 1993), *L. donovani* (Phelouzat *et al.*, 1995; Lawrence *et al.*, 1998) and bloodstream forms of *T. brucei* and *T. rhodesiense* (Goldberg *et al.*, 1997, 1999). The molecular identity of the AdoMet transporters in protozoan parasites remains totally unclear as they belong to a large membrane transporter family including amino acid transporters.

3.2.4. Distribution and Significance of the Methionine Recycling Pathways in Protozoa

(*a*) *Homocysteine methyltransferase* (*methionine synthase*) *in parasitic protozoa*. Our gene survey revealed that only two of the protozoan parasites included in this review possess a putative gene involved in methionine regeneration (Table 1): *Trichomonas vaginalis* possesses cobalamine-independent methionine synthase with high similarity to 5′-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase from *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, while *Trypanosoma brucei* has both cobalamine-dependent and cobalamine-independent methionine synthases. The observed redundancy of methionine synthases in *T. brucei* is unique, and has no precedent except for some bacteria, e.g. *Escherichia coli* and *Salmonella typhimurium.* Cobalamine-dependent and cobalamine-independent methionine synthases belong to totally distinct groups of proteins, suggesting that they have arisen by convergent evolution (Gonzalez *et al.*, 1992).

Methyltetrahydrofolate-dependent (cobalamine-dependent) methionine synthase activity was previously demonstrated in erythrocytic

stages of *Plasmodium falciparum* cultured *in vitro* (Krungkrai *et al.*, 1989). However, its biological role *in vivo* has been questioned based on the failure of metabolic labeling of methionine using [^{14}C]serine in *P. falciparum* (Asawamahasakda and Yuthavong, 1993). This agrees well with the lack of a putative gene encoding either 5′-methyltetrahydrofolate-dependent or independent homocysteine transmethylase in the *P. falciparum* genome database.

(*b*) *The presence of the MTA cycle in parasitic protozoa and the development of antiprotozoan compounds against the MTA cycle*. The MTA cycle has been receiving attention because this pathway is viewed as a potential target for the development of new antitrypanosomal drugs (Wang, C.C., 1995). The following reviews on polyamine metabolism regarding the MTA cycle should also be consulted: Marton and Pegg (1995), Muller, S. *et al.* (2001), Bacchi and Yarlett (2002), Heby *et al.* (2003) and Kaiser *et al.* (2003). Most of the previous studies related to the MTA cycle focused mainly on the polyamine biosynthetic pathway, rather than the MTA cycle per se. These studies demonstrated activities of the key enzymes in the pathway and effects of specific inhibitors targeted at the pathway. At least half a dozen enzymes, including AdoMetDC, MTAP, MTAN, MTRK, and aminotransferase are involved in this pathway as mentioned above (Figure 1). AdoMetDC, the first enzyme of the MTA cycle which generates DcAdoMet from AdoMet, has previously been demonstrated in *Trypanosoma cruzi* by Kinch *et al.* (1999) and in *Plasmodium falciparum* by Krause *et al.* (2000) and Muller, S. *et al.* (2000). MTAN and MTRK activities have also been reported in *Giardia duodenalis* and *Entamoeba invadens* by Riscoe *et al.* (1988). It has previously been reported that *P. falciparum* contained a significant amount of MTAN and MTRK activity, but no detectable MTAP activity (Riscoe *et al.*, 1988, 1989). *Trichomonas vaginalis* possesses MTAN, MTAP and aspartate aminotransferase as well as MAT, AdoMet:L-homocysteine methyltransferase, SAHH and ODC, but lacks AdoMetDC, spermidine synthase and spermine synthase (Yarlett and Bacchi, 1988b).

Trypanosoma b. brucei, *G. duodenalis*, *P. falciparum* and *Crithidia fasciculata*, a monoxenous parasite of arthropods, were also shown to possess activity of the final reaction of the MTA cycle: transaminative

conversion of KMTB into methionine using various amino acids as an amino donor (Berger *et al.*, 1996, 1998, 2001).

Our wide-ranging survey of AdoMetDC, MTAN, MTAP, and a putative aminotransferase involved in the MTA cycle revealed intriguing distributions of these enzymes among the parasitic protozoa (Table 1). AdoMetDC is present in *P. falciparum*, *Tryp. brucei* and *Tryp. cruzi*; MTAN is present in *E. histolytica*, *Trich. vaginalis* and *Tryp. cruzi*; and MTAP is present in *G. duodenalis*, *Trich. vaginalis* and three kinetoplastid organisms. This putative aminotransferase gene is probably present in all the species we investigated except *C. parvum.* However, unequivocal functional assignment of these putative AT is not possible since many related AT share significant homology. The limited distribution of the three major genes, i.e. AdoMetDC, MTAN and MTAP, indicates that the MTA cycle may not be operational in most of these parasites, except *Tryp. cruzi*. It is also worth noting that *Trich. vaginalis* and *Tryp. cruzi* probably possess both routes for the production of MTR-1-phosphate, one route involving a single step catalyzed by MTAP and the other route having two steps catalyzed by MTAN and MTRK. The absence of AdoMetDC, spermidine synthase and spermine synthase activities from *Trich. vaginalis* (Yarlett and Bacchi, 1988b) is consistent with the notion that the first step of the MTA cycle (AdoMetDC) and the subsequent polyamine synthesis are not functional, and other enzymes may be involved in the production of the aminopropyl donor and regeneration of methionine in this parasite.

Trypanosoma b. brucei MTAP showed enzymological properties that were quite different from those of mammalian MTAP (Ghoda *et al.*, 1988; Miller, R.L. and Toorchen, 1988). 5′-Deoxy-5′-(hydroxyethyl)thioadenosine (HETA) showed trypanocidal activity *in vivo* with no apparent toxicity to mammals (Bacchi *et al.*, 1991). Although significant MTAN and MTRK activity was demonstrated in *P. falciparum* by Riscoe *et al.* (1988, 1989) and HETA and other MTA analogs inhibited growth of malaria parasites (Sufrin *et al.*, 1995), the absence of the putative target enzymes from the database suggests that the actual target of these compounds may not be the MTA cycle. In addition, the MTA analogs 5′-deoxy-5′-*S*-isobutyladenosine and 5′-deoxy-5′-*S*-isobutyl-3-deazaadenosine showed no antimalarial

effect (Trager *et al.*, 1978, 1980), which agreed well with the absence of possible targets.

3.2.5. *S-Adenosylmethionine Decarboxylase (AdoMetDC) from Protozoan Parasites*

(*a*) *Unique bifunctional AdoMetDC/ODC from* Plasmodium. The molecular identity and enzymological uniqueness of *Plasmodium* AdoMetDC have recently been unveiled (Birkholtz *et al.*, 2003, 2004). *Plasmodium* AdoMetDC has an unusual structure. All known prokaryotic and eukaryotic AdoMetDC and ODC are independently transcribed to produce separate polypeptides. However, *P. falciparum* AdoMetDC and ODC are encoded in a single open reading frame and translated as a single 166-kDa protein with the AdoMetDC domain in the N-terminal region that is connected to the C-terminal ODC domain by a hinge region. *P. falciparum* ODC/AdoMetDC is also unique in its subunit structure, i.e. the assembly of domains in a heterotetramer consisting of a homodimeric ODC and a heterotetrameric AdoMetDC, dissimilar to mammalian ODC (homodimer or monomer) or AdoMetDC (homodimer) (Birkholtz *et al.*, 2003, 2004). It has previously been shown that AdoMetDC from *P. falciparum* showed a physiological K_m (33 μM) for AdoMet, and was inhibited by the known inhibitor of human AdoMetDC, methylglyoxal-bis(guanylhydrazone) (MGBG) with a K_i of 0.46 μM (Rathaur and Walter, 1987). MGBG also showed inhibitory effects on parasite growth when combined with chloroquine (Das *et al.*, 1997). MDL 73811, an enzyme-activated irreversible inhibitor of AdoMetDC, inhibited growth of the erythrocytic stage of chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* equally, with an IC_{50} of 2–3 μM. MDL 73811 at 1 μM inhibited >90% of the AdoMet DC activity in the parasite lysate (Wright *et al.*, 1991). Although *P. falciparum* has a bifunctional AdoMetDC–ODC enzyme, it lacks spermidine synthase as well as a pathway to synthesize trypanothione (Krause *et al.*, 2000; Muller, S. *et al.*, 2000). Thus, growth inhibition of the *P. falciparum* parasites by these AdoMetDC inhibitors is unlikely to be due to inhibition of polyamine biosynthesis, but to perturbation of AdoMet metabolism, as shown for trypanosomes

(see Section 3.2.5(b)). It was also shown that a growth defect of the AdoMetDC-deficient and ODC-deficient *Escherichia coli* line EWH331 was successfully complemented by expression of the malarial AdoMetDC/ODC protein, which verified its dual roles *in vivo* (Muller, S. *et al.*, 2000). The purified recombinant fusion protein containing the ODC domain was inhibited by two novel ODC inhibitors, CGP52622A and CGP54619A (Krause *et al.*, 2000). These results indicate that *Plasmodium* AdoMetDC/ODC is exploitable for the further development of novel antimalarial drugs.

(*b*) *Exploitation of AdoMetDC as a chemotherapeutic target in trypanosomiasis.* As explained in detail above (Section 3.2.3(c)), an irreversible inhibitor of ODC, DFMO (eflornithine) has been shown to be effective for treatment of both early and late-stage Gambian human African trypanosomiasis (Sjoerdsma and Schechter, 1984; Van Nieuwenhove *et al.*, 1985; Bacchi *et al.*, 1987). DFMO treatment also caused alteration in the AdoMet metabolism: AdoMet and DcAdoMet concentrations increased >50 and 1000-fold, respectively, while AdoMetDC activity, associated with the formation of spermidine and spermine in other eukaryotes, paradoxically declined in treated cells (Bacchi *et al.*, 1983; Yarlett and Bacchi, 1988a). This observation validated the premise that AdoMet metabolism is a good target for drug development. Additionally, it was demonstrated that *Trypanosoma brucei* AdoMetDC activity was irreversibly inhibited by Berenil (diminazene aceturate) and reversibly by pentamidine or MGBG (Karvonen *et al.*, 1985; Bitonti *et al.*, 1986). A number of other AdoMetDC inhibitors, including MDL 73811 (Bitonti *et al.*, 1990b), 5′-deoxy-5′-[(2-hydrazinoethyl)-methylamino] adenosine (MHZEA) and 5′-deoxy-5′-[(2-aminooxyethyl) methylamino] adenosine (MAOEA), have been further tested as potential trypanocidal drugs (Tekwani *et al.*, 1992a, b).

Irreversible inhibition of AdoMetDC by MDL 73811 was very effective against *T. brucei* infections in mice and rats (Byers *et al.*, 1991). In addition, combinations of MDL 73811 and DFMO showed a synergistic effect and cured mice infected with clinical isolates of *T. b. rhodesiense* (Bacchi *et al.*, 1992). The inactivation of trypanosomal AdoMetDC by MHZEA or MAOEA was irreversible, indicating that the compounds bind to the active site and form a covalent bond with

the enzyme (Tekwani *et al.*, 1992a). Ectopic overexpression of AdoMetDC by episomal transfection alleviated the toxic effects of MDL 73811 on wild-type *Leishmania donovani* (Roberts *et al.*, 2002), supporting the hypothesis that AdoMetDC is a target of this compound.

T. brucei AdoMetDC was relatively insensitive to activation by putrescine and was stimulated by only 50% over a 10 000-fold range of putrescine concentrations (Tekwani *et al.*, 1992a), unlike the situation in mammals and yeasts, in which AdoMetDC was stimulated several-fold by putrescine (Poso *et al.*, 1976). Furthermore, unlike the bacterial enzyme (Poso *et al.*, 1976), which is highly stimulated by Mg^{2+}, *T. brucei* AdoMetDC was not stimulated by 10 mM Mg^{2+} (Bitonti *et al.*, 1986).

The presence of AdoMetDC in *T. cruzi* was predicted based on growth inhibition by MDL 73811 (Yakubu *et al.*, 1993) and MGBG (Brun *et al.*, 1996), and a responsible gene has been identified (Persson *et al.*, 1998; Kinch *et al.*, 1999). MGBG is a potent inhibitor of human AdoMetDC, but a poor inhibitor of *T. cruzi* recombinant AdoMetDC (Persson *et al.*, 1998). Altogether, these results indicate that trypanosomal AdoMetDC differs significantly from its mammalian and bacterial counterparts.

3.3. Transsulfuration Reactions

3.3.1. Terminology and General Description of Forward and Reverse Transsulfuration Pathways

The forward transsulfuration pathway, previously demonstrated in bacteria, fungi and plants, is involved in the formation of methionine from cysteine. Since a part of this pathway, from homocysteine to methionine, is also part of the methionine-recycling pathway as explained in Section 3.2, we here describe only the two committed steps that lead to the formation of homocysteine from cysteine (Figure 2). These two steps are catalyzed firstly by cystathionine γ-synthase (CGS, EC 2.5.1.48), which synthesizes cystathionine from cysteine with *O*-activated-L-serine (*O*-acetyl-, *O*-phospho-, or *O*-succinyl-L-homoserine), and subsequently by cystathionine β-lyase (CBL, EC

4.4.1.8), which catalyzes an α, β-elimination of cystathionine to produce homocysteine and pyruvate. These two steps are followed by a reaction catalyzed by methionine synthase, described above, finally forming methionine.

The reverse transsulfuration pathway has previously been demonstrated in fungi and mammals, and generally includes the complete process leading to cysteine from methionine. Since the first three steps, i.e. methionine activation, transmethylation and hydrolysis, have been described in Section 3.2 as part of the methionine regeneration pathways, we describe here only the last two steps. These reactions are catalyzed sequentially by two PLP-dependent enzymes, cystathionine β-synthase (CBS, EC 4.2.1.22), which synthesizes cystathionine from homocysteine and serine, and cystathionine γ-lyase (CGL, EC 4.4.1.1), to finally form cysteine, α-ketobutyrate and ammonia (Figure 2). In the first reaction the alanyl moiety of serine is transferred to homocysteine by a β-replacement reaction to produce cystathionine, which is decomposed by the α- and γ-lyase activity of CGL.

3.3.2. Transsulfuration Pathway in Animals

Animals possess only the reverse transsulfuration pathway, which operates in the direction from methionine to cysteine. As explained in Section 3.2.2, homocysteine enters one of two pathways, methionine recycling and reverse transsulfuration. Homocysteine represents a key intermediate of methionine/cysteine metabolic pathways since it occupies a branch point leading to the production of cysteine and methionine. About half of the homocysteine formed is irreversibly converted into cysteine through transsulfuration (Finkelstein *et al.*, 1974; Mudd and Poole, 1975), while the remaining half of homocysteine is remethylated to regenerate methionine by the recycling pathway described above. Depletion of homocysteine is essential not only for proper flow of sulfur in the transsulfuration pathway and the methionine cycle, but also for scavenging this toxic intermediate, which has been implicated in pathological conditions associated with various genetic disorders such as deficiency of CBS or

homocysteine-methylating enzymes causing homocysteinuria and homocysteinemia (Mudd *et al.*, 1985; Stipanuk, 2004; Townsend *et al.*, 2004). Nearly a hundred mutations responsible for many pathological disorders have been identified in the CBS gene (Kraus *et al.*, 1999). Homocysteine was also shown to be a pro-oxidant, causing damage to vascular endothelia (De Bree *et al.*, 2002), and is known to be associated with increased cardiovascular risk (Wald *et al.*, 2002) and Alzheimer's disease (Miller, J.W., 1999). CBS contributes to keeping homocysteine and AdoHcy concentrations low, which is also essential for the efficient methylation capacity of the cell (Walker, R.D. and Duerre, 1975).

3.3.3. Transsulfuration Pathway in Bacteria, Fungi and Plants

In contrast to animals, bacteria, fungi and plants mainly operate the similar transsulfuration pathway in the opposite direction using a set of homologous enzymes at two steps, during which homocysteine is produced from cysteine. These organisms are also capable of *de novo* production of cysteine by sulfur assimilation (see below, Section 4). Cysteine produced by this anabolic pathway is converted into cystathionine by CGS, and further hydrolyzed by CBL finally to form homocysteine. These two enzymes also contain PLP as a cofactor, and belong to the so-called α-type PLP enzymes together with CGL, with high mutual homology at the primary sequence level (Eliot and Kirsch, 2004). Transsulfuration in plants and bacteria is similar in that the reaction proceeds only in the direction of cysteine to homocysteine (Giovanelli, 1987). In contrast, in fungi the reaction proceeds in both directions. The major difference between forward transsulfuration in plants and bacteria concerns the donors of the 4-carbon moiety of homocysteine. Plants utilize *O*-phosphohomoserine as the physiological donor of the 4-carbon moiety, while bacteria and fungi utilize either *O*-succinylhomoserine or *O*-acetylhomoserine (Giovanelli, 1987). The donor of the final methyl transfer reaction to homocysteine to produce methionine in bacteria, fungi and plants also differs from that of mammals (Griffith, 1987).

3.3.4. Transsulfuration Pathways in Parasitic Protozoa

(*a*) *A spectrum of the reverse transsulfuration pathway among parasitic protozoa.* We conducted a gene survey to evaluate the distribution of enzymes related to transsulfuration pathways among protozoan organisms. We previously demonstrated that *Entamoeba histolytica* lacks genes encoding enzymes of the transsulfuration pathway in both directions (Tokoro *et al.*, 2003). Our analysis using the latest genome database of *E. histolytica* confirmed lack of these enzymes. Similarly *E. histolytica*, *Giardia duodenalis*, *Trichomonas vaginalis*, *Plasmodium falciparum* and *Cryptosporidium parvum* lack both CBS and CGL of the reverse transsulfuration pathway. Lujan and Nash (1994) also demonstrated that the *G. intestinalis* lysate contained neither CBS nor CGL activity, which agrees well with the results of our gene survey. The presence of a transsulfuration pathway was demonstrated in bloodstream trypomastigotes of *Trypanosoma brucei* by Yarlett and Bacchi (1988a). It was shown that *T. brucei* bloodstream trypomastigotes rapidly incorporated [^{35}S]methionine and derivatized it into homocysteine, cystathionine and cysteine, suggesting the presence of an active reverse transsulfuration pathway (Bacchi *et al.*, 1995). Goldberg *et al.* (2000) also reported that cystathionine and cysteine concentrations increased when *T. b. rhodesiense* and *T. b. brucei* were incubated with saturating amounts of methionine. These studies, together with our previous report on molecular identification of CBS from *T. cruzi* (Nozaki *et al.*, 2001), indicate the presence of reverse transsulfuration pathways in trypanosomes in general. Our wide-ranging survey confirmed that both CBS and CGL genes for the reverse transsulfuration pathway are present in the genome of *T. brucei*, *T. cruzi* and *Leishmania major* (Table 1).

(*b*) *Redundancy of two pathways for cysteine synthesis and dual functions of CBS in Trypanosoma cruzi.* We previously showed by chromatographic separation of the lysates that *T. cruzi* possesses at least two separate CBS and CS activities. While CS activity is constitutively expressed, CBS activity is expressed in a stage-specific fashion. However, we could not attribute the CS activity to any putative gene in the

database (Nozaki *et al.*, 2001). The results of gene survey clarified this discrepancy. We unequivocally conclude that *T. cruzi* possesses both CS and CBS genes. There are two CS genes in the *T. cruzi* genome database, both of which clearly clustered with CS genes from other organisms. We also showed that these CBS genes are capable of complementing the CS-deficient *Escherichia coli* mutant cell line and the CBS-deficient *Saccharomyces cerevisiae* mutant cell line by ectopic expression (Nozaki *et al.*, 2001). These data, together with enzymological studies *in vitro* using recombinant CBS, indicate that *T. cruzi* CBS plays dual roles as both CS and CBS.

(*c*) *Multiplicity and biochemical features of unique CBS from* Trypanosoma cruzi. Genome survey revealed that *T. cruzi* possessed at least four CBS isoenzymes. However, the amino acid sequence of one CBS isotype was similar to, but distinct from, the CBS isotype we previously reported (Nozaki *et al.*, 2001), which contained two nucleotide substitutions that resulted in amino acid alterations (V14A, Y261C). In addition, the remaining three CBS isotypes were not previously found in our cDNA library. These isotypes differ from the major isotypes described above in a few amino acid residue substitutions, e.g. T19K–I71V–K210R, D93E and K210R. These data suggest two possibilities: (i) interstrain variations of the number and sequences of isoenzymes or (ii) CBS genes and proteins are extremely heterogeneous in *T. cruzi*. The biological significance of the presence of multiple CBS genes and their redundant functions in *T. cruzi* are currently unknown.

T. cruzi CBS lacks the 90–120-amino acid C-terminal extension present in CBS from all other organisms except *Leishmania* and other species of *Trypanosoma*. Trypanosomal CBS is one of the shortest CBS enzymes reported to date. Enzymological studies of CBS from *T. cruzi* revealed several unique aspects of this enzyme, including lack of activation by AdoMet, absence of detectable amounts of heme, and relative insensitivity to known non-specific inhibitors, e.g. aminooxyacetic acid and hydroxylamine. These data suggest that biochemical and physicochemical properties of *T. cruzi* CBS differ substantially from yeast and mammalian CBS (Nozaki *et al.*, 2001). *T. cruzi* CBS showed CBS and CS activities *in vitro*, which is in good

agreement with the functional rescue of the CS-deficient *Escherichia coli* strain and the CBS-deficient yeast strain.

(*d*) *Absence of the forward transsulfuration pathway from parasitic protozoa.* In contrast to the reverse transsulfuration pathway, the forward pathway appears to be totally absent from protozoan parasites. None of the protists discussed in this review possess either CGS or CBL homologs involved in the first two reactions of the forward pathway. However, since these enzymes belongs to the same α-family of PLP-dependent enzymes (Mehta and Christen, 1998) together with CGL and thus show moderate mutual homology, it is often difficult to predict gene function and to assign names for these putative genes based on sequence alignments and phylogenetic analyses. Therefore, further enzymological and functional characterization of these gene products must be conducted to unequivocally confirm the lack of the forward transsulfuration in the protozoan parasites.

4. SULFUR ASSIMILATORY *DE NOVO* CYSTEINE BIOSYNTHETIC PATHWAY

4.1. A General Scheme of the Cysteine Biosynthetic Pathway

As mentioned above, in mammals and fungi cysteine can be synthesized from methionine through the reverse transsulfuration sequence. The sulfur atom of cysteine is derived from methionine whereas carbon and nitrogen atoms of cysteine are derived from serine. Similarly, bacteria (Byrne *et al.*, 1988; Ogasawara *et al.*, 1994) and plants (Romer *et al.*, 1992; Saito *et al.*, 1992; Hell *et al.*, 1994; Noji *et al.*, 1994, 1998) are also capable of producing cysteine from serine. However, the sulfur donor of cysteine production in these organisms is inorganic sulfur, not organic compounds. Cysteine production utilizing inorganic sulfur is called sulfur assimilatory cysteine biosynthesis. This pathway is catalyzed by two steps, initiated by serine acetyltransferase (SAT, EC 2.3.1.30) to form *O*-acetylserine (OAS)

from L-serine and acetyl-coenzyme A. Subsequently, OAS reacts with sulfide to produce cysteine in an alanyl-transfer reaction by cysteine synthase (CS, OAS thiolyase, EC 2.5.1.47). Since SAT catalyses the formation of OAS from serine in the first reaction, SAT is located at the entry step from serine metabolism to cysteine biosynthesis. Thus, it is reasonable to assume that SAT plays a regulatory role in this pathway. SAT is in fact negatively regulated by allosteric inhibition by L-cysteine in bacteria (Kredich and Tomkins, 1966; Saito *et al.*, 1995; Noji *et al.*, 1998). Two isotypes of CS, CysK and CysM proteins, with different substrate specificities, were localized in the cytoplasm of prokaryotes. CysK prefers sulfide while CysM exclusively utilizes thiosulfate (Nakamura *et al.*, 1983). In plants, three isotypes, CS-A, CS-B and CS-C, are compartmentalized in the cytoplasm, chloroplasts and mitochondria, respectively (Saito *et al.*, 1992, 1993). Plant CSs have also been shown to catalyze the formation of β-substituted alanines besides cysteine, and are considered to be involved, at least in part, in detoxifying internal toxins like cyanide, pyrazole, and 3,4-dihydroxypyridine (Ikegami *et al.*, 1996; Maruyama *et al.*, 1998).

4.2. Presence of the Cysteine Biosynthetic Pathway in a Limited Range of Parasitic Protozoa

We previously demonstrated the presence of the cysteine biosynthetic pathway in parasitic protozoa for the first time by cloning and characterization of CS from *Entamoeba histolytica* (Nozaki *et al.*, 1998) and a closely related non-pathogenic species *E. dispar* (Nozaki *et al.*, 2000). We further demonstrated that SAT, which catalyzes a committed step of OAS formation, is also present in *E. histolytica*, *E. dispar* (Nozaki *et al.*, 1999) and *Trypanosoma cruzi* (Nozaki *et al.*, 2001). We also showed that *T. cruzi* possesses bifunctional CBS, which functions both *in vitro* and *in vivo* as do CS and CBS (Nozaki *et al.*, 2001) (also see above, Section 3.3.4(b)).

Survey of the genome databases of parasitic protozoa revealed very restricted distribution of these genes. As in the transsulfuration

pathway, *Giardia duodenalis*, *Plasmodium falciparum* and *Cryptosporidium parvum* appear to lack both CS and SAT genes (Table 1). The previous study by Lujan and Nash (1994) showed that [^{35}S]sulfate was not incorporated into *Giardia* proteins, suggesting that *G. duodenalis* lacks the capacity for *de novo* synthesis of cysteine from inorganic sulfur. This observation is consistent with the results of our gene survey. *P. falciparum* and *C. parvum*, which also lack a reverse transsulfuration pathway, are probably able to obtain these amino acids from erythrocytes and intestinal villus epithelial cells, respectively, as they live within these host cells. It has previously been shown that *Leishmania donovani* possesses an AdoMet transporter (Lawrence *et al.*, 1998) and probably the complete reverse transsulfuration pathway (see Section 3.3.4). Thus, the lack of CS and SAT in *Leishmania* reflects the fact that this organism does not need to depend on sulfur assimilatory cysteine biosynthesis for its cysteine source. In contrast to *T. brucei*, which lacks both CS and SAT, *T. cruzi* conserves this pathway. As explained in Section 3.3.4(b), *T. cruzi* possesses both CBS, which plays dual roles as CBS and CS *in vitro* and *in vivo*, and CS. This apparent redundancy of CS and CBS in *T. cruzi* might indicate the physiological importance of these metabolic steps for this protozoan parasite.

Trichomonas vaginalis also possesses at least seven putative CS genes. Our phylogenetic analysis revealed that, among these genes, six form a monophyletic clade with CS from *E. histolytica*, while one gene clusters with CS from other *Entamoeba* species, i.e. *E. dispar* and *E. moshkovskii* (personal observation). Serine sulfhydrase (synonymous with CBS) activity, which catalyzes the reversible interconversion of serine and cysteine, has previously been demonstrated in *T. vaginalis* by Thong and Coombs (1985, 1987). Several isoenzymes were separated by isoelectric focusing to verify their identities (Thong and Coombs, 1987). Although serine sulfhydrase activity was previously attributable to CBS in studies using chicken liver (Braunstein *et al.*, 1971) and *Aspergillus nidulans* (Pieniazek *et al.*, 1973; Stepien and Pieniazek, 1973), multiple CS, but not CBS, genes are present in the *T. vaginalis* genome database. Therefore, it is likely that some isotypes of putative *T. vaginalis* CS proteins are responsible for the serine sulfhydrase activity. Although the existence of multiple CS

isotypes needs to be further verified, the biological significance of the presence of multiple CS genes and redundant functions of these genes in *T. vaginalis* is currently unknown.

4.3. Possible Functions of the Cysteine Biosynthetic Pathway in *Entamoeba* and *Trypanosoma*

4.3.1. Contradiction Between the Presence of Cysteine Biosynthesis and the Apparent Requirement of Cysteine in Entamoeba histolytica

As mentioned above, among all representative protozoan parasites, only *Entamoeba histolytica* and *Trypanosoma cruzi* have the complete *de novo* cysteine biosynthetic pathway (see above; also Nozaki *et al.*, 1999, 2000, 2001). The presence of this pathway directly indicates that they are capable of production of cysteine *de novo*. However, the earlier finding of the apparent reliance of *E. histolytica* on extracellular cysteine appears to contradict this premise. It has been well established in axenic culture *in vitro* that *E. histolytica* apparently requires a high concentration of extracellular L-cysteine for growth, attachment, and antioxidative survival (Gillin and Diamond, 1980a, b, 1981a–c; Gillin *et al.*, 1984). Since these effects of L-cysteine could be produced by substituting D-cysteine, L-cystine, ascorbic acid, or thioglycolic acid, but not by thiomalic acids, methionine, taurine, glutathione, or other reducing agents (Gillin and Diamond, 1980b), these results indicate that extracellular cysteine is required not as an amino acid source but for redox control of the surface environment. We still do not have a reasonable explanation why oxidized L-cystine could be substituted for L-cysteine and why most of the reducing agents did not substitute for L-cysteine. One possible explanation for the apparent requirement of extracellular L-cysteine in the culture system is that L-cysteine synthesized *de novo* may not be efficiently utilized for the maintenance of the redox state of the surface thiol molecules, while extracellular L-cysteine or L-cystine may be utilized after reduction by an unidentified reductant, as previously suggested

(Gillin *et al.*, 1984). L-Cysteine is also essential for *Giardia duodenalis* (Gillin and Diamond, 1981a, b; Gillin and Reiner, 1982; Jarroll *et al.*, 1989; Adam, 1991), which lacks both CS and SAT. However, the requirement for L-cysteine is more stringent in *G. duodenalis* than in *E. histolytica*; e.g., L-cysteine is indispensable for both growth and protection against oxygen stress and cannot be substituted for by other compounds in *G. duodenalis*, while it can be replaced by, e.g., L-cystine or ascorbic acid in *E. histolytica*. Thus, the essential nature of extracellular cysteine is not solely attributable to, but is somehow related to, the presence or absence of this pathway. Among other *Entamoeba* species for which genome databases are available, *E. dispar*, the mammalian species that is most closely related to *E. histolytica*, and *E. moshkovskii* have both CS and SAT genes, while two others reptile *Entamoeba* species, *E. invadens* and *E. terrapinae*, lack both or one (SAT) of these two enzymes (personal observation). This suggests a limited distribution of the sulfur assimilatory cysteine biosynthetic pathway among *Entamoeba* species. In addition, cysteine biosynthesis is biologically important in *Entamoeba* species due to their reliance on iron–sulfur proteins for the electron-transport chain (Reeves *et al.*, 1980; Weinbach *et al.*, 1980; Beinert *et al.*, 1997; see below, Section 4.3.2). Cysteine and its *de novo* biosynthetic pathway might play an important role during tissue invasion by *E. histolytica*, when the parasites move from anaerobic (in the lumen of the cecum/colon) to highly oxygenated aerobic conditions (in mammalian tissues and organs, i.e. intestinal epithelium or oxygen-rich organs including liver, lung and brain). However, the requirement of cysteine for infection *in vivo* has not been tested.

4.3.2. *Roles of the Cysteine Biosynthetic Pathway in Antioxidative Defense Mechanisms and ISC Biosynthesis in* Entamoeba histolytica

The premise that extracellular or incorporated cysteine is important for cellular activities and homeostasis in *Entamoeba histolytica* prompted us to test if a *de novo* cysteine biosynthetic pathway plays a key role in redox regulation, or more specifically in antioxidative

defense mechanisms, in *E. histolytica* (Gillin and Diamond, 1981a). We tested whether the *de novo* cysteine biosynthetic pathway is associated with sensitivity to hydrogen peroxide. *E. histolytica* possesses superoxide dismutase to convert superoxide into hydrogen peroxide (Chen, J. *et al.*, 1996), and hydrogen peroxide is a major oxidative agent toxic to *E. histolytica* (Ghadirian *et al.*, 1986). An ameba cell line that was created by episomal transformation to express 5–10-fold higher CS activity than the wild type showed partial resistance to hydrogen peroxide, with a concomitant two-fold increase of intracellular cysteine concentration, while >10-fold overproduction of SAT, an allosteric enzyme negatively regulated by cysteine, neither increased cysteine concentrations nor altered sensitivity to hydrogen peroxide (Nozaki *et al.*, 1999). These data suggest that the amount of CS protein, but not of SAT, largely influences intracellular cysteine concentrations, and that cysteine or its unidentified derivatives probably inactivate hydrogen peroxide or its related toxic metabolites. Hence, although reductants of hydrogen peroxide and enzymes involved in the reaction [e.g. p34 NADPH:flavin oxidoreductase (Bruchhaus *et al.*, 1998), rubrethrin (Loftus *et al.*, 2005), or peroxiredoxin (Torian *et al.*, 1990; Tachibana *et al.*, 1991; Bruchhaus and Tannich, 1993)] are still unknown, the *de novo* cysteine biosynthetic pathway seems to participate in antioxidant defense. Thus, regulation of CS expression appears to control antioxidant activity in *E. histolytica*, which should be essential not only for inactivation of toxic reactive oxygen species produced by the host immune system during tissue invasion, but also for neutralization of highly reactive radicals produced during degradation within phagosomes of ingested host cells that contain high concentrations of iron (hemoglobin and iron–sulfur proteins in erythrocytes and bacteria).

As briefly described in Section 2.2.1, cysteine is also utilized for the production of ubiquitous iron–sulfur clusters. Iron–sulfur clusters (ISCs) are cofactors of proteins probably present in all living organisms (Beinert *et al.*, 1997). The ISCs play various important roles in electron transfer, redox regulation, nitrogen fixation, photosynthesis, biotin and thiamine biosynthesis, oxygen and iron sensing, and gene regulation (Beinert *et al.*, 1997). *E. histolytica* possesses a simplified

and non-redundant nitrogen fixation (NIF)-like system for the Fe–S cluster formation, composed of only a catalytic NifS component and a scaffold NifU component (Ali *et al.*, 2004b). There is no precedent for this NIF-like system in any other eukaryotes, including other parasitic and non-parasitic protozoa. The NIF system has been identified in, besides *E. histolytica* (Ali *et al.*, 2004b), only nitrogen-fixing bacteria and non-diazotrophic protobacteria including *Campylobacter jejuni* and *Helicobacter pylori* (Olson *et al.*, 2000). Thus, the NIF system appears to be involved in Fe–S assembly of the nitrogenase proteins in diazotrophic bacteria and that of non-nitrogenase Fe–S proteins in ε-proteobacteria and *E. histolytica* (Olson *et al.*, 2000; Ali *et al.*, 2004b). *E. histolytica* possesses at least a few essential iron–sulfur proteins including ferredoxin (Reeves *et al.*, 1980) and pyruvate:ferredoxin oxidoreductase (Reeves *et al.*, 1977; Rodriguez *et al.*, 1996), both of which play a crucial role in energy production and electron transport in this parasite (Reeves *et al.*, 1980; Weinbach *et al.*, 1980). In contrast to *E. histolytica*, all other parasitic protozoa so far studied, including *Trichomonas vaginalis* and *Giardia duodenalis* (Tachezy *et al.*, 2001; Tovar *et al.*, 2003), *Cryptosporidium parvum* (LaGier *et al.*, 2003), and *Plasmodium falciparum* (Ellis *et al.*, 2001; Wilson *et al.*, 2003) possess two other systems, the ISC and SUF (*Plasmodium* only) systems, for the formation of ISCs (Seeber, 2002; LaGier *et al.*, 2003; Ali *et al.*, 2004b). Therefore, the limited and shared distribution of the *de novo* cysteine biosynthetic pathway among the parasitic protozoa is apparently not directly associated with the systems for ISC formation.

4.3.3. *Roles of the Cysteine Biosynthetic Pathway in* Trypanosoma cruzi

Trypanosoma cruzi seems to be the only parasitic protozoon possessing two apparently redundant pathways for cysteine production, i.e. reverse transsulfuration and *de novo* cysteine biosynthetic pathways. Theoretically either of the two pathways should provide L-cysteine to fulfill its physiological demand. Fungi also possess both pathways. In *Saccharomyces cerevisiae*, cysteine is exclusively synthesized through

the transsulfuration pathway and OAS serves solely as a co-inducer of the sulfate assimilation pathway (Ono *et al.*, 1996). By analogy, in *T. cruzi*, the cysteine biosynthetic pathway may play a major role in the regulation of sulfate assimilation, while the reverse transsulfuration pathway mainly provides the necessary cysteine. In contrast, OAS serves as both a metabolic intermediate and a regulatory element in *Escherichia coli* (Kredich and Tomkins, 1966; Ostrowski *et al.*, 1987) and *Salmonella typhimurium* (Kredich *et al.*, 1979). Both CS and SAT are constitutively expressed in both insect-stage epimastigotes and mammalian-stage trypomastigotes/amastigotes of *T. cruzi*, while CBS is expressed exclusively in epimastigotes (Nozaki *et al.*, 2001). These results are consistent with the notion that the sulfur assimilatory cysteine biosynthetic pathway plays more 'housekeeping' roles, e.g. sulfate incorporation and activation, by controlling OAS abundance, as part of a complex mechanism to balance thiol concentration in *T. cruzi* (Nozaki *et al.*, 2001). In contrast, in the mammalian cytoplasm or bloodstream, the parasites are probably able to scavenge cysteine from their host. However, we cannot rule out the possibility that the sulfur assimilatory cysteine biosynthetic pathway also plays an indispensable role in *de novo* production of cysteine in epimastigotes. A biological role for these two pathways in trypanosomes could be unequivocally demonstrated by gene replacement or disruption (Cooper, R. *et al.*, 1993; Hariharan *et al.*, 1993) of SAT and CS genes.

4.3.4. *Other Possible Roles of the Cysteine Biosynthetic Pathway in Parasitic Protozoa and Possible Drug Development*

It has previously been indicated that the sulfur assimilatory cysteine biosynthetic pathway is involved in resistance to hydrogen sulfide (H_2S) in plants (Noji and Saito, 2002). They showed that a genetically manipulated *Arabidopsis thaliana* cell line that overexpressed watermelon SAT gene with a point mutation causing insensitivity to allosteric inhibition by L-cysteine, was more resistant to hydrogen sulfide in air than the wild-type cell line. However, it is not yet clear if the pathway is involved in the defense mechanisms against toxic gas under physiological conditions in plants. Whether or not the pathway

is involved in the detoxification of hydrogen sulfide or other possible alanyl acceptors in *Entamoeba histolytica* remains totally unknown. CS utilizes sulfide and various compounds as alanyl acceptors. The spectrum of these acceptors depends on the origin of CS. The spectrum of the alanyl acceptors for CS has been most extensively studied for plant enzymes. For instance, spinach CS utilizes a variety of alanyl acceptors to form the corresponding β-substituted alanines. These alanyl acceptors include L-cysteine, L-quisqualic acid, L-mimosine, L-willardiine, L-isowillardiine, β-(pyrazol-1-yl)-L-alanine, β-(1,2,4-triazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine (Ikegami *et al.*, 1996; Maruyama *et al.*, 1998). *E. histolytica* CS also uses sulfide, 1,2,4-triazol, isoxazolin-5-one, pyrazole and cyanide as alanyl acceptors; the specific activity against these acceptors is, however, 9–4000-fold less efficient than that against sulfide (Nozaki *et al.*, 1998). Although 1,2,4-triazole has been used as a herbicide targeting the plant CS, this compound did not show toxic effects on *E. histolytica* (personal observation), necessitating further screening of CS substrates with amebicidal activity. However, since CS and the sulfur assimilatory *de novo* cysteine biosynthetic pathway are absent from mammalian hosts, they represent a novel target for the development of new drugs against amebiasis, trichomoniasis and Chagas disease.

SAT from *E. histolytica* and *Trypanosoma cruzi* showed major biochemical differences from that of other organisms. While K_m values for acetyl coenzyme A (CoA) and serine are comparable, the specificity of allosteric feedback was notably different (Nozaki *et al.*, 1999, 2001). Enzymatic activity of SAT from *E. histolytica* and *T. cruzi* is negatively regulated by L-cysteine and L-cystine, and that of SAT from *T. cruzi* is also partially negatively regulated by glutathione, suggesting substantial differences in the binding of these inhibitors between protozoa and other organisms. While SAT from bacteria and plants is known to form a stable complex with CS, *E. histolytica* SAT and CS do not form a complex, as confirmed by biochemical co-purification and a yeast two-hybrid system (Nozaki *et al.*, 1999). Altogether, the sulfur assimilatory cysteine biosynthetic pathway in protozoan parasites reveals remarkable differences from that of other organisms.

5. DEGRADATIVE PATHWAYS OF SULFUR-CONTAINING AMINO ACIDS

5.1. Degradation of Sulfur-Containing Amino Acids in Mammals

As described above (Section 3.3.2), the majority of methionine is converted into homocysteine via AdoMet and AdoHcy. Homocysteine is either methylated to regenerate methionine in the methionine salvage pathway or used for transsulfuration to gain cysteine. Therefore, the reverse transsulfuration pathway can be considered one of the main methionine degradation pathways in mammals (Cooper, A.J., 1983; Walker, J. and Barrett, 1997). Methionine is also metabolized by the deamination reaction to form α-keto-γ-thiomethylbutyrate by L-amino-acid oxidase and aminotransferase. However, the enzyme responsible for this pathway is unknown to date; no methionine-specific aminotransferase has been demonstrated in mammals (Steele and Benevenga, 1979; Griffith, 1987).

Once methionine is converted into cysteine, the oxidative pathway is the principal route for cysteine catabolism in mammals, while a separate transaminative pathway also exists. Cysteine dioxygenase (CDO, EC 1.13.11.20; Figure 2) catalyzes the oxygenation of cysteine to 3-sulfinoalanine, a key intermediate of cysteine metabolism leading to hypotaurine, taurine, pyruvate, and sulfate in mammalian tissues (Yamaguchi and Hosokawa, 1987). The sulfur-containing amino acid biosynthesis and degradation in mammals has recently been reviewed by Stipanuk (2004). It was reported that CDO activity of rat liver responded to dietary protein content. CDO was thought to play an important role in the regulation of intracellular levels of methionine, cysteine and glutathione (Kohashi *et al.*, 1978). It was also suggested that CDO is involved in the prevention of free radical production by auto-oxidation of cysteine and dopamine in the brain (Parsons, R.B. *et al.*, 2001). The other cysteine degradative pathway in mammals is initiated by cysteine aminotransferase (CAT, EC 2.6.1.3), which deaminates cysteine to form 3-mercaptopyruvate (Figure 2). Sulfurtransferases (EC 2.8.1.1–5), widely distributed in prokaryotes and eukaryotes, catalyze the transfer of sulfane sulfur from a donor

molecule, such as thiosulfate or 3-mercaptopyruvate, to a nucleophilic acceptor, such as cyanide or mercaptoethanol. However, the natural sulfane donors and acceptors and the physiological functions of most sulfurtransferases remain uncertain (Nakayama, K. *et al.*, 2000; Bordo and Bork, 2002). The rhodanese family sulfurtransferases are thought to occur in many organisms, with the mammalian enzymes being the most extensively studied (Luo and Horowitz, 1994; Nandi *et al.*, 2000). Rhodanese from bovine liver plays a role in detoxifying cyanide by forming thiocyanate, which is harmless and excreted by the kidneys (Picton *et al.*, 2002). In organisms that possess a methionine biosynthetic pathway, cysteine is also degraded at least *in vitro* by CBL to thiocysteine, pyruvate and ammonia (Uren, 1987; Gentry-Weeks *et al.*, 1993). However, an *in vivo* role for CBL, or its related enzymes CGS and CGL, in degradation of sulfur-containing amino acids needs to be verified.

5.2. Peculiarities and Distribution of MGL among Parasitic Protozoa

None of the protozoan parasites discussed in this review possess the above-mentioned cysteine degradation pathways (Table 1). Instead, two protozoan parasites, *Entamoeba histolytica* and *Trichomonas vaginalis*, possess a unique enzyme known as methionine γ-lyase (MGL, EC 4.4.1.11) (Thong *et al.*, 1987b; Tokoro *et al.*, 2003). MGL catalyzes decomposition of methionine, homocysteine, cysteine, and also some substituted serine or homoserine analogs including OAS and *O*-acetyl-L-homoserine, by α- and β-elimination or α- and γ-elimination (Figure 2). MGL requires PLP as a cofactor for its activity and belongs to the α-family of PLP-dependent enzyme (Mehta and Christen, 1998), together with CGL, CGS and CBL. MGL shares high to moderate homology with these PLP-dependent enzymes: MGL1 of *E. histolytica* showed 39% identity with CGS from *Helicobacter pylori*, 37% identity with human CGL, and 26% identity with *Escherichia coli* CBL. The presence of an MGL gene or its encoded protein has also been demonstrated in a limited range of bacteria, including *Clostridium sporogenes* (Kreis and Hession, 1973), *Pseudomonas*

putida (Ito *et al.*, 1976), *P. taetrolens* (Zanin *et al.*, 1989), *Bacillus halodurans* (Nakayama, T. *et al.*, 1984), *Aeromonas* sp. (Nakayama, T. *et al.*, 1984), *Citrobacter intermedius* (Faleev *et al.*, 1996), and *Brevibacterium linenese* (Dias and Weimer, 1998). *T. vaginalis* and *E. histolytica* are the only two eukaryotic organisms known to possess MGL. Putative MGL genes have also been reported in several archaeal species of *Methanosarcina* (e.g. *M. mazei*, *M. acetivorans* and *M. barkeri*; Tokoro *et al.*, 2003). MGL has not been reported from fungi, plants or mammals.

Thorough genome search revealed that only *E. histolytica* and *Trichomonas vaginalis* are known to possess MGL, while all the other species, *Giardia duodenalis*, *Plasmodium falciparum*, *Cryptosporidium parvum*, *Trypanosoma brucei*, *Tryp. cruzi* and *Leishmania major*, lack an MGL gene. This limited distribution of MGL among parasitic protozoa suggests that MGL and its metabolic pathway play a specific role related to the unique metabolism shared by these two parasites. MGL activity has not been detected in crude extracts from other anaerobic protozoan parasites such as *E. invadens*, *Trichomonas foetus* and *Trichomitus batrachorum* (Thong *et al.*, 1987b; Lockwood and Coombs, 1991), suggesting that the presence of MGL is not directly associated with anaerobic metabolism. Although the genome databases of *E. invadens* and other *Entamoeba* species are still incomplete, putative MGL genes are present in *E. dispar* and *E. moshkovskii*, but probably not in *E. invadens* and *E. terrapinae* (personal observation). Our previous phylogenetic analyses indicate that *Entamoeba* MGL forms a monophyletic relationship with archaeal MGL (*Methanosarcina*), while *Trichomonas* MGL forms an independent clade with *Pseudomonas* and other bacterial MGL, suggesting that the origins of MGL are different in these two parasites. These data suggest that lateral gene transfers of MGL from the ancestral organism *Methanosarcina* to *Entamoeba*, and from eubacterial organisms to *Trichomonas*, occurred after the branching of *E. histolytica*/*E. dispar*/*E. moshkovskii* from the other *Entamoeba* groups.

MGL activity in *Trichomonas* was initially described as homocysteine desulfurase (EC 4.4.1.2) activity. This activity was claimed to be responsible for the breakdown of L-methionine in *T. vaginalis*, as occurs with bacterial MGL (Thong and Coombs, 1987; Thong *et al.*,

1987b). MGL of *T. vaginalis* was further characterized enzymologically after purification using conventional methods (Lockwood and Coombs, 1991), and later also by molecular cloning and biochemical characterization of recombinant MGL expressed in *Escherichia coli* (Han *et al.*, 1998; McKie *et al.*, 1998). These studies indicate that MGL catalyzes α,β- and α,γ-elimination reactions of a wide range of sulfur amino acids, e.g. methionine, homocysteine, cysteine and OAS, but not cystathionine. The lack of cystathionine lyase activity, together with the absence of several specific signature motifs shared by MGL from other organisms (Tokoro *et al.*, 2003), clearly indicates that MGL represents a new subfamily of the α-family of PLP-dependent enzymes. Interestingly, *T. vaginalis* possesses two MGL isoenzymes, which are different in substrate specificities and overall charge (isoelectric point). Similarly, *Esch. histolytica* also possesses two MGLs (Tokoro *et al.*, 2003) with distinct substrate specificities and isoelectric points. The two MGL isotypes from *E. histolytica* expressed in *Esch. coli* exhibited different colors and behaviors during chromatographic separation, suggestive of significant biochemical differences, e.g. the presence of a different prosthetic group and interaction with other proteins (personal observation). However, it is not known if the distinct enzymological properties of the two MGL isotypes, demonstrated *in vitro*, reflect differences in function *in vivo*, such as substrate preference and compartmentalization.

5.3. Functions of MGL in the Anaerobic Protozoan Parasites *Entamoeba histolytica* and *Trichomonas vaginalis*

Among the products of methionine degradation by MGL are α-keto acids, i.e. pyruvate and α-ketobutyrate, which provide a carbon skeleton to form other amino acids (e.g. pyruvate is involved in the formation of alanine). These α-keto acids are also key intermediates for energy metabolism (Lockwood and Coombs, 1991). Fermentation of pyruvate, forming acetate or ethanol, occurs via acetyl CoA, a product given by pyruvate:ferredoxin oxidoreductase, which thus plays an indispensable role in the energy metabolism of *Entamoeba histolytica*

and *Trichomonas vaginalis* (Reeves *et al.*, 1977). Pyruvate:ferredoxin oxidoreductase in these anaerobic protozoa utilizes various α-keto acids for the production of acetyl CoA and propionic acid (Upcroft and Upcroft, 1999). Furthermore, thiols, i.e. methanethiol and hydrogen sulfide, are also produced as a consequence of methionine degradation by MGL, and have been implicated in the pathogenesis of oral microorganisms (Lancero *et al.*, 1996). It was shown that exposure to low concentrations of these gases induced alteration of cell membrane permeability to ions as well as to larger molecules such as endotoxin (Ng and Tonzetich, 1984). These gases also hampered intracellular signaling pathways in oral tissues by increasing intracellular levels of cyclic AMP (Ratkay *et al.*, 1995) and/or by alteration of phosphorylation of specific proteins (Guan *et al.*, 1991). By analogy, these toxic gases may contribute to tissue damage caused by infections with *E. histolytica* and *T. vaginalis.* Methanethiol has also been identified as a major flavoring compound in many traditional fermented foods, including cheese (Dias and Weimer, 1998; Weimer *et al.*, 1999; Bonnarme *et al.*, 2001).

Since hydrogen sulfide, formed from cysteine or homocysteine by MGL, is reused by the sulfur assimilatory cysteine biosynthetic pathway to resynthesize cysteine, it is conceivable that the activity of these two pathways is coordinately regulated. MGL from *E. histolytica* and *T. vaginalis* does not react with cystathionine (Lockwood and Coombs, 1991; Tokoro *et al.*, 2003), whereas *Pseudomonas putida* and *Aeromonas* MGL utilize cystathionine (Esaki and Soda, 1987). The lack of cystathionine lyase activity in *E. histolytica* and *T. vaginalis* MGL seems to be reasonable since these protozoa lack both forward and reverse transsulfuration pathways, and thus do not produce cystathionine as an intermediate compound. *E. histolytica* and *T. vaginalis* possess MAT and SAHH, and thus produce homocysteine. Production of homocysteine does not appear to be beneficial for these parasites since they lack methionine recycling pathways (both methionine synthase and the MTA cycle; see above). Thus, it is conceivable that removal of AdoHcy via homocysteine by MGL is an absolute requirement for efficient methylation by AdoMet in these organisms. Thus, the removal of AdoHcy may be a primary role of MGL in these protozoan parasites.

5.4. MGL as a Target for the Development of Novel Drugs Against *Entamoeba histolytica* and *Trichomonas vaginalis*

Since MGL is present in *Entamoeba histolytica* and *Trichomonas vaginalis*, and absent from their mammalian hosts, it is an ideal target for the development of new drugs against these parasites. Our initial attempt to use inhibitors of *E. histolytica* MGL was not successful, as we found that a general inhibitor of PLP-dependent enzymes, propargylglycine, at 20 μM completely inhibited MGL activity in axenic cultures without causing growth inhibition for at least 48 hours. This indicates that MGL may not be essential for *E. histolytica* survival and growth in nutrient-rich conditions such as axenic media (e.g. BI-S-33). Coombs and Mottram (2001) and later ourselves (Tokoro *et al.*, 2003) hypothesized that a compound that is decomposed to yield a toxic product should serve as a drug against *E. histolytica*. A fluoro-substituted methionine analog, trifluoromethionine, was tested against *T. vaginalis* and *E. histolytica*, and showed significant cytopathic effects on these parasites *in vitro* (Coombs and Mottram, 2001; Tokoro *et al.*, 2003). Peritoneal administration of trifluoromethionine also cured mouse peritoneal infection by *T. vaginalis* (Coombs and Mottram, 2001) and also liver abscess caused by *E. histolytica* (personal observation). We are currently testing pro-drugs of trifluoromethionine with modifications that enable slow release and an extended half life.

The crystal structure of *T. vaginalis* and *Pseudomonas putida* MGL has been recently elucidated (Motoshima *et al.*, 2000, and the structure of *T. vaginalis* MGL has been deposited in the database). The *P. putida* MGL crystal revealed its spiral arrangement of subunits, with three functionally distinct domains and a quaternary arrangement, which is similar to the structure of CBS and CGL from *Escherichia coli* (Motoshima *et al.*, 2000). Structural analysis of MGL from *T. vaginalis* and *E. histolytica*, which is in progress, should facilitate structure-based designing of more effective inhibitors and 'suicide' compounds for these protozoan parasites.

6. SERINE METABOLIC PATHWAYS

6.1. Serine Metabolic Pathways in Eukaryotes

6.1.1. A General Scheme of Phosphorylated and Non-phosphorylated Serine Metabolic Pathways in Mammals

L-Serine is a key intermediate in a number of important metabolic pathways including the synthesis of proteins, phospholipids, and neurotransmitters, and is an intermediate in the metabolism of sulfur-containing amino acids (Snell, 1984; Giovanelli, 1987; Soda, 1987). L-Serine is also converted into L-cysteine, glycine, and L-methionine (Snell, 1984). Two major pathways are known for L-serine metabolism in mammals: phosphorylated and non-phosphorylated. These two pathways play mutually exclusive roles in L-serine metabolism (Snell, 1986). The phosphorylated pathway is the primary route for L-serine biosynthesis, while the non-phosphorylated pathway is involved in L-serine degradation and gluconeogenesis with a net flow in the reverse direction (Cheung *et al.*, 1969; Rowsell *et al.*, 1969; Chen, K.S. and Lardy, 1988). The phosphorylated pathway utilizes a glycolytic intermediate 3-phosphoglycerate (PHP), and includes three sequential reactions catalyzed by D-phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95), phospho-L-serine aminotransferase (PSAT, EC 2.6.1.52) and *O*-phospho-L-serine phosphatase (PSP, EC 3.1.3.3) (Figure 3). The non-phosphorylated pathway operates in the opposite direction, forming 2-phosphoglycerate from L-serine; it consists of three reactions catalyzed by L-serine:pyruvate aminotransferase (SPAT, EC 2.6.1.51), D-glycerate dehydrogenase (GDH, EC 1.1.1.29) and D-glycerate kinase (GK, EC 2.7.1.31) (Snell, 1986).

6.1.2. Regulation of the Phosphorylated Serine Metabolic Pathway

The L-serine biosynthetic pathway is affected by nutritional conditions in mammals (Snell, 1986). All three enzymes of the phosphorylated

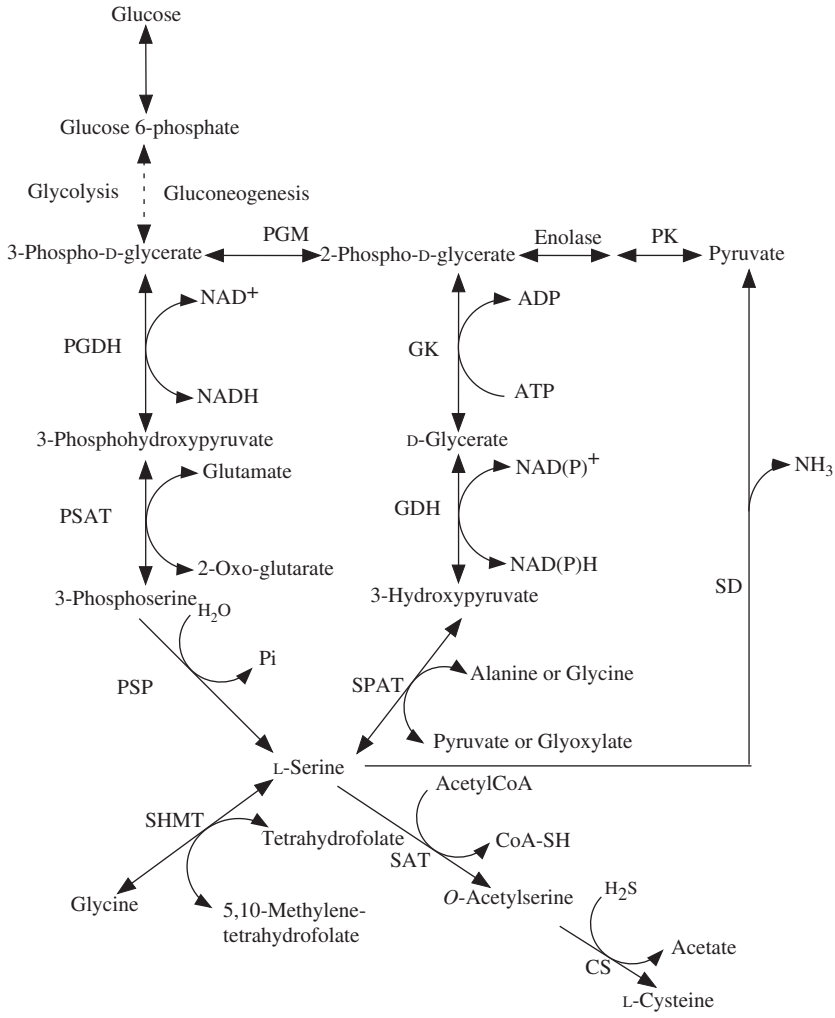

Figure 3 A general scheme of serine metabolic pathways. The abbreviations used are: CS, cysteine synthase (EC 2.5.1.47); GDH, glycerate dehydrogenase (EC 1.1.1.29); GK, glycerate kinase (EC 2.7.1.31); PGDH, phosphoglycerate dehydrogenase (EC 1.1.1.95); PK, pyruvate kinase (EC 2.7.1.40); PSAT, phosphoserine aminotransferase (EC 2.6.1.52); PSP, phosphoserine phosphatase (EC 3.1.3.3); SAT, serine *O*-acetyltransferase (EC 2.3.1.30); SD, serine dehydratase (EC 4.3.1.17); SHMT (GHMT), serine (glycine) hydroxymethyl transferase (EC 2.1.2.1); and SPAT (AGAT), serine pyruvate (alanine glyoxylate) aminotransferase (EC 2.6.1.51).

pathway in the liver are regulated in response to dietary and hormonal changes. The activity of PGDH and PSP is down-regulated by a high protein/low carbohydrate diet. Conversely, PGDH expression is induced by a low protein/high carbohydrate diet (Snell, 1984). The sulfur-containing amino acids methionine and cysteine also modulate PGDH expression and its activity (Fallon *et al.*, 1966; Achouri *et al.*, 1999). Hormonal factors such as glucagon and corticosteroids also influence PGDH and PSP activity and the corresponding mRNA level (Snell, 1984; Achouri *et al.*, 1999).

The pathway appears to be controlled by the cellular demand for L-serine rather than the supply of the precursor PHP (Snell and Fell, 1990). Intracellular concentrations of PHP are relatively high (0.3 μmol/g wet weight of rat liver) (Guynn *et al.*, 1986). Thus, availability of PHP cannot be a factor in determining overall L-serine production. Instead, the flux of L-serine synthesis is regulated by the reaction rate of PSP, which catalyzes the irreversible and rate-limiting step of L-serine biosynthesis (Fell and Snell, 1988; Snell and Fell, 1990). It has also been shown that enzymes of the phosphorylated pathway are up-regulated in proliferating tissues, such as neoplastic tissues, to meet the cellular demands for increased L-serine utilization (Snell *et al.*, 1988). The enzymes of the phosphorylated pathway are widely distributed in humans and high mRNA expression and activity of these enzymes are found in a variety of organs (Snell, 1984; Klomp *et al.*, 2000; Baek *et al.*, 2003). The high level of PGDH and PSAT expression indicates active L-serine biosynthesis and rapid cell proliferation in these tissues.

In plants, the phosphorylated serine pathway is regulated by environmental stress, i.e. high salinity, flooding and low temperature (Ho and Saito, 2001). For instance, mRNA expression of the PGDH gene was increased in conditions of high salinity, whereas PSAT mRNA expression was increased during flooding (Ho and Saito, 2001). PGDH activity from *Escherichia coli* (Sugimoto and Pizer, 1968), *Bacillus subtilis* (Saski and Pizer, 1975) and pea plants (Slaughter and Davies, 1968) was inhibited by the end product of the pathway, L-serine. Thus, PGDH also plays a critical role in the negative

regulation of this pathway. However, such allosteric inhibition by the end product was not reported for PGDH from other plants (Larsson and Albertsson, 1979; Ho *et al.*, 1999b), animals (Walsh and Sallach, 1965; Jaeken *et al.*, 1996a; Achouri *et al.*, 1997) and *Entamoeba histolytica* (see below; Ali *et al.*, 2004a). PSP is also regulated by feedback inhibition by L-serine at physiological concentrations in bacteria (Wang, W. *et al.*, 2002), mammals, plants and some other eukaryotes (Knox *et al.*, 1969; Ho *et al.*, 1999a).

6.1.3. *Serine–Pyruvate Conversion and Gluconeogenesis in Mammals*

In addition to the non-phosphorylated pathway described above, L-serine is also utilized by serine dehydratase (SD, EC 4.3.1.17) for the production of pyruvate and further gluconeogenesis. The contribution of these two routes to gluconeogenesis is influenced by dietary composition in animals (Snell, 1984), and is also organism- and tissue-dependent (Xue *et al.*, 1999a, b). Under starvation or hormonal action, SD favors energy metabolism since it directly produces pyruvate from L-serine (Xue *et al.*, 1999a). Although SPAT activity was shown to be elevated 6–10-fold by glucagon administration in rat liver, the flux through pyruvate accounted for 80–90% of gluconeogenesis, reinforcing the major contribution of SD to gluconeogenesis.

6.1.4. *Serine–Glycine Conversion by Serine Hydroxymethyltransferase*

L-Serine is converted into glycine by cytosolic serine hydroxymethyltransferase (cSHMT; EC 2.1.2.1, a synonym of glycine hydroxymethyltransferase), with concomitant generation of 5,10-methylene tetrahydrofolate from tetrahydrofolate (THF) (Figure 3). This reaction is unidirectional in physiological conditions, and provides the major source of one-carbon groups, e.g. formyl groups for purine synthesis and methyl groups for pyrimidine synthesis, remethylation

of homocysteine (see above) and many other methylation reactions involved in cellular homeostasis (Fu, T.F. *et al.*, 2001; Herbig *et al.*, 2002). Mitochondrial SHMT (mSHMT) operates in the opposite direction and is primarily involved in the production of L-serine from glycine (Appling, 1991). In neoplastic tissues, SHMT is significantly up-regulated together with several enzymes in the phosphorylated serine pathway (Snell *et al.*, 1988). These changes probably meet the high demand for L-serine required for nucleotide biosynthesis during rapid cell proliferation. SHMT has been shown to be a potential target for the development of anticancer chemotherapeutics, either by antagonizing the PLP cofactor or by direct inhibition of SHMT (Lin *et al.*, 1996; Renwick *et al.*, 1998).

6.1.5. *Human Genetic Disorders and Physiological Importance of the Serine Metabolic Pathway*

Genetic disorders of the metabolism of sulfur-containing and related amino acids are generally associated with neurological dysfunction (Pepplinkhuizen *et al.*, 1980; Waziri *et al.*, 1983, 1984; Tada *et al.*, 1992; Devor and Waziri, 1993; De Koning *et al.*, 2003). The first two cases of genetic neurological disorders in humans attributable to a defect in the phosphorylated L-serine pathway (PGDH and PSP) were reported by Jaeken *et al.* (1996a, b) and De Koning *et al.* (2003).

Genetic disorders of the non-phosphorylated serine pathway have also been well studied (Wadman *et al.*, 1976; Cochat & Rolland, 2002). In hyperoxaluria type 2, a defect in GDH activity causes accumulation of hydroxypyruvate (HP) and glyoxylate, which are converted into L-glycerate and oxalate by lactate dehydrogenase, resulting in the abnormal excretion of L-glycerate and oxalate in urine (Chalmers *et al.*, 1984; Van Schaftingen *et al.*, 1989; Giafi and Rumsby, 1998). In D-GK deficiency, D-glycerate cannot be phosphorylated to complete the pathway (Wadman *et al.*, 1976; Cochat and Rolland, 2002), causing D-glyceric aciduria (Van Schaftingen *et al.*, 1989). These genetic errors reinforce the physiological importance of L-serine metabolism in mammals.

6.2. A Spectrum of Serine Metabolic Pathways in Parasitic Protozoa

6.2.1. A Survey of Enzymes of Phosphorylated and Non-phosphorylated Serine Metabolic Pathways in Parasitic Protozoa

In order to examine how ubiquitous the serine metabolic pathways are in parasitic protozoa, we conducted a wide-ranging search of all the known enzymes involved in L-serine metabolism. Our analysis indicated that *Entamoeba histolytica* and *Trichomonas vaginalis* are similar in that both organisms possess both of the two L-serine pathways and the enzyme responsible for L-serine–pyruvate conversion (SD). One exception is that *E. histolytica* lacks an enzyme for L-serine–glycine conversion (SHMT) while *T. vaginalis* retains it (Table 2). In contrast, another anaerobic (microaerophilic) and amitochondrial protist, *Giardia duodenalis*, lacks all the pathways except SD (Ali *et al.*, 2003, 2004a) and utilizes L-serine for energy production, as does *E. histolytica* (Takeuchi *et al.*, 1979). Therefore, serine-associated energy metabolism is significantly diverse between these two groups of amitochondrial protozoa. The remarkable similarity of L-serine metabolism in *E. histolytica* and *T. vaginalis* is unexpected as *T. vaginalis* differs from *E. histolytica* and *G. duodenalis* in compartmentalization of energy metabolism. Energy production depends on hydrogenosomes, which are analogous to the mitochondria in higher eukaryotes in *T. vaginalis*, while *E. histolytica* and *G. duodenalis* lack this organelle (Muller, M., 1988, 1992). Since *Giardia* appears to be devoid of all known L-serine metabolic pathways except SD, it remains unclear how *G. duodenalis* maintains intracellular L-serine and glycine pools. It may be that *G. duodenalis* depends solely on uptake of L-serine from an extracellular milieu via as yet unidentified amino acid transporters. It has previously been shown that L-serine and arginine are incorporated from the external medium by *G.* '*intestinalis*' (a synonym of *G. duodenalis*) by Edwards *et al.* (1989). It has also been shown that L-serine is transported by a putative alanine transporter (Knodler *et al.*, 1994). It is worth mentioning that intracellular concentration of L-serine is significantly

Table 2 Enzymes involved in serine metabolism in parasitic protozoa[a]

Organisms	Enzymes							
	Phosphorylated pathways			Non-phosphorylated pathways			Serine–glycine conversion	Serine degradation
	PGDH	PSAT	PSP	SPAT	GDH	GK	SHMT	SD
E. histolytica	+	+	+	–	+	+	–	+
G. duodenalis	–	–	–	+[b]	–	–	–	+
T. vaginalis	+	+	–	–	+	+	+	+
P. falciparum	–	–	–	–	–	–	+	–
C. parvum	–	–	–	–	–	–	+	–
L. major	+	–	–	+[b]	–	–	+	–
T. cruzi	–	–	–	+	+	+	+	+
T. brucei	–	–	–	–	–	–	–	–

Accession numbers of putative homologs (either protein or nucleotide) are as follows: *E. histolytica*, PGDH (AB091512), PSAT (AB091513), PSP (191.m00122), GDH (AB091511), GK (92.m00143, 250.m00075), SD (4.m00604, 117.m00151, 221.m00087, 26.m00279); *G. duodenalis*, SPAT (EAA42011), SD (AAM94634); *T. vaginalis*, PGDH (39121.m00033), PSAT (61700.m00121), GDH (50194.m00035), GK (37903.m00037), SHMT (83992/133781 Nuc. No. 58883-60116), SD (37851.m00026, 57441.m00043); *P. falciparum*, SHMT (NP_701706); *C. parvum*, cSHMT (EAK89448), mSHMT (EAK89612); *L. major*, PGDH (AAM68972), SPAT (AAM68973), cSHMT (CAB94023), mSHMT (CAB72302); and *T. cruzi*, SPAT (8170.m00010), GDH (6876.m00003, 8304.m00012), GK (7853.m00017), SHMT (8251.m00015), SD (7107.m00007).

[a]See the legend of Figure 3 for abbreviations used in this table; + and – symbols denotes presence or absence of the gene.

[b]These putative SPAT showed highest homology (35–40% amino acid identity) to both 2-aminoethyl phosphonate:pyruvate aminotransferase and SPAT from various bacteria. A putative SPAT gene from *L. major* is denoted 2-aminoethyl phosphonate:pyruvate aminotransferase in the genome database.

lower in *G.* '*intestinalis*' (0.18 mM; Knodler *et al.*, 1994) than in two other amitochondriate protists, *E. histolytica* (3.2 mM; Bakker-Grunwald *et al.*, 1995) and *T. vaginalis* (1.15 mM; Knodler *et al.*, 1994), which may reflect the absence of major metabolism of serine by *Giardia*.

Both *Plasmodium falciparum* and *Cryptosporidium parvum*, like *Giardia*, lack both the phosphorylated and non-phosphorylated pathways (Gardner *et al.*, 2002; Abrahamsen *et al.*, 2004). Instead, these organisms possess an active L-serine–glycine interconversion pathway and apparently depend solely on this pathway for L-serine biosynthesis. The situation in kinetoplastids is more complicated. *Leishmania major* possesses a gene for PGDH only, and lacks all the other enzymes of the phosphorylated pathway. The biological significance, if any, of the solitary presence of PGDH but not the other enzymes of the pathway in *Leishmania* is unclear. In contrast to *Leishmania*, *Trypanosoma cruzi* seems to possess only enzymes of the non-phosphorylated, but not the phosphorylated, pathway, which is unique to this parasite. Although two contigs in the *T. cruzi* genome database contain putative GDH/PGDH/2-hydroxyacid dehydrogenase genes, our amino acid comparisons and phylogenetic reconstructions with GDH and PGDH from numerous organisms unequivocally demonstrated that they are most probably GDH. *Trypanosoma brucei* lacks all the enzymes of both phosphorylated and non-phosphorylated pathways. The biological significance of the evolutionary retention and loss of individual L-serine pathways remains largely unknown.

6.2.2. Distribution of Serine–Glycine and Serine–Pyruvate Conversion Pathways in Parasitic Protozoa

The conversion of L-serine to glycine occurs in most parasitic protozoa except *Entamoeba histolytica*, *Giardia duodenalis* and *Trypanosoma brucei*. The absence of this pathway in *E. histolytica* and *G. duodenalis* agrees well with the fact that these parasites lack folate metabolism, which gives rise to THF required as a hydroxylmethyl donor of SHMT (Alfadhli and Rathod, 2000). It is conceivable that SD, which is involved in conversion of L-serine into pyruvate and

ammonia, compensates for the lack of SHMT in these two organisms. It is intriguing that this enzyme is also missing from *Plasmodium falciparum*, *Cryptosporidium parvum* and *Leishmania major*, suggesting that the presence or absence of these two conversion pathways is mutually exclusive, with only two exceptions—*Trichomonas vaginalis* and *Trypanosoma cruzi*. However, we are not confident about the complete lack of serine metabolism in *Tryp. brucei* due to the incomplete nature of the genome database of this organism.

One group of parasites, *C. parvum*, *Tryp. cruzi* and *L. major*, have two types of SHMT (mitochondrial and cytosolic) while another group, *Trich. vaginalis* and *P. falciparum*, possesses only cytosolic SHMT. Since *Plasmodium* lacks serine biosynthetic pathways, its serine is probably derived solely extracellularly, e.g. by heme degradation. The wide (almost ubiquitous) distribution of SHMT strongly indicates the biological importance of L-serine–glycine conversion in the Apicomplexa and Kinetoplastida.

SHMT has been biochemically characterized only from *P. falciparum* (Alfadhli and Rathod, 2000). SHMT is associated with methylene tetrahydrofolate recycling, in which bifunctional dihydrofolate reductase–thymidylate synthase (DHFR–TS) was also extensively characterized as an important target of common antimalarial drugs including pyrimethamine and cycloguanil (Cowman, 1998). It is worth emphasizing that malaria parasites have conserved L-serine–glycine conversion while they have lost most of the metabolic pathways for other amino acids; only a limited set of enzymes involved in the conversion of L-serine to glycine, cysteine to alanine, and the interconversion between aspartate and aspargine, and glutamate and glutamine, are retained in the *P. falciparum* genome (Gardner *et al.*, 2002). *E. histolytica* and *G. duodenalis* lack SHMT and the conversion of L-serine into glycine. However, they possess PLP-dependent SD, which directly converts L-serine into pyruvate. It is worth mentioning that differentiation of SD and threonine dehydratase (EC 4.3.1.16) at the primary sequence level is not very reliable as putative SD homologs from protozoan parasites also showed significant homology to PLP-dependent threonine dehydratases, which deaminate both L-serine and threonine.

6.3. Unique Presence of Both Serine Metabolic Pathways in *Entamoeba histolytica* and *Trichomonas vaginalis*

Genome survey showed that both *Entamoeba histolytica* and *Trichomonas vaginalis* possess all the enzymes of the phosphorylated and non-phosphorylated pathways, except for SPAT in *E. histolytica* and PSP and SPAT in *T. vaginalis* (Table 2). Since SPAT is known to be localized in mitochondria or peroxisomes in mammals, both of which are missing from *E. histolytica*, it is conceivable that the amebic SPAT might have been lost during secondary loss of the mitochondria (Clark and Roger, 1995). SPAT activity, if present, may be attributable to an unidentified protein localized in a different compartment in these organisms. Since *E. histolytica* possesses a residual mitochondria-like organelle, the mitosome or crypton (Mai *et al.*, 1999; Tovar *et al.*, 1999), it is possible that SPAT or a SPAT-like protein is localized in this organelle in *E. histolytica*. In contrast, the biological significance of the lack of PSP and SPAT in *T. vaginalis*, which possesses hydrogenosomes, remains totally unresolved. We cannot at present be certain whether other ATs, present in the genome database, catalyze the conversion of L-serine into HP.

It is very striking that *E. histolytica* and *T. vaginalis* share many common features of sulfur-containing amino acid metabolism: (i) the presence of a sulfur assimilatory *de novo* cysteine biosynthetic pathway, (ii) the presence of MGL, (iii) lack of both the forward and reverse transsulfuration pathways, (iv) an MTAN-dependent methionine recycling pathway and (v) the presence of both phosphorylated and non-phosphorylated serine pathways. These shared characteristics should give us clues to the biological and physiological advantages of these metabolic pathways. The only major difference we found between *E. histolytica* and *T. vaginalis* by genome survey was the fact that SHMT is present in *T. vaginalis* and absent from *E. histolytic*a. The biological and evolutionary significance of the unique presence in *T. vaginalis* of SHMT, which contains a possible mitochondria/hydrogenosome-targeting signal, needs to be elucidated.

6.3.1. *The Phosphorylated Serine Pathway in* Entamoeba histolytica

Two major enzymes in the phosphorylated serine pathway, PGDH and PSAT, from *Entamoeba histolytica* were enzymologically characterized (Ali *et al.*, 2004a). The kinetic studies of recombinant PGDH showed an approximately eight-fold higher affinity for NADH than for NADPH, and specific activity was about three-fold higher with NADH than with NADPH in the reverse direction. The K_m for PHP and NAD^+ in the forward reaction was one order higher than those for 3-phosphohydroxypyruvate (PHP) and NADH in the reverse reaction (Ali *et al.*, 2004a). The K_m of *E. histolytica* PGDH for substrates was similar to that of mammalian PGDH (Walsh and Sallach, 1965; Achouri *et al.*, 1997), and one to two orders lower than that of bacterial PGDH (Sugimoto and Pizer, 1968). PGDH and PSAT, as well as GDH of the non-phosphorylated pathway, are not compartmentalized, and are likely to be cytosolic. While PGDH from all other organisms forms a homotetramer with a monomer size of 44–67 kDa, the amoebic PGDH exists in a homodimeric form (Ali *et al.*, 2004a).

Kinetic analyses of *E. histolytica* PSAT showed that the biochemical properties of this enzyme from *E. histolytica*, bacteria and mammals was similar in that (i) PSAT showed one order higher affinity to L-glutamate than to α-keto-glutarate, while affinity to PHP and *O*-phospho-L-serine was comparable and (ii) the specific activity in the forward reaction was one order higher than that in the reverse direction (Hirsch and Greenberg, 1967; Basurko *et al.*, 1999; Ali and Nozaki, unpublished study). When compared to plant PSAT, *E. histolytica* PSAT showed substantially different affinity toward substrates: K_m of PSAT from *E. histolytica* for PHP was two orders lower than that of PSAT from spinach and *Arabidopsis thaliana* (Saito *et al.*, 1997; Ho *et al.*, 1998), while K_m for glutamate is 5–10-fold higher. However, our current analysis of the kinetic parameters of *E. histolytica* and *A. thaliana* PSAT showed a similar K_m for PHP, but a seven-fold higher K_m for glutamate of *A. thaliana* PSAT. In addition, the specific activity of *A. thaliana* PSAT in the forward reaction was 1.5–3.7-fold higher. These data are consistent with the premise that *E. histolytica* PSAT is enzymologically superior to plant PSAT, which

may represent a biological advantage for *E. histolytic*a. Three substrate analogs, *O*-phospho-D-serine, *O*-phospho-L-threonine and OAS partially inhibited amebic PSAT activity, in contrast to mammalian and plant enzymes, which are not inhibited by these substrate analogs (Ho *et al.*, 1998; Basurko *et al.*, 1999).

6.3.2. *The Non-Phosphorylated Serine Pathway in* Entamoeba histolytica

GDH from all organisms including *Entamoeba histolytica* forms a homodimer (Rosenblum *et al.*, 1971; Greenler *et al.*, 1989; Izumi *et al.*, 1990; Ali *et al.*, 2003). The amebic GDH showed a strong preference toward NADPH and HP compared to $NADP^+$ and D-glyceric acid. We demonstrated that recombinant amebic GDH possesses two order higher affinities to NADPH than to NADH. However, specific activity was four-fold higher with NADH than with NADPH. The K_m for D-glyceric acid and $NADP^+$ in the reverse reaction was one order higher than those for HP and NADPH in the forward reaction (Ali *et al.*, 2003). *E. histolytica* GDH cannot utilize glyoxylate as substrate to produce glycolate, while GDH from mammals and plants can catalyze this reaction. Thus, the amebic GDH appears to be specific for the HP–glycerate conversion (Ali *et al.*, 2003).

The non-phosphorylated pathway is unlikely to be involved in energy metabolism in *E. histolytica* for the following reasons. First, gluconeogenesis does not occur in the ameba due to lack of an enzyme for the committed step (conversion of fructose-1,6-bisphosphate into fructose-6-phosphate) (McLaughlin and Aley, 1985). Second, the first enzyme of the pathway, SPAT, consumes pyruvate as an amino residue acceptor from L-serine in a transamination reaction to yield HP (Snell, 1984). Thus, the net yield of pyruvate from L-serine and pyruvate through the non-phosphorylated pathway is zero. In addition, one molecule each of NADPH and ATP are consumed to produce 2-phosphoglycerate from L-serine. However, this pathway is stimulated during starvation or by hormonal action, as has been demonstrated in mammals, and contributes substantially to energy metabolism during starvation (Xue *et al.*, 1999b). Thus, it is possible,

by analogy, that this pathway may also play a role in energy metabolism during starvation or encystation of the ameba.

6.3.3. *Origin of Serine Metabolic Pathways in* Entamoeba histolytica *and* Trichomonas vaginalis

Protein alignments and phylogenetic analyses of proteins involved in the phosphorylated pathway suggest that PGDH and PSAT from *Entamoeba histolytica* show a close kinship to those of bacteroides. This close kinship was also supported by the shared deletion and high amino acid identities (48–50% for PGDH and 56–59% for PSAT). *Trichomonas vaginalis* PGDH and PSAT appear to have ancestors distinct from those of the *E. histolytica* enzymes. For instance, *T. vaginalis* PGDH showed a close association with homologs from *Pyrococcus horikoshii* and *P. furiosus*, suggesting that *T. vaginalis* gained PGDH from an ancestral organism similar to archaea. On the contrary, *T. vaginalis* PSAT showed closest kinship to homologs from fungi and mammals (Ali and Nozaki, unpublished study). Interestingly, *T. vaginalis* PSAT possesses a 38-amino acid extension at the N terminus, which is not present in the amebic PGDH. This extension contains 11 positively charged and 16 hydrophobic residues, consistent with the mitochondria-targeting signal (unpublished observation). This finding suggests that *T. vaginalis* PSAT might be compartmentalized in hydrogenosomes. These data are consistent with the notion that most of the genes, if not all, encoding the enzymes involved in the phosphorylated pathway in *E. histolytica* were transferred from bacteroides or their ancestral organisms to *E. histolytica* or vice versa (i.e. from *E. histolytica* to bacteroides) by a single lateral gene transfer event. In contrast, the genes involved in the phosphorylated serine pathway in *T. vaginalis* were probably obtained from distinct ancestral organisms by at least a few independent lateral transfer events. It would be very interesting to know if PGDH and PSAT from *T. vaginalis* possess enzymological properties similar to or distinct from those of the *E. histolytica* enzyme. Lateral gene transfer has been well documented in *E. histolytica*, mainly for metabolic (fermentation) enzymes that were transferred from archaea or bacteria to the parasite (Field *et al.*, 2000; Nixon *et al.*, 2002).

Phylogenetic analysis of the non-phosphorylated pathway (Ali *et al.*, 2003) led us to a different scenario. *E. histolytica* GDH showed strong affinity (38–40% protein identity) to ε-proteobacteria including *Campylobacter jejuni* and *Helicobacter pylori*. This close kinship was also supported by the shared amino acid insertions, suggesting that amebic GDH was probably obtained from an ancestral organism of current ε-proteobacteria by lateral transfer. In contrast, *T. vaginalis* GDH showed its highest identity (32%) to *Escherichia coli*, *Bacillus subtilis* and human enzymes, while it was only 26% identical to *E. histolytica* GDH. Phylogenetic analyses also supported the premise of a close association of *Trichomonas* GDH with mammalian and γ-proteobacteria GDH. GK catalyzes phosphorylation to form 2-phosphoglycerate from D-glycerate utilizing ATP as a phosphate donor (Figure 3). *E. histolytica* and *T. vaginalis* GK showed highest identities (40–42%) to *Bacillus cereus*, *Esch. coli* (GK2 isotype), and 32% to *Haemophilus influenzae*, *B. halodurans* and *B. subtilis*. Phylogenetic analyses showed that *E. histolytica* and *T. vaginalis* GK belong to a single clade, suggesting that GK from *E. histolytica* and *T. vaginalis* may have had the same evolutionary origin, dissimilar to that of other enzymes of the phosphorylated and non-phosphorylated serine metabolic pathways (personal observation).

6.4. Biological Significance of Serine Metabolic Pathways in Parasitic Protozoa

Serine is a precursor of cysteine in both the reverse transsulfuration and sulfur assimilatory *de novo* cysteine biosynthetic pathways. Thus, in virtually all organisms, including protozoa, serine metabolic pathways contribute to the regulation of cysteine production. In the phosphorylated serine pathway, the primary substrate of the pathway, 3-phosphoglyceric acid, is an important intermediate of glycolysis. Thus, an overall flow of glycolysis also probably influences the cellular concentration of 3-phosphoglyceric acid and its availability for the serine metabolic pathway. In the sulfur assimilatory *de novo* cysteine biosynthetic pathway, since OAS is unstable *in vivo*, the production of OAS from acetyl CoA and serine by SAT appears to be

a bottleneck in cysteine production. The final enzyme of this pathway, CS, is negatively regulated by an intermediate of glycolysis (pyruvate) as well as by intermediates of L-serine metabolism (*O*-phosphoserine and HP) in *E. histolytica* (personal observation). Taken together, both intermediates of glycolysis and L-serine metabolism influence cysteine synthesis.

While serine metabolic pathways are absent from the majority of parasitic protists, as described above, there are several exceptions: *Entamoeba histolytica*, *Trichomonas vaginalis*, *Trypanosoma cruzi* and *Leishmania major*. In particular, the former two organisms possess both the serine metabolic pathways. It is conceivable that it is because L-serine metabolism plays such critical roles, including pyruvate production via SD and *de novo* cysteine synthesis, that dual pathways are retained in these parasites. The presence of the non-phosphorylated serine metabolic pathway in the amitochondrial protozoan parasites may be associated with the unique metabolism of anaerobic/microaerophilic parasites. Since both *E. histolytica* and *T. vaginalis* do not possess a functional tricarboxylic acid cycle and pentose-phosphate pathway, their major source of energy is glycolysis and fermentation. In addition, due to lack of lactate dehydrogenase, NADH formed during glycolysis is not reoxidized by the conversion of pyruvate into lactate in *E. histolytica*. Instead, acetyl CoA is anaerobically reduced to ethanol and CO_2 in *E. histolytica*, and to acetate in *Trichomonas* (Reeves, 1984 and McLaughlin and Aley, 1985). SD has also been implicated in the microaerophilic energy metabolism of *Entamoeba* as L-serine stimulates oxygen consumption and is converted into pyruvate in living cells and extracts (Takeuchi *et al.*, 1979). Since the non-phosphorylated pathway is not economically favored for the production of pyruvate (Section 6.3.2), there are probably other advantages of utilizing this pathway, e.g. NADH reoxidation. The physiological importance of the non-phosphorylated pathway, and in particular GDH, has been well demonstrated in L-glyceric aciduria (hyperoxaluria type II) by Giafi and Rumsby (1998) (Section 6.1.5). Since *E. histolytica* lacks lactate dehydrogenase and GDH does not catalyze glyoxylate reduction, the fate of HP, when GDH is absent, is not known. Altogether, GDH may play an important role in the maintenance of intracellular L-serine and HP concentrations, and also

in the control of downstream cysteine biosynthesis in these protozoa (Ali *et al.*, 2003). Although regulation of the non-phosphorylated pathway in gluconeogenesis depends on dietary composition in animals, regulation of this pathway in *E. histolytica* and *T. vaginalis* is totally unknown.

7. DEVELOPMENTAL STAGE REGULATION OF METABOLIC PATHWAYS OF SULFUR-CONTAINING AMINO ACIDS

7.1. Developmental Stage Regulation of Reverse Transsulfuration and *De Novo* Cysteine Biosynthetic Pathways in *Trypanosoma cruzi*

We currently have very limited knowledge of the developmental stage-specific regulation of metabolism of sulfur-containing amino acids in protozoan parasites. *Trypanosoma cruzi* possesses a complex life cycle consisting of intracellular or blood stages in mammalian hosts (amastigotes and trypomastigotes, respectively) and an extracellular stage in the alimentary tract of insect hosts (epimastigotes) (Brener, 1973). Environmental conditions such as availability of nutrition and oxidative stress drastically change during stage transitions. The reverse transsulfuration pathway and sulfur assimilatory *de novo* cysteine biosynthetic pathway are differentially regulated in two major developmental stages of *T. cruzi* (Nozaki *et al.*, 2001). Expression of CBS is stage-specific: CBS is exclusively expressed in epimastigotes, and no CBS activity has been detected in amastigotes or trypomastigotes. In contrast to CBS, CS is constitutively expressed in both mammalian and insect stages. It appears that stage-specific regulation of the reverse transsulfuration pathway in *T. cruzi* is causally connected to nutritional and environmental requirements associated with the complex life cycle of the parasite. Amastigotes or trypomastigotes reside in the cytoplasm of invaded host cells or in the bloodstream, respectively, where sulfur-containing amino acids are present in sufficient amounts and are readily available. Thus, it is conceivable that amastigotes and trypomastigotes rely on host

sulfur-containing amino acids or their intermediates, which are incorporated into the parasites. In contrast, epimastigotes live in the alimentary tract of insects, where sulfur-containing amino acids are not present in sufficient concentration. Thus, epimastigotes probably depend on two apparently redundant cysteine biosynthetic pathways. A large supply of cysteine may be required for the high demand of glutathione and trypanothione production in epimastigotes (Fairlamb and Cerami, 1992), in order to maintain redox potential in trypanosomes.

7.2. Developmental Stage Regulation of Sulfur-Containing Amino Acid Metabolism in *Plasmodium* and *Cryptosporidium*

Genes involved in sulfur-containing amino acid metabolism are also regulated in a stage-specific fashion in *Plasmodium falciparum*. We examined expression profiles of genes encoding enzymes involved in the methionine cycle (MAT, methyl transferase, SAHH, AdoMetDC–ODC, aspartate aminotransferase) and serine to glycine conversion (SHMT) using mRNA expression data based on DNA microarrays (glass slide and photolithographic oligonucleotide arrays) available on the PlasmoDB web site (http://plasmodb.org). As mentioned above, genes involved in forward and reverse transsulfuration as well as sulfur assimilatory pathways are absent from *Plasmodium*. Genes involved in the methionine cycle are constitutively expressed in all intraerythrocytic (meront) stages of *P. falciparum*; the level of expression generally peaks at the late trophozoite and early schizont stages except for aspartate aminotransferase, which is expressed continuously at comparable levels from ring to late schizont (meront) stages. Bifunctional AdoMetDC–ODC mRNA is 2–5-fold higher in sporozoites than in other stages, suggesting a specific role of this enzyme in the insect stage. The stage-specific regulation of MAT has also been described in *Cryptosporidium parvum* by Slapeta *et al.* (2003). MAT is continuously expressed throughout the life cycle, but its expression greatly increases in the late trophozoite and early meront stages of *C. parvum* (Slapeta *et al.*, 2003).

DNA microarray data showed that the expression of SHMT, which catalyzes serine–glycine conversion, is higher in late trophozoites, early schizonts and gametocytes than in sporozoites and early ring stages of *P. falciparum*. The stage-dependent expression of SHMT has also been shown independently by Nirmalan *et al.* (2002): SHMT mRNA was preferentially expressed in gametocytes and late trophozoites/schizonts. Since SHMT requires THF as a cofactor for its activity, SHMT metabolism is closely associated with the folate pathway, which has been viewed as a rational drug target for malaria (Ruenwongsa *et al.*, 1989; Rathod *et al.*, 1992). Two enzymes involved in the folate pathway have been extensively studied in *Plasmodium*. Thymidylate synthase (TS; EC 2.1.1.45), essential for *de novo* pyrimidine biosynthesis and DNA synthesis, and dihydrofolate reductase (DHFR) are known to exist as a DHFR–TS fusion protein in *Plasmodium*. The expression of DHFR–TS is constant during intraerythrocytic development of *P. falciparum* (Nirmalan *et al.*, 2002). The folate pathway and folate-dependent formation of thymidylate are critical for replication of malaria parasites. The malaria parasites salvage purines, rather than using folate-dependent synthesis (Sherman, 1979). However, malaria parasites are unable to salvage pyrimidine (Sherman, 1979), and entirely depend on the thymidylate cycle for pyrimidine biosynthesis during replication. This fact may explain why expression of DHFR–TS and SHMT are up-regulated during the transition from ring to trophozoite/schizont stages, in which DNA content drastically increases (about 14-fold per parasite in 48 hours) (Smeijsters *et al.*, 1994). All folate, synthesized *de novo* or salvaged, must be first reduced to THF by DHFR and then converted by the transfer of a hydroxymethyl moiety to THF to form 5,10-methylene tetrahydrofolate, catalyzed by SHMT. 5,10-Methylene tetrahydrofolate is then utilized to convert deoxyuridine monophosphate into deoxythymidine monophosphate catalyzed by TS, with concomitant formation of 7,8-dihydrofolate, which needs to be stoichiometrically recycled to THF by DHFR. Thus, it is conceivable that SHMT expression synchronizes with that of DHFR–TS due to the cellular requirement of THF cofactor essential for SHMT activity. It is also intriguing that SHMT expression is high in gametocytes as well as in trophozoites and schizonts. This may reflect the fact that DNA

synthesis occurs during gametocyte maturation, development of microgametes and replication following fertilization, all of which occur shortly after ingestion by the mosquito (Janse *et al.*, 1988).

8. REMAINING QUESTIONS AND FUTURE PERSPECTIVES

Despite the biological significance of the ubiquitous sulfur-containing amino acids, the distribution of the metabolic pathways of these amino acids is extremely diverse among parasitic protozoa. This level of heterogeneity was totally unexpected, and should help us in elucidating the mechanisms of retention or loss of these metabolic pathways in parasitism-associated evolution. A number of important and unsolved questions remain. (i) All the parasitic protozoa which have been studied, except for *Entamoeba histolytica* and *Trichomonas vaginalis*, apparently lack enzymes or pathways responsible for degradation of toxic intermediates of sulfur-containing amino acids, e.g. MGL and CDO. It remains totally unknown how they evade the effect of these toxic metabolites. For instance, how does *Plasmodium falciparum*, which lacks a complete methionine cycle, the MTA cycle, and both of the transsulfuration pathways, detoxify or eliminate homocysteine in erythrocytes? (ii) *Giardia duodenalis* lacks both the sulfur assimilatory *de novo* cysteine biosynthetic and reverse transsulfuration pathways. How does the parasite acquire sufficient methionine and cysteine in the small intestine? If *Giardia* acquires cysteine solely by uptake from the small intestine, it must possess an efficient cysteine transporter. (iii) Sulfur assimilatory *de novo* cysteine biosynthesis and both phosphorylated and non-phosphorylated serine metabolic pathways occur in a very limited range of organisms, i.e. *E. histolytica* and *Trich. vaginalis*, but not in other parasitic protozoa studied including the anaerobic/amitochondriate protist *G. duodenalis.* What are the biological advantages (or the selective pressure in nature) leading to the retention of these pathways in the former two species and to their elimination in the latter? (iv) Why is the reverse transsulfuration pathway conserved solely in kinetoplastids? (v) What is the biological interpretation of the striking similarity

between sulfur-containing amino acid metabolism in general in *E. histolytica* and *T. vaginalis*?

We have now a list of model organisms in which to study the specific function of each pathway. For instance, *P. falciparum* lacks most of the pathways discussed in this review, but selectively retains methionine activation and SHMT, highlighting the physiological significance of these processes in *Plasmodium*. On the other hand, *Trypanosoma cruzi* is the only known parasitic protozoon that possesses the non-phosphorylated serine pathway but not the phosphorylated pathway. It is conceivable that these pathways play an indispensable role for these organisms. Thus, *Plasmodium* and *Tryp. cruzi* should provide good models to study the biological roles in parasitism and pathogenesis of serine–glycine conversion and the non-phosphorylated serine metabolic pathway, respectively.

Only a few enzymes and pathways that are present in parasites, but absent from their mammalian hosts, have been characterized so far. All of these parasite-specific enzymes and pathways could be rational targets for new chemotherapeutic agents. In particular, (i) sulfur assimilatory *de novo* cysteine biosynthesis, (ii) MTAN in the methionine cycle and (iii) degradation of sulfur-containing amino acids by MGL, represent the most appealing targets. Considering the biological importance of sulfur-containing amino acids in parasites, either the antagonistic inhibition of these enzymes or the subversive conversion of synthetic substances (e.g. trifluoromethionine) into toxic substrates by these enzymes should be damaging for the parasites. There are already several lead compounds available for these studies. However, needless to say, further exploitation of these potentially useful drug targets requires multidisciplinary collaboration between organic and inorganic chemistry, biochemistry, structural and cellular biology, and medicine.

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The Use and Implications of Ribosomal DNA Sequencing for the Discrimination of Digenean Species

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ABSTRACT

In just over a decade, the use of molecular approaches for the recognition of parasites has become commonplace. For trematodes, the internal transcribed spacer region of ribosomal DNA (ITS rDNA) has become the default region of choice. Here, we review the findings of 63 studies that report ITS rDNA sequence data for about 155 digenean species from 19 families, and then review the levels of variation that have been reported and how the variation has been interpreted. Overall, complete ITS sequences (or ITS1 or ITS2 regions alone) usually distinguish trematode species clearly, including combinations for which morphology gives ambiguous results. Closely related species may have few base differences and in at least one convincing case the ITS2 sequences of two ''good'' species are identical. In some cases, the ITS1 region gives greater resolution than the ITS2 because of the presence of variable repeat units that are generally lacking in the ITS2. Intraspecific variation is usually low and frequently apparently absent. Information on geographical variation of digeneans is limited but at least some of the reported variation probably reflects the presence of multiple species. Despite the accepted dogma that concerted evolution makes the individual representative of the entire species, a significant number of studies have reported at least some intraspecific variation. The significance of such variation is difficult to assess *a posteriori*, but it seems likely that identification and sequencing errors account for some of it and failure

to recognise separate species may also be significant. Some reported variation clearly requires further analysis. The use of a ''yardstick'' to determine when separate species should be recognised is flawed. Instead, we argue that consistent genetic differences that are associated with consistent morphological or biological traits should be considered the marker for separate species. We propose a generalised approach to the use of rDNA to distinguish trematode species.

1. INTRODUCTION

Few parasitologists now question the value of molecular data in defining and separating species of parasites. Although the techniques continue to evolve, the basic methods are now well established and are used in many laboratories. The attractions of a molecular approach for identifying parasites are compelling; molecular data has the capacity to allow comparisons that remove confounding factors of age, host or geographically based variation. In the work that we review below, DNA sequence information has been reported for about 155 species from 19 families of trematodes. In some of the papers, quite long-standing problems of identity have been resolved. However, despite these advances, molecular approaches to the identification of trematodes have problems. Predominant among these are questions pertaining to the interpretation of results and what should be considered best-practice for the field.

Here we review the work that has been done so far in the discrimination of trematodes by the use of molecular approaches, consider the interpretation of data and make general recommendations for future work. The review focuses on the internal transcribed spacer region of ribosomal DNA (ITS rDNA), in its entirety and as its constituent components, because this has become the default region of choice in taxonomic studies of digeneans and we see no reason to expect this to change.

2. THE DIGENEA

The Digenea is the largest and most successful group of internal metazoan parasites. As at 2001, the subclass comprised some 150

recognised families containing nearly 2700 nominal genera and about 18 000 nominal species (Cribb *et al.*, 2001). As adults, digeneans display a multiplicity of morphological form. Variation is seen in the form and position of the suckers, the digestive tract and the reproductive system. Digenean life cycles are complex, involving both free-living and parasitic stages, one to four hosts, usually three distinct generations and alternating sexual and asexual reproductive generations (Cribb *et al.*, 2003). The mainstay of digenean systematics is morphological examination of sexual adults from vertebrates (Blair *et al.*, 1996).

3. SPECIES DISTINCTION: TRADITIONAL AND MODERN APPROACHES

Studies of morphology, epidemiology and behavioural characteristics, host and geographic distribution, cross-breeding studies and studies of physiology and biochemistry have all been used in the process of identifying closely related animal species (Coleman and Mai, 1997). In digenean taxonomy, however, the vast majority of species have been described entirely on the basis of their adult morphology and by reference to their host and geographic distributions. However, morphology alone may be insufficient to unequivocally identify many species. Factors that render the differentiation of digenean species difficult include: the small size of adult stages and a paucity of taxonomic characters, combined with uncertainty over the validity of those characters (Luton *et al.*, 1992; Leon-Regagnon *et al.*, 1999; Schulenburg *et al.*, 1999; Maldonado *et al.*, 2001); high-morphological similarities between closely related species (i.e. cryptic species) (Tkach *et al.*, 2000); an apparent time-lag between primary genetic speciation and morphological differentiation (Jousson *et al.*, 2000); phenotypic plasticity (especially age and host-induced variation) (Galazzo *et al.*, 2002); a lack of conserved and hard structures (Jousson and Bartoli, 2001) and the fact that many digenean life cycle stages, e.g. cercariae and metacercariae, lack distinctive morphological characters, making their association with adults by morphological characters alone impossible (Jousson *et al.*, 1998b, 1999; Bartoli

and Jousson, 2003). These problems can lead to underestimates or overestimates of parasite diversity; typically, variation must be interpreted by an "expert" and a judgement made. Such judgements may have no truly objective basis. Thus, trematode taxonomy is fertile ground for the subjective activities of "lumpers" and "splitters".

The use of host and geographic distribution, cross-breeding studies, epidemiological and behavioural characteristics and physiology to distinguish species also has shortcomings. These problems have been discussed by McManus and Bowles (1996) in some detail and will not be reviewed here. As an alternative to these classical approaches, molecular tools (usually DNA sequencing) are now thought to allow quick and accurate identification of genetically distinct but morphologically similar species.

The advent of modern molecular techniques, such as polymerase chain reaction (PCR) (Mullis *et al.*, 1986; Saiki *et al.*, 1986), has meant that DNA sequence data are often used to test hypotheses formulated using the traditional methods of species distinction. As observed by Morgan and Blair (1995), genetic variation at the nucleotide level provides the highest resolution for investigating inheritable differences. Thus, molecular techniques, especially primary sequence comparisons, are used for life cycle elucidation, examination of potential cryptic species, the examination of species complexes and their phylogeographical genetic structure and phylogenetic studies.

3.1. Advantages and Disadvantages of Using Molecular Techniques for Systematic Studies

There are four main advantages of using DNA for systematic studies (Nadler, 1990; McManus and Bowles, 1996). First, as a consequence of the fact that different genes, and different segments within certain genes, evolve at disparate rates, it is possible to answer a wide range of taxonomic questions at many phylogenetic levels. Secondly, only the number of non-repetitive nucleotide positions in the genome limits the number of potential characters for systematic inference. Thirdly, the very nature of DNA molecules implies that non-heritable

(phenotypic) variation is not a problem. Lastly, molecular techniques are now fast, reliable and relatively inexpensive.

Although molecular techniques may provide apparently objective insights into the systematic position of digeneans, there are several problems associated with these methods. First, there are problems associated with obtaining and analysing sequence data sets including, potentially, a lack of primary material from which to work (due to low prevalence of infection within hosts), inability to sample a wide range of sites throughout the geographical range of the parasite for comparison purposes, incorrect identification of samples (identification problems associated with cryptic species for instance), laboratory contamination, errors in nucleotide sequences incorporated during sequencing and not understanding the underlying parameters and assumptions of alignment programs necessary to achieve the "optimal" sequence alignment. Secondly, there is the potential problem of convergence, especially for anciently diverged taxa, as there are only five base possibilities (the four nucleotides and a gap: some computer programs, e.g. PAUP (Swofford, 1998), allow gaps, which are inserted into sequences during multiple sequence alignments, to be considered as a "New State" and analysed as a 5th base) for each site (Nadler, 1990; McManus and Bowles, 1996). Lastly, although molecular methods have the advantage of precision and speed, their nature means that it is possible to bypass study of the biology of parasites. Molecular analysis cannot provide much of the information gained from traditional approaches.

4. GENES AND SPACERS

Selecting an appropriate gene or spacer region requires careful consideration of the purpose of the study, different genes and observed rates of evolution. Over time all genomes, both nuclear and mitochondrial, accumulate mutations. However, some regions are more susceptible to nucleotide changes than others. Regions apparently unconstrained by function, such as introns and non-coding regions, usually evolve more rapidly than coding regions. Those regions coding for particular functions (e.g. the translation of mRNA) accumulate

fewer spontaneous mutations, as preservation of function is vital to an organism's survival (McManus and Bowles, 1996; Gasser, 2001). Consequently, different regions of a genome evolve at different rates. The challenge therefore is to achieve a balance. If a gene or spacer region displays too little variation, genuinely distinct groups will not be differentiated whereas if too much variation is present, difficult or questionable alignments will fail to yield robust systematic inferences. According to Hillis and Dixon (1991), systematic relationships based on highly divergent sequences are not robust because levels of homoplasy (resulting from convergences and parallelisms) increase as the probability for change at each base position increases, and, the number of alignments that are "nearly good enough" becomes prohibitively large. These authors therefore suggest that the best regions for study should be greater than 70% but less than 100% similar. Generally, suitable genes are chosen on the basis of prior knowledge gleaned from studies of closely related organisms. Ribosomal DNA (rDNA) genes and mitochondrial genes are overwhelmingly the most popular choices for systematic investigation of trematodes.

4.1. Ribosomal DNA

Ribosomal RNA genes and their spacer regions (transcribed and non-transcribed) are collectively called ribosomal DNA (rDNA). rDNA is a multigene family whose array contains hundreds of tandemly repeated copies, or paralogues, of the transcription unit, the non-transcribed spacer and two internal transcribed spacers within the nucleolar organiser region of eukaryotic genomes (Figure 1). It is within this organiser region that the rDNA array folds, forming helices that provide the appropriate recognition and docking signals for the molecular complex that processes the primary transcript (Hillis and Davis, 1986; Hillis and Dixon, 1991; Michot *et al.*, 1993; Capowski and Tracy, 2003; Coleman, 2003; Goertzen *et al.*, 2003).

Ribosomal DNA is useful for taxonomic studies because it includes regions with varying rates of evolution, from highly conserved (18S, 5.8S and 28S) to highly variable (transcribed and non-transcribed or intergenic spacer regions). Consequently, it is possible to find suitable

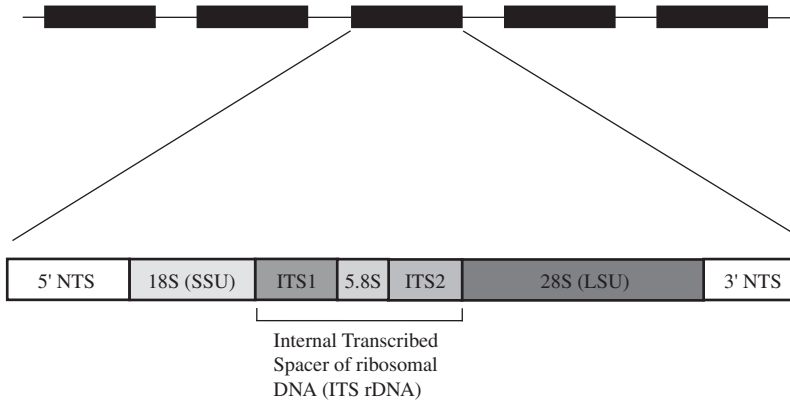

Figure 1 Diagrammatic representation of ribosomal DNA within the nucleolar organiser region of the eukaryotic genome (tandem repeats (black boxes), the transcription unit, the non-transcribed spacers and the two internal transcribed spacers) (not to scale).

gene regions to answer systematic questions at many taxonomic levels (Hillis and Davis, 1986; Hillis and Dixon, 1991). rDNA is also useful because, as a multigene family, the entire rDNA repeat tends to evolve in a concerted fashion (through unbiased gene conversion and unequal crossing-over). This process is thought to rapidly homogenise and fix new variants that arise through random genetic drift and natural selection within reproductive units (Morgan and Blair, 1995), making the individual representative of an interbreeding group (Hillis and Davis, 1986, 1988; Hillis and Dixon, 1991; McManus and Bowles, 1996; Coleman, 2003). Also, the necessity for the region to fold into its secondary structure to maintain correct function within the ribosome, an action dependent on the primary sequence, means that the region must stay relatively conserved* (Coleman and Mai, 1997). Maintenance of this secondary structure is preserved by compensatory mutations between paired nucleotides (Hillis and Dixon, 1991;

*It has been suggested that conservation of secondary structure allows for the identification of positional homology and application of ITS2 sequence data to broad systematic questions (Goertzen *et al.*, 2003). It should be noted however that possible rapid evolution in regions corresponding to loops might lead to gene saturation. This according to Stevens and Schofield (2003) leads ultimately to reversals, homoplasy and inaccurate phylogenetic inference.

Michot *et al.*, 1993; Morgan and Blair, 1998; Coleman, 2003). A further advantage to using this region is that the tandemly repeated copies provide a large number of target sequences for PCR amplification (Jousson *et al.*, 1998a).

The 18S rRNA, or small subunit ribosomal gene, is among the slowest evolving sequences found in living organisms and is used to infer deep phylogenetic relationships among ancient lineages (Hillis and Davis, 1986). The 28S rRNA, or large subunit rRNA gene, is larger than 18S and shows a faster rate of evolution through its different domains than does the 18S gene. However, the 28S rRNA gene does possess regions with levels of gene conservation similar to those of 18S (Hillis and Dixon, 1991). Both regions have been used to detect species boundaries within digenean families (Kaukas and Rollinson, 1997; Schulenburg and Wagele, 1998; Snyder and Tkach, 2001; Leon-Regagnon and Paredes, 2002) but this is not common. This review will focus on the internal transcribed ribosomal spacer region (ITS rDNA) because it is by far the most frequently exploited region for the exploration of species boundaries in digeneans.

4.1.1. Internal Transcribed Spacer rDNA (ITS1, 5.8S rRNA Gene and ITS2) Region

The internal transcribed spacer includes two spacers, ITS1 and ITS2, separated by the 5.8S rRNA gene. Typically the region is thought to be relatively conserved within a species or genus and therefore useful for determining species boundaries (Morgan and Blair, 1995; Jousson *et al.*, 1998b). This region (as a whole or as its individual constituent components) has been used to explore species boundaries in at least 19 digenean families. The ITS is not usually analysed as a whole; rather it is broken into its two main components, the internal transcribed spacers 1 and 2. The 5.8S rRNA gene has levels of gene conservation similar to the 18S rRNA gene (although Hershkovitz and Lewis (1996) found the region to have divergences roughly twice those of 18S). However, according to Hillis and Dixon (1991), Hershkovitz and Lewis (1996) and Coleman (2003) the gene region is too short to effectively infer robust phylogenies across large time scales.

4.1.1.1. Internal transcribed spacer 1 (ITS1) of rDNA. The nature of the ITS1 rDNA spacer has allowed characterisation of digeneans at different taxonomic levels. The high variability of the 5′ end allows the entire ITS1 sequence to identify species, and the more conservative 3′ end can provide information on relative systematic position (Schulenburg *et al.*, 1999).

Much of the variation in this region is due to the presence of tandemly repeated elements located at the 5′ end of the spacer. Such repeats are known from the Haematoloechidae, Mesometridae, Opecoelidae, Schistosomatidae, Strigeidae and the Telorchiidae. Most ITS1 regions that possess these repeats are composed of three elements: first, the short 5′ end (the length of which varies depending on the species or species complex); secondly, a tract containing "X" nucleotides that may be repeated "Y" times and may be composed of two or more parts; and thirdly, the 3′ region that usually lacks repeats, although repeat elements have been found in this region in *Schistosoma margrebowiei*, *S. mattheei* (Kane and Rollinson, 1994; Kane *et al.*, 1996) and *Trichobilharzia szidati* (Dvorak *et al.*, 2002). The number of nucleotides that constitute a repeat, and the number of times this repeat is present, varies with the species and the family. Often these tandem repeats are composed of two parts, usually designated "a" and "b". Picard and Jousson (2001), however, found repeat elements in *Trichobilharzia* cercariae composed of three elements with a "ba-$(ca)_5$" repeat pattern configuration. The order and size (in nucleotides) of these components is again dependent on the species and the family to which it belongs (Van Herwerden *et al.*, 1998, 1999).

4.1.1.2. Internal transcribed spacer 2 (ITS2) of rDNA. The second internal transcribed spacer (ITS2 rDNA) is generally considered to contain fewer variable sites than the ITS1. Generally it does not contain repeat elements, although they have been found in the Echinostomatidae (Morgan and Blair, 1995). It is highly variable in length both within and between families and is known for its relatively high sequence conservation at the species level. This means that satisfactory alignment above the genus level may be a problem and has meant that this region is not often used for phylogenetic inference at the family, order or higher taxonomic levels (Coleman, 2003). As a result, some

authors (Michot *et al.*, 1993; Morgan and Blair, 1998; Coleman, 2003) have suggested that once the secondary structure of this region has been established, it may aid the alignment of ITS2 sequences at taxonomic levels above that of the genus. However, a study by Morgan and Blair (1998) of 38 digenean species from nine families found that satisfactory sequence alignments between families were not possible. This was despite the authors determining a four-domain secondary structure model for digenean ITS2, which supported the model proposed by Michot *et al.* (1993). Morgan and Blair (1998) showed that there was substantial length variation associated with different families. This study therefore suggests that although knowing the secondary structure of ITS2 may aid alignment within a family, a combination of high sequence conservation and great variation in sequence lengths, both within and between families, may result in the region being restricted to systematic studies at the genus and family levels only.

5. STUDIES USING ITS rDNA TO DISTINGUISH DIGENEAN SPECIES

We have found 63 studies that have reported partial or complete ITS rDNA of digenean species (literature searched to 1st May, 2004). Only 14 of these analysed the entire ITS. The ITS varies from 820 to 1125 nucleotide sites depending on the family. Eight studies found evidence of intraspecific variation, which was generally less than 1.0% (although four found intraspecific variation of 1.2–3.2%). Of the 63 studies, 24 (including six that analysed the entire region) analysed only partial or complete ITS1; 13 found intraspecific variation, which was again usually less than 1.0%, although there are exceptions (Luton *et al.*, 1992; Galazzo *et al.*, 2002; Jousson and Bartoli, 2002). Of the 63 studies, 44 analysed ITS2; 16 found intraspecific variation that ranges from 0.3 to 3.5%.

Below we review the findings of these studies as they relate to the 19 families of digeneans. Our approach here is to summarise the literature, but to make no attempt to reinterpret the conclusions reached in any of the studies. Approaches to analysis are considered with reference to selected studies in Section 6. We refer to ‘‘replicate

sequences'' as sequences obtained from separate individual worms, *not* repeat sequencing of the same material to check accuracy.

5.1. Apocreadiidae

Lo *et al.* (2001) sequenced the ITS2 from *Schistorchis zancli* from the fish *Zanclus cornutus* from the Great Barrier Reef and French Polynesia. Despite more than 6000 km of deep ocean separating the two sites, they found zero differences (266 nucleotides compared) between sequences from the two localities.

5.2. Bivesiculidae

Cribb *et al.* (1998) sequenced the ITS2 for two genera and four species of adult bivesiculids, and specimens of an immature bivesiculid to attempt to elucidate the life cycle of the latter. The sequences from the immature bivesiculids from the intestine of *Thalassoma lunare* (Labridae) were identical to replicate sequences obtained from adults of *Bivesicula claviformis* from *Epinephelus fasciatus* (Serranidae). Intrageneric variation ranged from 8.1 to 11.6% (23–33 base differences) and intergeneric variation from 16.0 to 36.0% (41–101 base differences).

5.3. Cladorchiidae

Itagaki *et al.* (2003) reported ITS2 sequences from three common Japanese bovine amphistomes, *Calicophoron calicophorum*, *Orthocoelium streptocoelium* and *Homalogaster paloniae*. They found no intraspecific variation for *C. calicophorum* (284 nucleotides, seven replicates, four locations), *O. streptocoelium* (284 nucleotides, 10 replicates, three locations) and *H. paloniae* (285 nucleotides, two replicates, one location). Interspecific variation between the three species ranged from 4.2 to 5.3%.

5.4. Didymozoidae

Two studies (Anderson and Barker, 1998; Anderson, 1999) have explored didymozoids of platycephalid fishes from Queensland waters

using ITS2 sequences. Anderson and Barker (1993) had previously used restriction fragment length polymorphism (RFLP) analysis[†] of the entire ITS region to distinguish six didymozoid species. Anderson and Barker (1998) reported ITS2 sequences from 11 species from four genera (seven *Indodidymozoon* species, two *Helicodidymozoon* species, *Rhopalotrema elusiva* and *Neometadidymozoon polymorphis*) and found differences between all combinations of species distinguished by morphological criteria. Interspecific variation ranged from 0.5 (2 base differences over 342 sites) to 8.0%; intergeneric variation ranged from 3.0 to 19.0%; there was no mention of replicate sequences.

Anderson (1999) compared ITS2 sequences from four putative didymozoid metacercariae to those of adult didymozoid species obtained by Anderson and Barker (1998) and found the sequence of one to be identical to the sequence from the adult of *I. pearsoni*.

5.5. Diplostomidae[‡]

Overstreet *et al.* (2002) studied two *Bolbophorus* species from the channel catfish, *Ictalurus punctatus*, and the American white pelican, *Pelecanus erythrorhynchos*. They reported intraspecific variation over the entire ITS of 0.6% for *Bolbophorus* sp. and 3.2% for *B. damnificus*. Interspecific sequence divergence between the adult stages of the two *Bolbophorus* species from *P. erythrorhynchos* reached 13.5% (over 1036 sites). These levels of variation were confirmed by experimental infection with nestling and de-wormed adult pelicans in conjunction with sequence data from the 18S, 28S and CO1 gene regions.

Galazzo *et al.* (2002) found 0.0–0.8% intraspecific variation in the ITS1 and ITS2 from three North American species of *Diplostomum*.

[†]RFLP analysis is an indirect approach to estimating variation at the nucleotide level using restriction endonucleases. DNA is digested with a restriction enzyme. The resulting DNA fragments are separated by electrophoresis. The cut DNA fragments are visualised directly by end labelling (Maniatis *et al.*, 1982) or after transfer to a nitrocellulose filter (Southern, 1975). The presence or absence of restriction sites in the DNA under consideration will change the lengths of the fragments. These length variations are referred to as restriction fragment polymorphisms.

[‡]For the purposes of this review, *Bolbophorus* is considered co-familial with *Diplostomum*, as presented by Niewiadomska (2002). This is despite Overstreet *et al.* (2002) assigning *Bolbophorus* to the family, Bolbophoridae.

Five *Diplostomum baeri* sequences were identical; four *D. huronense* sequences were identical whereas a fifth had a single transition. Six of nine specimens identified as *D. indistinctum* were identical. The other three differed at three sites in the ITS1, while one differed at a further five sites in ITS1 and a single site in ITS2. The three specimens exhibiting intraspecific variation had been identified only tentatively (Galazzo *et al.*, 2002).

Niewiadomska and Laskowski (2002) examined six Eurasian *Diplostomum* species. Interspecific variation between four species ranged from 9 to 32 base differences (1.3–4.7% divergence), but *D. spathaceum* and *D. parviventosum* had identical ITS1 sequences. Niewiadomska and Laskowski (2002) did not synonymise the two species, as the two were said to be readily distinguishable based on morphological differences between adult, cercarial and metacercarial stages.

5.6. Echinostomatidae

Morgan and Blair (1995) sequenced the entire ITS rDNA of six recognised *Echinostoma* species and *Echinostoma* sp. I and II from Africa, Asia, Europe and North and South America. They found no intraspecific variation over 1042 sites between replicate sequences for any species. They also found no interspecific variation between *Echinostoma caproni*, *E. liei* and *Echinostoma* sp. II, and on this basis, synonymised these three species. Interspecific variation within the 37-collar–spine group ranged from 1.1 to 3.7%, while outside this group variation reached as high as 19.2%.

Sorensen *et al.* (1998) sequenced the ITS from *E. revolutum* from Eurasia and North America and *E. trivolvis* from Indiana to determine the levels of intraspecific variation within North American *Echinostoma* species. They found intraspecific variation of 0.9% between sequences from *E. revolutum* from different continents (no variation between sequences from North America) and 0.6% sequence divergence between two isolates of *E. trivolvis* taken from the same pond in Indiana. Interspecific variation in the same study ranged from 0.8 to 2.9% (8–29 of 1006 sites). Despite finding levels of intraspecific variation higher than some interspecific variation found

in the same study, Sorensen *et al.* (1998) concluded that variation of 0.6 and 0.9% did not invalidate the integrity of the two species.

Grabda-Kazubska *et al.* (1998) sequenced ITS1 from rediae and cercariae (stages easily discriminated by morphology and chaetotaxy) to discriminate species of *Echinoparyphium*, *Pseudoechinoparyphium*, *Neoacanthoparyphium* and *Hypoderaeum*. By counting the number of base differences figured in their Table II, it is possible to determine that interspecific variation between species of *Echinoparyphium* in this study ranged from 1.6 to 3.2% (6–12 of 376 sites). Levels of intergeneric variation range from 4.8 to 12.2% (18–46 base differences).

Maldonado *et al.* (2001) studied a species of *Echinostoma* obtained from the rodent *Nectomys squamipes* from Brazil. The ITS2 was identical to that of *E. paraensei* (isolated from the snail *Biomphalaria glabrate* from Brazil). Consequently, but also in the light of light and scanning electron microscopy, the new Brazilian isolate was identified as *E. paraensei*.

5.7. Fasciolidae

Adlard *et al.* (1993) sequenced ITS2 from *Fasciola hepatica* from Australia, Hungary, Mexico and New Zealand, *F. gigantica* from Indonesia and Malaysia, *Fasciola* sp. from Japan and *Fascioloides magna* from the United States. They found geographical variation within *F. hepatica* was limited to a single base substitution in the Mexican isolate (0.4% variation, 263 nucleotides compared). There was no sequence variation between replicate sequences from *F. gigantica* (213 nucleotides compared). There were six base differences (2.8%, 213 nucleotides compared) between *F. hepatica* and *F. gigantica*. Intergeneric variation ranged from 13.2% (38 of 289 sites—*F. hepatica* and *F. magna*) to 16.0% (34 of 214 sites—*F. gigantica* and *F. magna*) (differences in lengths of sequences compared were due to incomplete sequences obtained for *F. gigantica*). Sequences from the unidentified Japanese isolate differed from *F. gigantica* at one base and consequently Adlard *et al.* (1993) identified this form as *F. gigantica*.

Hashimoto *et al.* (1997) obtained ITS2 from *F. hepatica* from Australia, *F. gigantica* from Malaysia and *Fasciola* sp. from Japan to

elucidate the taxonomic status of the Japanese *Fasciola* species. Their findings confirmed those of Adlard *et al.* (1993).

Itagaki and Tsutsumi (1998) obtained ITS2 sequences from the Japanese triploid form of *Fasciola* (seven), *F. hepatica* from Uruguay (three) and *F. gigantica* from Zambia (two) and Indonesia (two) to determine the nature of the Japanese triploid form. They found no intraspecific variation among *F. hepatica* replicate sequences from Uruguay. In contrast, three different ITS2 sequences were obtained for *F. gigantica*. Two sequences from Indonesia were identical (also identical to sequences from *F. gigantica* from Malaysia obtained by Adlard *et al.* (1993) and Hashimoto *et al.* (1997)). However, the two Zambian isolates differed from each other at four sites and one of these differed from the Indonesian isolate at six sites. A comparison of the ITS2 sequences from the seven *Fasciola* isolates from Japan revealed two distinct sequence types. The first was identical to those of *F. hepatica* from Uruguay and the second was similar to sequences from *F. gigantica* from Indonesia and Japan reported by Adlard *et al.* (1993) and Hashimoto *et al.* (1997).

Mas-Coma *et al.* (2001) compared complete ITS1 and two sequences (433 and 364 nucleotides long) for *F. hepatica* from the Northern Bolivian Altiplano and Spain and found no base differences. Comparison of these sequences to those of Adlard *et al.* (1993), Hashimoto *et al.* (1997) and Itagaki and Tsutsumi (1998) showed the Spanish and Bolivian sequences to differ from all previous sequences in at least one base position.

Huang *et al.* (2004) sequenced ITS2 for *F. hepatica* from France and *F. hepatica*, *F. gigantica* and a *Fasciola* sp. from three locations in China. They found no intraspecific variation for *F. hepatica* or *F. gigantica*. The sequences from *F. hepatica* differed from those of *F. gigantica* at six of 362 nucleotide sites (1.7%). The *Fasciola* sp. differed from *F. hepatica* and *F. gigantica* at five sites (1.4%).

5.8. Haematoloechidae

Leon-Regagnon *et al.* (1999) partially sequenced the ITS and 28S rRNA of several *Haematoloechus* species described as Mexican endemics. They combined their data sets (1836 bp) and found

sequence divergence of 1.7% between *H. macrorchis* (1 replicate) and *H. longiplexus* (1 replicate). Based on what they interpreted as a low interspecific sequence divergence and a lack of distinguishing morphological traits between the two species, they proposed *H. macrorchis* (Mexico) as the junior synonym of *H. longiplexus* (Nebraska, USA). It was suggested that the 1.7% could represent differentiated populations or apparent affinities for different host species. In the same study a *Haematoloechus* isolate, Pulcher I (from *Ambystoma lermaensis*), was considered conspecific with *H. complexus*, as the 1.2% divergence between these isolates was less than that of *H. longiplexus* over the same region. Another isolate, Tuxtlas I (Los Tuxtlas, Mexico), and *H. medioplexus* (Nebraska, USA) differed by 2.0% and were also considered conspecific, partly because they have a continuous distribution from Los Tuxtlas to Nebraska.

Snyder and Tkach (2001) studied the ITS from eight North American and European *Haematoloechus* species and found interspecific sequence variation as low as 0.5% between *H. medioplexus* and *H. varioplexus*, which are easily distinguishable on morphological grounds. They found no intraspecific variation within *H. longiplexus*, *H. varioplexus*, *H. variegatus* and *H. abbreviatus* and just 0.1% between three isolates of *H. asper* from the same population.

5.9. Lepocreadiidae

Lo *et al.* (2001) reported ITS2 sequences from two species of Lepocreadiidae, *Preptetos laguncula* and *Neohypocreadium dorsoporum*, from French Polynesia and the Great Barrier Reef. Replicates of *P. laguncula* were identical. Replicates of *N. dorsoporum* from *Chaetodon vagabundus* from French Polynesia and *C. ephippium* from the Great Barrier Reef were also identical, but specimens from *C. flavirostris* and *C. vagabundus* from the Great Barrier Reef each had a single base difference (0.3%) relative to the first two samples.

5.10. Leucochloridiidae

Casey *et al.* (2003) sequenced the entire 5.8S and ITS2 and partially sequenced the 28S and ITS1 from the green-banded brood sac of

Leucochloridium paradoxum and the brown-banded brood sac of *L. variae* from Poland, Denmark and Norway. They found no intraspecific variation between replicates from the three geographical locations over the entire region for both species and 6.8% (72 of 1059 sites) interspecific variation. They found 8.2% interspecific variation in the ITS1 and 5.1% interspecific variation in the ITS2 between the two species.

5.11. Mesometridae

Jousson *et al.* (1998b) analysed ITS sequences to identify cercariae of the Mesometridae. Complete ITS rDNA sequences were obtained from five adult mesometrid species and compared to complete ITS1 sequences from five putative mesometrid cercariae. Sequences from cercariae isolated from three species of *Rissoa* were almost identical to those from the adult of *Centroderma spinosissima* (0.0–0.1% intraspecific variation). Sequences from cercariae taken from *Vermetus triqueter* and *Barleeia rubra* corresponded to those from adults of *Elstia stossichianum* (no variation) and *Wardula capitellata* (0.4% intraspecific variation), respectively. Within the ITS1, interspecific variability ranged from 6.6 to 19.1%, slightly higher than for the ITS2, 3.4–15.1%. Jousson *et al.* (1998a) later used this same data to construct a molecular phylogeny for these parasites.

5.12. Monorchiidae

Bartoli *et al.* (2000) sequenced *Cercaria cerastodermae* I from the cockle *Cerastoderma edule*, adult trematodes from the experimentally infected sparid *Diplodus sargus* and adult specimens of *Monorchis parvus* from naturally infected *D. annularis*. Sequences from the three sources differed at two of 1050 base positions (0.2% sequence divergence). A comparison of the 0.2% variation to interspecific variation of 7.3–7.5% (74–76 of 1050 sites), between *M. parvus* and *M. monorchis*, suggested that *Cercaria cerastodermae* I corresponded to the adult of *M. parvus* found in *D. annularis*.

Jousson *et al.* (2000) investigated *Monorchis parvus* from *Diplodus vulgaris*, *D. sargus* and *D. annularis* from the north-western

Mediterranean. Analysis of ITS1 sequences from specimens taken from each host species showed the presence of two distinct taxa; one restricted to *D. vulgaris* and *D. sargus* and the other to *D. annularis*. Intraspecific variation within each group was no greater than 0.7% (634 sites) while interspecific variation reached 3.8%. The authors concluded that *M. parvus* was composed of two cryptic species. However, no new species was proposed.

Jousson and Bartoli (2002) analysed the ITS1 from *Monorchis parvus* and *M. monorchis*, from sparid fishes. Sequences from *M. parvus* again divided into two host-defined groups separated by 3.5% divergence (21 base differences). One group was restricted to *Diplodus sargus* and *D. vulgaris* and the other to *D. annularis*, suggesting a host-associated species complex (supporting their previous study). However, as a consequence of the morphological homogeneity between the adult stages of each group, Jousson and Bartoli (2002) continued to identify both as *M. parvus*. The sequences from *M. monorchis* from *Spondyliosoma cantharus* and *D. puntazzo* were identical, but differed from apparent *M. monorchis* sequences from *Parablennius gattorugine* by 12.9%. This high divergence was supported by analysis of morphological characters and morphometrics. The authors subsequently described the new species, *M. blennii*, from *P. gattorugine*. Interspecific variation in this study ranged from 4.1% (over 601 bases) between *M. parvus* and *M. monorchis* to 14.7% (over 973 bases) between *M. parvus* and *M. blennii*.

5.13. Opecoelidae

Jousson *et al.* (1999) reported the entire ITS from 16 adult opecoelid species, eight opecoelid cercariae and four opecoelid metacercariae. They initially reported intraspecific variation ranging from 0.1 to 1.5%. Cercariae collected from species of *Tricolia*, *Haliotis*, *Clanculus* and *Gibbula* were found to correspond to *Allopodocotyle pedicellata* (0.1% difference), *Cainocreadium labracis* (0.7%), *Podocotyle scorpaenae* (0.2%) and *Helicometra fasciata* (1.1%), respectively. Sequences from cercariae from *Columbella rustica* and *Mitrella scripta* differed from those of adult *Opecoeloides furcatus* at 1.5 and 0.8%,

respectively. Jousson *et al.* (1999) contrasted this difference to the sequence divergence between two apparently closely related opecoelids, *O. furcatus* and *Poracanthium furcatum*, whose divergence was significantly higher (15.0–17.8%). However, later study (Jousson and Bartoli, 2000) showed that 1.5% divergence differentiated *O. furcatus* into two taxa, one from each intermediate host species.

Jousson *et al.* (2000) analysed ITS sequences from opecoelid species from *Diplodus vulgaris*, *D. sargus* and *D. annularis*. Sequences from *Macvicaria crassigula* revealed two well-defined host-dependent groups. Interspecific variation between the two groups was 2.6% but no new species was proposed.

Jousson and Bartoli (2000) also found evidence for two cryptic species associated with *Opecoeloides furcatus*. ITS1 sequences from adults of this species from *Gaidropsarus mediterraneus* and cercariae from *Columbella rustica* were 840 bp long, whereas those of adults from *Mullus surmuletus* and cercariae from *Mitrella scripta* were 900 bp long. The difference was due to a 60-nucleotide repeat, present twice in the 900 bp sequence. They concluded that the 840 bp sequences corresponded to a new species, *Opecoeloides columbellae*, and the 900 bp sequences corresponded to the "real" *O. furcatus*. The authors concluded that inclusion of distantly related Mediterranean opecoelids in their previous study (Jousson *et al.*, 1999) had meant that the ITS1 region was too variable to be aligned unambiguously. This had led them to the spurious conclusion that cercariae from *Columbella rustica* and *Mitrella scripta* both belonged to *O. furcatus*. In a later study, Bartoli and Jousson (2003) found that interspecific sequence divergence between the two *Opecoeloides* species over the entire ITS was 1.5%, significantly lower than that between other congeneric opecoelids, e.g., *Cainocreadium labracis* and *C. dentecis*, which had 7.5% variation (Jousson and Bartoli, 2001).

5.14. Paragonimidae

The Paragonimidae has been studied more than any family except the Schistosomatidae. This is attributable to the socio-economic importance of the family, but also because one species, *Paragonimus*

westermanii, has both diploid (2*n*) and triploid (3*n*) forms and probably consists of a complex of cryptic species. In overview, 12 studies use ITS sequence data for consideration of 19 *Paragonimus* species, two *Pagumogonimus* species and two *Euparagonimus* species. One study considers the ITS1, but comments only on the nature of the repeat elements. Eleven studies make use of ITS2; this region varies from 287 to 364 nucleotides in length, exhibits intraspecific variation that ranges from 1 to 10 base differences, and interspecific variation that ranges from less than 6 to 48 base differences depending on the comparisons made. Typically, species recognised by morphology have been corroborated by these analyses, but two species have been synonymised as a result of them. Three studies conclude that *P. westermanii* may be composed of a complex of species across Asia, although no formal move to divide this species has been made.

Van Herwerden *et al.* (1999) are the only authors to consider the ITS1 region of paragonimid species. They found repeats in paragonimid ITS1 rDNA to be composed of two sub-repeats, ''a'' and ''b''. Five sequenced clones of *P. ohirai* had $(ab)_2$a-type repeats, as did the single clone of *P. macrorchis*. Within each of the diploid and triploid forms of *P. westermanii* there were three repeat types (''a''; ''a,del''; ''del,a'' for the diploid form and ''a''; ''aba''; ''$(ab)_2$a'' for the triploid form (see Van Herwerden *et al.*, 1999, Figure 1, p. 69); each form represents intra-individual variation. The intra-individual variation within *P. westermanii* was not present in the *P. ohirai* group. Numbers of base differences were not given in this study.

Blair *et al.* (1997b) investigated the geographic structure of diploid and triploid forms of *P. westermanii* collected from 14 localities in seven Asian countries. Their findings are presented in Table 1. The authors suggested that ITS2 was a good marker for specific relationships within *Paragonimus*, but less useful for investigating intraspecific genetic structure.

Blair *et al.* (1997a) sequenced the ITS2 from the three species of the *Paragonimus ohirai* group; *P. ohirai*, *P. iloktsuenensis* and *P. sadoensis*. They found no base differences between the sequences from these species and concluded that *P. iloktsuenensis* and *P. sadoensis* should be regarded as junior synonyms of *P. ohirai*. Sequences from

Table 1 Pairwise differences among ITS2 sequences

Species	Origin	1	2	3	4	5	6	7
P. westermani	1 Hyogo, Japan	—	1 (0.3%)	7 (1.9%)	6 (1.7%)	35	25[a,b]	25[a]
	2 Karapai, Taiwan		—	8 (2.2%)	7 (1.9%)	36	26[a,b]	26[a]
	3 Leyte, Philippines			—	1 (0.3%)	38	29[a,b]	29[a]
	4 Sungai Wa, Malaysia				—	37	28[a,b]	28[a]
P. ohirai	5 Tanegashima, Japan					—	27[a,b]	27[a]
P. miyazakii	6 Miyazaki, Japan						—	0[b]
P. skrjabini	7 China, Sichuan							—

Notes: Modified from Blair *et al.* (1997). Figures within parentheses indicate sequence divergences between each comparison (not included in the original paper). All isolates from Japan, China and Korea had sequences identical to that from Hyogo, Japan. Both Taiwanese isolates had identical sequences.

[a]Ignoring 1 "N" in *P. miyazakii*.

[b]Ignoring a deletion 2 nucleotides long in *P. miyazakii* and *P. skrjabini*.

the *P. ohirai* group differed at 29 and 35 sites from *P. miyazakii* and *P. westermanii*, respectively (8.0 and 9.6%).

The status of *Euparagonimus* and *Pagumogonimus* relative to *Paragonimus* was investigated by Blair *et al.* (1999). Sequences from representatives of each genus were taken from two previous studies (Blair *et al.*, 1997b, 1998). Interspecific variation in *Paragonimus* ranged from 16 to 43 base differences, while intergeneric variation was only 16–44 base differences. *P. miyazakii* (Japan) and *Pg. skrjabini* (China) possessed identical ITS2 sequences. The non-monophyly of representatives of *Pagumogonimus*, and the close relationship between *P. miyazakii* (Japan) and *Pg. skrjabini* led these authors to conclude that *Pagumogonimus* was not a natural taxon, although *P. miyazakii* and *Pg. skrjabini* were not synonymised.

Chang *et al.* (2000) sequenced ITS2 to evaluate its value in diagnosis of paragonimiasis. Sequences were obtained from eggs in the sputum of a human patient and from eggs collected from two dogs previously infected with *P. westermanii* and *P. skrjabini*. Sequence

comparison showed the unidentified eggs to be identical to *P. westermanii* and to differ by 8.0% from *P. skrjabini*.

Iwagami *et al.* (2000) investigated the geographic structure of *P. westermanii* in Asia by sequencing diploid and triploid forms from 23 localities in six countries. Their results are presented in Table 2. As a result of these findings, the authors came to a conclusion similar to that of Blair *et al.* (1997b); populations of *P. westermanii* can be divided into two possible species, one in northeast Asia and the other in southern Asia. Comparison of *P. westermanii* sequences to those of *P. miyazakii* and *P. ohirai* revealed 27–34 (9.4–11.9%) and 38–43 (13.2–15.0%) base differences, respectively.

Ryu *et al.* (2000) sequenced 363 bases of ITS2 from seven adults of *Paragonimus* collected from Anhui Province, China. Six sequences were identical to a previously sequenced Chinese isolate of *P. westermanii*. The remaining sequence was identical to *P. ohirai*. The mitochondrial genes CO1 and ND1 supported these interpretations. This study confirmed findings of Blair *et al.* (1997b) and Iwagami

Table 2 Pairwise differences among ITS2 sequences

Species	Origin	1	2	3	4	5	6	7
P. westermani	1 Chiba, Japan	—	1 (0.3%)	7 (2.4%)	8 (2.8%)	10 (3.5%)	27	38
	2 Myaoli, Taiwan		—	8 (2.8%)	9 (3.1%)	11 (3.8%)	28	39
	3 Kuala Pilah, Malaysia			—	7 (2.4%)	9 (3.1%)	31	41
	4 Leyte, Philippines				—	10 (3.5%)	31	42
	5 Thailand					—	34	43
P. miyazakii	6 Rokuroshi, Japan						—	32
P. ohirai	7 Kinosaki, Japan							—

Notes: Modified from Iwagami *et al.* (2000). Figures within parentheses indicate sequence divergences between each comparison (not included in the original paper). All isolates from Japan, China and Korea had sequences identical to that from Chiba, Japan. Three Taiwanese isolates had identical sequences, as did three Malaysian isolates and two Philippine isolates.

et al. (2000), who also found all *P. westermanii* sequences from different geographical locations in China to be identical.

Sugiyama *et al.* (2002) sequenced rDNA from paragonimid metacercariae provisionally identified as *P. westermanii* and *P. miyazakii* from freshwater crabs from Wakayama Prefecture, Japan. ITS2 sequences were 287 and 285 nucleotides for the putative *P. westermanii* and *P. miyazakii*, respectively. No intraspecific variation was observed for either species between six replicates (each) from different host individuals. The sequences were identical to those reported previously for *P. westermanii* and *P. ohirai*. Interspecific variation over the ITS2 was 9.4% (27 of 287 sites).

Cui *et al.* (2003) obtained ITS2 sequences for five species of *Paragonimus* not previously sequenced in other systematic studies. They found interspecific variation ranging from 1.4% between *P. bangkokensis* and *P. menglaensis* to 8.8% between *P. paishuihoensis* and *P. hokuensis*. No mention was made of intraspecific variation.

Iwagami *et al.* (2003a) studied metacercariae of *P. mexicanus* from Guatemala and Ecuador. Two ITS2 sequences were both 285 nucleotides in length but differed at a single site (0.4% divergence). Interspecific variation (comparison of *P. mexicanus* to *P. westermanii*) was observed at 33 sites (11.5% divergence).

Iwagami *et al.* (2003b) determined the identities of two *Paragonimus* species from Sri Lanka using ITS2 sequences (CO1 sequences were also used). Metacercariae were collected from freshwater crabs from six localities. Sequences from metacercariae were compared to sequences from 10 *Paragonimus* species from eight countries in Asia and the Americas. The Sri Lankan metacercariae had two sequence types that differed at 23 sites (8.0% divergence). The Type 1 sequence was closest to *P. westermanii* (Malaysia, Philippines and Thailand), with 5.2–5.9% sequence divergence (15–17 sites). Type 2 sequences were closest to *P. siamensis* from Thailand, diverging by 2.1% (six sites). Intraspecific variation between *P. westermanii* sequences used in this study ranged from 2.4 to 3.1% (seven to nine sites). The identity of worms with Type 1 sequences remained unclear, while those with Type 2 were suggested to be either *P. siamensis* or a sibling of the Thai species.

Park *et al.* (2003) examined intraspecific polymorphism in diploid and triploid forms of *P. westermanii* from 15 geographical locations,

including three from Korea. Diploid and triploid ITS2 sequences were 363 nucleotides in length and varied at nine sites (2.5% divergence). Sequences from Korean *P. westermanii* were almost identical to each other with the diploid form (Haenam) and the triploid form (Youngam and Bogil-Island) differing at two sites (0.6% divergence).

5.15. Plagiorchiidae

Tkach *et al.* (2000) studied three *Plagiorchis* species belonging to the *P. vespertilionis* group from European bats. They found no intraspecific variation between replicate sequences from the three species regardless of host or geographical location. Interspecific variation among the three species ranged from 2.9 to 12.8%. Tkach *et al.* (2000) also sequenced *P. elegans* from the intestine of birds. Sequence divergence between *P. elegans* and the three species from bats was higher (15.2%, 197 of 1297 sites) than that observed between species from bats alone.

5.16. Sanguinicolidae

Nolan and Cribb (2004a) found no intraspecific variation between five replicate sequences from adult and four replicate sequences from cercarial stages of the blood fluke *Paracardicoloides yamagutii* (344 nucleotides) from southeast Queensland, Australia. Thirteen replicate sequences from *Plethorchis acanthus* (414 nucleotides) were identical, as were nine replicate sequences from an unidentified sanguinicolid cercaria (351 nucleotides). Intergeneric variation was greater than 40% and made effective alignment of sequences impossible.

Nolan and Cribb (2004b) sequenced rDNA to test the distinctness of *Pearsonellum pygmaeus* from the type-species of the genus, *P. corventum*, and found 29 base differences (5.7% sequence divergence). Four replicate sequences from *P. corventum* from the southern Great Barrier Reef were identical.

5.17. Schistosomatidae

Owing to the significant socio-economic importance of this family and the ability of certain of its species to interbreed and produce

viable hybrids, the schistosomes are the most frequently studied digenean family. We have found 14 studies that have used partial or complete ITS rDNA sequences to explore the systematic status of 26 schistosome species, particularly of the genus *Schistosoma* (17 species). Six studies utilise the ITS1 for systematic study of the *Schistosoma haematobium* group, *Trichobilharzia* species and the interrelationships of Asian *Schistosoma* species. The remaining studies use the ITS2, often using the same sequences obtained from previous studies for broader systematic comparisons.

In overview, six studies use ITS1 data and find sequence length variation ranging from 929 to 2290 bp, depending on the species and the number and size of repeat elements. Eight studies use ITS2; length varies from 286 to 398 bp. Intraspecific variation over the ITS2 ranges from 0 to 2 base differences. Interspecific variation ranges from 0 to 42 base differences between African species, 1 to 39 base differences between Asian species and 40 to 90 base differences between the two groups. Three species (*S. intercalatum*, *S. bovis* and *S. curassoni*) have been reported with identical ITS2 sequences.

The first study using ITS1 (Kane and Rollinson, 1994) attempted to determine the nature of the size variation observed in this region and establish if it could be used to differentiate *Schistosoma haematobium*, *S. intercalatum* and *S. mattheei* (Zambian isolate). It was found that *S. haematobium* and *S. intercalatum* possessed two repeat elements (72 base pairs each) and *S. mattheei* possessed four (72, 77, 78 and 80 base pairs). Actual numbers of base differences between these species were not given. However, by counting the number figured in the ITS1 alignment, it is possible to determine sequence divergence between the species. There are nine base differences between *S. haematobium* and *S. intercalatum* (0.8% divergence over 1086 nucleotides, although it may be lower as three bases are unidentifiable), 182 base differences between *S. haematobium* and *S. mattheei* (16.8% sequence divergence) and 176 base differences between *S. intercalatum* and *S. mattheei* (16.2% sequence divergence). Intraspecific variation was reported between successive repeating elements rather than between replicate ITS1 sequences from different isolates of the same species. Between repeat elements one and two of

S. haematobium there were 12 base differences (of a total of 72), and between elements one and two of *S intercalatum* there were 13 (of 72). Between the four repeat elements of *S. mattheei* there were 10–15 base differences (of a total of 72–80 bases).

Kane *et al.* (1996) studied the ITS1 from *Schistosoma mansoni*, *S. margrebowiei*, *S. spindale* and *S. mattheei* (South African isolate). Again, numbers of repeating elements were used to distinguish species; *S. margrebowiei* had four repeat elements (three tandem and one towards the 3′ end of the ITS1), *S. mansoni* had two repeat elements (in common with *S. haematobium* and *S. intercalatum*), *S. spindale* had seven repeat elements and the South African *S. mattheei* isolate had five repeat elements (four tandem and one towards the 3′ end of the ITS1). Intraspecific variation was also determined between replicate sequences from the same *Schistosoma* species from different geographical locations. ITS1 sequences from five geographical isolates of *S. mansoni* were identical except for one base ambiguity. Five geographical isolates of *S. haematobium* differed at one base position and two replicate sequences from *S. intercalatum* were identical except for three ambiguous bases. The ITS1 from the *S. mattheei* isolate from South Africa contained five repeat elements in contrast to the four previously found in the Zambian isolate of *S. mattheei* (Kane and Rollinson, 1994) and in *S. margrebowiei* (Kane *et al.*, 1996). The authors suggested that such intraspecific variation in the ITS1 might be unique to *S. mattheei*.

Blair *et al.* (1997c) determined the relationships between *Schistosoma malayensis* and other Asian and African *Schistosoma* species. Although they stated that there was evidence of intraspecific variation within Asian species, no data were provided. They reported that one of the three clones of *S. malayensis* varied at a single site in the ITS2.

Van Herwerden *et al.* (1998) explored variation in the number of repeating elements of *Schistosoma malayensis* (four repeats), *S. mekongi* (four repeats) and *S. japonicum* (two repeats). Although they presented information on variation within the ITS1 within individuals and between strains and species, due principally to the presence of the internal repeats, they were more concerned with nucleotide variation between each repeat element for a single species than

between whole replicate sequences. For instance, *S. malayensis* possesses four repeats, each composed of two sub-repeats (a~90 nucleotides; b~15 nucleotides). Intra-individual variation between repeat a_1, a_2, a_3 and a_4 within *S. malayensis* ranged from 1.3 to 7.5%. Intraspecific sequence variations within repeat a_1 across multiple molecular weight products were less than the intra-individual variation observed between repeats a_1 and a_2. Interspecific variation between repeat "a" of *S. malayensis* and that of *S. mekongi* ranged from 1.3 to 8.8%. Similar comparisons were made for repeat "b". Consequently it is not possible to comment on the levels of intra- and interspecific sequence variation within and between the three species.

Picard and Jousson (2001) and Dvorak *et al.* (2002) studied European *Trichobilharzia* species, comparing sequences from cercariae isolated from *Lymnaea* spp. to an adult tentatively identified as *Trichobilharzia regenti*. The authors found that the ITS1 from two cercariae from *L. auricularia* and four cercariae from *L. ovata* gave a single product 1100 bp in length and two cercariae from *L. ovata* and the adult of *T. regenti* had a PCR product 1400 bp long. The observed variations were due to repeat elements composed of three sub-repeats (a, 75 bp; b, 30 bp; c, 40 bp); the 1100 bp sequence had a repeat structure of $b(ab)_3$ and the 1400 bp sequence had a repeat type $ba\text{-}(ca)_5$ repeated six times. The sequence divergence between the sequences types reached 11.0% (89 base differences). Within-type divergences were 5.6% (47 base differences) for type 1100 and 1.8% (16 base differences) for type 1400. Intra-individual variation reached 0.6% for type 1100 and 1.0% for type 1400.

Dvorak *et al.* (2002) studied three *Trichobilharzia* species to clarify the status of the unusual *T. regenti*. Like Asian and African *Schistosoma* species, European *Trichobilharzia* also have repeating elements present at the 5′ end of the ITS1. They found that *T. regenti* had six repeats (111 + 112 + 111 + 111 + 112 + 85 nucleotides), *T. szidati* two repeats (not tandem and 43 and 31 nucleotides long) and *T. franki* three tandem repeats (101 + 100 + 101 nucleotides). Dvorak *et al.* (2002) also gave base differences between replicate sequences; sequences from *T. franki* were identical, while those of *T. szidati* differed at two base positions (laboratory versus wild strain).

Despres *et al.* (1992) were the first to study the ITS2. They sequenced six African *Schistosoma* species and found no intraspecific variation for replicate sequences from either *S. mansoni* or *S. bovis*. They found the sequences from *S. intercalatum*, *S. curassoni* and *S. bovis* to be identical, but did not synonymise these species. Interspecific variation between the African species ranged from 0 to 28 base differences (0.0–7.9%). *Schistosoma haematobium* and *S. intercalatum*, two species known to produce viable second generation hybrids naturally and under laboratory conditions, differed at four base positions (1.1% divergence). African and Asian species varied at 40–50 base positions (11.3 and 14.1%). Despres *et al.* (1995) expanded this work by including a single sequence from *S. hippopotami* in South Africa; prior to this study *S. mansoni* and *S. hippopotami* were considered potentially synonymous. Despres *et al.* (1995) found 6.5% sequence divergence between the two species and concluded that they were distinct. This conclusion was supported by finding two species of the *S. mansoni* group (lateral-spined egg group) to be more distant from *S. hippopotami* (also of this group) than either was from species of the *S. haematobium* group (terminal-spined egg group).

Bowles *et al.* (1993) studied geographical isolates of *Schistosoma japonicum* from China, the Philippines and Japan, and found no intraspecific variation between replicate sequences. ITS2 sequences from *S. japonicum* differed from those of *S. mekongi* and *S. mansoni* in 4.9 and 11.3% of nucleotide sites respectively. The latter two species differed from each other in 10.9% of nucleotide sites.

Michot *et al.* (1993) determined the secondary structure of the ITS2 from *Schistosoma mansoni*, *S. haematobium*, *S. intercalatum* and *S. japonicum*. They found interspecific sequence divergences between African species ranging from 4 to 28 base differences (1.1–7.8%). Variation between African and Asian species was considerably higher, however, ranging from 86 to 90 base differences (24.0–25.1%). The number of differences between African species found in this study is comparable to those found by Despres *et al.* (1992). However, the number of base differences between African and Asian species found by Michot *et al.* (1993), 86–90 bases, was considerably higher than reported by Despres *et al.* (1992) (40–50 bases).

Bowles *et al.* (1995) used ITS2 to test hypotheses associated with the grouping of *Schistosoma* species, groupings traditionally based on egg morphology, intermediate host-specificity and geographic origin. They obtained sequences from four African and two Asian *Schistosoma* species as well as from *Schistosomatium douthitti*. There was no intraspecific variation between two replicate sequences from *Sc. douthitti* or three replicate sequences from *S. japonicum*. They found five base differences between sequences from *S. haematobium* and *S. intercalatum* (*S. haematobium* group), three between *S. mansoni* and *S. rodhaini* (*S. mansoni* group), 32 between *S. japonicum* and *S. mekongi* (*S. japonicum* group) and up to 145 base differences between *Schistosoma* species and *Sc. douthitti*.

Agatsuma *et al.* (2001) studied *Schistosoma sinensium* from Thailand and China, comparing ITS2 sequences to those obtained by Bowles *et al.* (1995). Intraspecific variation between the Thai and Chinese isolates of *S. sinensium* was 0.7% (two base differences over 294 nucleotides). This level of variation was higher than that observed between *S. malayensis* and *S. mekongi*, 0.4% (one base difference over 286 nucleotides). Consequently, in the context of differences in snail host specificity and differences in the size of eggs between the two geographical isolates, Agatsuma *et al.* (2001) suggested that there was a species complex associated with *S. sinensium*.

Agatsuma *et al.* (2002) sequenced the ITS2 from four species of the *Schistosoma indicum* group (*S. indicum*, *S. spindale*, *S. nasale* and *S. incognitum*) and compared these to previously obtained sequences. Interspecific variation within the group ranged from 1.6 to 8.1%. They found 12.6–16.7% sequence divergence between *S. indicum* group sequences and those of the *S. japonicum* group. Sequence divergences of 4.8–9.4% were found between the *S. indicum* group and both the *S. haematobium* and *S. mansoni* groups from Africa.

Agatsuma *et al.* (2004) sequenced the ITS2 from *Bivitellobilharzia nairi* to determine its taxonomic position. Alignment of 13 *B. nairi* sequences (from seven male worms and six female worms) revealed one sequence to contain a TA insertion (0.6% over 329 sites) and another replicate sequence to vary at a single site only (0.3%).

5.18. Strigeidae

Bell *et al.* (2001) determined the inter-relationships of *Ichthyocotylurus variegatus*, *I. platycephalus*, *I. erraticus* and *I. pileatus* by analysing three data sets, ITS1, ITS2 and CO1. Interspecific variation in the ITS2 between the four species was 1–3 base differences (0.3–1.0%) and confined to the last 14 bases of the spacer region. PCR amplification of the ITS1 produced five or six different molecular weight products ranging from 300 to 1800 nucleotides in length. The authors chose to sequence the most abundant and ubiquitous of these products (800 bp). Partial sequencing of the other different molecular weight products revealed these to represent either intra-individual or intraspecific variation within the gene. A comparison of sequences from *I. variegatus* from two different Scottish fish revealed a single base transversion over 784 base positions; both samples exhibited a common base transversion relative to samples from a Finnish fish. Bell *et al.* (2001) found no evidence of intraspecific variation in the ITS1 sequences from the other three species. Interspecific variation between the four species ranged from 1.9 to 3.1% (15–24 of 784 sites). Intergeneric variation between *Ichthyocotylurus* and *Apatemon* was 24.7%.

Bell and Sommerville (2002) used three nucleotide data sets to explore the systematic status of *Apatemon gracilis* from four fish species and *A. annuligerum* from two geographical locations. They found that ITS2 sequences were 292 nucleotides long and identical within a species (regardless of host or geographical location) and also between species. As in their previous study, Bell *et al.* (2001) found evidence of a number of different molecular weight products after PCR amplification of the ITS1. Again, only the most abundant and ubiquitous product (700 bp) was isolated and sequenced. There was no intraspecific variation in the ITS1 from *A. annuligerum* (2 repeat elements) over 688 sites. Intraspecific variation between replicate sequences from *A. gracilis* was a result of a varying number of repeat elements. For instance, *A. gracilis* from the stone loach possessed three repeats; specimens from salmon parr possessed two repeats and specimens from rainbow trout possessed two tandem repeats of (TCGGCT) and a third repeat of (ATACCTCGACC), not present in

other *A. gracilis* sequences. Aside from intraspecific variation as a consequence of a difference in number of repeat elements, no intra- or interspecific specific sequence variation was observed (*A. gracilis* from salmon parr and *A. annuligerum* both possess two repeat elements and were identical along 688 nucleotides). Consequently, *A. annuligerum* was considered synonymous with *A. gracilis*. This conclusion was supported by CO1 sequences, which were identical in length and composition for both putative species.

5.19. Telorchiidae

The study by Luton *et al.* (1992) of two *Dolichosaccus* species from Australia was the first to use sequence data in a taxonomic study of digeneans. It remains the only such study of telorchiids. Over the entire ITS, they found 0.3 and 2.5% intraspecific variation between replicate sequences from *D. symmetrus* and an unidentified *Dolichosaccus* sp. (respectively) between two localities. Interspecific variation over the same number of nucleotides between the two species was 30.7% (541 of 1762 sites). The most conspicuous difference between the sequences from the two species was the length of the ITS1, owing mainly to the presence of several repeats approximately 100 nucleotides long in *Dolichosaccus* sp. that are present only once in *D. symmetrus*. The multiple presence of these repeats ultimately leads to a larger interspecific sequence divergence when just ITS1 sequences are compared, 75.4% (488 of 647 sites). When the 3′ end of the ITS1 is compared between the two species only, interspecific variation is 16.7% (63 of 378 sites). Replicate sequences from ITS2 for *D. symmetrus* and *Dolichosaccus* sp. were identical and interspecific variation reached 20.2% (51 of 252 sites).

6. THE INTERPRETATION OF ITS rDNA SEQUENCES

We have reviewed 63 studies that report partial or complete ITS sequences to identify about 155 named digenean species from 19 families. Some of these sought sequence matches between candidate

life cycles stages and others used sequence data to determine the distinctness of species, whose validity was not necessarily clear on the basis of morphological comparisons. A few explored geographic variation. All have needed to consider what levels of sequence variation constitute intra- and interspecific variation. Here, we review levels of variation reported as intra- and interspecific variation and how it can be best interpreted.

The implied expectation in the literature is that ITS sequences will be identical for replicates of the same species, recognisably different for species of the same genus, and significantly more different for species of different genera or families. As discussed in Section 4.1, this expectation is based on the fact that the entire rDNA repeat is thought to evolve in a concerted fashion (making the individual representative of the entire group) while the necessity for the region to fold into its secondary structure to maintain correct function within the ribosome means that the region must stay relatively conserved. It thus follows that we expect ITS sequences from trematodes to show very little intraspecific variation. This expectation is generally met. For approximately 33 species, there is evidence of replicate sequences but no intraspecific variation having been found. However, "trouble" is met in the two following ways:

- Species are sometimes reported as having sequences identical to those of other closely related species.
- Species are sometimes reported as having intraspecific variation.

6.1. Identical Sequences, Different Species

Given that ITS sequences are generally sought with a view to exploring the validity of species boundaries, it is no surprise that identical sequences tend to be taken as evidence of the same species. In studies of the Echinostomatidae (Morgan and Blair, 1995), Paragonimidae (Blair *et al.*, 1997a) and Strigeidae (Bell and Sommerville, 2002) replicate sequences for parts of the ITS region were repeatedly found to be identical for putatively different species. In each case, it was concluded that there was no other compelling evidence that

required that separate species should continue to be recognised and the species were synonymised. These studies are examples of the highly effective application of sequencing technology to improve our understanding of the taxonomy of trematodes.

There are also three cases in which putatively different species have been found to have identical ITS sequences, but have continued to be recognised as distinct. Each of these is worthy of special consideration.

Niewiadomska and Laskowski (2002) found no differences between ITS1 sequences from *Diplostomum spathaceum* and *D. parviventosum* (Diplostomidae). They did not synonymise the two species because they considered them distinguishable by morphological differences between adult, cercarial and metacercarial stages. However, in addition to the two sequences for each species lodged by these authors on Genbank (which are all identical as reported) there are a further four otherwise unpublished ITS1 sequences for *D. spathaceum*. These differ from each other at 0–2 sites and from the sequences from Niewiadomska and Laskowski at 22–24 base positions. It thus seems at least possible that the Niewiadomska and Laskowski sequences for *D. spathaceum* were reported in error and that the two species are indeed easily separated by ITS sequences.

Despres *et al.* (1992) reported identical ITS2 sequences for *Schistosoma intercalatum*, *S. curassoni* and *S. bovis*, but did not synonymise these species. We examined GenBank and found nine ITS2 sequences for *S. bovis* (AF146030; AF146032-146035; LO3651-3653; LO3657), one for *S. curassoni* (LO3654) and two for *S. intercalatum* (LO3655; U22166). In a new alignment, we added six sequences for *S. bovis*, a single ITS2 sequence for *S. intercalatum* (U22166), the only sequence for *S. curassoni* and sequences for *S. haematobium* (AF146038), *S. mansoni* (U22168) and *S. hippopotami* (AY029182) (to delimit the 5′ and 3′ ends of ITS2). We found a single base difference between *S. intercalatum* and *S. bovis*. On this basis, we believe that further systematic work will show all three species may possess distinct ITS2 sequences.

Blair *et al.* (1997b) and Blair *et al.* (1998) found identical ITS2 sequences for *Paragonimus miyazakii* from Japan and *Pagumogonimus skrjabini* from China. However, they found substantial

differences in the mitochondrial cytochrome *c* oxidase subunit I gene (CO1). These two species were also described as having significant morphological differences.

Thus, of the three studies that recognise different species despite the recovery of identical sequences from the ITS region, only that of paragonimids has a robust and convincing data set. However, this finding is of great importance because it establishes that genuinely distinct species may not necessarily differ in at least one of the internal transcribed space regions of rDNA. In some respects, this should not be surprising because other pairs of species that have been characterised convincingly have as few as one base difference so that there appears to be no reason to think that the ITS region is guaranteed to find differences between species.

6.2. Interspecific Variation

With the exception of the three cases discussed above (two potentially spurious), all reported combinations of congeners have differences in some part of their ITS sequences or are now considered synonymous. Levels of interspecific variation are typically substantial so that there is usually little doubt as to the separation of the species involved. However, a number of studies have found very small differences between some species, especially in the ITS2. Agatsuma *et al.* (2001) found just one base difference between the ITS2 sequences from *Schistosoma malayensis* and *S. mekongi* (Schistosomatidae). This difference correlates with the two species having different biological attributes (Greer *et al.*, 1988) and differing in other molecular markers (Agatsuma *et al.*, 2001). Bell *et al.* (2001) found just 1–3 base differences between combinations of four species of *Ichthyocotylurus* (Strigeidae). Species distinction was confirmed by molecular data from CO1 and ITS1. Anderson and Barker (1998) reported just two base differences between ITS2 sequences in one combination of 2 of 11 didymozoids from Queensland flatheads. The two species occurred in different but sympatric hosts and had slightly differing morphologies. Morgan and Blair (1995) found two base differences in the ITS2 from *Echinostoma trivolvis* and *E. paraensei* (Echinostomatidae). They

concluded on the basis of divergences over the entire ITS (not on ITS2 alone) that the two species were distinct. Bowles *et al.* (1995) found just three base differences between *Schistosoma mansoni* and *S. rodhaini* (Schistosomatidae). Snyder and Tkach (2001) found interspecific sequence variation of only four differences over 851 sites over the entire ITS between *Haematoloechus medioplexus* and *H. varioplexus* (Haematoloechidae), which are easily distinguishable by morphology. Other reported combinations of congeners differ at progressively larger numbers of sites in ITS2 (see Section 5).

Levels of interspecific difference between congeners over the ITS1 range from 0.8% (9 of 1086 bases) for *Schistosoma haematobium* and *S. intercalatum* (Kane and Rollinson, 1994) to 75.4% between *D. symmetrus* and *Dolichosaccus* sp. from two locations in central Queensland (Luton *et al.*, 1992). Except for the schistosomes, all combinations of congeners are reported to vary at greater than 1.0%. Because of the frequent importance of repeats in the analysis of ITS1 sequences, percent differences are often far less meaningful than a description of differences in the repeat structure.

6.3. Intraspecific Variation

Intraspecific variation of some sort has been reported for many species. We suspect that some reported intraspecific variation is spurious, resulting from error or from the failure to recognise the presence of more than one species. Genuine intraspecific variation may occur in a sample from a single site or perhaps over the geographic range of the species.

6.3.1. Error

We think it is likely that error is common. The first kind of error is brought about by the correct sequencing of incorrectly identified specimens. This can occur by the failure to appreciate that more than one species is present in a sample or that two different samples represent the same species. In this we do not refer to the problems of cryptic species, which may only be detected when a molecular approach is taken. Rather, we refer to plain errors of misidentification,

mislabelling and contamination. To some extent these problems can be controlled by the rigorous retention of morphological vouchers for all sequenced samples and, ideally, their lodgement in museums. The most convincing way for this to be done is by retaining half the specimen for morphology and sequencing the other half; we have seen only one report of this having been done for trematodes (Galazzo *et al.*, 2002). That such errors occur is shown at least by our own work; we have detected several cases of mislabelling and misidentification in our own studies (although as far as we know we have detected these prior to publication).

The second kind of error is sequencing error; the correct sample is sequenced, but the sequence is not a true reflection of the species. Sequences are now usually generated by automatic sequencing machines that return a chromatogram and a ''call'' of each base position. For a myriad of reasons (Innis *et al.*, 1988), there may be one or more bad ''calls'' for a given sequence. In our experience, bad sequences are generally recognised by the combination of the quality of the sequence itself and by reference to other sequences. A poor sequence may have many base positions that cannot be called and a generally poorly resolved chromatogram (our own sequence database has many tags saying ''bad read, do not use''). However, ''poor'' may be subjective, and it is entirely possible for a generally excellent sequence to have an area where the read is not reliable. *In this context, we think it essential that at least a minimum 85% sequence match should be found between forward and reverse sequences to confirm an entire sequence before it is used.*

Poor sequences may also be recognised as such by sequence replication. Many of the apparently more careful studies refer to many identical replicates. The advantage of multiple replicates is that they draw attention to unconvincing (or interesting) base differences. However, if there is no confirmation of a sequence for a given host/parasite/locality combination, then anomalous (and potentially spurious) bases may remain undetected. We suspect that small amounts of error are rife in the published literature. Many studies have reported variation in one or two base positions. Tellingly, we are unaware of any study where such a low difference has been confirmed by the reported re-sequencing of the samples.

In the main, very low levels of variation are considered inconsequential and are ignored; if two target species differ at 15 sites, does it matter that there may be intraspecific variation in a few base positions? Perhaps typically the answer is that the variation is indeed inconsequential. However, we conclude that it does matter because, as we have seen above, variation, especially in the ITS2, ranges from high and very clear levels of difference to negligible difference and rarely to no difference at all.

6.3.2. Failure to Recognise Multiple Species

We suspect that several studies have wrongly interpreted variation as intraspecific when it is in fact an indication of the presence of multiple species. Many studies report at least some variation that is interpreted as intraspecific variation. This ranges from as few as one base difference in one replicate to as much as 3.2% variation over the entire ITS region. We have seen above (Section 6.2) that convincing pairs of species may be separated by very small differences. Here, we consider the five largest levels of variation that have been interpreted as intraspecific variation.

Luton *et al.* (1992) found 0.9 and 6.8% intraspecific variation over the entire ITS for replicate sequences from *Dolichosaccus symmetrus* and *Dolichosaccus* sp. (Telorchiidae) from cane toads from two Queensland localities. There is no other sequence data available for the Telorchiidae with which to contrast these results. It is noteworthy, however, that in a morphology-based study Barton (1994) subsequently reported three species of *Dolichosaccus* from cane toads from the same area. The two studies did not refer to each other's specimens, but it seems likely that the exceptionally wide divergence reported by Luton *et al.* (1992) related to the presence of a third species in the system. It is noteworthy also that at the time there was no other information about levels of variation in the rDNA ITS region for trematodes. We therefore believe that this system is worthy of further examination.

Sorensen *et al.* (1998) sequenced the ITS from *Echinostoma revolutum* (Echinostomatidae) from Eurasia and North America and

reported intraspecific variation of 0.9%. They suggested this variation could also represent genetically differentiated cryptic species. Morgan and Blair (1995) found no intraspecific variation over the entire ITS rDNA between replicate sequences for six recognised *Echinostoma* species. Therefore, we conclude that Sorensen *et al.* (1998) may indeed have examined cryptic species.

Leon-Regagnon *et al.* (1999) partially sequenced the ITS and 28S rRNA of several species of *Haematoloechus* (Haematoloechidae) from Mexico and the United States, and reported what they interpreted as intraspecific variation of 1.7, 1.2 and 2.0% for three species. However, Snyder and Tkach (2001) found interspecific sequence variation in the ITS from eight North American and European *Haematoloechus* species to be as low as 0.5% between species that are easily distinguishable on morphological grounds and found no intraspecific variation in four species and just 0.1% in the fifth. Based on their own findings, Snyder and Tkach (2001) suggested the cautious use of Leon-Regagnon *et al.* (1999) data.

Overstreet *et al.* (2002) reported intraspecific variation of 3.2% over the entire ITS for *Bolbophorus damnificus* (Diplostomidae). In contrast, Galazzo *et al.* (2002) found no or negligible intraspecific variation for most replicates in the ITS2 from three North American species of *Diplostomum* and only minor variation for some specimens identified only tentatively. In addition, Niewiadomska and Laskowski (2002) found only 1.3–4.7% divergence among six Eurasian *Diplostomum* species (difficulties with the sequences from *D. spathaceum* and *D. parviventosum* are ignored here). These levels of variation make that found by Overstreet *et al.* (2002) appear anomalously large and may suggest that further study is required on *Bolbophorus damnificus* (despite the corroborating evidence from experimental infection and sequence data from different gene regions).

Jousson and Bartoli (2002) analysed morphological, biological and molecular data for two species of *Monorchis*, *M. parvus* and *M. monorchis*, both from multiple Mediterranean fishes. Within each host–parasite combination intraspecific variation did not exceed 0.3% (1–2 base differences over 601 sites). *M. parvus* sequences formed a monophyletic group consisting of two host-associated clades separated by 3.5% sequence divergence. The two taxa were also

separated by morphometric data. However, the observed differences were attributed to host-related phenotypic plasticity. This led the authors to identify both as *M. parvus*. On the basis of these findings and those of their previous studies on opecoelids (Jousson and Bartoli, 2000) the authors proposed, "*the degree of morphological variation between species is not necessarily correlated with the amount of genetic divergence for marine digeneans*". On balance, however, we reject the interpretation that forms of *M. parvus* differing at 3.5% can be a single species.

We conclude that the evidence relating to each of these studies, suggests that (1) there has been error in sequencing or identification of the specimens, (2) there has been an unconvincing interpretation of the significance of the variation (i.e. only one rather than at least two species) or (3) that there is an exceptional case of intraspecific variation that has not been sufficiently characterised. We predict that for most of these cases there will prove to have been more than one species involved.

6.3.3. Geographical Variation

There have been relatively few studies of genetic variation in trematodes over their geographical range. The most detailed work has been on the groups of greatest economic importance (Fasciolidae, Paragonimidae and Schistosomatidae). For at least 16 species, ITS sequences from relatively widely separated localities have proven identical (Table 3). These reports suggest that, frequently, what is considered to be single species with a wide range is exactly that.

For the Paragonimidae, there have been three studies of *P. westermanii* that report variation associated with geographical distribution (Blair *et al.*, 1997b; Iwagami *et al.*, 2000; Ryu *et al.*, 2000). These studies used ITS2 sequences and reported either no or very little variation for samples from China, Japan, Korea and Taiwan (Taiwanese samples differed consistently at one position). Samples from Thailand, Malaysia and the Philippines differed from the northern group at 6–10 sites; Malaysian and Philippine samples differed from each other at one base and from the Thai population at 8–9 bp. The

Table 3 Sixteen species, from which ITS2 rDNA sequences, from isolates of relatively widely separated geographical localities, have proven identical

Species	Family	Spacer	Separation	Reference
Schistorchis zancli	Apocreadiidae	ITS2	Great Barrier Reef and French Polynesia (6000 km)	Lo *et al.* (2001)
Bivesicula claviformis; *Bivesicula unexpecta*	Bivesiculidae	ITS2	Northern and Southern Great Barrier Reef (1188 km)	Cribb *et al.* (1998)
Calicophoron calicophorum; *Orthocoelium streptocoelium*; *Homalogaster paloniae*	Cladorchiidae	ITS2	Iwate, Aomori, Akita and Hyogo Prefecture, Japan	Itagaki *et al.* (2003)
Preptetos laguncula; *Neohypocreadium dorsoporum*	Lepocreadiidae	ITS2	Great Barrier Reef and French Polynesia (6000 km)	Lo *et al.* (2001)
Leucochloridium paradoxum; *Leucochloridium variae*	Leucochloridiidae	ITS	Poland, Denmark and Norway	Casey *et al.* (2003)
Plagiorchis vespertilionis; *Plagiorchis muelleri*; *Plagiorchis koreanus*	Plagiorchiidae	ITS	Golaya Pristan district; Khersan Region, Velika Pisarevka district, Sumy Region; Kiev Region; town of Nizhin, Chernigiv Region (all localities in Ukraine)	Tkach *et al.* (2000)
Schistosoma mansoni; *Schistosoma bovis*	Schistosomatidae	ITS2	Brazil, Guadeloupe, Venezuela, Ivory Coast, Senegal; and Niger, Italy (respectively)	Despres *et al.* (1992)
Schistosoma japonicum	Schistosomatidae	ITS2	China, Indonesia, Japan, Taiwan and The Philippines	Bowles *et al.* (1993)
Schistosoma mansoni	Schistosomatidae	ITS1	Brazil, Egypt, Kenya and Senegal	Kane *et al.* (1996)

consensus of these authors was that *P. westermanii* could be divided into two or three species, one in northeast Asia and the other two in southern Asia. These findings were supported by COI sequences and biologically by the family of snail host used by each group (members of the southern group use thiarids and members of the north eastern group use pleurocerids). Given these studies, it is safe to conclude that *P. westermanii* as conceived of prior to the advent of molecular studies is not a ''stable'' species. However, there has been no formal move to propose such a division on the basis of these data.

For the Schistosomatidae replicate sequences from species from different geographical localities have typically been identical. Two cases are noteworthy, however, Kane and Rollinson (1994) and Kane *et al.* (1996) studied the ITS1 from *S. mattheei* from South Africa and Zambia and found five repeat elements in the South African isolate in contrast to four in the Zambian isolate. Agatsuma *et al.* (2001) compared ITS2 sequences from *S. sinensium* from Thailand and China and found two base differences. This was more than the single base difference that separates *S. malayensis* and *S. mekongi*. There are differences in snail host specificity and in the size of eggs between the two geographical isolates of *S. sinensium*. Agatsuma *et al.* (2001) suggested that there is a species complex associated with *S. sinensium*, although no formal move to divide the species has been made.

For the Fasciolidae, ITS2 sequences have been generated for *Fasciola hepatica* from Australia, China, France, Hungary, Mexico, New Zealand, Spain, Uruguay and Bolivia (Adlard *et al.*, 1993; Hashimoto *et al.*, 1997; Itagaki and Tsutsumi, 1998; Mas-Coma *et al.*, 2001; Huang *et al.*, 2004). ITS2 sequences were mainly identical (Australia, China, France, Hungary, New Zealand and Uruguay). The Mexican isolate had a single base substitution and the Spanish and Bolivian samples differed from all other sequences in at least one base position. The ITS2 from *F. gigantica* has been sequenced for samples from China, Indonesia, Malaysia and Zambia (Adlard *et al.*, 1993; Hashimoto *et al.*, 1997; Itagaki and Tsutsumi, 1998; Huang *et al.*, 2004). There were two different sequences obtained for Zambian samples. Sequences from China, Indonesia, Malaysia and one from Zambia were identical. The second Zambian isolate differed from the

other at four sites and one of these differed from the Indonesian isolate at six sites.

Overall, where variation has been investigated over a substantial geographical range there have been three types of outcomes. Most commonly the sequences are identical. Far less commonly consistent differences are found that have been interpreted as probably being the reflection of the presence of separate species. Also uncommon is minor variation that has been interpreted in the literature as genuine geographical variation. We suspect that in some cases this variation may be a pointer to the presence of distinct species. In such circumstances there are two paths open for further investigation. Further molecular analysis would be to sequence more samples (and extra genes) from localities intermediate between the sites already sampled to see how (or if) the sequences change gradually over their range. Conceivably, different sequences may grade into each other (indicative of a single species) or there might be an abrupt boundary or overlap zone between the two (indicative of separate species). The second approach would be to explore the biological basis of difference between the populations—are there differences in morphology, behaviour or host-specificity? At present, the weight of evidence suggests that genetic geographical variation between isolates of trematode species is not common; at least it is not routinely revealed by sequencing of the ITS region. ITS sequences from species are typically identical at different localities and where they differ, it appears that the best interpretation is often that separate species are present.

6.3.4. "Real" Intraspecific Variation

Because high-quality studies that use multiple replicates usually find little or no intraspecific variation, we infer that low levels of variation reported where there are few replicates may frequently be the result of sequencing error. Because the level of variation between species may be very low (exceptionally no variation at all), especially for ITS2 sequences, we conclude that it is likely that some reported intraspecific variation is actually interspecific variation. Where significant geographical variation has been reported, the most compelling

interpretations have suggested that it is indicative of the presence of separate species. Very low levels of geographical variation may represent genuine intraspecific variation but this requires further exploration. We conclude that, for the present, there is no compelling evidence of significant intraspecific variation in ITS sequences, especially where the sequences have been derived from sympatric samples. We argue that where variation is found it should be taken as a signal that further investigation is necessary.

So far no molecular studies have revealed or suggested the presence of combinations of cryptic (morphologically inseparable) species infecting a single host species at a single locality. Multiple sympatric congeners are by no means rare for digeneans so it can be expected that ultimately such systems will be identified. It is conceivable that some of the intraspecific variation reported in the literature at present represents exactly this.

6.3.5. The Recognition of Species

We have argued above that some of the intraspecific variation reported in the literature probably reflects the presence of separate species. If we are right, such misinterpretations may have arisen because of a combination of inadequate replication and the inappropriate use of "yardsticks" of comparison.

This question raises the issue of when genetic differences can be interpreted as reflecting the presence of more than one species. We believe that the answer to this question can only be resolved by reference to studies in relatively simple and controlled conditions. The key, in our view, is the study of samples collected in sympatry, which removes any possible issues of geographical variation. If, at a given location, trematode populations associated with different hosts, different morphology or different ecology are consistently characterised by a genetic trait in the form of base differences in ITS sequences, then, we think that in all probability the distinction is a marker for separate species, regardless of how many differences there are. We suspect that many such small but important differences remain to be recognised.

The basis for this view is established by reference to hypothetical data. Suppose that one form of trematode, always found in Genus A species A at a given locality, has 20–21 oral spines and a second at the same locality but in Genus B species A, always has 29–30. Morphological taxonomists would, with very few exceptions, conclude that two species are present. A few might wonder if the identity of the host affected morphology, but with such a substantial morphological difference this would not be a major concern. If we then examined specimens from Genus A species B and found that they had 22–23 spines then legitimate argument as to whether different species should be recognised might ensue; the outcome might well be recourse to a molecular study.

Suppose then that we sequence the ITS1 or ITS2 from one specimen of each of the three forms from the three hosts mentioned above. We might find that the sample from Genus B species A differs at 20 sites from the specimens from the other two hosts, which differ from each other at only a single (different) position. The tempting conclusion would be that, based on the morphological (spine number), biological (host) and genetic (base) differences between the specimens from Genus B relative to those from Genus A, there are just two trematode species in the system. The minute morphological and genetic differences seen between specimens from Hosts A and C could be dismissed as intraspecific variation or sequencing error. However, it is entirely possible for such an interpretation to be quite wrong. False interpretation would arise from a combination of the use of an inappropriate "yardstick" and from a lack of replication in the data set. Suppose that we chose to explore this system further and sequenced 10 replicates from each host and returned 10 identical sequences for each of the three forms. We would then be forced to conclude that we had three species in the system. Species A and C might be best considered cryptic species because of the similarity of their morphology, but they differ consistently in their host and consistently in their genotype while being sympatric and thus potentially having the opportunity for genetic exchange.

In the hypothetical system described above, the large genetic difference between Species A and B has initially misled us into assuming that all sets of species differences will be comparable. This

expectation is unsound because some species will have diverged far more recently in evolutionary time than others and thus we can expect levels of genetic difference to vary dramatically, even within a single genus. That the sequence differences may be very small between genuinely distinct species is shown by several studies reviewed earlier. For this reason we argue that the idea that a "yardstick" of levels of variation can be developed is seriously flawed. Rather than seeking to apply a yardstick, the better approach is to test for consistent *difference* between sequences and to seek a biological, morphological or ecological basis for any distinction that may be suggested.

Thus, wherever studies have reported intraspecific variation we suspect that there is room for the further careful consideration of the system. The intraspecific variation reported in the literature ranges from single base positions (Galazzo *et al*., 2002) through to as many as 31 positions (Overstreet *et al.*, 2002). At the lower end of the scale we suspect that most of the variation reported is intraspecific variation or sequencing error. As the number of differences increases this becomes less likely, but each system needs careful consideration and the application of many replicates. We also note that "careful consideration" requires substantial work and if there is no *a priori* reason (such as a difference in hosts or in morphology) to do that work, then often there may be little reason to pursue the study.

7. IMPLICATIONS OF ITS SEQUENCING FOR DIGENEAN TAXONOMY

The goal of any taxonomic studies whether morphological, molecular or otherwise is to lead to an accurate reflection of biological reality and to develop an effective naming system for use by the scientific community. An important goal of any system of classification should be stability; continual (especially unnecessary) changing of names is not welcomed by the scientific community at large. It is timely to consider, therefore, the overall impact of molecular taxonomic approaches (at least those using rDNA sequences) on digenean taxonomy.

7.1. Implications for Species Richness

At present there are thought to be about 18 000 described trematode species. The studies reviewed here have reported sequence information for about 155 named species, or less than 1% of them, and representatives of fewer than half the families. In this respect, molecular approaches cannot be said to have revolutionised the field. Overall, it is pleasing that most of the existing morphological taxonomy has been corroborated by the molecular studies; there is no evidence that existing taxonomy is comprehensively inaccurate. However, molecular studies have now contributed to the proposal of four convincing synonymies of named species and have helped separate about eight combinations of species (Despres *et al.*, 1995; Blair *et al.*, 1997b; Iwagami *et al.*, 2000; Jousson and Bartoli, 2000; Jousson *et al.*, 2000; Agatsuma *et al.*, 2001; Galazzo *et al.*, 2002; Jousson and Bartoli, 2002), which otherwise might not have been recognised as distinct (although not all of these new species have been named formally).

Thus, as might have been predicted, molecular approaches have the capacity to solve problems on the margin of the resolving power of traditional taxonomic approaches. Often they give corroboration to hypotheses that are just suspicions. Studies involving rDNA sequencing so far have led to the recognition of far fewer new synonymies than they have potentially new species. The data set is still small, but if this pattern persists for the Digenea as a whole, it would suggest that morphological taxonomy typically underestimates the true species-richness of trematodes.

In some of the cases described above, we suspect that the pairs of species could have been recognised as such without the use of molecular techniques. Are molecular approaches then replacing morphology-based approaches and should this be seen as good, bad or unimportant? Our view is clear. Morphological and molecular approaches will always work best when they are used to support and augment each other. Ambiguous morphological or biological differences that remain unexplored from the point of view of molecules are unsatisfactory to exactly the same extent as are genetically distinguished but biologically or morphologically undifferentiated species. Which approach (morphological or molecular) should come first is a

matter for the individual circumstance, although in our experience it is knowledge of biological questions formed from the study of morphology and biology that usually prompt an augmentative molecular study.

7.2. Implications for Morphological Taxonomy

An important benefit of the molecular work reviewed above should be reciprocal illumination of processes in morphological taxonomy, not just by testing its predictions, but by exploring levels of geographical variation that are supported by molecular tests. To this end we here consider the morphological variation reported for three pairs of species that have been separated, in part, by differences in ITS sequences. In each case the work was all done by the same authors so that preparation and figuring methods are directly comparable.

Jousson *et al.* (2000) and Jousson and Bartoli (2002) found that *Monorchis parvus* from three species of *Diplodus* (Sparidae) from the Mediterranean was actually two species, one restricted to *Diplodus vulgaris* and *D. sargus* and one to *D. annularis*, which differed by 3.8% (Jousson *et al.*, 2000) and 3.5% (Jousson and Bartoli, 2002) over ITS1. Jousson *et al.* (2000) specifically concluded that they considered this system to represent a pair of cryptic species, but they did not name a new species. Differences between the two forms are certainly subtle (Figure 2). The two forms differ somewhat in body shape, in the size of the testis, the position of the ovary and slightly in the arrangement of terminal genitalia.

Jousson and Bartoli (2000) reported two cryptic species associated with *Opecoeloides furcatus* (Opecoelidae) in Mediterranean fishes. ITS1 sequences from the two species differ by an extra 60 nucleotide repeat in one form and 13 other scattered base differences. Figure 3 shows "*habitus*" figures of the two species reconstructed from mean data. The two species are clearly distinct in their typical overall size and in the lobation of the testes.

Nolan and Cribb (2004b) sequenced the ITS2 from a new sanguinicolid species, *Pearsonellum pygmaeus*, to test its separation from the type species of the genus, *P. corventum*. Both species occur

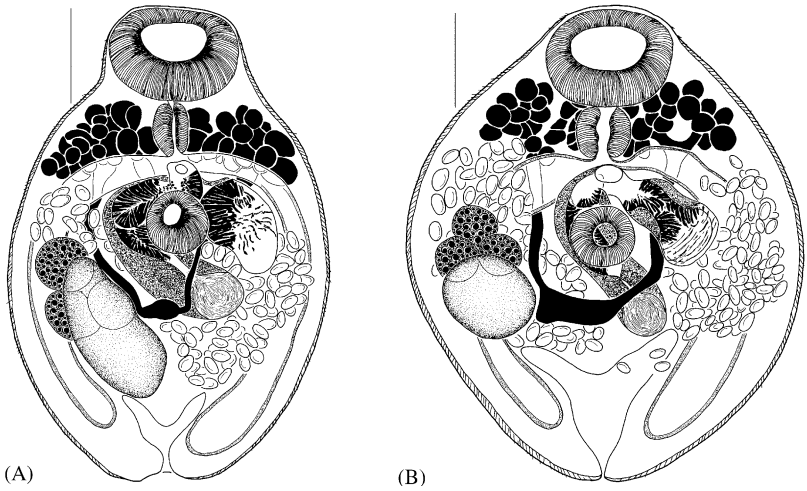

Figure 2 *Monorchis parvus* from (A) *Diplodus sargus*, (B) *D. annularis*. Ventral view. Scale bars: (A,B) 100 μm. (Redrawn from Jousson and Bartoli, 2002.)

sympatrically in serranid fishes (but different genera) on the Great Barrier Reef. They found 29 base differences between the species. The two species are morphologically similar, but *P. pygmaeus* differs from *P. corventum* in the smaller overall body size, the relative distance of the brain from the anterior end and the relative lengths of the oesophagus and the testis. This species also differs with respect to the distribution and shape of the testis, the position of the uterus with respect to the ovary and the size and shape of the ovary (Figure 4).

This small sample of studies is both encouraging and challenging. The opecoelid and sanguinicolid pairs of species show that the kinds of characters used routinely in taxonomy appear to be reliable. The monorchiid pairs show that differences may indeed be quite subtle.

7.3. When to Name a New Species?

Should a pair of species distinguished convincingly only by genetic differences be described with separate names? This problem has already been encountered to some extent and it is likely that it will become more common in the future. Jousson *et al.* have reported

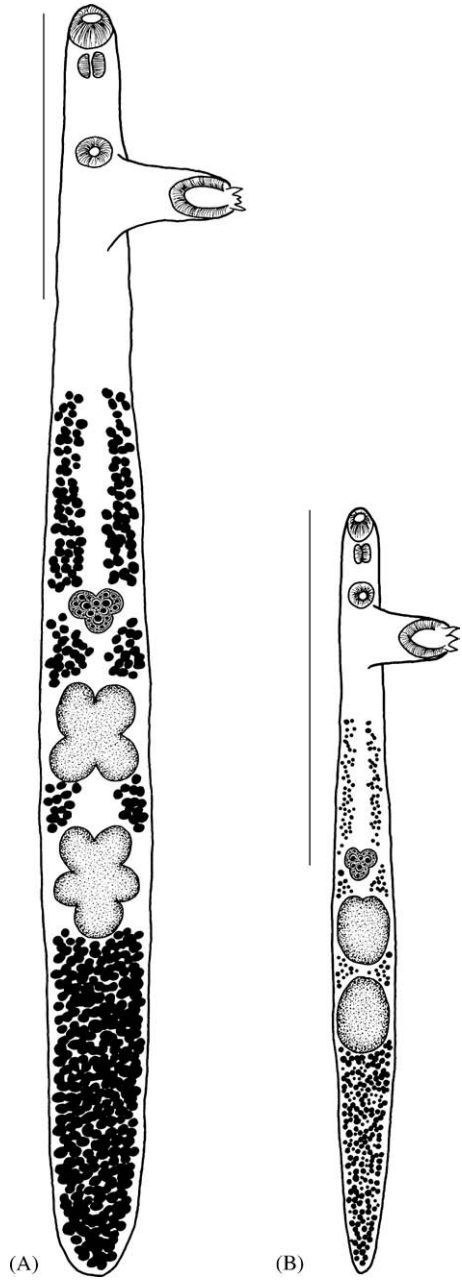

Figure 3 Habitus of (A) *Opecoeloides furcatus*, (B) *O. columbellae*. Scale bars: (A,B) 1000 μm. (Redrawn from Jousson and Bartoli, 2000.)

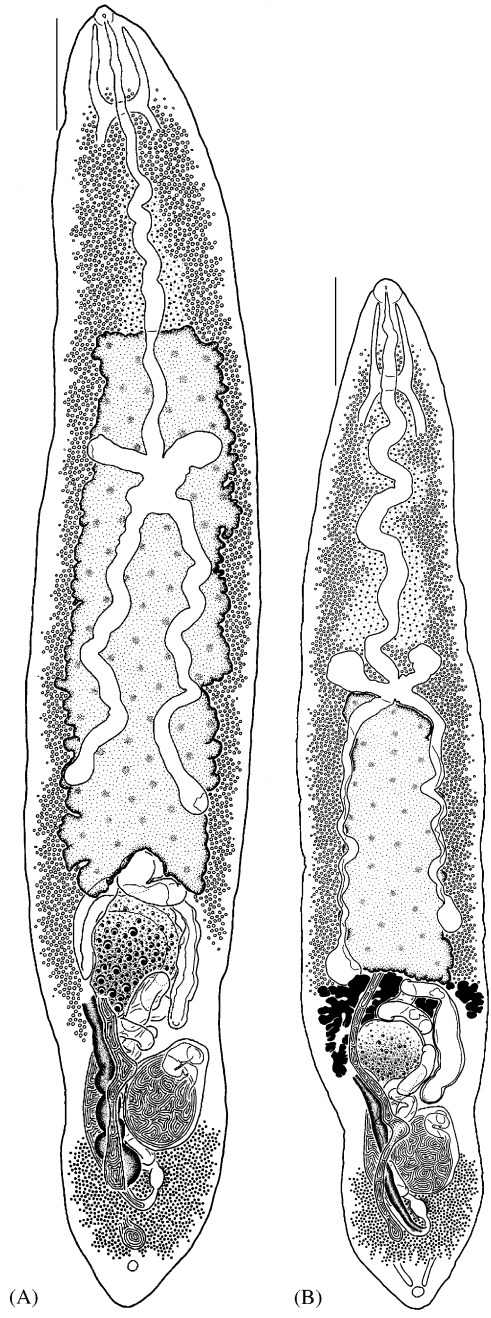

Figure 4 (A) *Pearsonellum corventum* from *Plectropomus leopardus*. (B) *Pearsonellum pygmaeus* from *Cromileptes altivelis*. Scale bars: (A,B) 100 μm.

what they explicitly consider to be two pairs of species with clear genetic differences and with clear differences in hosts but in each case did not name the second new species. In one case (Jousson and Bartoli, 2002) they stated that, in effect, they were unwilling to describe the new species because it could not be distinguished by morphology. The reason for this approach is clear. If a species can only be identified by its genetic sequence and perhaps by its host (and host is always a problematic marker of identity), then the classification is made difficult for those who need to identify the parasites. The disadvantage of not naming species is that the true species richness of a system tends to be undervalued and the true complexity of a biological system is not represented by the taxonomic system. We incline to the view that species that are characterised convincingly should be named, even if the characterisation is entirely genetic. If the characterisation *is* entirely genetic, then we see this as a signal that more work probably needs to be done to understand the morphology and biology of the species in question.

An important component of any classification system is its stability and its utility; names of species that are used widely, especially those of economic importance, should not be changed unless there is a compelling reason to do so. On this basis, we accept that changes within important genera such as *Fasciola*, *Paragonimus* and *Schistosoma* should only be made with considerable caution. In this context we do note, however, that splitting species and providing new names may have some advantages. The advantages may be in the knowledge that a socio-economically important parasite has a limited distribution and perhaps peculiar biological attributes that enhance the capacity to control it. Except for the small number of trematodes of economic importance, the great majority of trematode species are of interest to biology because of their significance in biological diversity (in its broadest possible sense). Most trematode species will only be considered by biologists whose interests should always lie with the most accurate possible classification. We therefore advocate the bold proposal of new species and new synonymies with the understanding that all our scientific work is hypothesis-based, and that the eternal battle between ''lumpers'' and ''splitters'' should lead to an approximation of scientific truth.

8. CONCLUSIONS

8.1. Is ITS Sequencing Effective?

Molecular approaches are generally added to studies that consider species boundaries of trematodes because they are seen as bringing objectivity to the study and because they cut through problems of phenotypic variation. In large part they do just this. Overwhelmingly, studies using ITS rDNA have confirmed the distinctness of well-accepted trematode species and they have also confirmed that morphologically variable species are indeed single species. In addition, in several cases, they have revealed the existence of cryptic species, which were either unknown or only suspected.

Most investigations that use the ITS from rDNA for the distinction of species use the ITS1 or ITS2 rather than the entire ITS. Only 14 of 63 studies reviewed used the entire region. Yet of these 14, only four found interspecific variation of $<1.0\%$ (including one study where species were synonymised). Of the 24 studies that used partial or complete ITS1, only three found interspecific variation of $<1.0\%$. In fact, all studies that use either the ITS or ITS1, with the exception of those of Hashimoto *et al.* (1997), Bell *et al.* (2001), Bell and Sommerville (2002) and Overstreet *et al.* (2002), base their conclusions solely on data obtained from rDNA sequence data. This is in sharp contrast to the number of studies using the ITS2 region, the number finding interspecific variation of $<1.0\%$ and the number relying on sequence data from mitochondrial genes to support their findings.

Does this mean that ITS2 is too conserved between closely related species to allow the conclusive distinction of species? Morgan and Blair (1995) think so, as do Blair *et al.* (1997b). We conclude that this assessment is harsh. Given the number of studies that have used ITS2 and that have also used data from other gene regions (whether nuclear or mitochondrial) for support, it is true that ITS2 is too conserved to unequivocally distinguish all combinations of species. The greatest divergences in the ITS occur in ITS1. This region has the potential to identify variation that is not detected by ITS2. Given the variation revealed by ITS1, we believe it is desirable to sequence the entire ITS to

improve the chances of distinguishing digenean species. However, it should be noted that ITS2 has successfully discriminated species from many digenean families and it can be expected to continue to do so.

8.2. Remaining Problems

There remain several significant issues to be considered when using rDNA sequence data for distinguishing species.

- Despite the established view that the ITS region shows minimal variation within species because of concerted evolution and the need to maintain biological function, minor variation is indeed reported commonly. The amount of variation that is real and that can be attributed to sequencing error or the presence of multiple species requires active investigation.
- The level of genetic variation between clearly distinct species is often large (numerous base differences). It thus becomes attractive to set such levels of variation as a "yardstick" that can be applied to variation wherever it is found. This approach is flawed, however, because, almost by definition, molecular approaches are valuable when comparing species that are not clearly distinct. Species that are not clearly distinct can be expected to be more closely related than distinct ones, so that a "yardstick" drawn from obviously different species is not likely to discriminate between cryptic species. Any level of consistent genetic variation that is associated with a biological attribute such as differing hosts or morphology is likely to be significant.
- In just one clear case, sequencing of the rDNA ITS2 revealed no differences at all between what are considered to be good species for strong biological reasons and for differences identified by other genetic analysis. It is essential, therefore, that lack of variation should not necessarily be interpreted as meaning that only one species is present. Nonetheless, identical sequences are typically an indication of the presence of one species and in some cases where this has been so it was thought no further study was required.
- Geographical variation in the genotype of species probably occurs, but it is not well understood, and some of the variation that has

been reported as geographical variation probably relates to interspecific variation. Distinguishing between intraspecific geographic variation and interspecific variation remains a substantial challenge.

8.3. An Effective Study

We conclude that the characteristics of an effective taxonomic study to explore trematode species boundaries will incorporate:

1. *An understanding of the species in question*. Molecular data will be of little value in the absence of information about (some of) morphology, host range, life cycle and geographic distribution.
2. *Breadth*. A sound study will sequence as many combinations of parasite species, hosts species and geographical localities as is possible.
3. *Replication*. The more replicates that a study contains, the greater the confidence can be had in the veracity of the results. This becomes of greatest importance when the differences between species are small.
4. *Voucher specimens*. If it is not possible to examine a morphological voucher for a given molecular sample then one of the principle methods of checking and understanding interesting or spurious results is lost.
5. *Discarding of suspect data*. Information from suspect collections (such as dubious localities, host identifications or samples lacking morphological vouchers) or low quality sequences should be discarded. Just as in classical morphological taxonomy, spurious records are essentially impossible to disprove after publication and only high-quality data should be reported.
6. *Sequencing the entire ITS region*. Traditionally, the ITS2 has been considered a good place to start. This remains true, but ITS2 alone will not necessarily give complete illumination of a system. Whereas either ITS1 or ITS2 will typically discriminate trematode species, the use of both regions increases the discriminating power significantly.
7. *Addition of extra genes*. Using molecular data from other gene regions (such as the mitochondrial COI, NDI and ND4) will test

and (usually) corroborate results based on rDNA sequences. These regions are under different selection pressures and different rates of evolution; parallel results will give weight to conclusions based on ITS1 or ITS2 and give answers to a wider range of taxonomic questions.

8. *Interpreting a system in terms of the combination of morphological, biological and molecular data*. In an ideal study, the addition of molecular resolution will clarify previous uncertainty about characters such as host distribution and morphology. Where these sources of data conflict (e.g. "good" species with identical sequences or divergent sequences from samples that lack an indication of morphological or biological difference) then such conflict should be made clear and should be seen as the basis for the need for further study.
9. *Willingness to propose change*. There is rarely a pressing or immediate need for new species to be proposed or for species to be synonymised. However, we advocate that where the evidence has become compelling, those scientists should not hesitate to propose taxonomic change, recognising that their proposals remain hypotheses.

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Advances and Trends in the Molecular Systematics of the Parasitic Platyhelminthes

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ABSTRACT

The application of molecular systematics to the parasitic Platyhelminthes (Cestoda, Digenea and Monogenea) over the last decade has advanced our understanding of their interrelationships and evolution substantially. Here we review the current state of play and the early works that led to the molecular-based hypotheses that now predominate in the field; advances in their systematics, taxonomy, classification and phylogeny, as well as trends in species circumscription, molecular targets and analytical methods are discussed for each of the three major parasitic groups. A by-product of this effort has been an ever increasing number of parasitic flatworms characterized genetically, and the useful application of these data to the diagnosis of animal and human pathogens, and to the elucidation of life histories are presented. The final section considers future directions in the field, including taxon sampling, molecular targets of choice, and the current and future utility of mitochondrial and nuclear genomics in systematic study.

1. INTRODUCTION

From new developments in systematic theory and molecular biological techniques, notably the polymerase chain reaction, emerged the field of molecular phylogenetics and its effect has had an immediate and far-reaching impact on biology. Beyond simply coming to the fore of systematic biology, the field has facilitated the integration of comparative evolutionary thought throughout the sub-disciplines of biology, and our inferences are now richer for it. With regard to the Platyhelminthes, and particularly to the parasitic groups (the Neodermata), research over the past decade forms a significant body of

literature with the systematics of few major taxa remaining that have not been investigated at least preliminarily using molecular data. To some extent, however, studies have been concentrated at two extremes, aiming either to circumscribe major lineages and estimate their interrelationships, or to circumscribe species and strains, particularly in those of medical or economic importance. Fewer studies have addressed the systematics of intermediary taxonomic groups such as families and orders, although focus is increasingly moving in this direction.

This paper reviews the advances that have been made through molecular studies in our understanding of the systematics and evolution of the three obligately parasitic groups of flatworms: the Cestoda (tapeworms), Digenea (flukes) and Monogenea. In each of these taxa, new phylogenetic hypotheses, evolutionary scenarios and in some cases revised classifications have been proposed based on molecular data, mostly stemming from the ribosomal DNA (rDNA) genes. In attempting a comprehensive review of the literature that has led to these advances, we cannot give adequate attention to their many biological implications or the corresponding morphological arguments for or against the results based on molecules. The nature of the work and the many hypotheses that predate the field of molecular systematics make it equally difficult to avoid reference to morphology altogether, and at least some consideration of these issues is necessarily found in the text. In addition, it is not possible to provide comprehensive coverage of the many ways in which molecular data are being used for the diagnosis of medically important taxa, most studies of which do not bear directly on issues in systematics or evolution.

Ongoing technical advances in sequencing and associated laboratory procedures have led to an ever increasing expansion of available genetic data and ways in which such data may be applied to the study of systematics. The characterization and study of complete mitochondrial genomes, for example, are now readily possible and the characterization of nuclear genomes and their associated transcriptomes are being sought for a number of key platyhelminth taxa (e.g. *Schistosoma* and *Echinococcus*). The impact of genomic data has yet to make a tangible mark on the study of platyhelminth systematics, but may well introduce significant changes to the field in the near future. Work in

this area, as well as current practices in the analysis of molecular systematic data, are considered briefly in the final section.

1.1. DNA Taxonomy

Molecular data are being used not only to hypothesize ancestor–descendant relationships among living organisms in the pursuit of a comprehensive 'tree of life' (or perhaps 'ring of life'; Rivera and Lake, 2004), but for the circumscription of our most basic of biological entities: species. Thus among the many species concepts that have been proposed and debated, typically with little or no direct influence over the practical, i.e. morphological, recognition of species, DNA-based taxonomy is quickly coming to the fore, and with it new criteria for delineating and testing species boundaries. A considerable number of the studies reviewed herein are based at least implicitly on such an approach, although the questions addressed and the results reported concern their contribution to the understanding of higher interrelationships, classification and related areas relevant to systematics broadly.

There is no doubt that species circumscription, as the foundation of practical systematic biology, has benefited immensely from the incorporation of molecular data. On the other hand, it could also be seen to have been made more complex because of the effectively continuous spectrum of variation available from the genome. Proponents of 'DNA taxonomy' argue for supremacy of their methods over traditional morphological species concepts citing reproducibility, efficiency and cost as being principal advantages (Tautz *et al.*, 2003), or as justified simply by the theoretical impossibility of describing all of Earth's organisms using traditional methods (Blaxter and Floyd, 2003). In truth, however, both the theoretical and practical subjectivity of species boundaries remain as much (more so?) a problem for DNA-based taxonomy as they have been using traditional, morphologically based criteria, and this, along with many other factors (Lipscomb *et al.*, 2003; Seberg *et al.*, 2003), would suggest that a purely DNA-based approach to species circumscription makes sense only in the absence of other data (see also Spakulová, 2002, for a review of species-level issues and concepts in helminthology).

Whatever the long-term future and practice of DNA taxonomy may be, we already see that the combined application of morphological and molecular methods has become commonplace in the description of new species and will come to significantly improve our understanding of both genetic and morphological variability at the species boundary. Practical and theoretical considerations of the approach and its application to the parasitic platyhelminthes are not considered further here; a companion paper by Nolan and Cribb (this volume) provides an evaluation of these issues as applied to the Digenea.

1.2. The Platyhelminthes as Lophotrochozoan Bilaterians

Traditional views on the position of the Platyhelminthes within the animal kingdom held that they were a basal, 'primitive group' exemplifying the first appearance of bilateral symmetry among the triploblastic animals (e.g. Hyman, 1951). Results stemming from the analysis of 18S rDNA quickly overturned this and other deeply entrenched ideas in animal evolution (e.g. Halanych *et al.*, 1995; Aguinaldo *et al.*, 1997; Carranza *et al.*, 1997), and such a pivotal position in the evolution of the Bilateria is now postulated for the acoelomorph flatworms alone (Ruiz-Trillo *et al.*, 1999). The uniqueness of the acoelomorph flatworms is supported by multiple lines of evidence including both ribosomal and protein-coding genes (Ruiz-Trillo *et al.*, 1999, 2002; Jondelius *et al.*, 2002; Giribet, 2003; Telford *et al.*, 2003), Hox gene signatures (Cook *et al.*, 2004), and unique mitochondrial codon usage (Telford *et al.*, 2000). The revised Platyhelminthes (minus the acoelomorphs) in turn appears to be more derived and is grouped within the Lophotrochozoa, one of the three main branches of the Bilateria as inferred from 18S rDNA (Aguinaldo *et al.*, 1997) and other genes (e.g. Eernisse and Peterson, 2004; see Figure 1A). Although the interrelationships of the Lophotrochozoa remain contentious, the circumscription of a monophyletic Platyhelminthes and its new position in the animal kingdom are significant advances toward a better understanding of the origins and diversification of the phylum.

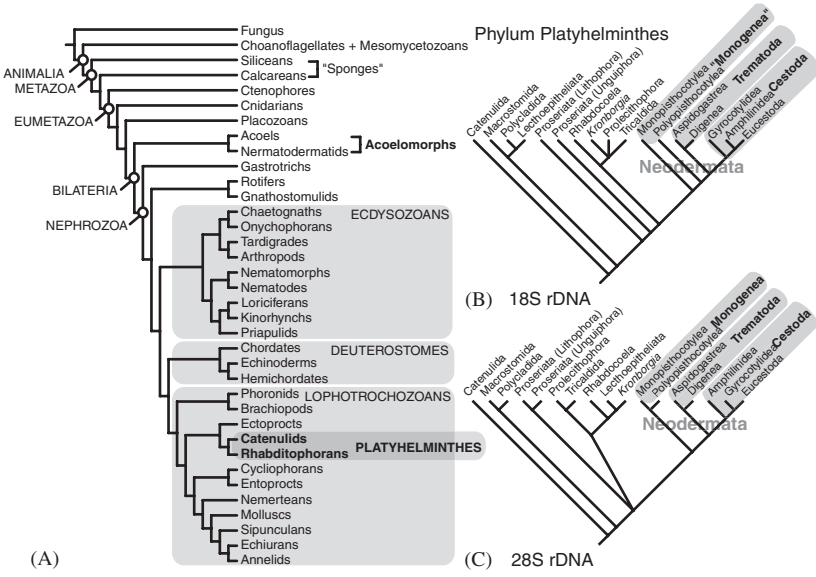

Figure 1 Phylogenetic position and interrelationships of the phylum Platyhelminthes. (A) The platyhelminths as members of the Lophotrochozoa based on a combined analysis of morphology, SSU and Myosin II by Eernisse and Peterson (2004). Note position of the acoelomorphs at the base of the Bilateria. (B), (C) Interrelationships of the Platyhelminthes as estimated by Bayesian analysis of complete SSU or complete LSU from Lockyer *et al.* (2003a). Although monophyly of the parasitic Platyhelminthes (the Neodermata) has been strongly and consistently supported, interrelationships within the Neodermata remain confused and alternatives to the scenarios shown here have also been supported by molecular studies.

1.3. Interrelationships and Position of the Neodermata

Phylum-level molecular phylogenetic analyses of the Platyhelminthes have consistently shown that the obligately parasitic groups, Cestoda (Gyrocotylidea + Amphilinidea + Eucestoda), Monogenea (Monopisthocotylea and Polyopisthocotylea) and Trematoda (Aspidogastrea + Digenea) form a derived clade (Baverstock *et al.*, 1991; Blair, 1993b; Rohde *et al.*, 1993; Littlewood *et al.*, 1999b; Litvaitis and Rohde, 1999; Littlewood and Olson, 2001), as was previously proposed by Ehlers (1984) who coined the term Neodermata in reference to the replacement of the larval epithelium by a

syncitial tegument. However, the interrelationships of the Neodermata have been and remain unsettled by the application of molecular data. Traditionally held views derived from Janicki (1920) and Bychowsky (1937) proposed that the presence of a 'cercomer' united the Monogenea with the Cestoda into the 'Cercomeromorphae', to the exclusion of the Trematoda. While this scenario has been supported by some molecular phylogenies based on 18S (e.g. Littlewood and Olson, 2001), others have suggested a closer relationship between the Cestoda and Digenea (e.g. Litvaitis and Rohde, 1999), including a recent study based on complete 18S and 28S rDNA (Figures 1B and C) that advocates rejecting the cercomer theory due to the questionable homology among such structures (Lockyer *et al.*, 2003a). The difficulty in resolving this basic three-taxon statement is further confounded by the likely paraphyly of the Monogenea (Mollaret *et al.*, 1997; Justine, 1998; Littlewood *et al.*, 1999b). Although supported by a number of morphological features (Boeger and Kritsky, 2001), the two main branches of the group, Monopisthocotylea and Polyopisthocotylea, show greater differences than similarities in their anatomy and biology (Justine, 1998; Euzet and Combes, 2003) and rate of sequence divergence (Olson and Littlewood, 2002). Indeed, it may be that the synapomorphies (shared *derived* characters) identified by Boeger and Kritsky (2001) are in fact symplesiomorphies (shared *ancestral* characters) simply retained in the more derived lineage of 'monogeneans' whose most recent ancestor is shared with either the Cestoda or Digenea (in which such features were lost). Thus other than support for the monophyly of the Neodermata itself, the interrelationships of the primary neodermatan lineages as estimated by molecular data remain controversial, precluding robust inferences regarding the condition of the 'proto-typical' parasitic flatworm.

1.4. Abbreviations

The following abbreviations are used throughout the text: 18S, small subunit rDNA; 28S, large subunit nuclear rDNA; bp(s), base pair(s); *cox1*, cytochrome *c* oxidase subunit 1; gDNA, total genomic DNA; ITS1/ITS2, internal transcribed spacers 1 or 2; mtDNA, mitochondrial

DNA; *nad1*, nicotinamide adenine dinucleotide dehydrogenase subunit 1; PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

2. MOLECULAR SYSTEMATICS OF THE CESTODA

The Cestoda comprises more than 5000 described species (Georgiev, 2003) including the aetiological agents of hydatidiosis and cysticercosis (*Echinococcus* and *Taenia*, respectively). As adults, they are enteric parasites of all classes of vertebrates and utilize arthropods as first intermediate hosts. They have been recognized since the time of ancient Greece (Grove, 1990) and their diversity has been well documented in the last century; systematic treatments include Yamaguti (1959), Schmidt (1986) and most recently Khalil *et al.* (1994), who recognized 14 major lineages at the Linnean rank of order. Nevertheless, the application of molecular data has challenged ideas based on morphology and host associations and has led to fundamental changes in our understanding of cestode relationships and evolution. Early studies in the molecular systematics of the Cestoda were reviewed by Mariaux and Olson (2001) and we have minimized repetition of their review here.

2.1. Interrelationships of the Major Lineages of Cestodes

Prior to molecular systematics, scenarios presented to explain the evolution of the cestodes were almost entirely lacking in agreement. Disagreement stemmed not only from the general pattern of evolution, but in the homology and evolutionary importance of morphological structures, and in the number and taxonomic composition of the major lineages themselves. The absence of segmentation in groups such as the Caryophyllidea, for example, could be justifiably argued as evidence of their 'primitive' condition or as having become secondarily lost; morphology and life history traits are themselves insufficient to arbitrate between the two interpretations. Unique

features of groups such as the Nippotaeniidea or Haplobothriidea only help to establish their dissimilarity to other groups; characters unambiguously uniting two or more groups to the exclusion of others are few and far between. Works by Mariaux (1998) and Olson and Caira (1999) were early attempts to overcome the limitations of morphology and examine the composition and relative positions of the major lineages of cestodes as had been defined in Khalil *et al.* (1994). Their studies (see Figure 2) differed in the representation of higher (ordinal) taxa and in the gene regions analyzed, making them more complementary than compatible, but together provided the initial evaluations of the major questions in cestode evolution. While limited in taxonomic representation and support from the data, a number of important general conclusions were consistent: the monozoic (Caryophyllidea and Spathebothriidea) and difossate (Diphyllidea, Pseudophyllidea and Trypanorhyncha) orders are basal to a clade of tetrafossate (four-part scoleces) groups, with the orders Cyclophyllidea, Nippotaeniidea and Tetrabothriidea forming a derived clade within the tetrafossate clade (Figure 2). In addition, the orders Pseudophyllidea and Tetraphyllidea were found to be paraphyletic, with the Diphyllobothriidae independent of the remaining 'Pseudophyllidea', and the Tetraphyllidea forming multiple lineages within the tetrafossate clade. The relative positions of the difossate lineages, as well as those within the tetrafossate clade were inconsistent between studies (Mariaux, 1998; Olson and Caira, 1999) and among gene regions and methods of analysis (Olson and Caira, 1999).

Thus a reasonably solid and morphologically independent foundation for the group was forged and available for further testing. Kodedová *et al.* (2000), for instance, expanded the 18S data of Olson and Caira (1999) with additional taxa representing the Caryophyllidea, Proteocephalidea and Pseudophyllidea (Figure 2). Considered only from the interrelationships of the orders themselves, the improved taxon representation of these groups had the unfortunate effect of reducing the resolution (using parsimony analysis), although a derived tetrafossate clade was still supported. Similarly, Hoberg *et al.* (2001) combined morphological data with a few additional sequences added to the work of Mariaux (1998) and added further support of the conclusions described above. The same year, Olson

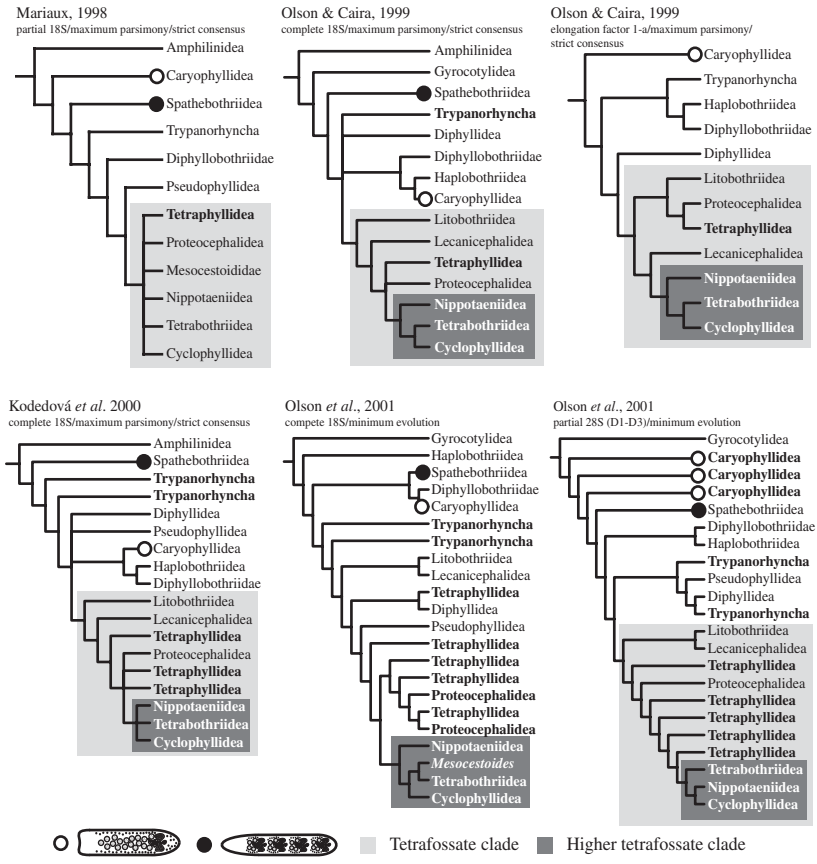

Figure 2 Molecular hypotheses of the inter-ordinal relationships of the tapeworms showing broad support for the derived tetrafossate and higher-tetrafossate clades. The positions of the Caryophyllidea and Spathebothriidea and their implications for the evolution of strobilation in the cestodes are highlighted with open and filled circles, respectively. Orders found to be paraphyletic indicated in bold type (Note: the Diphyllobothriidae is labelled as a lineage separate of the 'Pseudophyllidea' throughout the figure.)

et al. (2001) more than doubled the number of exemplar taxa used in previous studies, complemented complete 18S data with partial (D1–D3; ~1400 bp) 28S data, and re-evaluated a suite of morphological characters derived from the studies of Hoberg *et al.* (2001) and Justine (2001). Their analyses (Olson *et al.*, 2001; see Figure 2), provided additional support for the general conclusions stated above as

well as greater insight into the problems of paraphyly, but also showed how labile the internal nodes of the tree were. For this reason, no formal revision of the classification was made, although recommendations were mooted by the authors. Figure 2 shows the positions of the Caryophyllidea (open circles) and Spathebothriidea (closed circles) as estimated in some of the works discussed above. In most of these, their positions are basal with respect to the fully segmented 'strobilate' groups, implying that they are primarily monozoic (non-segmented). However, strong and consistent support for their positions has not been obtained, and more derived positions are also recovered, albeit with an equal lack of support.

Below the class level, the interrelationships of few higher taxa (e.g. orders, families) have been examined even preliminarily using molecular data. A notable exception is the Proteocephalidea for which a concerted effort has been made. de Chambrier *et al.* (2004), for example, presented a comprehensive treatment, expanding the earlier work of Zehnder and Mariaux (1999). Their work (de Chambrier *et al.*, 2004) combined morphology with partial 28S data for 75 taxa and showed that a number of genera, including the highly speciose *Proteocephalus*, were paraphyletic and such taxa were formally amended by the authors. Unexpectedly, their work also revealed that neotropical species, which show the greatest present day diversity, were derived and therefore the neotropics could not be the centre of origin for the group as had been postulated previously. These works have been expanded further by Hypsa *et al.* (2005) who added ITS2 and partial (V4) 18S data to the sequences analyzed by de Chambrier *et al.* (2004), and also examined the utility of secondary structural characters (of ITS2). Considerable agreement is found between the two studies and the additional data helped primarily to resolve the more derived parts of the tree.

Olson *et al.* (1999) made a preliminary examination of the lineages that make up the 'Tetraphyllidea', parasites of sharks and rays, revealing clades specific to their hosts groups (i.e. sharks, cownose rays and diamond rays). This and other works (i.e. Olson and Caira, 1999; Olson *et al.*, 2001) show that the Rhinebothriinae (sensu Euzet, 1994) appears as the most basal of the 'tetraphyllidean' lineages, and a recent analysis by Caira *et al.* (in press) has shown that this is followed

by a lineage representing the Cathetocephalidae. Beyond that results are less consistent, albeit the phyllobothriid (non-hooked) lineages generally appear basal to onchobothriid (hooked) lineages, the latter of which also includes the monophyletic Proteocephalidea.

Future works on the constituent cestode groups similar to that on the Proteocephalidea are needed, especially to resolve relationships among the difossate lineages Diphyllidea, 'Pseudophyllidea' and Trypanorhyncha, and to subdivide the 'Tetraphyllidea' into monophyletic lineages. Although a few works have addressed interrelationships in the Cyclophyllidea (e.g. Mariaux, 1998; von Nickisch-Rosenegk *et al.*, 1999; Foronda *et al.*, 2004), no attempt at a comprehensive estimate has been made. It is in this group particularly, being more speciose than all other cestode orders combined and containing the most important cestode pathogens of man, that a large-scale analysis is needed. Ongoing work by the present authors and by many of our colleagues in cestodology is currently underway and aims to provide more comprehensive studies of a number of orders including the Cyclophyllidea, Lecanicephalidea, Pseudophyllidea, Tetraphyllidea and Trypanorhyncha. In turn, such studies will provide a wealth of new data for re-examining the interrelationships of the orders themselves.

2.2. Inter- and Intraspecific Variation in the Cestodes

The Cyclophyllidea, especially the Taeniidae, contains the vast majority of cestode species reported from man (Ashford and Crewe, 2003) and are thus widely studied at the inter- and intraspecific levels. For example, molecular data have allowed the recognition of a new Asian species of taeniid from Man, *Taenia asiatica*, which occurs sympatrically alongside *T. saginata* and *T. solium* (Eom *et al.*, 2002), and PCR methods for their differentiation have been recently developed (Gonzalez *et al.*, 2004). Moreover, Yamasaki *et al.* (2002), using mitochondrial genes and two techniques capable of detecting single-base changes, found that Asian *T. solium* could be readily differentiated from American/African isolates, suggesting the possibility of yet another distinct Asian lineage. Variation in *cox1* was examined in seven species of *Taenia* and two species of *Echinococcus*, including 10 isolates

of *T. taeniaeformis* and six isolates of *E. multilocularis*, by Okamoto *et al.* (1995). High interspecific variation was found among the taeniid species and isolates of *T. taeniaeformis*, but no variation was found among those of *E. multilocularis*, regardless of host or location.

Kamenetzky *et al.* (2000) developed a technique to extract usable gDNA from both fertile and non-fertile hydatid cysts of *Echinococcus granulosus* and found that cyst fertility was not correlated with strain, as both types recovered from the intermediate host proved to be of the same genotype. Similarly, analysis of *nad1* sequences of *E. granulosus* cysts from domestic and wild animals as well as humans in Poland, Ukraine and Slovakia (Kędra *et al.*, 1999, 2000b; Tkach *et al.*, 2002b) has demonstrated that fertile and infertile cysts of the pig strain of *E. granulosus* may be found in both animals and humans. However, Obwaller *et al.* (2004) showed high levels of intraspecific strain differentiation in *E. granulosus* using *cox1* and *nad1* that corresponded largely to their host associations (e.g. sheep, horse, cattle, pig, etc.). Comparison of complete mitochondrial genomes (see Section 6.4.1) similarly link strain variation to host association (Le *et al.*, 2002b). Previously Kędra *et al.* (2000a, 2001) showed genetic variability in *nad1* sequences of *E. multilocularis* and *T. hydatigena*, from different geographic regions.

Genetic variation in *Mesocestoides*, primarily parasitic in canids, was studied by Crosbie *et al.* (2000) among isolates from domestic dogs and coyotes and showed a number of distinct genetic signatures using 18S and ITS2. *Hymenolepis nana* isolates from man and rodents in Western Australia could be differentiated using COI, albeit not with the more conserved ITS1 or paramyosin genes (Macnish *et al.*, 2002b). Attempts to infect mice with human isolates were unsuccessful (Macnish *et al.*, 2002a), further supporting the presence of two independent strains or species.

Fewer reports have dealt with cyclophyllidean species of wild animals. Genetic variation in species of the genus *Rodentolepis* (Hymenolepididae) from the Pyrenean mountains of Spain and France was examined using allozyme electrophoresis and morphometrics. Casanova *et al.* (2001) were able to discriminate between *R. straminea* and *R. microstoma* in murid rodents, and Santalla *et al.* (2002) examined intraspecific variation in *R. asymmetrica* from several species of voles,

finding minor morphometric, but no genetic variation. Another pair of studies provides extensive analysis of genetic and morphometric variation, as well as biogeography in anoplocephalid cestodes of Holarctic collared lemmings (*Dicrostonyx*): Haukisalmi *et al.* (2001) uncovered cryptic species of *Paranoplocephala*, and Wickström *et al.* (2001) showed cospeciation of *Andrya arctica* within the Holarctic region, but not in the host split between Eurasia and North America. Using *cox1* data, evidence for multiple cryptic species of anoplocephalids was found in *Paranoplocephala omphalodes* in voles (Haukisalmi *et al.*, 2004), *P. arctica* in lemmings (Wickström *et al.*, 2003) and in the genus *Progamotaenia* in Australian macropodid marsupials (Hu *et al.*, 2005).

Outside the Cyclophyllidea, only a few members of the Pseudophyllidea are known from man (Ashford and Crewe, 2003) and all belong to the family Diphyllobothiidae, the only pseudophyllidean family infecting homeothermic tetrapods and now thought to represent an independent evolutionary lineage (Mariaux, 1998; Olson and Caira, 1999; Olson *et al.*, 2001; see below and Figure 2). The diphyllobothriid *Spirometra* is the aetiological agent of sparganosis, the result of infection with the plerocercoid stage of these canid/felid adult worms. Its extensive host and geographic ranges make it likely to be a complex of species, and Zhu *et al.* (2002) used SSCP to detect single-base variation in the *cox1* gene in populations stemming from different second intermediate hosts. Somewhat surprisingly, only two haplotypes were detected, differentiating specimens from non-amphibian and amphibian hosts in Australia.

Like *Spirometra*, *Ligula intestinalis* is more commonly encountered in the larval stage. The global ubiquity of *Ligula* plerocercoids in freshwater fishes and observed differences in pathological reactions of fish species also suggest the possibility of a large species complex. To this end, Olson *et al.* (2002) used the entire ITS region and partial 28S to examine *Ligula* populations in two different sympatric fish hosts in Northern Ireland. Within-host populations were identical, whereas consistent between-host differences were found in all gene regions (except the 5.8S which was invariant), with the ITS-2 showing the most variation. The same rDNA gene regions and sequences were used by Luo *et al.* (2003a) to examine the possible synonymy of

Ligula with *Digramma*. Their comparisons showed that intergeneric divergences were only marginally larger than intra-generic (0.5–2% vs. 0–1.2%) divergences. Confounding their judgement, however, were parsimony results that supported separated clades for each genus. The same year, Li and Liao (2003) provided additional evidence for the synonomy of *Ligula* and *Digramma*, finding no variation in the 28S and COI genes, and only 0.7 and 7.4% variation in the quickly evolving ITS1 and *nad1* genes, respectively. Subsequently, Logan *et al.* (2004) examined the interrelationships of the diphyllobothriid genera *Digramma*, *Diphyllobothrium*, *Ligula* and *Schistocephalus* using ITS2. Their results further corroborated those above (i.e. Li and Liao, 2003; Luo *et al.*, 2003a), and provided additional evidence for the likely species complex represented by *L. intestinalis*. Thus the long-standing question of the validity of *Digramma* appears to be answered in the negative.

Other studies on pseudophyllideans have targeted species in commercially important freshwater and marine fishes. For example, following a series of studies examining genetic variation in *Proteocephalus* (Král'ová, 1996; Král'ová and Spakulová, 1996; Král'ová *et al.*, 1997), Král'ová *et al.* (2001) used direct sequence comparison and PCR–RFLP to differentiate the salmonid cestodes *Eubothrium crassum* and *E. salvelini*, and more recently extended this to *E. rugosum* (Král'ová-Hromadová *et al.*, 2003). Snábel *et al.* (2004) approached the same problem via allozyme variation, showing *E. crassum* and *E. salvelini* to be distinct entities restricted to their respective fish hosts. In all cases, these species could be readily diagnosed by RFLP analysis of the ITS region. Much finer variation was studied by Luo *et al.* (2003b) in the fish cestode *Bothriocephalus acheilognathi* using microsatellite markers. Their work represents one of the few population genetics studies on cestodes (Wickström *et al.*, 2001; Snábel *et al.*, 2004) and follows an earlier, phylogenetically based study of the same species collected from the basin area of the Yangtze River in China (Luo *et al.*, 2002). Using eight microsatellite markers, they showed that heterozygosity was best accounted for by host species, rather than location, and genetic variation was higher than might be expected among sub-populations from sympatric host species, taken together as evidence of cryptic speciation (Luo *et al.*,

2003b). More recently the genus *Bothriocephalus* was shown to be paraphyletic based on ITS2 and 18S without the inclusion of a number of additional genera such as *Anantrum* and *Clestobothrium* (Skeríková *et al*., 2004). Moreover, karyological variation in number and morphology has been recently documented in *Bothriocephalus* spp. by Petkeviciute (2003).

In the Proteocephalidea, Zehnder and de Chambrier (2000) and Zehnder *et al*. (2000) provided examples of the combined morphological and molecular approach to taxonomy. Similarly, Skeríková *et al*. (2001) showed the monophyly of the European species of *Proteocephalus*, a cosmopolitan genus previously shown to be non-natural (i.e. non-monophyletic, Zehnder and Mariaux, 1999; de Chambrier *et al*., 2004), but found no evidence for host–parasite co-evolution. Rosas-Valdez *et al*. (2004) examined the monophyly of the subfamily Corallobothrinae using 28S and showed it to be a non-natural division of the Proteocephalidae, although the genera *Corallobothrium*, *Corallotaenia* and *Megathylacoides* formed a 'North American' clade with strong support.

3. MOLECULAR SYSTEMATICS OF THE DIGENEA

Comprising ~18 000 nominal species, the Digenea is by far the most speciose of the three main groups of parasitic Platyhelminthes, and may well be the largest group of internal metazoan parasites of animals (Cribb *et al*., 2001). Despite a vast literature on the group extending back well into the eighteenth century, the classification and phylogeny of the Digenea remained unstable and the interrelationships of numerous digenean taxa, at both higher and lower taxonomic levels, unclear. Thus the origin and evolution of the digeneans and their remarkable diversity of life cycles have long been subjects of inquiry. Basic problems such as identifying the most 'primitive' extant digenean lineage have elicited heated debates without resolution (for instance, Gibson, 1987; Brooks, 1989; Brooks *et al*., 1989; Pearson, 1992; Brooks and McLennan, 1993). At lower taxonomic levels, differentiating species and genera has been problematic in many families, and thus the families themselves lack clear morphological

boundaries. Studying digenean life history is especially challenging due to their complex sequence of ontogenetic stages, hosts and niches, and data on the ontogeny of most digenean groups are fragmentary or lacking entirely (Yamaguti, 1975). Thus the adoption of molecular techniques was especially welcomed by researchers working on their systematics, evolution and ecology.

Unlike the Cestoda, where considerable effort has focused on resolving the interrelationships of higher-level taxa (i.e. orders), studies of digeneans have generally concentrated on lower taxa and only in recent years have more comprehensive works appeared. This is explained in part by the extraordinary diversity of the digeneans, and thus the significantly greater time and resources required to generate a molecular database sufficient for analysis of the class as a whole. Nevertheless, data are now available for the major groups and most significant families, enabling both diagnostic and systematic research in the group to advance more rapidly.

3.1. Non-Sequence-Based Works

Publications based on DNA sequence data represent the mainstream of modern molecular systematics and thus form the majority of works covered here. However, a substantial number of works dealing with species differentiation and systematics were published before PCR and sequencing became routine techniques. By far the most commonly used non-PCR-related technique is alloenzyme electrophoresis (e.g. Andrews and Chilton, 1999), although other methods, such as thin layer chromatography and DNA hybridization, have also been applied to digeneans. The vast majority of these works has been devoted to differentiating among populations or species in medically important taxa such as the schistosomes (Fletcher *et al.*, 1980; Write and Ross, 1980; Viyanant and Upatham, 1985; Walker *et al.*, 1985, 1986, 1989a, b; Woodruff *et al.*, 1985; Yong *et al.*, 1985; Bobek *et al.*, 1991; Kaukas *et al.*, 1994; Webster *et al.*, 2003), paragonimids (Agatsuma, 1981; Agatsuma and Suzuki, 1981; Agatsuma and Habe, 1986; Agatsuma *et al.*, 1988, 1992), fasciolids (Blair and McManus, 1989) and opisthorchiids (Pauly *et al.*, 2003). Studies have also been

carried out on members of the genus *Echinostoma* (Bailey and Fried, 1977; Voltz *et al*., 1988), *Halipegus* (Goater *et al*., 1990) and recently Vilas and co-authors published a series of articles devoted to population and species-level genetic variability in the hemiurid genus *Lecithochirium* (Vilas *et al*., 2000, 2002a–c, 2003a, b, 2004a, b).

The RAPD technique has been used in several studies on schistosomatids and diplostomids (Barral *et al*., 1993; Kaukas *et al*., 1994; Laskowski, 1996; Mone *et al*., 2003) and liver flukes (Morozova *et al*., 2002; Semyenova *et al*., 2003), while RFLP analysis has been applied to studies of species differentiation in the Didymozoidae (Anderson and Barker, 1993) and the introduction of *Schistosoma mansoni* to America (Després *et al*., 1993). These approaches have been largely superseded by direct sequence analysis and thus their application to the systematics of flatworms has become increasingly rare.

3.2. Interrelationships of the Major Lineages of Digeneans

All publications devoted to the molecular phylogenetics of the Platyhelminthes have included at least some representative digenean taxa (Baverstock *et al*., 1991; Blair, 1993b; Blair and Barker, 1993; Rohde *et al*., 1993, 1995; Blair *et al*., 1998; Campos *et al*., 1998; Littlewood *et al*., 1998a, b, 1999a, b; Litvaitis and Rohde, 1999; Littlewood and Olson, 2001; Lockyer *et al*., 2003a), but specifically these works do not address the interrelationships of the Digenea, and in recent years the accumulation of sequence data has enabled more comprehensive studies of digenean phylogeny. The first such analysis was that of Cribb *et al*. (2001) based on complete 18S and including a total of 75 digenean taxa representing 55 families (plus five aspidogastrean outgroup taxa). Unlike the cestodes, for which comparisons at this taxonomic level using 18S reveal excessive variation in specific variable regions (e.g. V4 and V7), 18S sequences of disparately related digeneans show far less variability. The result is that analysis of 18S typically produces exceedingly short internodes and low nodal support. Nevertheless, the basic patterns revealed by Cribb *et al*. (2001) have been subsequently corroborated. For example, the

Diplostomata *sensu* Olson *et al.* (2003a), uniting a clade including the blood groups Schistosomatidae, Sanguinicolidae and Spirorchidae with the Clinostomidae, together with the Brachyolaimoidea and Diplostomoidea, forms one of the primary lineages in a basal split in the Digenea (Figure 3). This rejected the traditional trichotomy implied by their classification into three large groups and showed definitively the polyphyletic nature of the traditional grouping 'Echinostomida' (whereas the composition of the Plagiorchiida and Strigeida were, with exceptions, largely supported by molecular data). Re-examination and coding of morphological characters were also done by Cribb *et al.* (2001) and showed greater congruence with the results from molecules than had previous such analyses (e.g. Brooks *et al.*, 1985; Pearson, 1992), albeit considerable differences were found. Tkach *et al.* (2000a, b) presented phylogenies of the suborder Plagiorchiata based on 28S and demonstrated its derived phylogenetic position in relation to all other major digenean lineages. It was also found that the Renicolidae belongs to the most derived clade of digeneans, along with the Eucotylidae whose systematic position was previously enigmatic. Although members of both families are parasitic in the kidneys of birds, their general morphology is so distinct that no one had previously hypothesized their close affinity.

The most comprehensive phylogenetic analysis of the Digenea to date was based on a combination of complete 18S and partial 28S and included 163 members of 77 families representing all major groups (Olson *et al.*, 2003a; Figure 3). As discussed above, their work recognized two major clades, the Diplostomida, corresponding to the Strigeida *sensu lato*, and the Plagiorchiida, comprising the traditional Plagiorchiida along with the members of the 'Echinostomida', the latter of which was found to be a polyphyletic taxon. The Diplostomida includes only one nominal superfamily, whereas the Plagiorchiida now comprises 13 superfamilies of which the Xiphidiata is the largest and most derived and members of which share the presence of a penetrating stylet in their cercariae. Due to the basal dichotomy, no one group is seen to occupy the most basal position in the tree. However, at the base of the Plagiorchiida two lineages were found, each of which had been hypothesized previously as the progenitors of the Digenea: the Transversotrematidae and Bivesiculidae. However,

Figure 3 Hypothesis of digenean interrelationships and revised classification based on Bayesian inference of 18S and 28S data by Olson *et al.* (2003a). In contrast to traditional digenean classification, their scheme recognizes two main lineages, the Diplostomida and Plagiorchiida, as indicated on the tree. Expanded resolution of the Schistosomatoidea and the genus *Schistosoma* represents a consensus of compatible trees found in the publications shown, all of which analysed a subset of the same data. Taxa found to be paraphyletic indicated in bold type.

support for their placement was weak. Within the Diplostomida, the position of the Clinostomidae within the Schistosomatoidea (comprising the blood dwelling groups Sanguinicolidae, Schistosomatidae and Spirorchidae) seems surprising, although it confirms existing

views based on shared features of their larval morphology (e.g. the presence of a dorsal cercarial body fin-fold).

Cribb *et al.* (2003) re-evaluated the evolution of digenean life cycles in light of the hypothesis presented in Olson *et al.* (2003a) and suggested that the two-host life cycle of blood flukes was the result of secondary abbreviation, whereas the active penetration of miracidia into snail hosts and infection of definitive (fish) hosts by the passive feeding on cercariae are plesiomorphic behaviours. No evidence of strict co-evolution between digeneans and their mollusc hosts was found. With respect to vertebrate parasitism, the recognition of the Diplostomida (all of which are parasitic in tetrapods except the Sanguinicolidae) forced the authors to conclude that the digeneans acquired vertebrate definitive hosts three separate times in their evolutionary history.

3.3. Interrelationships of Genera and Families

Interestingly, molecular systematic studies aimed at these taxonomic levels tend to produce the most conclusive results; studies of higher taxa suffer from higher levels of homoplasy, whereas studies targeting inter- and intraspecific questions must deal with the subjectivity of delineating fine-scale genetic variation. Early publications were devoted to molecular variability among fasciolids and paragonimids (Blair, 1993a), and comparison of 18S sequences in gyliauchenids (Blair and Barker, 1993), lepocreadiids and fellodistomids (Lumb *et al.*, 1993). Barker *et al.* (1993) used 18S sequences to test the phylogenetic hypothesis of Brooks *et al.* (1985) that *Heronimus* is the most basal extant digenean lineage. Although based on a very limited set of taxa, Barker *et al.* (1993) refuted this hypothesis and this result was corroborated a decade later by Olson *et al.* (2003a).

Anderson and Barker (1998) used ITS2 sequences to infer the phylogeny of members of the Didymozoidae, perhaps the most unusual and morphologically difficult group. Blair *et al.* (1998) studied phylogenetic relationships within the Hemiuroidea; a group with a complex taxonomic history and controversial views regarding its structure and content. No significant difference was found between

the phylogenies obtained based on a morphological matrix and analysis of sequences of the V4 domain of 18S (Neefs *et al.*, 1990), which showed the Hemiuroidea to be a monophyletic group comprising at least two primary lineages. Moreover, no support for the placement of the Azygiidae within the Hemiuroidea was found. Fernández *et al.* (1998a, b) published works on the molecular phylogeny of the families Campulidae and Nasitrematidae based on mtDNA and 18S sequences. In part, the molecular data supported the hypotheses that the Campulidae are phylogenetically closest to Acanthocolpidae and not to the Fasciolidae, indicating a host-switch from fish to mammals. Another host-switching event was hypothesized for the digenean parasites of marine mammals based on analysis of mitochondrial ND3 sequences of the genus *Lecithodesmus* (Fernández *et al.*, 2000). In this case the parasites were captured by one group of cetaceans from another.

Grabda-Kazubska *et al.* (1998) and Kostadinova *et al.* (2003) addressed the interrelationships of several echinostomatid taxa. In the latter paper, sequence data confirmed morphological identifications of specimens representing different stages of the life cycle and suggested that *Echinoparyphium aconiatum* probably does not belong to this genus. Both *nad1* and ITS data failed, however, to provide sufficient resolution of the species of *Echinostoma* used in the study. Analysis of the family Mesometridae by Jousson *et al.* (1998) revealed general trends in the evolution of this small group of digeneans parasitic in marine fish, such as the regression of the pharynx, a change in the body shape from elongated to subcircular, and the development of an accessory holdfast organ. Blair *et al.* (1999b) examined the paragonimid genera *Paragonimus*, *Euparagonimus* and *Pagumogonimus* using sequences of COI and ITS2, and showed that at least the type species of *Pagumogonimus*, *P. skrjabini*, clearly belongs to the genus *Paragonimus*, making the genus *Pagumogonimus* a junior synonym. In the same analysis, the position of *Euparagonimus cenocopiosus* was not stable and it appeared as either the sister taxon of *Paragonimus*, or was found within the latter genus (thus suggesting all three genera are synonymous). Hall *et al.* (1999) used the V4 region of the 18S to examine the family Fellodistomidae and showed that members of the group actually represent three separate families:

Fellodistomidae, Faustulidae and Tandanicolidae. Use of bivalve molluscs as intermediate hosts, which has been traditionally considered a robust synapomorphy for the members of Fellodistomidae, was instead considered by the authors as an indicator of multiple host-switching events within the radiation of the Digenea.

A series of molecular phylogenetic studies based on the partial 28S sequences by Tkach and co-authors was devoted to resolving the phylogenetic relationships and taxonomic status of a number of taxa traditionally circumscribed within the suborder Plagiorchiata. Tkach *et al.* (1999, 2001a) showed the close affinity of four genera (*Macrodera*, *Leptophallus*, *Metaleptophallus* and *Paralepoderma*) from European snakes and their molecular results were corroborated by cercarial morphology and chaetotaxy, and the presence of an external seminal vesicle in adult worms. In subsequent publications, Tkach *et al.* (2001a, c, 2002a, 2003) studied the phylogenetic positions and interrelationships of the families Macroderoididae, Omphalometridae, Microphallidae, Lecithodendriidae, Prosthogonimidae and Pleurogenidae. Among other conclusions, it was shown that several genera (e.g. *Glypthelmins*, *Haplometra*) allocated to the Macroderoididae by previous authors, were not closely related and form independent lineages within the Plagiorchioidea. Alternatively, members of the Omphalometridae exhibited little sequence divergence despite the great differences in their size and body shape. Within the Microphalloidea, development of a seminal vesicle lying freely in parenchyma seems to have occurred independently at least twice. In turn, the Pleurogenidae has been found to be a family distinct from the Lecithodendriidae.

3.4. Inter- and Intraspecific Variation in the Digeneans

3.4.1. Systematics of Schistosoma *and Related Blood Flukes*

The Schistosomatidae is by far the best studied group of digeneans, and early studies on their molecular systematics have been summarized by Rollinson *et al.* (1997). Although the vast majority of effort in schistosome research concerns immunological, epidemiological

and other medical aspects of the species that infect human populations (i.e. *Schistosoma* species), significant advances in our understanding of the origins and diversification of these species and their close relatives in animal populations have been made in recent years (e.g. Combes *et al*., 1992; Després *et al*., 1992, 1993; Bowles *et al*., 1995; Rollinson *et al*., 1997; Snyder and Loker, 2000; Snyder *et al*., 2001; Agatsuma, 2003; Lockyer *et al*., 2003b; Morgan *et al*., 2003; Snyder, 2004). Much of this work involves testing the validity of described species, and genetic analyses of isolates have also revealed new lineages. For example, ITS2 has been used to verify the species status of *S. hippopotami* (e.g. Després *et al*., 1995), and genetic analysis of cercariae from its type locality (Lake Edward in western Uganda) has identified a third potential lineage in hippos (Morgan *et al*., 2003). Population-level variability has been analysed in *S. japonicum* (Bowles *et al*., 1993; van Herwerden *et al*., 1998) and *S. mansoni* (Curtis *et al*., 2002). In the latter work, microsatellite loci showed moderate genetic differentiation among *S. mansoni* populations within a single village in Brazil. Picard and Jousson (2001) characterized ITS sequences from cercariae causing swimmer's itch in Europe and although multiple sequences were found, some were identical to that of *Trichobilharzia regenti* recovered as adults from a mallard duck. Advances in the molecular systematics of European *Trichobilharzia* have been recently summarized by Dvorak *et al*. (2002) and Horak *et al*. (2002).

Snyder and Loker (2000) and Snyder *et al*. (2001) provided the first comprehensive phylogenetic assessment of the Schistosomatidae and were thus able to test its geographic origin, as well as the composition and interrelationships of genera and suprageneric lineages within the family. Their work first suggested an Asian origin of the genus *Schistosoma* and this was subsequently supported by Lockyer *et al*. (2003b; see Figure 3) through more extensive sampling, including 30 taxa and three genes (18S, 28S and COI), as well as morphological and biogeographic data. The avian schistosomes *Austrobilharzia* and *Ornithobilharzia* were shown to form the sister lineages of the mammalian genus *Schistosoma*, whereas *Orientobilharzia* appears to be a junior synonym of *Schistosoma*. Other taxonomic changes included the synonymy of *T. ocellata* and *T. szidati*. Although a majority of

known schistosomatid taxa has been analyzed in the works above, the phylogenetic position of the only schistosome known from reptiles, *Gryphobilharzia* from Australian freshwater crocodiles, remains undetermined and has not been reported on since its original description by Platt *et al.* (1991). Recent collections (S. Snyder, pers. comm.) of specimens suitable for molecular analysis should mean that its phylogenetic position will be determined soon.

Snyder (2004) recently added 18S and partial 28S sequences of representatives of eight genera of spirorchiids from freshwater and marine turtles. His work confirmed the basal position of the spirorchiids in relation to the schistosomatids, corroborating Olson *et al.* (2003a) who had included only a single spirorchiid representative. The wide representation presented by Snyder (2004) showed that the Spirorchiidae was a paraphyletic taxon (Figure 3) that should be subdivided taxonomically. Spirorchiids of freshwater turtles were found to be basal to those of marine turtles, mirroring the evolution of their hosts for which the marine forms are thought to have evolved from freshwater ancestors.

3.4.2. *Systematics of the Liver Flukes* Fasciola *and* Paragonimus

Intraspecific variability and its use for species/population diagnostics in *Paragonimus* have been intensively studied using nuclear and mitochondrial markers (e.g. Agatsuma *et al.*, 1994b; Blair *et al.*, 1997a, b, 1999b; van Herwerden *et al.*, 1999, 2000; Iwagami *et al.*, 2000, 2003a, b; Agatsuma *et al.*, 2003; Cui *et al.*, 2003a; Park *et al.*, 2003) and early works were summarized in a paper by Blair *et al.* (1999b). Blair *et al.* (1997b) provided molecular evidence for the synonymy of three species of *Paragonimus*. Based on the comparison of ITS2 and COI sequences, they concluded that *P. iloktsuinensis* and *P. sadoensis* should be considered junior synonyms of *P. ohirai* despite differences in metacercarial morphology among these forms. Further works devoted to the molecular identification and differentiation among species of *Paragonimus* have been published by Ryu *et al.* (2000), Sugiyama *et al.* (2002) and Iwagami *et al.* (2003a, b). Several works dealing with the differentiation of nominal species of *Paragonimus* in

China and south-eastern Asia have been published (Cui *et al.*, 2003a, b; Chen *et al.*, 2004). Among other findings, it was concluded that *P. hokuoensis* and *P. szechuanensis* are genetically close to *P. skrjabini*, and the latter two species are most probably synonymous. van Herwerden *et al.* (1999) demonstrated that molecular markers should be used with caution, at least in some cases, as their study involving numerous clones of *P. westermani* and related species showed intra-individual variability among ITS1 sequences greater than that between individuals of the species complex(!) At the same time, sequence variation within individuals of *P. ohirai* was minimal. Similarly, van Herwerden *et al.* (2000) discovered at least two lineages of the mitochondrial *nad1* gene within individual worms that confounded the use of this gene for phylogenetic inference. Iwagami *et al.* (2000) summarized the state of molecular phylogeographic studies of *P. westermani* in Asia. Since then, several works on the subject have been published that address the possible geographic origin of triploid forms in *P. westermani* (Agatsuma *et al.*, 2003; Park *et al.*, 2003), variability among *P. skrjabini* from several provinces in China (Cui *et al.*, 2003a) and among *P. mexicanus* from Guatemala and Ecuador (Iwagami *et al.*, 2003a).

Due to the small number of species, fewer works have been devoted to the intrageneric systematics and phylogenetics of *Fasciola*. Adlard *et al.* (1993) and Agatsuma *et al.* (1994a) studied variability among populations, and Hashimoto *et al.* (1997) demonstrated that the Japanese species he examined should all be considered *F. gigantica*. Huang *et al.* (2004) compared ITS2 sequences from several samples of *Fasciola* in China and France. They were able to differentiate between *F. hepatica* and *F. gigantica* but found an intermediate genotype exhibiting sequences similar to both species and taken as evidence of heterogeneity in ITS2.

3.4.3. Systematics of Animal Flukes

Unlike the literature on species of medical and economic importance, molecular systematic literature on the majority of digeneans is less focused taxonomically and consists typically of isolated studies or

series of studies stemming from individual research groups. Thus, other than being united by resolving questions of taxonomic boundaries and patterns of evolution, there are few common themes to unite them. Among the first papers dealing with the molecular differentiation of digeneans of (non-human) animals was the work of Luton *et al.* (1992) on two species of *Dolichosaccus* in Australia. Sorensen *et al.* (1998) reported their results on the intraspecific variation in the ITS region of 37-collar-spined echinostomes from North America. Jousson *et al.* (2000) and Jousson and Bartoli (2001, 2002) investigated cryptic speciation in digeneans of the genera *Macvicaria*, *Monorchis* and *Cainocreadium* parasitic in marine fishes and found a pair of morphologically similar species in each case, prompting the description of a new species of *Monorchis*. In another investigation combining morphological, molecular and life cycle data, Overstreet *et al.* (2002) described a new species of *Bolbophorus* from the American white pelican and channel catfishes in the south-eastern United States. Their data set included sequences from a mixture of four nuclear and mitochondrial genes making their taxonomic conclusions well corroborated. Levy *et al.* (2002) and Dzikowski (2003) also addressed the problem of differentiating among *Bolbophorus* species, whose metacercariae cause significant losses to channel catfish farming in the Mississippi River delta. In these works, analysis of 18S as well as morphology were used to show that *B. damnificus*, *B. levantinus* and *B. confusus* were distinct species. Platt and Tkach (2003) used 18S, ITS and 28S in addition to morphological characters, to differentiate species of *Choanocotyle* from Australian turtles. Recently, Dzikowski *et al.* (2004b) used 18S and ITS to demonstrate the validity of two *Clinostomum* species, *C. marginatum* and *C. complanatum*, parasitic in aquatic birds in the Old and New Worlds, respectively.

Bell and Sommerville (2002) compared ITS and COI sequences between metacercariae of *Apatemon annuligerum* and *A. gracilis* originating from different localities and fish hosts. The authors concluded that the species should be synonymized due to the lack of genetic variability, finding only a short repeat motif in ITS1. Bell *et al.* (2001) and Bell and Sommerville (2002) reached the same conclusion regarding synonymy of two strigeid species, *Ichthyocotylurus erraticus* and *I. pileatus*. Bray *et al.* (1999) examined two fellodistomid genera

from deep sea fishes, *Lepidapedon* and *Steringophorus*, and despite inconsistency among trees obtained using different DNA fragments, as well as apparent host-switching in the evolutionary history of genus *Steringophorus*, were able to conclude that the genera have most likely radiated in the deeper waters off the continental shelf.

Galazzo *et al*. (2002) showed that *Diplostomum* species from North America and Europe form two clades, and that species identified as *D. baeri* on both continents most likely represent distinct species. Niewiadomska and Laskowski (2002) attempted to differentiate among six species of *Diplostomum* using ITS and morphology, but found inconsistency among the phylogenetic analyses based on molecules as well as the morphology of different life cycle stages. Given the widespread and economically important nature of the genus, there is a clear need for additional sampling and greater taxonomic resolution. Tkach *et al*. (2000b) examined morphological and molecular differences among three species of the genus *Plagiorchis* parasitic in European bats. Molecular and morphological characteristics show reliable differences between the recently described *Plagiorchis muelleri* and the type species of this large cosmopolitan genus, *P. vespertilionis*, which has now been re-described and its neotype established.

Echinostoma and related genera have been traditional models in digenean studies due to their ubiquity and impact on our health and economy; 16 species of the family Echinostomidae have been reported from man (Ashford and Crewe, 2003). Nevertheless, the taxonomy of echinostomids remains unclear, especially among species belonging to the 37-collar-spine group. Morgan and Blair (1995, 1998a, b, 2000) have published a series of works on the interrelationships of *Echinostoma* species using sequences of several mitochondrial and nuclear DNA regions. They demonstrated that Australian species are more diverse than was previously thought, analysed the relative merits of various DNA regions for phylogenetic inference and differentiation, and provided a useful review on the molecular biology of the group.

The common frog lung flukes (family Haematoloechidae) have also been the subject of a number of recent studies as their cosmopolitan distribution, and parasitism of some of the best studied vertebrates (frogs) makes them an interesting and convenient model for studies in phylogenetics and historical biogeography. Leon-Regagnon *et al*.

(1999) used partial ITS and 28S to examine the taxonomy and interrelationships of Mexican species of *Haematoloechus*, and Snyder and Tkach (2001) demonstrated the presence of three distinct evolutionary lineages within the Holarctic *Haematoloechus* of North America and Europe. The latter results confirmed the taxonomic validity of *Haematoloechus abbreviatus* and demonstrated that the presence or absence of extracecal uterine loops is labile, and therefore a poor character for the differentiation of genera. Two of three clades revealed by phylogenetic analysis were comprised of both European and North American species, indicating that lineages of *Haematoloechus* arose before the breakup of Laurasia and radiated after Eurasia and North America split. Later, Leon-Regagnon and colleagues (2001, 2002, 2003) used molecular markers to support the differentiation of two new species, *H. meridionalis* and *H. danbrooksi*, and presented an updated molecular phylogeny of the genus including several additional North American and two African species. Their study generally supported the conclusions of Snyder and Tkach (2001) and further revealed that African *Haematoloechus* appears to have evolved after the separation of Gondwana and Laurasia.

Growing concern regarding amphibian population declines and correlation of some of these events with limb deformities caused by metacercariae of the psilostomid digenean *Ribeiroia* has provoked interest in the taxonomy and systematic position of the genus. In the taxonomic part of their review of *Ribeiroia*, Johnson *et al*. (2004) used sequences of ITS2 to compare isolates from multiple localities in the USA, Puerto Rico, Guadeloupe and Kenya. Their results suggest the genus comprises three species, one of which (*R. marini*) was represented by two subspecies.

Casey *et al*. (2003) explored differences among green- and brown-banded sporocysts within the genus *Leucochloridium* occurring in Europe. They showed that each sporocyst colour morph is species specific (*L. paradoxum* or *L. variae*) with no intraspecific differences across samples from different European countries. Criscione and Blouin (2004) chose three species of digeneans parasitic as adults (*Deropegus aspina*, *Plagioporus shawi*) or metacercariae (*Nanophyetus salmincola*) in salmonids in the north-western United States as model taxa to investigate the effect of life cycle patterns on the distribution

of genetic variation within and among populations. The authors demonstrated that species with entirely aquatic life cycles had more structured populations with lower gene flow than did species with both aquatic and terrestrial phases in their life cycles, owing presumably to the higher dispersion ability of the latter. In the course of the same study, they were able to differentiate a genetically distinct form of *Deropegus aspina* that probably represents a cryptic species.

4. MOLECULAR SYSTEMATICS OF THE MONOGENEA

The Monogenea is the smallest of the three parasitic groups, encompassing less than half the diversity of the Cestoda and roughly one tenth that of the Digenea based on the number of described genera (Caira and Littlewood, 2001), although estimates suggest they may be far more diverse than appreciated at present (Whittington, 1998). They are primarily ectoparasites of fishes and, with notable exceptions, only rarely produce significant pathological effects to their hosts. Human beings are not hosts of monogeneans. Characteristically, they are skin parasites that erode the epidermis using proteolytic enzymes (Monopisthocotylea), or have become specialized to feed on blood (Polyopisthocotylea). However, a fascinating array of endoparasitic species have colonized the various internal cavities of their hosts that open to the exterior (Kearn, 1994). Perhaps their most fundamental distinction from the other parasitic flatworms is their reproduction, exhibiting both direct life cycles and viviparity. The best-known genus, *Gyrodactylus*, is responsible for enormous economic loss to fish farming, particularly in northern Europe, and has been the subject of intense study; almost half of the reports dealing with the molecular systematics of monogeneans concern this genus solely. From this work has evolved an implicitly DNA-based system of taxonomy now widely employed to help discriminate among the hundreds of described congeners. Outside the family Gyrodactylidae, the taxonomic focus of research has reflected both the varied interests of the workers themselves, as well as the need to establish a molecularly based ground plan for the class and its two constituent groups.

Recent multi-authored taxonomic keys for the Cestoda (Khalil *et al.*, 1994) and Digenea (Gibson *et al.*, 2002) have helped to guide molecular phylogenetic investigations by providing up-to-date working classifications, and thus circumscriptions of higher taxa (e.g. orders, families and genera). This has allowed greater consistency by ensuring that authors using the same taxonomic names are indeed describing the same entities when phylogenetic hypotheses are tested, whereas studies preceding such keys did not have this advantage. Unfortunately, such a key is neither available nor underway for the Monogenea.

4.1. Non-Monophyly of the Monogenea

Molecular data have not generally supported the monophyly of the Monogenea. From the earliest reports to the present (Baverstock *et al.*, 1991; Blair, 1993b; Mollaret *et al.*, 1997; Campos *et al.*, 1998; Littlewood *et al.*, 1999b; Litvaitis and Rohde, 1999; Littlewood and Olson, 2001), two monophyletic lineages representing the Monopisthocotylea and Polyopisthocotylea were shown to have evolved independently, with one or the other lineage sharing a more recent common ancestor with either the cestodes or trematodes. Relative positions of the two lineages and their relationship to the cestodes and trematodes vary relative to the data analysed, yielding little consensus among data sets (Justine, 1998; Lockyer *et al.*, 2003a). A notable exception was found by Lockyer *et al.* (2003a) who showed support for monophyly using the combination of complete 18S and 28S rDNA (Figure 1). However, results from independent analyses of the two genes and from different methods of analysis showed inconsistencies in relation to their monophyly. In general, molecular evidence against recognition of the 'Monogenea' has been a consistent finding despite the lack of support for alternate positions of the two independent lineages.

Few authors had previously questioned the validity of the Monogenea and the traditional classification can no doubt still be found in any current textbook of parasitology. However, significant differences in the morphology and ultrastructure, behaviour, nutrition and ecology of the Monopisthocotylea and Polyopisthocotylea have been

mooted previously (Justine, 1991, 1998; Euzet and Combes, 2003) and it is likely that most authors have, as expressed by Euzet and Combes (2003), simply chosen to emphasize the similarities rather than the differences between the two groups. Indeed, the weight of available evidence strongly favours paraphyly of the 'Monogenea' and thus now, in our opinion, the burden of proof is on the side supporting monophyly. See Justine (1998) for an excellent account of the molecular (and other) studies leading to this conclusion, and Euzet and Combes (2003) for detailed and compelling arguments based on fundamental differences in their biology.

4.2. Interrelationships of the Monopisthocotylea and Polyopisthocotylea

Only a handful of studies to date have sought to resolve the broader interrelationships of the Monopisthocotylea or Polyopisthocotylea using molecular data (e.g. Mollaret *et al.*, 2000a; Jovelin and Justine, 2001; Olson and Littlewood, 2002; Simková *et al.*, 2003), although numerous studies have addressed selected groups within these lineages (see below). Initial studies (e.g. Mollaret *et al.*, 1997) relied on partial (~500 bp) 28S data and this trend was continued by Mollaret *et al.* (2000a) and Jovelin and Justine (2001), the latter of whom also examined COI data but determined the gene to be saturated for such levels of comparison. Olson and Littlewood (2002) consolidated and augmented the different fragments of available 28S data (domains D1, D2, or both), as well as complete 18S data, in an attempt to provide more comprehensive estimates (see Figure 4). Their work illustrated the highly fragmentary nature of the available data, necessitating analysis of subsets of the taxa that taken together represented roughly half of the described families *sensu* Boeger and Kritsky (2001). Consistent patterns have emerged from these data that also provide independent support for inferences made on the basis of morphology (i.e. Boeger and Kritsky, 1993, 1997, 2001). For example, the general pattern of evolution in the Polyopisthocotylea reveals a basal split between radiations in tetrapods (i.e. Polystomatidae) and in fishes (Littlewood *et al.*, 1998b; Mollaret *et al.*, 2000a; Olson and Littlewood,

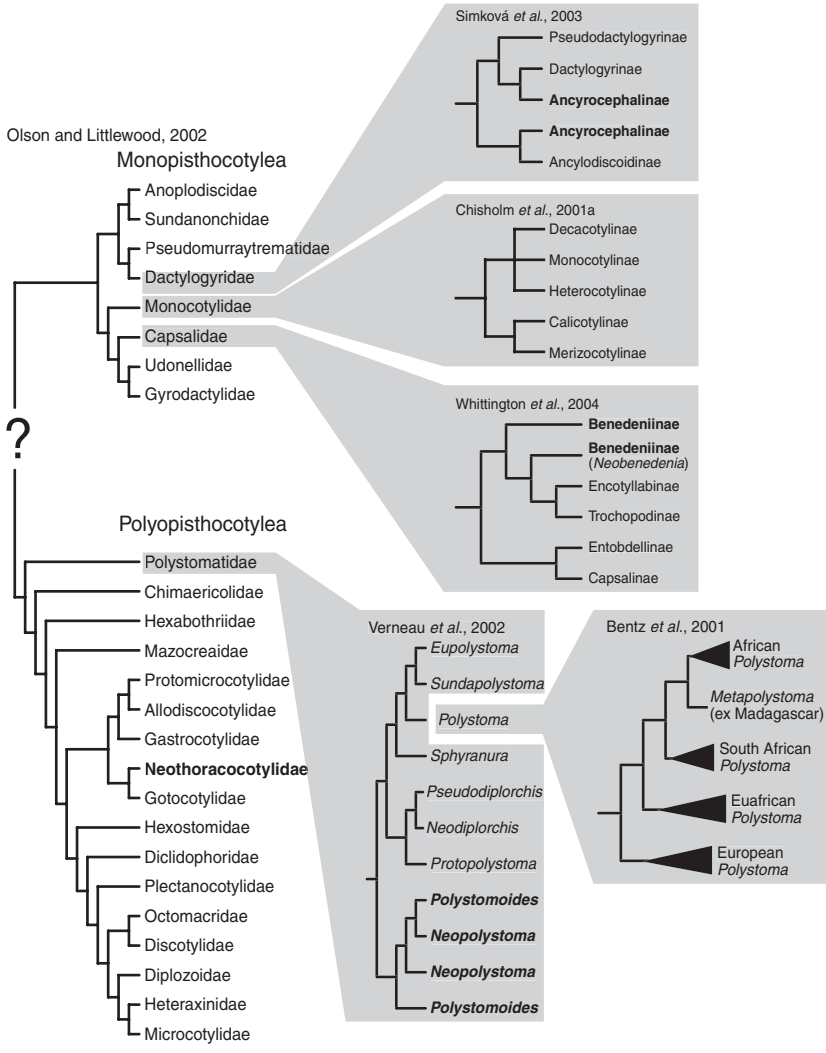

Figure 4 Phylogenetic hypotheses of the monopisthocotylean and polyopisthocotylean 'monogeneans' and a selection of available phylogenetic hypotheses for constituent groups. Taxa found to be paraphyletic indicated in bold type.

2002), with the latter clade showing a progressive series of lineages starting with polyopisthocotylean parasites of holocephalans (i.e. Chimaerocolidae), higher elasmobranchs (Hexabothriidae) and those of teleosts (Jovelin and Justine, 2001; see Figure 4).

Whereas the interrelationships of the Polyopisthocotylea may be described as largely pectinate (i.e. a hierarchy of nested clades), the Monopisthocotylea tends to show a bifurcating pattern (i.e. a series of splits). Thus, the Gyrocotylidae (plus *Udonella*, see below) is the sister group to the Capsalidae, which together form a sister group to the Monocotylidae, and these in turn form a sister group to the Dactylogyridae + Diplectanidae (Mollaret *et al*., 2000a; Olson and Littlewood, 2002; Simková *et al*., 2003). Beyond this, a more significant (and perhaps not entirely independent) difference is seen in the average divergence rate of the Monopisthocotylea, which is four times faster than that of the Polyopisthocotylea (as inferred from 18S rDNA; Olson and Littlewood, 2002). This increased divergence is also correlated with at least two cases of explosive radiation in the monopisthocotyleans, namely in the genera *Dactylogyrus* and *Gyrodactylus*. Such large differences in divergence rates can be problematic for parsimony and statistically based analyses alike and may be confounding our ability to resolve the 'Monogenea' as monophyletic using rDNA. However, given the many fundamental differences between the two groups, it is more likely a reflection of their independent evolutionary trajectories.

4.3. Systematics of Select Groups

A number of papers has used molecular data to examine the positions of poorly known or enigmatic taxa, some building on the comparative data resulting from the studies discussed in the preceding section and others necessarily generating data specific to the level of inference required. Mollaret *et al*. (2000b), for example, examined the positions of the genera *Sundanonchus*, *Thaparocleidus* and *Cichlidogyrus* and showed the validity of the Sundanonchidae. Justine *et al*. (2002) later added members of the Bothitrematidae and Neocalceostomatidae and found a strong association among *Bothitrema*, *Anoplodiscus* and *Sundanonchus*, each representing different families, whereas the position of the Neocalceostomatidae remained labile. Bentz *et al*. (2003) examined the position of *Euzetrema*, a monopisthocotylean genus unusually found in tetrapods, but found its position relative to the taxa parasitizing actinopterygian and chondrichthyan fishes equivocal

with regard to arbitrating between the possibilities of host capture or co-evolution. Desdevises *et al.* (2000) examined species-level genetic variation and host associations in the relatively large genus *Lamellodiscus* and showed an interesting example of morphological variation in the absence of genetic variation. Desdevises (2001) later showed through molecular analysis that the monotypic genus *Furnestinia* was invalid and should be synonymized with *Lamellodiscus*. Matejusová *et al.* (2001b) were able to differentiate genera and species of the unique Diplozoidae, but were subsequently unable to correlate differences in morphology in species of *Paradiplozoon* with variation in ITS2. Restriction digests of ITS2 were used subsequently to differentiate European species of diplozoids (Matejusová *et al.*, 2004).

Congruence between morphological and molecular estimates of phylogeny in the Monocotylidae (parasites of chondrichthyans; sharks, rays and chimaeras) was examined by Chisholm *et al.* (2001a, b; see Figure 4) whose revised classification (Chisholm *et al.*, 1995) was generally supported by partial 28S data. Within the family, comparison of *Calicotyle* species using 28S (Chisholm *et al.*, 2001c) suggested that species in the genus are not strictly host specific, although phylogenetic analysis showed that species clades reflected associations at least to the level of host genus. The commonly reported *C. kroyeri* was suggested to represent a convenient identity for members of the genus found in the cloaca of skates (*Raja* spp.), as divergences among species from different host species were in some cases greater than those among separate parasite species. Also using 28S, Whittington *et al.* (2004) provided a preliminary investigation of the interrelationships of the Capsalidae (see Whittington, 2004 for a recent review of the group) and showed that the genus *Neobenedenia* was closer to the *Encotyllabe* and *Trochopus* than to *Benedenia*, thus showing the Benedeniinae to be a paraphyletic subfamily (see Figure 4).

4.3.1. Udonella *as a Monopisthocotylean Monogenean*

An important question readily resolved through analysis of molecular data was the phylogenetic position of *Udonella*, a small genus of flatworm hyperparasites of caligid copepods that in turn parasitize

marine fishes. To some, the genus represented a potentially pivotal position in the evolution of the parasitic Platyhelminthes (e.g. the sister group to the Neodermata), whereas others considered it an aberrant monopisthocotylean whose phylogenetic position therefore had little bearing on the origin of parasitism in the phylum (Littlewood *et al*., 1998b). Its position was first examined using rDNA by Littlewood *et al*. (1998b) who clearly showed its affinities to the Gyrocotylidae within the Monopisthocotylea, and this result has been supported by all subsequent analyses including members of both of these taxa (e.g. Olson and Littlewood, 2002; Simková *et al*., 2003). Hypothesizing a position of *Udonella* outside the Monopisthocotylea (let alone outside the Neodermata, e.g. Zamparo *et al*., 2001), in light of the data accumulated in recent years is simply unfounded.

4.3.2. Systematics of the Dactylogyridae

Simková *et al*. (2003, 2004) examined the position and speciation of the extraordinarily diverse Dactylogyridae. Their work (Simková *et al*., 2003; Figure 4) showed that the group is recently derived within the Monopisthocotylea and tested the validity of the subdivisions of the family, rejecting the Ancyrocephalinae as paraphyletic. Subsequent work (Simková *et al*., 2004) examined the intrageneric relationships of *Dactylogyrus*, suggesting that members of the Cyprininae were the original hosts and that the diversification of the genus was the result of sympatric speciation with some (albeit seemingly minor) degree of niche specialization/separation on the gills. A very different situation regarding mode of speciation and niche specialization appears to be the case in the polystomes.

4.3.3. Systematics of the Polystomatidae

The Polystomatidae is the sole family of monogeneans to parasitize tetrapod hosts (primarily freshwater anurans and chelonians) and thus presents an interesting case in the evolution of the Polyopisthocotylea (Kearn, 1994), which has been addressed by a handful of molecular investigations. Verneau *et al*. (2002; see Figure 4) estimated

their interrelationships based on partial 18S using a large sampling of taxa and showed that the polystomes had strong affinities to their hosts groups, with those of chelonians forming a sister group to an anuran clade split between archaeobatrichian and neobatrician hosts. According to the authors, molecular clock estimates of the nodes corresponded well with the divergence of the major host groups and suggested an origin of the Polystomatidae (*sensu stricto*) of ~353 mya. The position of the unique *Sphyranura* (a parasite of caudate amphibians) appeared embedded within the clade including anuran parasites, and thus did not support recognition of a separate family (i.e. Sphyranuridae). Their results agreed with the findings of Sinnappah *et al.* (2001) who postulated that *Sphyranura* was a neotenic genus restricted (partly) to neotenic hosts and in fact represented the pleisiomorphic condition of the group (a 'missing link'). The position of *Concinnocotyla*, a parasite of Australian lungfish, was also examined by Verneau *et al.* (2002) but it fell outside the group altogether. However, support for the positions of both of these genera has been weak and requires additional study. Within the speciose genus *Polystoma*, Bentz *et al.* (2001) showed a single colonization event of Africa from European stock (Figure 4). Embedded within the African clade of *Polystoma* species was the genus *Metapolystoma* from Madagascar, the validity of which was therefore rejected. Earlier, Littlewood *et al.* (1997) had studied speciation in *Polystoma* and related genera in order to test whether the species infecting different sites of the same host (e.g. oral cavity vs. urinary bladder, etc.) resulted from sympatric speciation. Analysis of 28S and COI strongly supported allopatric speciation and showed that site specificity was a stronger predictor of relatedness than was host specificity.

4.4. Systematics and Diagnostics in *Gyrodactylus*

Gyrodactylus is the most intensively studied group of monogeneans due to their economic importance in aquaculture, species richness and ubiquity. Members of the Gyrodactylidae, and particularly *Gyrodactylus*, create an enormous economic strain on fish farming in the UK, Scandinavia and elsewhere, and not surprisingly, much of the

work on gyrodactylids therefore involves differentiating pathogenic from non-pathogenic forms; a non-trivial task with over 400 described species in the genus (Harris *et al*., 2004) and 50-fold more species predicted to be undescribed (Bakke *et al*., 2002). Morphological diagnoses are based on subtle quantitative and qualitative differences of the sclerotized elements of the haptor, making it both difficult and potentially unreliable (but see the morphometric methods of Shinn *et al*., 2001, 2004). Early efforts to characterize and differentiate gyrodactylids molecularly employed RFLP analysis of 5.8S and ITS sequences (Cunningham *et al*., 1995b, c; Cunningham and Mo, 1997), although the 18S (Cunningham *et al*., 1995a), the intergenic spacer region between ribosomal gene arrays (Collins and Cunningham, 2000), the 28S (Cunningham *et al*., 2000; Matejusová and Cunningham, 2004), and more recently the *cox1* gene (Meinilä *et al*., 2002, 2004) have also been investigated for their utility in discriminating among species of *Gyrodactylus*. Among these genes and gene regions, the ITS1–5.8S–ITS2 between the 18S and 28S genes has proven to be the target of choice for inter- and intraspecific diagnosis, particularly because of the number of comparative sequences now available (e.g. Cable *et al*., 1999; Harris *et al*., 1999; Harris and Cable, 2000; Zietara *et al*., 2000; Bruno *et al*., 2001; Matejusová *et al*., 2001a, 2003; Huyse and Volckaert, 2002; Zietara *et al*., 2002; Zietara and Lumme, 2002; Huyse *et al*., 2003; Lindenstrøm *et al*., 2003; Zietara and Lumme, 2003; Huyse and Malmberg, 2004; Meinilä *et al*., 2004). Differences in the models of nucleotide substitution employed by various authors make genetic divergence estimates difficult to compare, but in general, variability in the region is highest in the ITS1, followed by ITS2 and 5.8S (the short 5.8S is often found to be invariant at the inter- and intra-specific levels, but has been shown to be useful for sub-generic diagnosis; Zietara *et al*., 2002). In addition, minor geographic, as well as intra-specific variation has been shown through comparison of this region among disjunct populations (e.g. Matejusová *et al*., 2001a).

To a certain extent, the reliance on DNA for species discrimination has meant that an implicit system of DNA taxonomy based on ITS data has become the standard for species circumscription of gyrodactylids (e.g. Harris and Cable, 2000; Bruno *et al*., 2001; Cunningham

et al., 2001; Lindenstrøm *et al*., 2003; Zietara and Lumme, 2003; Huyse and Malmberg, 2004; Huyse *et al*., 2004). While not explicit in the use of 'DNA taxonomy', the necessity and justification for such a system in *Gyrodactylus* (Zietara and Lumme, 2003) is effectively the same as that proposed by researchers studying other groups of organisms (e.g. Tautz *et al*., 2003). Using DNA, both morphologically distinguishable, as well as 'cryptic' (Huyse and Volckaert, 2002; Zietara and Lumme, 2002) species have been described and variation in the ITS region is frequently guiding the re-examination of morphology, rather than being used simply to confirm morphological differences previously observed.

Species circumscription based on DNA has been widely applied to the salmonid parasite *Gyrodactylus salaris*, which has been a known cause of mortality in Atlantic salmon (*Salmo salar*) in Norway since the 1970s and remains a significant economic problem to the region (Mo, 1994). Distinguishing the variants of *G. salaris* and the documentation of the pathological differences among these strains or species and the various fish species they infect has been studied intensively in a number of laboratories. Sterud *et al*. (2002), for example, documented significant pathological differences between *G. salaris* and the nominal species *G. thymalli*, and Lindenstrøm *et al*. (2003) showed host preference in controlled infection experiments between *G. salaris* and a laboratory variant founded from a single individual. In both cases, morphology of the variants fell within ranges exhibited by *G. salaris sensu* Malmberg, 1957, and genetic distances based on the ITS region failed to show marked differences. Indeed, attempts to discriminate *G. salaris* from *G. thymalli* based on a variety of methods and gene regions have shown insufficient variation to support the specific status of *G. thymalli* (Cunningham, 1997; Cunningham *et al*., 2003; Hansen *et al*., 2003; Meinilä *et al*., 2004). Using *cox1* data, for example, Hansen *et al*. (2003) found 12 haplotypes among 76 specimens of *G. salaris* and *G. thymalli* from 32 host populations. Phylogenetic analysis of the haplotypes showed no support for reciprocal monophyly of the two 'species'. Thus, by the standards established by these and other monogenan researchers, the use of DNA taxonomy in the case of *G. salaris*/*thymalli* may result in a species definition that fails to reflect significant differences in host

response and pathology, a situation that is unlikely to be readily accepted by workers whose concerns involve the recognition of the highly pathogenic strains (e.g. Sterud *et al*., 2002). It remains to be seen if more sensitive methods will yield support for the recognition of *G. thymalli*.

A number of recent reports have built on and contributed to the growing number of characterized gyrodactylid sequences in order to address large-scale patterns of speciation in the genus, as well as the mechanisms that explain these patterns and account for their enormous radiation. In populations of both *G. salaris* as well as other species of *Gyrodactylus* studied, host-switching appears to be the predominant mode of speciation (Zietara and Lumme, 2002; Huyse *et al*., 2003; Meinilä *et al*., 2004), which in turn may be driving further speciation events (Zietara *et al*., 2002). Host-switching between families (Zietara *et al*., 2002) as well orders (Huyse *et al*., 2003) of fishes has been shown, as have instances of sympatric speciation, although speciation through allopatry is more commonly implied by the patterns observed (Huyse *et al*., 2003; Meinilä *et al*., 2004). New phylogenies have also allowed a reassessment of the systematics and nomenclature of the Gyrodactylidae. For example, the well-established sub-generic divisions of the group based on features of the excretory system (Malmberg, 1970) is rejected by both 5.8S (Zietara *et al*., 2002; Huyse *et al*., 2003) and ITS (Huyse *et al*., 2003; Matejusová *et al*., 2003) data, although such nomenclatural problems are hardly surprising in a species group of this size. Taxonomic issues aside, the studies above demonstrate that *Gyrodactylus* may be used as an interesting model system for studying speciation in animal systems generally.

5. BEYOND SYSTEMATICS: MOLECULAR DIAGNOSTICS

The complexity and variation of life history strategies in cestodes and digeneans remain among the most compelling and intriguing aspects of the biology of these parasites. Elucidating their life cycles was a cornerstone of the field for much of the early part of the twentieth

century, by which time the basic ontogenetic and host sequences had been worked out for most major groups. Since then, new information on life cycles diminished as parasitologists turned first to experimental, and later molecular questions and approaches. Today, it would be difficult to justify the labour and expense involved in a trial-and-error approach to life cycle elucidation using *in vivo* systems or *in vitro* cultivation (e.g. Chambers *et al*., 2000). However, the accumulation of molecular data, or 'barcodes' (Besansky *et al*., 2003; Hebert *et al*., 2003; Moritz and Cicero, 2004), of adult sequences has enabled a direct and efficient means of identifying larval ontogenetic stages and thus inferring complete life cycles (see also McManus and Bowles, 1996 for an early review including other approaches to molecular diagnostics). Coupled with phylogenetic analysis, broader affinities may be discerned even in cases where exact matches are not attainable through direct sequence comparison. 'Barcoding' and its use in diagnostics is a new and somewhat contentious field, if only because it may be confounded in practice by issues of 'DNA taxonomy' (see Section 1.1, also Moritz and Cicero, 2004). Although its application to the parasitic Platyhelminthes is limited at present, in the coming years we anticipate the method to produce a rapid acceleration in studies of host associations and life cycles.

Precise identification of larval flatworm parasites is generally not possible on the basis of comparative morphology alone. At present, our knowledge of host utilization and specificity is restricted largely to the definitive host, and fully elucidated life cycles are exceptionally few in comparison to the number of adult species described. In the Cestoda, at the family level, and at the ordinal level in some groups, a single complete life cycle is unknown (Beveridge, 2001), and in the Digenea, perhaps half of the groups remain in a similar state of ignorance (Cribb *et al*., 2003). Obviously, the utility of molecular diagnostics is directly proportional to the number of species for which sequence data have been characterized, and workers interested in diagnosing cestode or digenean parasites should concentrate on the 18S and 28S genes in order to maximize the number of species for which comparative sequences are currently available (e.g. Mariaux, 1998; Olson *et al*., 1999, 2001, 2003a; Tkach *et al*., 2001b; Lockyer *et al*., 2003b; de Chambrier *et al*., 2004). The extraordinary growth of

publicly available genetic repositories and linked databases dedicated to biodiversity (e.g. the Global Biodiversity Information Facility, www.gbif.org; Consortium for the Barcode of Life, barcoding.si.edu) will rapidly increase our understanding of helminth life history, host associations and trophic interactions by making identifications simple and efficient, and thus making large-scale biotic survey and inventory of both larval and adult helminths feasible for the first time.

5.1. Ecological Diagnostics and Life Cycle Studies

5.1.1. Cestoda

In the Cestoda, Brickle *et al.* (2001) identified larval tetraphyllidean parasites of an important commercial squid fishery using partial 28S sequences and found that they differed from the adult species infecting the local skate population, the only elasmobranches endemic to the Falkland Islands. These data were used subsequently by Agustí *et al.* (2005) who examined the larval cestodes of dolphins in the Mediterranean. Like Brickle *et al.* (2001), they found larval worm sequences exceedingly similar to that of *Clistobothrium montaukensis*, a tetraphyllidean cestode of lamniform sharks, suggesting that members of this genus are widespread both in terms of geography and intermediate host range. Dezfuli *et al.* (2002) used partial sequencing of 18S to match procercoids with adults of the spathebothriid *Cyathocephalus truncatus*, and to discriminate between these procercoids and cysticercoids of *Microsomacanthus pachycephala* (Hymenolepididae) in co-infected amphipods (*Echinogammarus stammeri*) in Northern Italy. They stressed the importance of using such data where co-infection of hosts may confound the identities (albeit larval discrimination between caryophyllidean and cyclophyllidean cestodes could be readily done on the basis of morphology). Reyda and Olson (2003) used partial 28S rDNA data to verify the identity of proteocephalidean tapeworms encysted within the parenchyma of adult tetraphyllidean worms in freshwater rays in Peru. Although a handful of similar reports of cestode–cestode hyperparasitism in South America had been published previously, molecular

data provided the first independent evidence that proteocephalidean 'hyperparasites' could infect other groups of cestodes. Moreover, thanks to a large reference database stemming from previous works on the phylogeny of the Proteocephalidea (Zehnder and Mariaux, 1999), a far more precise identification of the cysts was made than would have been possible based on their morphology alone.

5.1.2. Digenea

The complexity of digenean life cycles makes their elucidation via molecular diagnostics of even greater utility than for the cestodes, and the earliest and most abundant of such works have been in the Digenea. Life cycles in the digeneans of marine animals have been especially difficult to elucidate due to the expense and difficulties of the animal husbandry required. With the accumulation of sequence data on adult forms, identification of cercariae found in snails and metacercariae from second intermediate or paratenic hosts is now as easy for marine species as it is for freshwater or terrestrial (save perhaps the collection of specimens themselves). For example, Cribb *et al.* (1998) demonstrated a three-host life cycle in the unusual Bivesiculidae, and Anderson (1999) used DNA to identify metacercariae of *Indodidymozoon pearsoni*, a member of the Didymozoidae. Schulenburg and Wägele (1998) examined digenean metacercia in the isopod *Cyathura carinata* using ITS1 and 18S, but were unable to identify these precisely due to a lack of available comparative data at the time. Jousson *et al.* (1998, 1999), Jousson and Bartoli (2000), Bartoli *et al.* (2000) and Bartoli and Jousson (2003) demonstrated the life cycles of a number of marine digeneans belonging to the families Monorchidae and Opecoeloidae. Mone *et al.* (2003) detected larval stages of *S. mansoni* for the first time in Oman using RAPD. Recently, Hertel *et al.* (2003, 2004) used tandemly repeated DNA sequences characterized in *Echinostoma* and *Schistosoma* in order to differentiate among congeneric species. Similarly, Hust *et al.* (2004) compared ITS region sequences of cercariae belonging to two microphallid species (*Maritrema subdolum* and an undetermined species)

and designed species-specific primers enabling their reliable differentiation. Dzikowski *et al.* (2004a) identified different life cycle stages of several species belonging to the family Heterophyidae and outlined their phylogenetic position using newly obtained and previously published sequences of 18S. Their work resolved the life cycle of a widely distributed heterophyid, *Pygidiopsis genata*. The works on the molecular and morphological differentiation among species of *Bolbophorus* discussed previously (i.e. Levy *et al.*, 2002; Overstreet *et al.*, 2002; Dzikowski *et al.*, 2003), also included comparative analysis of DNA sequences obtained from both adult and larval stages of the parasites. Galazzo *et al.* (2002) identified metacercarial stages of the genus *Diplostomum*, a genus most commonly encountered as metacercariae, and as a result, rarely identified to species. Donald *et al.* (2004) obtained ITS2 sequences from digenean larvae recovered from topshells (Mollusca: Trochidae) and found that they represented a mixture of species whose taxonomic positions could not be discerned due to a lack of comparative data. Fortunately, such reports can be readily re-investigated once the appropriate data become available.

5.2. Clinical Diagnostics

In clinical situations, identification of helminths may be problematic despite recognition of disease agents and the numerous methods that continue to be developed for diagnosing species of medical and economic importance (see review by Ito, 2002). Under some circumstances, conditions may be present that result in aberrant parasite development and/or unrecognized pathology, and that create opportunity for rare or unknown zoonotic diseases to establish. Such was the case in an early report of a 'possible mutated sparganum' (Conner *et al.*, 1976) in a Hodgkin's patient being treated with a regime of immunosuppressive drugs. A more recent case involving an AIDS patient (Santamaría-Fríes *et al.*, 1996) was similarly difficult to diagnosis due to a metastatic disease process that showed no morphological indication of having resulted from a metazoan parasite. Both cases were subsequently re-investigated using molecular sequencing techniques (Olson *et al.*, 2003b), demonstrating that the common, and

generally benign human dwarf tapeworm, *H. nana*, was capable of producing lethal pathological consequences in the case of the AIDS patient, and that the Hodgkin's patient's disease was the result not of an infection with a sparganum, but with a cyclophyllidean cestode *not* belonging to one of the well-known genera previously reported from man. These applications show the immediate utility of genetically characterized animal helminths, and the need for such data in identifying the aetiological agents of emerging diseases. We necessarily spend the greatest resources on those species known to be a threat to our health and economy, but it is difficult to predict which parasite species may become the cause of zoonotic disease when conditions for such infections become favourable. Thus, the broader our knowledge and characterization of helminth biodiversity, the more likely we will be to identify the agents of opportunistic, rare and emerging disease.

6. FUTURE DIRECTIONS

6.1. Taxonomic Considerations

Early molecular phylogenetic studies of the parasitic flatworms, as in other taxa, have tended to address questions from the bottom up; that is, from the basal divergences among major lineages to subsequent investigations of divergences in more restricted and recent clades. The result is that we have a phylogenetic framework, based on one or more genes, for the phylum, the Neodermata, and for each of the major neodermatan groups. While these hypotheses require further corroboration, effort is increasingly needed to fill out the branches of the trees by providing significantly greater representation of the diversity encompassed by the constituent groups. In the Cestoda, only the order Proteocephalidea has received such comprehensive treatment (e.g. Zehnder and Mariaux, 1999; de Chambrier *et al*., 2004), although molecular phylogenetic investigations of other orders are presently underway. Priority areas must be seen to include the Cyclophyllidea, which not only contains the most important cestode pathogens of man, but also a greater diversity than all other cestode orders combined. Moreover, the diversity of life cycles

(including the transition to terrestrial cycles), host associations and biogeographic patterns provide a large number of interesting questions yet to be addressed in a molecular phylogenetic context. Other priority areas include the circumscription of a monophyletic Tetraphyllidea, resolving interrelationships among difossate groups, and corroborating the positions of basal taxa.

Although the recent analysis by Olson *et al.* (2003a) included representatives of 77 digenean families, a large number of omissions remain; according to Gibson *et al.* (2002) the Digenea comprises 140 families. Among unattended taxa there are many smaller families with unknown phylogenetic affinities, including the Mesotretidae, Rhytidodidae and Urotrematidae, as well as families crucial to the full elucidation of digenean evolution such as the Allocreadiidae, Gymnophallidae, Liolopidae, Paramphistomidae and Ptychogonimidae. The basal clade formed by the superfamilies Brachylaimoidea, Diplostomoidea and Schistosomatoidea merits further support in order to verify existing hypotheses on the evolution of their life cycles. Also needed in future investigations are detailed studies of the internal phylogenies of families, clarification of their taxonomic content, and the allocation of numerous genera with presently unclear affinities. The Aspidogastrea (see Figure 1) is routinely confirmed as the sister group to the Digenea, but has otherwise yet to be examined in detail with molecular tools. Although small in number, they are equal in age to the Digenea and have left an interesting suite of host associations that should be investigated in a molecular phylogenetic context.

Work on monogeneans remains dominated by studies on *Gyrodactylus*, and unusually, the broader interrelationships of the group have been arguably studied more intensively with morphology than molecules (e.g. Boeger and Kritsky, 1993, 1997, 2001). While a number of groups outside of the Gyrodactylidae have also been studied in some detail (see Section 4.3), few comprehensive treatments have been published and the work of Olson and Littlewood (2002) illustrates the fragmented nature of the available molecular data for the group as a whole. A more coordinated effort within the monogenean research community would help to establish the phylogenetic groundplan of the group(s) and thus link together the more restricted clades (e.g. monocotylids, polystomatids, etc.) in which interrelationships have

been studied. Equally important is firmly establishing the relative positions of the monopisthocotylean and polyopisthocotylean lineages relative to the cestode and digenean clades, a problem that has yet to yield to analyses of rDNA. It also remains to be seen to what extent a formal DNA-based taxonomy will be adopted in the genus *Gyrodactylus* and whether or not such criteria can be extended to other groups of monogeneans.

6.2. Analytical Considerations

Ancient divergences, rapid diversification and convergent evolution are all potential sources of error that confound phylogenetic estimation. Being cumulative, these effects become more pronounced with the age of divergence. Thus, future studies, particularly at higher taxonomic levels, should aim not only to increase the number of taxa and gene loci, but to become increasingly mindful that multiple substitutions in the sequence data must be compensated for. Statistical methods in phylogenetic reconstruction such as maximum likelihood and Bayesian inference necessarily incorporate various models of nucleotide or amino acid substitution and thereby allow for more realistic treatment of the data (Huelsenbeck *et al.*, 2001; Holder and Lewis, 2003). Fortunately, Bayesian analysis has proven computationally feasible even with large datasets (e.g. Olson *et al.*, 2003a). Statistical methods are also available for choosing an appropriate model based on the data to be analysed (Posada and Crandall, 1998; Posada and Buckley, 2004), with the selection criterion being the minimization of the number of estimated parameters. Incorporating complex substitution cost matrices in parsimony analysis is significantly more difficult, whereas unweighted parsimony makes no attempt to account for multiple substitutions and its greatest utility is in providing a least-change estimation. Given advances in statistical methods, distance analyses (e.g. neighbour joining, minimum evolution), despite the ability to incorporate substitution models into pair wise distance estimates, are phenetic and should be avoided when the estimation of phylogeny, rather than overall genetic similarity, is sought. The development and refinement of analytical techniques in molecular

systematics continues rapidly and reviews on current practices abound (e.g. Lewis, 2001; Whelan *et al.*, 2001; Holder and Lewis, 2003).

6.3. Molecular Targets

Ribosomal data have provided the basis for molecular phylogenetic investigations of animals, protists and prokaryotes since some of the earliest works in the field (botanists have favoured the *rbcl* (rubisco large subunit) gene from the chloroplast genome); the current basis of animal interrelationships rests largely on 18S data alone (e.g. Aguinaldo *et al.*, 1997). As in other taxonomic groups and as this review shows, 18S, and later 28S, have been widely employed in studies of the parasitic platyhelminths and now provide the most diverse reference database of molecular characters. The ITS regions have been used widely as well in cases requiring high levels of variation, albeit the highly variable regions of the 18S and 28S genes may be equally suited to such analyses. The mitochondrial genes tend to be faster evolving than those of the nuclear genome (Simon *et al.*, 1994) and, with exceptions, are generally not useful for studies of the parasitic platyhelminths due to the presumed antiquity of the group. Reliance on ribosomal and mitochondrial genes in light of questions left unresolved has led eventually to a search for additional nuclear protein-coding markers and a corresponding weariness of the utility of rDNA. However, comparative analysis of additional such genes has failed to produce alternative markers with wide utility, and thus the rDNA genes have, for better and for worse, continued to be the most popular markers for making phylogenetic inferences at a variety of taxonomic levels. New markers, and perhaps new classes of data, will surely come to light as the emerging field of genomics matures.

6.4. Genomics

6.4.1. Mitochondrial Genomes

Technical advances in PCR, cloning and automated sequencing technology have made large-scale sequencing projects feasible even in

modestly sized and funded laboratories. No complete genome of a platyhelminth has been characterized to date, but a multi-national effort has been underway for some time that will make *S. mansoni* the first flatworm for which this is achieved (see http://www.nhm.ac.uk/hosted_sites/schisto/). On the other hand, mitochondrial genomes (Figure 5), which average ~14 000 bp in flatworms (Le *et al*., 2002a), have been completed for a handful of cestodes and digeneans, but a wider representation will be published in the near future (D.T.J. Littlewood, pers. comm.). Characterized mitochondrial genomes in the cestodes include *E. granulosus* (Le *et al*., 2002b), *H. diminuta* (von Nickisch-Rosenegk *et al*., 2001; see Figure 5), *T. crassiceps* (Le *et al*., 2000) and *T. solium* (Nakao *et al*., 2003). In the Digenea, a number of shistosome species (Blair *et al*., 1999a; Le *et al*., 2001b), *Fasciola hepatica* (Le *et al*., 2001a) and *Paragonimus westermani* (Le *et al*., 2000) have all been completed (see recent review by McManus *et al*., 2004). Although largely similar to other metazoan mitochondrial genomes, the *atp*8 gene is missing (Le *et al*., 2002a) and minor differences are known to exist in their genetic code (Nakao *et al*., 2000; Telford *et al*., 2000). These data will have many uses, especially in diagnostics and strain differentiation. The phylogenetic utility of large-scale changes such as gene order seems dubious (Le *et al*., 2000) but it remains to be seen if the large number of potentially conserved amino acid changes will help elucidate early divergences in platyhelminth evolution.

6.4.2. Nuclear Genomes and Transcriptomes

There can be little doubt that in due course the characterization and, more importantly, understanding of nuclear genomes, as well as their expressed products (transcriptomes), will have a profound impact on our concept of species, and thus on systematics. In the short term, a seemingly limitless number of new targets will be revealed that may be useful for phylogenetic studies (e.g. Philippe *et al*., 2004), and access to a suite of targets will allow for the generation of datasets better tailored to the question at hand. In the longer term, it is easy to see genomics affecting the practice of systematics more profoundly, in

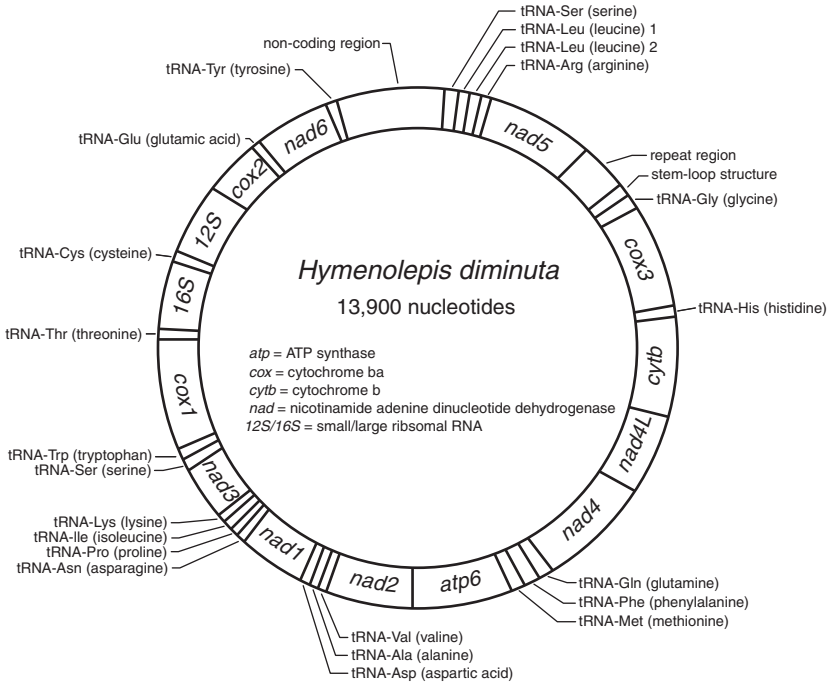

Figure 5 Mitochondrial genome of *H. diminuta*, adapted from von Nickisch-Rosenegk *et al.* (2001). Mitochondrial gene order in *H. diminuta* appears to be typical of mtDNA in the parasitic Platyhelminthes generally. (Note: All genes are encoded on the same strand and the direction of transcription is clockwise.)

a manner similar to the advent of molecular systematics which required the development and adoption of new analytical and theoretical tools, and which stands to change the practice of systematics more profoundly still through DNA-based taxonomy. At present, however, the impact of genomics on the field of systematics, and certainly on platyhelminth systematics, is marginal. Instead, we see phylogenetic techniques being used to better understand the genome (Hardison, 2004), rather than platyhelminth genomes being used to understand organismal relationships. Among the parasitic Platyhelminthes, *Schistosoma* is the only taxon for which a complete genome project has been initiated, although EST (expressed sequence tag) projects aiming to characterize site or stage-specific transcripts are

ongoing for a number of medically important digeneans. In the cestodes and monogeneans, no genome project has been initiated, but a large-scale EST project for *Echinococcus* is underway (see www.sanger.ac.uk/Projects/Helminths). The full fruits of these endeavours, and the ways in which they will contribute to our understanding of platyhelminth evolution is yet on the horizon.

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Wolbachia Bacterial Endosymbionts of Filarial Nematodes

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ABSTRACT

Filarial nematodes are important helminth parasites of the tropics and a leading cause of global disability. They include species responsible for onchocerciasis, lymphatic filariasis and dirofilariasis. A unique feature of these nematodes is their dependency upon a symbiotic intracellular bacterium, *Wolbachia*, which is essential for normal development and fertility. Advances in our understanding of the symbiosis of *Wolbachia* bacteria with filarial nematodes have made rapid progress in recent years. Here we summarise our current understanding of the evolution of the symbiotic association together with insights into the functional basis of the interaction derived from genomic analysis. Also we discuss the contribution of *Wolbachia* to inflammatory-mediated pathogenesis and adverse reactions to antifilarial drugs and describe the outcome of recent field trials using antibiotics as a promising new tool for the treatment of filarial infection and disease.

1. THE CELLULAR ENVIRONMENT OF *WOLBACHIA* IN NEMATODES

1.1. Habitat

Initial descriptions of bacterial-like structures using electron microscopy and more recent studies by immuno-histology have provided a comprehensive description of the distribution of *Wolbachia* in nematode tissues (McLaren *et al*., 1975; Kozek, 1977; Kozek and Figueroa, 1977; Taylor *et al*., 1999; Peixoto *et al*., 2001; Hoerauf *et al*., 2003a; Kramer *et al*., 2003; McGarry *et al*., 2004). They are found throughout all the stages of the life cycle of the nematode although they occur in varying proportions between individual worms and different developmental stages (Kozek, 1977; Kozek and Figueroa, 1977; McGarry *et al*., 2004). In adult nematodes, *Wolbachia* is predominantly found throughout the hypodermal cells of the lateral cords (Figure 1). The bacteria occur within host-derived vacuoles in variously sized discrete groups ranging from a few organisms, often

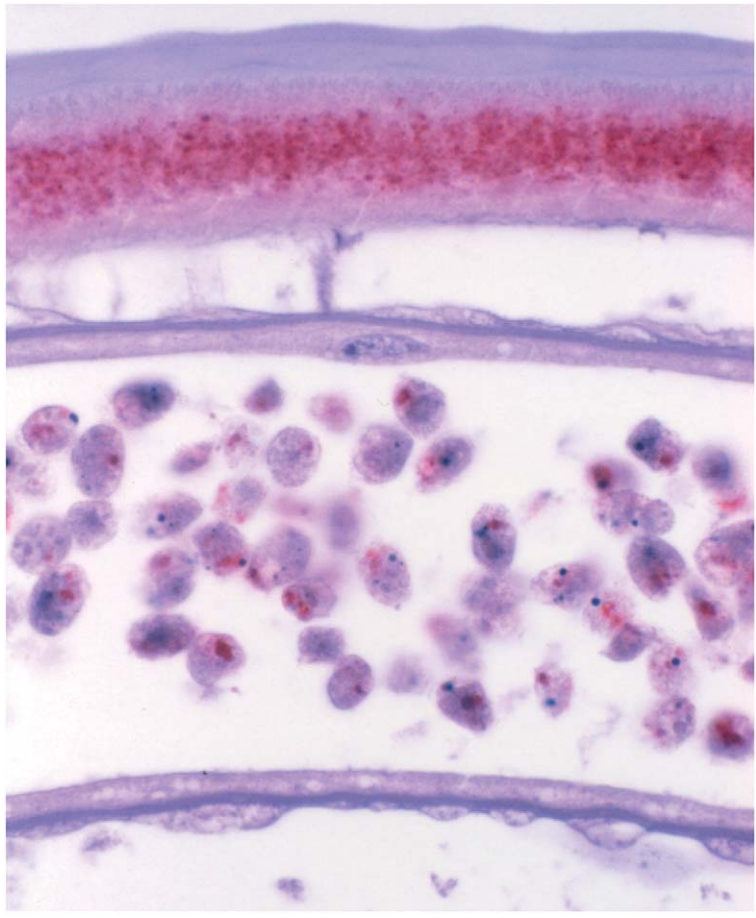

Figure 1 *Wolbachia* (stained red) distributed throughout the lateral hypodermal cord cells and embryos within the uterus of *Onchocerca volvulus*. (Image courtesy of D.W. Büttner.)

clustered around hypodermal nuclei, to areas where they almost completely fill the cellular environment reminiscent of bacteriocyte-like structures. In females, *Wolbachia* is also present in the ovaries, oocytes and developing embryonic stages within the uteri, whereas they have not been demonstrated in the male reproductive system (Taylor and Hoerauf, 1999; Sacchi *et al.*, 2002; Kozek, 2005). This suggests that the bacterium is vertically transmitted through the cytoplasm of the egg and not through the sperm (Kozek, 1977; Kozek

and Figueroa, 1977; Taylor *et al*., 1999). The concomitant phylogeny of *Wolbachia* with that of the host filariae (Casiraghi *et al*., 2001) provides indirect evidence that transmission has been at least predominantly vertical.

1.2. Growth and Population Dynamics

We know little of the mechanisms of reproduction and growth of the bacteria but structural observations and studies on the population dynamics of the bacteria within different developmental stages of the nematode have unravelled a more complex pattern of cell division at the individual and population level (McGarry *et al*., 2004; Kozek, 2005). The bacterial cells are plieomorphic and range in size from 0.2 to 4 μm in length (Kozek, 2005). In addition to the typical binary fission commonly used by bacteria, an alternative more complex cycle has been suggested in the form of small dense coccoid stages, which appear to form in the parent organism through condensation of the cytosol with features similar to the formation of elemental bodies in the Chlamydiae (Kozek, 2005). At the population level, quantification of bacterial numbers in different developmental stages has been studied in *Brugia malayi* (McGarry *et al*., 2004; Fenn and Blaxter, 2004). The numbers of bacteria remain static in microfilariae and the mosquito-borne larval stages (L2 and L3), with the lowest ratios of *Wolbachia*/nematode DNA. However, within the first week of infection of the mammalian host, bacteria numbers increase dramatically and the bacteria/worm ratio is the highest of all life-cycle stages. The rapid multiplication continues throughout L4 development, so that the major period of bacterial population growth occurs within the first month of infection of the definitive host. Microscopy confirms that there are few bacteria in mosquito-derived L3 but many, in large groups, in L4 collected 1–3 weeks after infection. It appears that the large clusters of bacteria observed throughout the hypodermal cord of adult worms originate from this rapid period of division, which thereafter are maintained at that level as demonstrated in adult male worms up to 15 months of age. In females, bacterial numbers increase further as the worms mature and as the ovary and embryonic larval

stages become infected (McGarry *et al.*, 2004). Further studies on the dynamics of population levels in other filarial species are going on and should serve to further define the key features of the symbiotic association. Individual worms appear to vary widely in their bacterial load, which may reflect a dynamic change of population size over time or if constant the potential for a selective advantage in terms of longevity or fecundity in worms carrying more bacteria. A recent study comparing the different 'forest' and 'savanna' strains of *O. volvulus* of West Africa found a significantly greater ratio of *Wolbachia* DNA to nuclear DNA in the severe, ocular disease causing 'savanna' strain, supporting the role of the bacteria in the pathogenesis of ocular onchocerciasis (Higazi *et al.*, 2005).

One might predict that an obligate symbiont that provides a continuous essential resource for its host would show a constant regulated population. The changes in the dynamics of *Wolbachia* populations throughout the life cycle may therefore illustrate the points at which the symbiotic relationship is critical. The rapid increase in bacterial numbers during the period of larval and embryonic development is consistent with a role for the bacteria in these processes, two processes compromised by antibiotic treatment. The pattern of population growth would also be compatible with a role in evasion of mammalian immunity and for the long-term survival of adult worms. It also can explain the differential activity of bacteriostatic antibiotic treatment on distinct developmental stages, in which larval and embryonic development are associated with rapidly dividing bacteria and are affected soon after antibiotic treatment, whereas the more slowly dividing populations in adults take longer to deplete and for the consequences to show. This is a feature observed following doxycycline treatment of *Wuchereria bancrofti* (see Section 3.3).

2. TAXONOMY AND DIVERSITY OF THE GENUS *WOLBACHIA*

The genus *Wolbachia* includes a group of intracellular bacteria found in arthropods (insects, spiders, mites and crustaceans) and in filarial nematodes (Werren, 1997; Taylor and Hoerauf, 1999; Bandi *et al.*,

2001a, b; Stevens *et al*., 2001). This genus belongs to the family Anaplasmataceae in the order Rickettsiales (alphaproteobacteria). Presently there is a single valid species in the genus *Wolbachia*, i.e. *Wolbachia pipientis*. The other species previously assigned to this genus (*W. persica* and *W. pipentis*) do not belong to the alphaproteobacteria and should be removed from the genus *Wolbachia* (Dumler *et al*., 2001; La Scola *et al*., 2005).

Despite the existence of a single valid *Wolbachia* species, this genus encompasses a wide variety of molecular diversity. Based on the analysis of different genes (16S rDNA; ftsZ; dnaA; wsp), six main phylogenetic lineages of *Wolbachia* have been described (Lo *et al*., 2002; Casiraghi *et al*., 2003), which are provisionally indicated as supergroups A–F (Figure 2). Supergroups A and B include most of

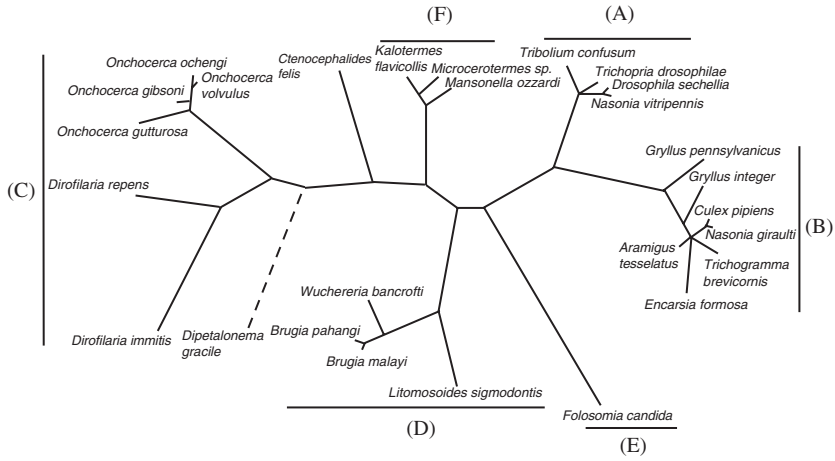

Figure 2 *Wolbachia* phylogeny based on ftsZ gene sequences. The unrooted tree was obtained using the maximum likelihood (ML) approach. Species names at the nodes are those of the host species. Representatives of *Wolbachia* supergroups A–F are shown (Lo *et al*., 2002). In addition, *Wolbachia* from a flea (*Ctenocephalides felis*) and from a recently examined filarial species (*Dipetalonema gracile*) are included, since these bacteria cannot be assigned to the above supergroups (Fischer *et al*., 2002a; Casiraghi *et al*., 2004). The branch leading to *Wolbachia* from *D. gracile* is broken, since the ftsZ gene sequence is not available for this bacterium (the positioning is based on Casiraghi *et al*., (2004) and on unpublished GroEL gene sequences; M. Casiraghi, pers. comm.).

the wolbachiae thus far detected in arthropods, while supergroups C and D include most of those found in filarial nematodes. The remaining two supergroups are less well known. Supergroup E encompasses wolbachiae from collembola; supergroup F includes those found in termites and in the filarial nematode *Mansonella ozzardi*. In addition, unpublished results indicate that further main lineages of *Wolbachia* may be described in the future, collecting wolbachiae found in both arthropods (e.g. some flea species) and filarial nematodes (M. Casiraghi *et al*., unpublished).

Which taxonomic status should be attributed to the above supergroups of *Wolbachia*? Bacterial strains showing over 3% nucleotide differences in their 16S rDNA genes are frequently assigned to different species (Stackebrandt and Goebel, 1994). The level of divergence between the six main supergroups of *Wolbachia* is around 3%. This might suggest that the six main lineages could be elevated at the species rank. However, the overall molecular diversity observed in the genus *Wolbachia* is probably still to be uncovered. The overall phylogeny of *Wolbachia* that currently appears as a tree with six main branches might become saturated in the future as a result of the detection and characterization of new *Wolbachia* lines found in new host species. Should *Wolbachia* tree become a bush, definition of the species boundaries might become more difficult. Based on this reason and in the absence of a firm reconstruction of the branching order of the main lineages, there is a general consensus of the *Wolbachia* community to maintain a single species name (Bandi *et al*., 2003), until new data are generated in different research areas (comparative genomics; molecular phylogenetics; screening for *Wolbachia* in new hosts, etc.).

Besides the issue of whether the different lineages of *Wolbachia* should be elevated to the species rank or maintained in the same species, the divergences between these lineages are certainly not negligible. For example, at the 16S rDNA level, the *Wolbachia* found in the mosquito *Culex pipiens* (supergroup B) and in the filarial nematodes *W. bancrofti* (supergroup C) are more different than rickettsiae assigned to different species (e.g. *Rickettsia ricketsii* vs *R. conorii*; *R. prowazekii* vs *R. ricketsii*). It should also be noted that, based on the rates of molecular evolution estimated for bacteria, the

evolutionary separation between the main lineages of *Wolbachia* may have occurred 50–100 million years ago (Werren *et al*., 1995a; Bandi *et al*., 1998).

2.1. *Wolbachia* in Arthropods

Wolbachia has been detected in most of the insect orders thus far screened, as well as in a variety of other arthropods (mites, crustaceans, spiders). Particularly in insects this bacterium appears widespread, with estimated proportions of infected species ranging from ~20 to ~75% (Werren *et al*., 1995b; Jeyaprakash and Hoy, 2000). These estimates have been obtained through polymerase chain reaction (PCR) screening of field-collected insect specimens, and phenotypic effects associated with the presence of *Wolbachia* had usually not been determined in the context of these prevalence studies. However, in most of the host–*Wolbachia* system thus far characterized, the presence of this bacterium is usually associated with various kinds of alterations of the host reproduction: parthenogenesis, killing of male embryos, feminization of genetic males and cytoplasmic incompatibility (CI) (Stouthamer *et al*., 1999). In addition, there is a strain of *Wolbachia*, which appears needed for host reproduction (Dedeine *et al*., 2001), and another one whose behaviour is more similar to that of "traditional" pathogens, with invasion of the host tissues and, eventually, the death of the host (Min and Benzer, 1997). The kind and strength of effect that *Wolbachia* will determine on the host is linked to the genotypes of the bacterium and the host, on the density of the bacteria and on the interaction between the different strains coinfecting a given host (Bordenstein *et al*., 2003b; Ikeda *et al*., 2003).

2.2. *Wolbachia* in Nematodes

Before we discuss the distribution of *Wolbachia* among filarial species, we should briefly summarize the classification of these nematodes. Filarial nematodes belong to the order Spirurida. The common names "filariae" and "filarial nematodes" are generally used to refer to a coherent group of spirurid nematodes that Anderson and Bain (1976)

have collected into the superfamily Filarioidea, composed of the families Filariidae and Onchocercidae. These families encompass, respectively, two and eight subfamilies (Filarinae and Stephanofilarinae; Waltonellinae, Setariinae, Oswaldofilariinae, Icosiellinae, Splendidofilariinae, Lemdaninae, Onchocercinae and Dirofilariinae). Within the order Spirurida, the groups that appear more closely related to the Filarioidea are the Thelaziodiea and Habronematoidea (Anderson, 2000). The relationships among the 10 subfamilies of the Filarioidea are not firmly established, even though there is some evidence for a deep branching of the Filariidae (Anderson, 2000; Casiraghi *et al.*, 2004). Within the Onchocercidae, the lineages leading to the Setarinae, and Waltonellinae appear deep (Figure 3), while the evolutionary radiation of the subfamilies Onchocercinae and Dirofilariinae might have occurred after the splits of these two lineages (Casiraghi *et al.*, 2004). It must be emphasized that the Onchocercinae and Dirofilariinae do not form monophyletic lineages. The genera in these subfamilies are frequently intermixed, and might be collected into a single subfamily (Casiraghi *et al.*, 2004). It is also important to note that all the filariae that cause known diseases in humans are members of these two subfamilies.

Based on the data thus far obtained, the presence of *Wolbachia* in filarial nematodes appears restricted to the subfamilies Onchocercinae and Dirofilariinae, with 16 positive species out of the 21 examined (Table 1). Even though only a limited number of specimens and species have been examined for the remaining subfamilies of filarial nematodes and for the closely related superfamily Thelazioidea, these groups have consistently been found negative. Within the Onchocercinae and Dirofilariinae, there are both positive and negative species. Negative species are thus both outside the group encompassing the positive ones, as well as inside this group. Based on the available information, two different scenarios can be hypothesized: (1) the association between *Wolbachia* and filarial nematodes was established after a single infection along the lineage leading to the Onchocercinae/Dirofilariinae, and current negative species in these subfamilies are the results of secondary losses; (2) the association between *Wolbachia* and filarial nematodes was established several times along various lineages of the Onchocercinae/Dirofilariinae; in

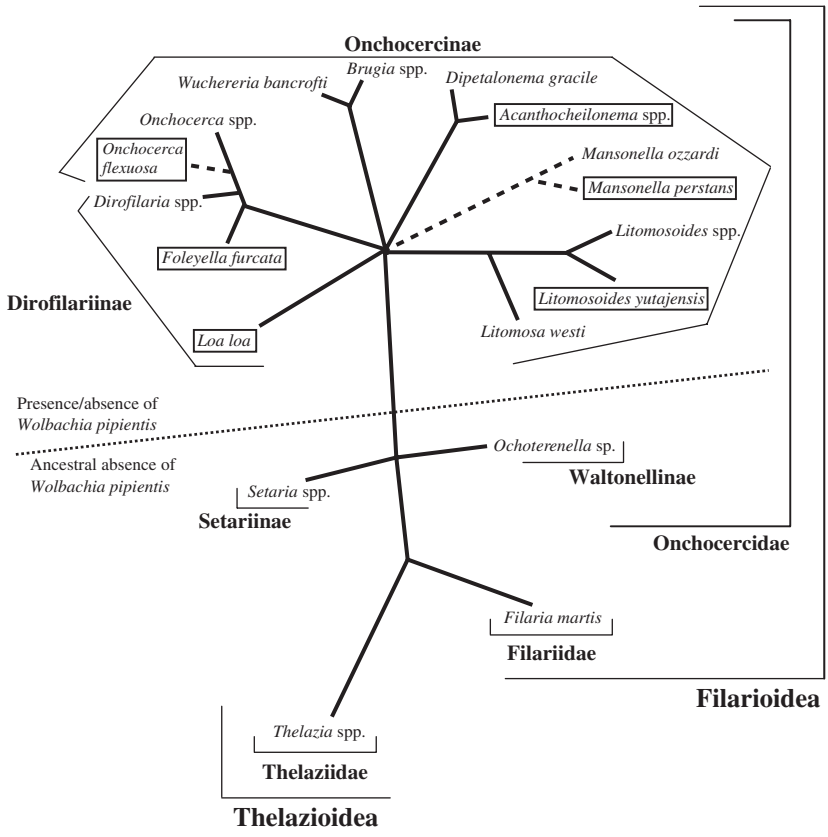

Figure 3 A hypothetical scenario of the evolution of *Wolbachia* infection in filarial nematodes. The tree represents the phylogeny of filarial nematodes (Filarioidea) reconstructed in Casiraghi *et al*. (2004). *Wolbachia* could have been ancestrally absent in the sister groups of filarial nematodes (represented in the tree by *Thelazia* spp.) and in the deep-branching filarial lineage Filariidae. Within the family Onchocercidae, *Wolbachia* could also have been ancestrally absent in the lineages Setariinae and Waltonellinae. During the evolution of the family Onchocercidae, *Wolbachia* could have been acquired on the lineage leading to the subfamilies Onchocercinae and Dirofilariinae, and then lost along the lineages leading to *Acanthocheilonema* spp., *Loa loa*, *Onchocerca flexuosa*, *Litomosoides yutajensis*, *Mansonella perstans* (outlined in boxes). The positions of *Mansonella* spp. and *Onchocerca flexuosa* are based only on their taxonomic affiliations and are thus indicated by dashed lines (gene sequence from these species have not yet been used in phylogenetic analyses). Classification of filarial nematodes is based on Anderson and Bain (1976) and Bain *et al*. (1982). (Reproduced from Casiraghi *et al*., 2004.)

Table 1 Presence or absence of *Wolbachia* from filarial nematodes

Present[a]	Absent
Brugia malayi[1,2]	*Acanthocheilonema viteae*[1,6]
Brugia pahangi[1,2]	*Acanthocheilonema reconditum*[4]
Brugia timori[3]	*Filaria martis*[4]
Dipetalonema gracile[4]	*Foleyella furcata*[4]
Dirofilaria immitis[5]	*Litomosoides yutajensis*[4]
Dirofilaria repens[5]	*Loa loa*[4,11–13]
Litomosa westi[4]	*Mansonella perstans*[12]
Litomosoides sigmodontis[1,6]	*Ochoterenella sp.*[4]
Litomosoides brasileiensis[4]	*Onchocerca flexuosa*[8,14]
Litomosoides galizai[4]	*Setaria equina*,[4,15]
Litomosoides hamletti[4]	*Setaria labiatopapillosa*[4]
Mansonella ozzardi[7]	*Setaria tundra*[4]
Onchocerca volvulus[8]	
Onchocerca ochengi[1]	
Onchocerca gutturosa[1]	
Onchocerca gibsoni[1]	
Onchocerca lupi[9]	
Onchocerca cervicalis[10]	
Wuchereria bancrofti[1,2]	

Source: 1, Bandi *et al*. (1998); 2, Taylor *et al*. (1999); 3, Fischer *et al*. (2002b); 4, Casiraghi *et al*. (2004); 5, Sironi *et al*. (1995); 6, Hoerauf *et al*. (1999); 7, Casiraghi *et al*. (2001); 8, Henkle-Dührsen *et al*. (1998); 9, Egyed *et al*. (2002); 10, GeneBank accession AY095210; 11, Büttner *et al*. (2003) 12, Grobusch *et al*. (2003); 13, McGarry *et al*. (2003); 14, Plenge-Bönig *et al*. (1995) 15, Chirgwin *et al*. (2002).

[a]The species listed as positive for *Wolbachia* are those for which positive PCR and gene sequence data have been obtained (see references below); in addition, there is microscopical evidence (electron microscopy and/or immunohistochemical staining) that further filarial species of the genus *Onchocerca* harbour *Wolbachia*: *O. armillata*, *O. dukei, O. fasciata*, *O. tarsicola*, *O. jakutensis, O. lienalis* (Franz *et al*., 1987; Franz and Copeman 1988; Plenge-Bönig *et al*., 1995; Determann *et al*., 1997; Henkle-Dührsen *et al*., 1998; M. J. Taylor and D.W. Büttner, unpublished).

this case, negative species in this subfamilies could represent either a primitive absence of the symbiosis or the effect of a secondary loss. Even though the available information does not allow one to choose one of the two scenarios, the close relationship of some positive and negative species in the Onchocercinae/Dirofilariinae supports the hypothesis that at least in a few occasions the association with *Wolbachia* was lost during evolution. Outside the subfamily Onchocercinae/Dirofilariinae, there are no other nematodes in which *Wolbachia* has thus far been found (e.g. Bordenstein *et al*., 2003a). The results of only one screening for *Wolbachia* in nematodes outside

the Spirurida has been published, but the examination of non-filarid nematode species has been performed in several laboratories with no evidence for the presence of *Wolbachia* (C. Bandi, unpublished; T.J.C. Anderson, pers. comm.; G. Favia, pers. comm.).

The apparent singularity of the Onchocercinae and Dirofilariinae among all the other nematodes, i.e. the high prevalence of species infected by *Wolbachia*, seems to support the hypothesis of a single acquisition of the association with this bacterium. However, phylogenetic analysis splits the *Wolbachia* of filarial nematodes into three different supergroup, C, D and F (Figure 1). The branching order of *Wolbachia* supergroups is still to be established; should future analyses show that the supergroups encompassing nematode *Wolbachia* form a monophyletic lineage, the hypothesis of a single acquisition would be supported; should the origin of these supergroups be polyphyletic, this hypothesis would be weakened (even though, in this case, one might also suggest a single acquisition of *Wolbachia* by filarial nematodes, followed by some events of horizontal transmission to arthropods). In this respect, there is only one result of phylogenetic analyses, which appears firmly established: the F supergroup encompasses *Wolbachia* from both filarial nematodes (i.e. *Mansonella* spp.) and arthropods (i.e. termites), and this result is supported by the analysis of all of the genes thus far examined (M. Casiraghi *et al*., unpublished). The relatively close relationship between *Wolbachia* from *Mansonella* spp. and termites suggests that a further event of horizontal transmission of *Wolbachia* between nematodes and arthropods might have occurred, in addition to the original transmission event that presumably established the association in nematodes.

2.3. Distribution and Phylogeny of *Wolbachia* in the Onchocercinae and Dirofilarinae

Among the Onchocercinae and Dirofilariinae, *Wolbachia* occur in the main agents of human and animal filariases. Examples of filariae with *Wolbachia* are *Onchocerca volvulus* (the agent of river blindness) *W. bancrofti* and *Brugia malayi* (agents of lymphatic filariasis), and *Dirofilaria immitis* (the agent of canine and feline heartworm disease).

Remarkable exceptions among the filariae of humans are *Loa loa* and *Mansonella perstans* (Brouqui *et al*., 2001; Büttner *et al*., 2003; Grobusch *et al*., 2003; McGarry *et al*., 2003). In species harbouring *Wolbachia*, the prevalence appears to be 100% (i.e. all the individuals examined have been shown to be positive). Moreover, the symbiosis appears stable along evolutionary times: main branches of filarial evolution are composed of species harbouring *Wolbachia* (Casiraghi *et al*., 2001). This is shown for example by the phylogenetic lineage encompassing the genera *Onchocerca* and *Dirofilaria*, in which there is only one negative species (*Onchocerca flexuosa*) out of the numerous species thus far examined (Casiraghi *et al*., 2004). It is also noteworthy that there are no indications for multiple infection of a single host, or for the presence of different ''types'' of *Wolbachia* in a given filarial species. In summary, there is overall evidence that *Wolbachia* symbiosis with filarial nematodes has been stable and species-specific for long evolutionary periods, while there is evidence for only sporadic losses of the symbiosis during the evolution of a few species.

With few exceptions, the distribution and phylogenetic patterns of *Wolbachia* infection in arthropods is in general remarkably different from the one observed in filarial nematodes. In general, not all populations within a species, and not all individuals within a population are infected by *Wolbachia*. In addition, at the high taxonomic level, the phylogeny of *Wolbachia* usually does not match that of the host species. There are indeed several examples of distantly related insect species whose wolbachiae are closely related. In addition, several studies have provided evidence for the horizontal transmission of *Wolbachia* between insect species. Multiple *Wolbachia* infections are also known to occur in insects. The distribution and phylogeny of *Wolbachia* in arthropods thus indicate that the infection is less species-specific than in nematodes, with frequent events of horizontal transmission. Finally, in arthropod *Wolbachia* there is strong evidence for the occurrence of genetic recombination (Werren and Bartos, 2001; Jiggins *et al*., 2001). The pattern is again different in nematode *Wolbachia*, in which genetic recombination does not appear to occur (Jiggins, 2002; Casiraghi *et al*., 2003). An absence of recombination among strains of symbiotic bacteria might be a

consequence of an absence of horizontal transmission and multiple infections. Under this perspective, there is an overall consistency of the information available about the population structure of *Wolbachia* in nematodes.

3. EVIDENCE FOR DEPENDENCE

3.1. Indirect Evidence

Vertical transmission can lead to the establishment of different kinds of host–symbiont relationships, or specializations (Werren and O'Neill, 1997; Bandi *et al*., 2001a; Dedeine *et al*., 2003). An obvious pathway is towards mutualistic symbiosis: the symbiont will increase its own fitness by increasing the fitness of the host that is involved in its transmission. Another possible outcome is to become a reproductive parasite: by manipulating host reproduction, the symbiont can reduce the fitness of those members of the host species, which are not involved in its transmission. There is however no intrinsic conflict between mutualistic symbiosis and reproductive parasitism: for example, a maternally inherited microorganism could be beneficial towards females (the host sex, which is responsible for transmission to the offspring) while being detrimental towards males (which are not involved in transmission). In arthropods, *Wolbachia* appears in most of the known cases to act as a reproductive parasite. There are however examples of arthropods in which *Wolbachia* is needed for host reproduction (i.e. in the parasitic wasp *Asobara tabida*; Dedeine *et al*., 2001) and where reproductive parasitism and mutualism coexist (i.e. in the mosquito *Aedes albopictus*, where a CI-inducing *Wolbachia* strain also increases female fecundity; Dobson *et al*., 2002). Which kind of role could we expect for *Wolbachia* in filarial nematodes? In this section we will discuss the indirect evidence, which suggests that the association between *Wolbachia* and filarial nematodes is obligatory.

There are three main aspects in which nematode and arthropod *Wolbachia* appear different, suggesting that this bacterium in the two hosts should have adopted different evolutionary strategies: (1) the

different rate of horizontal transmission, (2) the efficiency of vertical transmission and (3) the prevalence of multiple infection. In general, the pattern observed in arthropods indicates that the association is less stable and species-specific than in nematodes, with evidence for horizontal transmission, multiple infection and recombination between strains. The patchy distribution of *Wolbachia* in arthropods also suggests that losses of the association with this bacterium could be more frequent than in nematodes.

In nematodes, the distribution and phylogenetic patterns of *Wolbachia* appear more similar to those generally observed in obligatory symbionts (100% prevalence; consistency of host–symbiont phylogenies; main phylogenetic branches in which the association is observed in all—or almost all—species; no evidence for multiple infections). It must be highlighted that current hypotheses on the evolution of virulence of microorganisms suggest that multiple infections hamper the evolution of obligatory/mutualistic symbiosis (mainly for the competition for the host resource among unrelated strains/clones), while a strict vertical symbiont transmission, with no chances for multiple infections, should lead to virulence reduction *via* kin-selection (Herre *et al*., 1999). In conclusion, in addition to the above resemblances between nematode *Wolbachia* and the most well-known obligatory bacterial symbionts of insects (e.g. *Buchnera* in aphids and *Blattabacterium* in cockroaches; Baumann *et al*., 1995 and Lo *et al*., 2003), also from the theoretical point of view the association between *Wolbachia* and filarial nematodes might be expected to be obligatory or, at least, not parasitic.

Gene sequence analysis has provided a further indication that the association between *Wolbachia* and filarial nematodes is not parasitic. A comparison of the rates of nucleotide substitutions of the *Wolbachia* surface protein (WSP) has evidenced an excess of non-synonymous substitutions in the symbiont of arthropods (Baldo *et al*., 2002; Jiggins *et al*., 2002). Selective pressures for variation of symbiont proteins interacting with the host cells/tissues/fluids are expected to occur in those cases in which the symbiont has some detrimental effect on the host (i.e. it is a parasite), and indicate that the host and the symbiont are engaged in an arms race. It is intriguing that no bias towards non-synonymous substitutions is observed in

nematode WSP. This lack of evidence for an arms race in nematode *Wolbachia* seems to suggest that the association with the host is more "peaceful" than in arthropods. According to some authors this might also be suggestive of a mutualistic interaction (Jiggins *et al*., 2002; Baldo *et al*., 2002).

3.2. Direct Evidence

As discussed above, the information available on the distribution, phylogeny and molecular evolution of *Wolbachia* suggests that filarial nematodes and their endosymbionts are reciprocally dependent. Experimental work using antibiotics have provided direct evidence for the existence of this dependence. Indeed, tetracycline and derivates, which are effective against bacteria of the order Rickettsiales, have been shown to have detrimental effects on filarial nematodes, which harbour *Wolbachia*, and no effects on the *Wolbachia*-free filaria *Acanthocheilonema viteae* (Hoerauf *et al*., 1999; McCall *et al*., 1999). Papers suggesting that tetracycline might have anti-filarial activity have been published earlier (Bosshardt *et al*., 1993), even though the possibility that these anti-filarial properties were mediated by effects on *Wolbachia* has been discussed only recently (Genchi *et al*., 1998; Bandi *et al*., 1999; Hoerauf *et al*., 1999, 2003a; McCall *et al*., 1999; Langworthy *et al*., 2000; Taylor and Hoerauf, 2001). More recently, other antibiotics with anti-rickettsial properties have been demonstrated to also have anti-filarial properties (see Table 2 and Section 3.3).

Various antibiotics have been tested for their anti-filarial activity, both *in vivo* and *in vitro*. The studies have involved various filarial species and hosts, with different effects depending mainly on the developmental stage of the filaria (Table 2). In most cases, the effects are sublethal with inhibition of embryogenesis and infertility (*Brugia pahangi*, *Dirofilaria immitis*, *Litomosoides sigmodontis*, *Onchocerca volvulus*—Bosshardt *et al*., 1993; Genchi *et al*., 1998; Bandi *et al*., 1999; Hoerauf *et al*., 1999, 2000a, b; Townson *et al*., 2000), inhibition of third-stage larval development and *in vivo* prophylaxis (*B. pahangi*, *L. sigmodontis* —Bosshardt *et al*., 1993; Hoerauf *et al*., 1999; McCall

Table 2 The effects of antibiotics on filarial nematodes

Species	Host/model	Antibiotic	Parasitological effects	Reference
Acanthocheilonema viteae[a]	Jird/*Mastomys*	Tetracycline	None	1, 2
Brugia malayi	*In vitro*	Tetracycline/ Rifampicin	Reduced motility, viability and microfilarial release Inhibits L3/L4 moult and motility	3, 4, 5
Brugia pahangi	Jird/*in vitro*	Tetracycline	Inhibits larval development, embryotoxic, prophylactic, macrofilaricidal	2, 5, 6, 7, 8, 9
	In vitro	Rifampicin	Reduced adult viability and microfilarial release, embryotoxic, macrofilaricidal	6
Dirofilaria immitis	Dog/*in vitro*	Tetracycline	Embryotoxic, inhibits L3/L4 moult and transovarial transmission	5, 7
Litomosoides sigmodontis	Mouse/Cotton rat	Tetracycline/ Rifampicin	Inhibits larval development Infertility and stunted growth	1, 10
Onchocerca gutturosa	*In vitro*	Tetracycline/ Rifampicin	Reduced motility and viability	6
Onchocerca lienalis	Mouse	Tetracycline/ Rifampicin	Reduced microfiladermia	6
Onchocerca ochengi	Cow	Oxytetracycline	Embryotoxic, macrofilaricidal	11
Onchocerca volvulus	Human	Doxycycline	Embryotoxic, sustained amicrofiladermia following ivermectin treatment	12
Wuchereria bancrofti	Human	Doxycycline	Amicrofilaremia, macrofilaricidal	13

Source: 1, Hoerauf *et al.* (1999); 2, McCall *et al.* (1999); 3, Rao and Weil (2002); 4, Rao *et al.* (2002); 5, Smith and Rajan (2000); 6, Townson *et al.* (2000); 7, Bandi *et al.* (1999); 8, Casiraghi *et al.* (2002); 9, Chirgwin *et al.* (2003a, b); 10, Volkmann *et al.* (2003); 11, Langworthy *et al.* (2000); 12, Hoerauf *et al.* (2000a, b, 2003a); 13, Taylor *et al.* (2005).

[a]Free of *Wolbachia*.

et al., 1999), stunting of adult worm growth (*L. sigmodontis*—Hoerauf *et al*., 1999). However, in *Onchocerca ochengi*-infected cattle, protracted treatment has been shown to be macrofilaricidal (Langworthy *et al*., 2000) and shorter term treatment of humans infected with *O. volvulus* showed a trend towards frequent degeneration or death of adult worms (Hoerauf *et al*., 2003a). Recent data from trials with *W. bancrofti*, infected humans also provide evidence for a strong macrofilaricidal effect (Taylor *et al*., 2005).

There is general agreement that the anti-filarial effects of antibiotic therapy are a result of activity against *Wolbachia* because antibiotics have no effect on the *Wolbachia*-negative filaria *A. viteae* (Hoerauf *et al*., 1999; McCall *et al*., 1999) and because the anti-bacterial effect precedes the anti-filarial effects (Langworthy *et al*., 2000; Hoerauf *et al*., 2003a). Interestingly, a recent study found that irradiation of *B. malayi* leads to reductions in bacterial populations with dose-dependent effects on worm motility, viability and arrested development of embryogenesis in a similar manner to treatment with antibiotics, potentially attributing the effects of irradiation to the loss of the endosymbionts (Rao *et al*., 2005). Other studies have suggested that a chemically modified tetracycline maintains the capacity of blocking the L3–L4 moult in *B. malayi* without apparent depletion of *Wolbachia* (Rajan, 2004). However, the activity of this modified compound against *Wolbachia* was monitored only with non-quantitative PCR (other studies have shown that real time PCR has to be used to assess the extent of reduction of *Wolbachia*, Hoerauf *et al*., 2003b). If this compound did indeed not show activity against *Wolbachia*, it may indicate that the *in vitro* moulting assay may also be sensitive to effects by tetracyclines that are independent from their activity against *Wolbachia*, as the duration of treatment (<2 weeks) is too short to reduce *Wolbachia* beyond the threshold where they become undetectable by immunohistology, and are reduced by $>95\%$ before anti-parasitic effects occur (Hoerauf *et al*., 2003a, b). Alternatively, the activity may inhibit *Wolbachia* dependent processes (e.g. through inhibition of protein synthesis), which occur independently of the depletion of bacteria. Further studies using aposymbiotic species and more precise measures of bacterial viability would be required to address these issues. In general, the activity of antibiotics

against filarial nematodes *in vitro* often appear to induce effects more rapidly than can be achieved *in vivo* (e.g. Townson *et al.*, 2000; Rao and Weil, 2002; Rao *et al.*, 2002).

3.3. Antibiotic Therapy as a New Treatment for Human Filariasis

Initial trials using a six-week course of doxycycline treatment against *O. volvulus* were effective at depleting the bacteria and resulted in a block of embryogenesis, which persists for up to two years after the start of treatment. The apparent permanent block in embryogenesis was reflected in sustained reductions in skin microfilariae, the cause of onchocercal disease (Hoerauf *et al.*, 2000a, 2001, 2003a). Depletion of *Wolbachia* by doxycycline has also been demonstrated in human lymphatic filariasis patients infected with *W. bancrofti*. Doxycycline administered for 6 weeks at 200 mg/day resulted in a reduction of $>95\%$ of *Wolbachia* levels, assessed from blood microfilariae, compared to pre-treatment levels (Hoerauf *et al.*, 2003b). This treatment led to a chronic decline in microfilarial loads, followed by an amicrofilaremia, which was highly significant at 12 months (Hoerauf *et al.*, 2003b) and sustained for almost 2 years (A. Hoerauf *et al.*, unpublished), in contrast to the control treatment with ivermectin. Although direct proof is lacking owing to the unavailability of adult worms, these data suggest that the mode of action of doxycycline is equivalent to that observed in animal models and human onchocerciasis, namely a block in embryogenesis in the adult female worms when *Wolbachia* are absent or at least below a certain threshold.

Importantly, a recent placebo-controlled trial in humans infected with *W. bancrofti* has demonstrated a clear macrofilaricidal effect of doxycycline (Taylor *et al.*, 2005). When administered for 8 weeks at 200 mg/day, the treatment resulted in a complete amicrofilaremia in 28/32 patients assessed and a lack of scrotal worm nests as determined by ultrasonography in 21/27 patients (also known as 'filarial dance sign', Dreyer *et al.*, 1994; Mand *et al.*, 2003). In the other patients, the number of worm nests declined. This was significantly

different from placebo patients where lack of worm nests was only observed in 3/27. Since published (Dreyer *et al*., 2002a, b) and unpublished (S. Mand and A. Hoerauf) data demonstrate that scrotal worm nests are stable over time and only a small number of male patients do not show scrotal worm nests, the lack of worm nests in doxycycline-treated patients are highly unlikely to be due to a spontaneous loss. The observed loss of worm nests, suggesting death of adult worms, was corroborated by a significant decline in circulating filarial antigen levels as a second measure of adult worm loss in doxycycline-treated individuals compared to no change in placebo groups (Taylor *et al*., 2005).

Loss of worm nests was observed at 14 months after the onset of treatment in this study, and this fits well with the fact that circulating filarial antigen levels continuously declined over this time. This suggests that macrofilaricidal effects induced by doxycycline need more than a year to manifest. This is underscored also by the above-mentioned study of 6-week doxycycline treatment, where we failed to observe a significant loss of worm nests after 12 months, but could easily detect it after 18 and 22 months, accompanied by stronger reduction in circulating filarial antigen levels and lymphatic vessel dilation (A. Hoerauf *et al*., unpublished). New, placebo-controlled studies where doxycycline was administered for 6 weeks at 200 mg/day confirm the above data and prove for the first time by a longitudinal observation using ultrasonography that worm nests that were detected pre-treatment disappeared by 18 months after doxycycline, while nests in placebo patients remain stable (S. Mand and A. Hoerauf *et al*., unpublished observation). Again, this was corroborated by an even higher reduction in circulating filarial antigen levels after 24 months. A three-week course of doxycycline also abolishes microfilaremia for over a year and reduced the severity of adverse reactions to ivermectin and albendazole, but failed to show macrofilaricidal effects even after 24 months (J. Turner *et al*., unpublished observation). Since real-time PCR showed a higher residual *Wolbachia* gene copy number per microfilaria than did the 6 weeks course, the data suggest that embryogenesis may be blocked at a lower degree of *Wolbachia* reduction than required for macrofilaricidal effects.

The results obtained from human trials so far have unequivocally demonstrated the superior efficacy of doxycycline both for onchocerciasis and lymphatic filariasis. The major difficulty at present is the required length of treatment, as well as the known contra-indications of doxycycline (not to be given to children <9 years of age and pregnant or breast-feeding women), which makes this approach currently unsuitable for mass treatment. Nevertheless, the trial outcomes argue clearly for the use of doxycycline for selected indications including (i) treatment of individuals that leave an endemic area for a long period, because the filarial stages that are causative for pathology (microfilariae in onchocerciasis, adult worms in bancroftian filariasis) will be depleted (onchocerciasis) or at least dramatically reduced (bancroftian filariasis); and (ii) hyperreactivity to microfilariae, as observed in the 'Sowda' form of onchocerciasis and in tropical pulmonary eosinophilia, where the re-appearance of microfilariae following microfilaricidal therapy with ivermectin or diethylcarbamazine (DEC), respectively, is particularly undesirable.

More indications are likely to appear on completion of current trials. Studies are underway in Indonesia that indicate a similar success of doxycycline against brugian filariasis, as predicted from animal models and for the treatment of onchocerciasis in populations co-infected with *Loa loa* currently excluded from mass drug administration due to rare encephalopathy reactions to ivermectin. In addition, our pilot studies have shown a beneficial effect on lymphatic pathology in bancroftian filariasis: thus, supratesticular lymphoceles induced by the adult worms have shown to be significantly reduced in size (A. Hoerauf and M.J. Taylor, unpublished), and lymphedema patients treated with doxycycline showed reduced degrees of their pathologies (grading according to Dreyer *et al*., 2002a, b) compared to placebo patients who were only supervised to perform standard lymphedema management (Dreyer *et al*., 2002). Currently, larger studies are underway to add proof to these preliminary findings.

Finally, a growing concern is the apparent existence of *O. volvulus* worms that show a "suboptimal response" to ivermectin (Awadzi *et al*., 2004a, b). While a clear resistance mechanism has not yet been established, there is plenty of evidence for resistance of gastro-intestinal helminths of livestock against ivermectin (Wolstenholme *et al*.,

2004). Treatment with ivermectin is known to cause a loss of polymorphism at certain loci of the β-tubulin, γ-aminobutyric-acid-receptor, glutamate-gated chloride channel and ATP-binding-cassette (ABC) transporter genes of ivermectin-resistant *Haemonchus contortus*. Intriguingly, a loss of polymorphisms at some of these loci, notably ABC transporter genes, P-glycoprotein and β-tubulin, has also been observed after treatment of *O. volvulus* with ivermectin (Ardelli and Prichard, 2004; Eng and Prichard, 2005). If resistance to *O. volvulus* did indeed occur during the envisaged 30 year + mass treatment for onchocerciasis, there is currently no real alternative drug to use, given that suramin is not an option in a field setting and DEC has known contra-indications (Awadzi, 2003). Since new developments may take up to 10 years from the first high-throughput screens, waiting for short-term breakthroughs in this field is highly unrealistic. Thus, while no one hopes that this scenario will happen, it cannot be excluded that one may have to consider early application of an anti-wolbachial treatment against the spread of resistance. Again, studies are being planned to formally prove that anti-wolbachial treatment is an option for worms with a suboptimal response to ivermectin. In the context of lymphatic filariasis, similar concerns exist not only for ivermectin resistance but also for albendazole and DEC. A recent study has identified single nucleotide polymorphisms of β-tubulin associated with benzimidazole resistance in populations of bancroftian filariasis from Ghana and Burkina Faso (Schwab *et al*., in press). The putative resistance alleles occur in ~26% in untreated populations rising to 60% after one round of treatment and 86% after two rounds, suggesting the selection for resistance in these populations.

3.4. Genomic Insights into the Nature of the Symbiosis

The recent completion of genome sequencing and annotation of the metabolic pathways of *Wolbachia* from *B. malayi* have identified important candidates for the dependency of the symbiosis (Foster *et al*., 2005). In comparison with insect *Wolbachia* and related *Rickettsia*, the genome of *Wolbachia* from *B. malayi* is drastically reduced in size,

a feature common to the lifestyle of other endosymbiotic bacteria. However, *Wolbachia* contain more intact metabolic pathways, which may be important in contributing to the welfare and fecundity of its host. The ability to provide riboflavin, flavin adenine dinucleotide (FAD), haem and nucleotides are likely to be the bacterial contribution, whereas the host nematode provides amino acids required for bacterial growth with the exception of the only amino acid synthesized by the bacteria, *meso*-diaminopimelate, a major component of peptidoglycan. The cell wall biosynthesis pathways are devoid of genes required for the biosynthesis of lipopolysaccharide (LPS) in common with the related *Wolbachia* from *Drosophila* (Wu *et al.*, 2004) and *Ehrlicha* and *Anaplasma* sp. (Lin and Rikihisa, 2003). In addition, an unusual peptidoglycan structure is suggested with some possible similarities to peptidoglycan-derived bacterial cytotoxins. Other features include a common type IV secretion system and an abundance of ankyrin domain-containing proteins, which could regulate host gene expression as suggested for *Ehrlichia phagocytophilia AnkA* (Park *et al.*, 2004). Glutathione biosynthesis genes may be a source of glutathione for the protection of the host nematode from oxidative stress or immunological effector molecules. Haem from *Wolbachia* could be vital to worm embryogenesis as there is evidence that moulting and reproduction are controlled by ecdysteroid-like hormones (Warbrick *et al.*, 1993), whose synthesis requires haem. Depletion of *Wolbachia* might therefore halt production of these hormones and block embryogenesis. Alternatively, or in addition, *Wolbachia* may be an essential source of nucleotides during embryogenesis. Thus, the completion of the *w*Bm genome offers a wealth of information, which may help to understand the molecular basis for the endosymbiosis between filarial nematodes and *Wolbachia*. We now know which metabolites might be provided by *Wolbachia* to the nematode and which are required by the endobacteria from the nematode. This may open up the exciting possibility to find and test drugs already registered for use in humans, which might inhibit key biochemical pathways in the *Wolbachia* that could lead to sterility or killing of the adult worms.

Genomic sequencing has also been useful in re-analysing the origin of a catalase gene, previously attributed to *Wolbachia* from

Onchocerca sp. (Henkle-Duhrsen *et al*., 1998), but which instead derives from pseudomonad contamination of the cDNA libraries from which it was originally cloned (Foster *et al*., 2004). The multiple effects of antibiotic depletion on the nematode and the dynamics of bacterial populations in different developmental stages suggest the worms have become dependent on the bacteria for a diverse range of biological processes and may have distinct stage-specific function. Further studies incorporating biochemical and functional genomic approaches should help unravel the role of these different metabolic pathways throughout the nematode life cycle and identify those suitable as targets for novel anti-symbiotic therapy.

4. *WOLBACHIA*-MEDIATED ACTIVATION OF INFLAMMATION

4.1. Lymphatic Filariasis

Soluble extracts of *B. malayi* adults or microfilariae induce a potent innate inflammatory response *in vitro* and *in vivo* (Taylor *et al*., 2000, Saint-André *et al*., 2002, Gillette-Ferguson *et al*., 2004). The activation of innate inflammation requires CD14 and Toll-like receptor 4 (TLR-4) pattern recognition receptors and the activity is lost following antibiotic depletion of bacteria and absent from soluble extracts derived from aposymbiotic species (*A. viteae* and *L. loa*, Taylor *et al*., 2000 and unpublished).

Inflammatory responses also occur following anti-filarial drug treatment particularly in patients with high parasite burdens. Severe adverse reactions are associated with the increase in systemic pro-inflammatory cytokines and inflammatory mediators (Haarbrink *et al*., 2000). PCR and immunoelectron microscopy analysis of plasma samples following the treatment of *B. malayi* with DEC showed the persistent presence of *Wolbachia* in patients with severe systemic inflammation (Cross *et al*., 2001). In animal models the production of TNFα following the chemotherapy of *B. malayi* microfilariae only occurred in mice with an intact TLR-4 receptor, suggesting that the release of *Wolbachia* is responsible for this inflammation (Taylor

et al., 2000). Recently, clinical trials have provided further evidence to support the role of *Wolbachia* in the presentation of adverse reactions. In patients infected with *W. bancrofti*, prior treatment with a 3-week course of doxycycline to deplete *Wolbachia* prevented moderate adverse reactions to albendazole and ivermectin, whereas in individuals in the placebo group levels of *Wolbachia* released into plasma were related to the incidence of adverse reactions, levels of plasma pro-inflammatory cytokines and pre-treatment microfilarial load (J. Turner *et al*., unpublished observation).

Further effects of TLR-4 mediated responses have been reported following infection of mice with *Litomosoides sigmodontis* (Pfarr *et al*., 2003). In C3H/HeN mice infection results in adult female worm development including females containing mature microfilariae, although free microfilariae are not detected. Infection of TLR-4 mutant C3H/HeJ mice produced worms with an increased fertility and the production of microfilariae. These observations suggest TLR-4-mediated immune responses regulate worm fertility either by inhibiting embryogenesis (a process dependent on *Wolbachia*) or direct killing of released microfilariae. Therefore, the inflammatory stimulatory activity released by dead worms is derived from endosymbiotic *Wolbachia* bacteria, rather than the nematode. This finding led to a proposed mechanism by which repeated exposure to *Wolbachia*-mediated inflammation may lead to damage of the infected lymphatics and tolerization of innate immunity, leading to susceptibility to the opportunistic infections commonly associated with lymphoedema and elephantiasis (Taylor *et al*., 2001). Indeed, experimental infections of monkeys with *B. malayi* have been reported, in which antibody responses to WSP develop prior to and throughout episodes of lymphoedema (Punkosdy *et al*., 2001). Moreover, human antibody responses to WSP are elevated in individuals with hydrocoele and lymphoedema suggesting acquired immune responses to *Wolbachia* maybe associated with the development of chronic pathology (Punkosdy *et al*., 2003). The pilot studies mentioned above in human lymphoedema patients benefiting from doxycycline treatment further underscore these findings and suggest that anti-wolbachial chemotherapy, in addition to its anti-parasitic activity, may also have a beneficial effect on pathology.

4.2. Onchocerciasis

Inflammatory activity derived from *Wolbachia* has also been demonstrated in *Onchocerca* sp. (Brattig *et al*., 2000, Saint-André *et al*., 2002). As with lymphatic filariasis, *Wolbachia* are released into the blood following anti-filarial chemotherapy, with peak DNA levels correlating with clinical reaction scores and increased levels of TNFα, neutrophils and anti-bacterial peptides (Keiser *et al*., 2002). *Wolbachia* are also responsible for the recruitment and activation of neutrophils in the granulomatous response infiltrating adult worm subcutaneous nodules and disappear when *Wolbachia* are cleared using doxycycline (Brattig *et al*., 2001; Volkmann *et al*., 2003). These studies suggest that living worms may release *Wolbachia* and/or their products, possibly from uterine debris (Kozek, 2005), which promote inflammatory responses adjacent to the worms.

Neutrophil-mediated inflammation is also a feature of ocular pathology following death of microfilariae in the cornea. In a mouse model of ocular inflammation, increases in stromal thickness and haze and neutrophil-mediated keratitis developed in response to *O. volvulus* and *B. malayi* extracts containing *Wolbachia*, but was reduced or absent when parasite extracts derived from doxycycline-treated *O. volvulus* or *Wolbachia*-free species (*A. viteae*) were used (Saint-André *et al*., 2002). The recruitment of neutrophils into the cornea was also dependent on TLR-4 together with reduced expression of platelet endothelial cell adhesion molecule (PECAM) and the chemokines macrophage inflammatory protein-2 and keratinocyte-derived chemokine (KC), suggesting their upregulation by *Wolbachia* stimulates the recruitment of neutrophils to the cornea. Further studies in this model show that microfilariae injected into the cornea become surrounded by neutrophils after 18 h (Gillette-Ferguson *et al*., 2004). Immuno-electron microscopy revealed that the major WSP was prominent within neutrophil phagolysomes and associated with activation. Moreover, whole bacteria were shown to directly stimulate purified neutrophils to secrete TNFα and chemokines. Studies in TLR-2,-4,-9 and MyD88 knockout mice showed that stromal haze and neutrophil-mediated inflammation induced by whole bacteria and soluble extracts of *O. volvulus* in wild-type mice

were abolished in the absence of MyD88 and partially dependent on TLR-2 and -4 but unaffected by the absence of TLR-9 (E. Pearlmann *et al.*, unpublished observation). Intriguingly, a recent finding that strains of *O. volvulus* responsible for severe ocular disease contain higher levels of *Wolbachia* than the mild form, which results in little ocular disease, further supports the association of *Wolbachia* with ocular pathology (Higazi *et al.*, 2005).

4.3. Dirofilariasis

Genomic sequencing has shown that *Wolbachia* lack the genes required for LPS biosynthesis (Wu *et al.*, 2004; Foster *et al.*, 2005). The search for the molecular nature of the stimulatory activity has therefore focused on other candidate molecules found on the surface membrane of the bacteria. A series of experiments using purified recombinant WSP from *D. immitis Wolbachia* have shown that it can activate innate inflammatory activity. WSP activates IL-8 transcription and stimulates chemokinesis in canine neutrophils (Bazzocchi *et al.*, 2003). Further studies showed that WSP induces pro-inflammatory cytokine production from murine macrophages and dendritic cells and human whole blood cell cultures (Brattig *et al.*, 2004). The stimulation of innate responses was dependent upon both TLR-2 and TLR-4 in transfected human fibroblastoid cells and murine gene knockout and mutant cells. WSP also stimulated anti-inflammatory IL-10 and prostaglandin E_2 responses and IFNγ production from peripheral blood cells together with IgG1 antibody responses from onchocerciasis-infected individuals. These experiments suggest WSP is a major inducer of the inflammatory activity of *Wolbachia*. Whether it is the exclusive inflammatory molecule from *Wolbachia* is the subject of ongoing studies in this and other filarial species.

Taken together, these studies show *Wolbachia* are intrinsically pro-inflammatory, as demonstrated by the ability to activate innate cellular immunity. Whole bacteria or their products induce activation of macrophages and monocytes, neutrophils or dendritic cells leading to the secretion of pro-inflammatory cytokines and chemokines, through TLR-2, TLR-4 and MyD88 dependent signalling pathways.

Evidence to suggest that *Wolbachia* stimulates inflammatory activity in people infected with filariasis comes from studies on the systemic inflammatory adverse reactions to anti-filarial chemotherapy.

4.4. *Wolbachia* Serology

Antibody responses to a number of *Wolbachia* antigens have been observed in both human and animal filariasis, including the major surface protein (WSP), HSP60, HtrA-type serine protease and aspartate aminotransferase (Bazzocchi *et al*., 2000; Punkosdy *et al*., 2001, 2003; Simon *et al*., 2003; Morchon *et al*., 2004; Brattig *et al*., 2004; Lamb *et al*., 2004; Kramer *et al*., 2005, in press). These studies illustrate the exposure of *Wolbachia* to the acquired immune system and suggest both these and additional antigens, both specific to *Wolbachia* and cross-reactive to other bacterial antigens are a feature of the immune response to filarial infection.

In dogs and cats infected with *D. immitis*, *Wolbachia* has been detected in a number of organs and tissues including the glomerular capillaries of the kidney and within inflammatory cells of the lungs and liver (Kramer *et al*., 2005, in press) and raise antibody responses to WSP (Bazzocchi *et al*., 2000; Morchon *et al*., 2004; Kramer *et al*., 2005, in press). Also in people diagnosed with pulmonary dirofilariasis, IgG responses to WSP are elevated in clinical cases compared with *D. immitis* sero-positive 'healthy' individuals and donors from non-endemic areas, suggesting a possible sero-diagnostic test for pulmonary dirofilariasis (Simon *et al*., 2003).

Studies on WSP serology in human filariasis show an intriguing association with the presentation and duration of chronic disease in lymphatic filariasis (Punkosdy *et al*., 2003). Moreover, longitudinal analysis in both primate and human cases show a transient elevation in WSP reactivity is temporally associated with the onset of lymphoedema and suggests immune responses evoked by *Wolbachia* may trigger the development of disease (Punkosdy *et al*., 2001, 2003). The death of adult worms, a critical factor in the development of chronic disease, is associated with the release of endosymbionts into the blood, an event likely to lead to the activation of WSP immune

reactivity. Depletion of *Wolbachia* by antibiotic treatment had no effect on lymphatic granulomatous lesions in a primary infection of *B. pahangi* in jirds and was associated with the lack of appreciable WSP antibody reactivity (Chirgwin *et al*., 2003). These findings are not unexpected as multiple exposures to infective larvae are associated with the development of WSP reactivity related to clinical lesions (Punkosdy *et al*., 2001, 2003). Additional studies have shown that exposure to infective larvae in particular also stimulate and maintain WSP responses, suggesting the death of developing larvae may also trigger disease progression (Lamb *et al*., 2004). A concept supported by epidemiological studies showing the level of exposure to transmission is associated with acute and chronic disease (Kazura *et al*., 1997; Michael *et al*., 2001). Therefore, further analysis of the antigens and regulation of acquired immune responses to *Wolbachia* is important in order to understand their role in disease pathogenesis.

5. FUTURE ADVANCES/CONCLUDING REMARKS

Over the past six years, there has been a considerable increase in our knowledge of the biological significance of *Wolbachia* for their filarial hosts. We now know a great deal about their role in parasite fertility and survival and in the induction of immune responses associated with pathology and adverse reactions to classical microfilaricidal drugs. Most importantly, the proof of principle for a new chemotherapeutical approach, i.e. doxycycline against onchocerciasis and bancroftian filariasis, has been established. This advance has seen rapid progress from animal studies into use for humans in only a few years, and without the enormous costs usually necessary for the development of novel drugs.

Much more remains to be learned about the molecular basis of this fascinating interaction. The sequencing and annotation of the genome of the *Wolbachia* endosymbiont in *B. malayi* has provided us with a wealth of information for future definition of the essential components in this symbiosis. Targeting *Wolbachia* for treatment will allow exploitation of critical bacterial pathways with the potential to identify drugs that have fewer adverse reactions than those directed

against eukaryotic pathways. It may also circumvent one of the inherent problems of tropical medicine, the lack of a drug market that drives drug development, because drugs may already exist that can be predicted to act against *Wolbachia*, once we have learned what makes them so essential for their host.

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The Biology of Avian *Eimeria* with an Emphasis on their Control by Vaccination

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ABSTRACT

Studies on the biology of the avian species of *Eimeria* are currently benefiting from the availability of a comprehensive sequence for the nuclear genome of *Eimeria tenella*. Allied to some recent advances in transgenic technologies and genetic approaches to identify protective antigens, some elements are now being assembled that should be helpful for the development of a new generation of vaccines. In the meantime, control of avian coccidiosis by vaccination represents a major success in the fight against infections caused by parasitic protozoa. Live vaccines that comprise defined populations of oocysts are used routinely and this form of vaccination is based upon the long-established fact that chickens infected with coccidial parasites rapidly develop protective immunity against challenge infections with the same species. Populations of wild-type *Eimeria* parasites were the basis of the first live vaccines introduced around 50 years ago and the more recent introduction of safer, live-attenuated, vaccines has had a significant impact on coccidiosis control in many areas of the world. In Europe the introduction of vaccination has coincided with declining drug efficacy (on account of drug resistance) and increasing concerns by consumers about the inclusion of in-feed medication and prospects for drug residues in meat. The use of attenuated vaccines throughout the world has also stimulated a greater interest in the vaccines that comprise wild-type parasites and, during the past 3 years worldwide, around 3×10^9 doses of each type of vaccine have been used. The need for only small numbers of live parasites to induce effective protective immunity and the recognition that *Eimeria* spp. are generally very potent immunogens has stimulated efforts to develop other types of vaccines. None has succeeded except for the licensing, within several countries in 2002, of a vaccine (CoxAbic vaccine; Abic, Israel) that protects *via* the maternal transfer of immunoglobulin to the young chick. Building on the success of viral vaccines that are delivered *via* the embryonating egg, an *in ovo* coccidiosis vaccine (Inovocox™, Embrex Inc.) is currently in development. Following successful field trials in 2001, the product will be ready for Food and Drug Administration approval in 2005 and a manufacturing plant will begin production for sale in late 2005.

Limited progress has been achieved towards the development of subunit or recombinant vaccines. No products are available and studies to identify potential antigens remain compromised by an absence of effective *in vitro* assays that correlate with the induction of protective immunity in the host. To date, only a relatively small portfolio of molecules has been evaluated for an ability to induce protection *in vivo*. Although *Eimeria* are effective immunogens, it is probable that to date none of the antigens that induce potent protective immune responses during the course of natural infection has been isolated.

1. INTRODUCTION

1.1. *Eimeria* spp. and the Need for their Control

Coccidiosis, an intestinal disease of intensively reared livestock, is caused by coccidial parasites of the genus *Eimeria*; protozoa within the taxonomic family Eimeriidae (Levine, 1982) and the phylum Apicomplexa (Levine, 1970). This phylum contains many other protozoa of medical and/or veterinary importance including the malarial parasites *Plasmodium* spp., the zoonotic organisms *Cryptosporidium parvum* and *Toxoplasma gondii* and the more recently described *Neospora caninum*, which is an important cause of abortion in cattle. Most apicomplexans are obligate intracellular parasites that are transmitted to new host cells by invasive extracellular stages, which are equipped with a specialised array of cytoskeletal elements and secretory organelles known as the apical complex, after which the phylum is named.

Economically, the most important members of the genus *Eimeria* are the seven species that infect the ~30 billion chickens reared annually worldwide, viz. *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. praecox*, *E. necatrix* and *E. tenella*. Infections with *E. acervulina*, *E. maxima* and *E. tenella* are diagnosed frequently in intensively reared poultry (McDougald *et al.*, 1997) and their control is usually accorded a higher priority over the other four species, especially in chickens raised for meat (broilers). Accurate figures for the costs associated with clinical and subclinical coccidiosis and their

control are not generally available. However recent calculations of the financial impact of prophylaxis and estimates of production losses due to disease, put the costs of coccidiosis to the poultry industry in Great Britain to at least £38 millions per annum (Williams, 1999). By extrapolation, the costs worldwide could be expected to be around £1500 million (equal to US$2400 million) per annum.

Control of the avian coccidia is essential to the poultry industry because, for the most part, large flocks of chickens are kept on the floor at high stocking densities (often more than 25 000 chickens in a single building) in warm environments. These conditions are highly favourable for the transmission, replication and accumulation of *Eimeria* spp. in very large numbers. Organisationally, the poultry industry comprises both broiler and egg production and the two sectors have different requirements for the control of *Eimeria* spp. The biggest and most intensive sector is the production of broilers, which are reared to around six weeks of age before slaughter. Control of *Eimeria* in these birds can be achieved without an absolute need for the host to develop a protective immune response and prophylactic chemotherapy has generally been the sole method of control for the past 50 years. Virtually all broiler chickens reared throughout the world are given anticoccidial drugs from the time of hatch until a few days before slaughter.

In the egg-production sector, the situation is quite different. Prophylactic anticoccidial drugs must be withdrawn once hens approach the point of lay to prevent any carryover of drugs into eggs destined for consumers. While prevention of coccidiosis during the initial period of rearing has always been comparatively easy, through the use of anticoccidial drugs, a greater difficulty has been to ensure that hens develop immunity to each of the major species of *Eimeria* by the time they come into lay and anticoccidial drugs are removed. Thus for many years, poultry producers used strategies of husbandry during the initial rearing period that were intended to expose flocks to limited infections with *Eimeria* spp. so that immunity could develop and protect the birds against parasite challenge during the (anticoccidial drug-free) laying period. Historically, this balance between infection and immunity has been difficult to achieve without attendant risks of clinical disease. Strategies to achieve this balance have included the

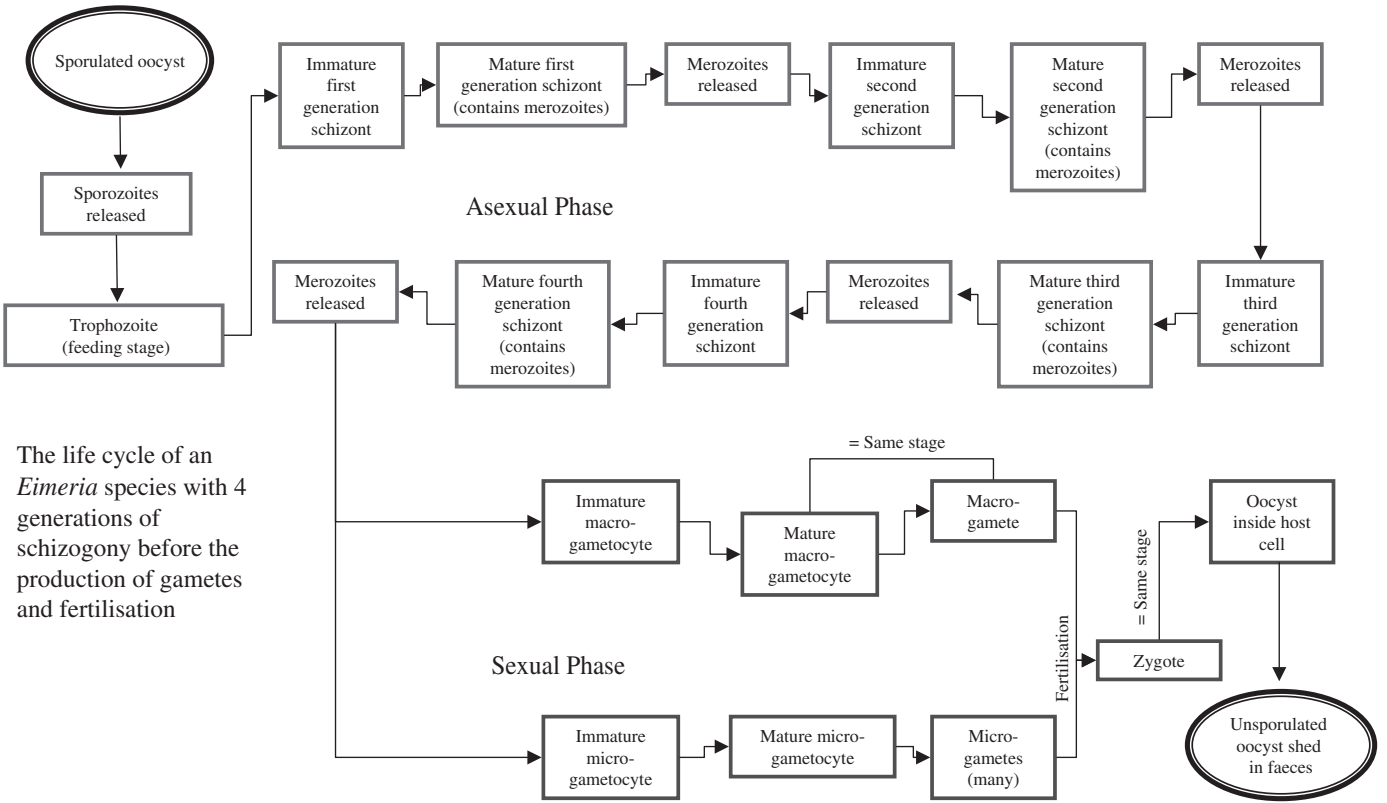
administration of suboptimal concentrations of effective drugs, the use of drugs that are not totally effective or even no prophylactic control at all (with a need for rapid drug therapy if disease occurred). With most of these approaches, there was an ever-present risk that birds would eventually succumb to severe disease (e.g. McDougald *et al*., 1990) and this remained a difficult issue to resolve satisfactorily until the introduction of live-attenuated vaccines. These products have provided an excellent remedy to the problem and the attenuated populations of *Eimeria* that are now used provide a margin of safety that eliminates any risk of disease.

While the impact and rapid uptake of attenuated coccidiosis vaccines was immediate in the egg-production sector (both in flocks kept for breeding and eggs for consumption), the increasing use of vaccines in broiler chickens has generally been more gradual. However, in the past few years some broiler-specific products have been introduced and it is highly probable that sales of these vaccines will continue to rise considerably over the next few years.

1.2. Life Cycles of *Eimeria* spp., Including the Relevance of Different Endogenous Stages to the Induction of Protective Immunity

Eimeria species are transmitted between hosts by the faecal–oral route. The developmental life cycle consists of an exogenous phase in the environment during which oocysts excreted from the chicken undergo differentiation (sporulation) and become infective, and an endogenous phase in the chicken intestine during which three or four discrete and expansive rounds of asexual reproduction (schizogony), depending on the species, are followed by sexual differentiation (gametogony), fertilisation and, finally, shedding of the unsporulated oocysts (Figure 1).

During the endogenous phase of the life cycle, each successive developmental stage of the parasite expresses a wide spectrum of antigens that are presented to the immune system of the host (McDonald *et al*., 1988; Tomley, 1994). The earlier endogenous life cycle stages are the most important for the induction of protective immune responses as shown from several independent lines of



The life cycle of an *Eimeria* species with 4 generations of schizogony before the production of gametes and fertilisation

Figure 1 Life cycle of a species of *Eimeria* with four generations of schizogony

research including exposure of animals to partial infections by transferring infected mucosa between hosts, e.g. *E. maxima* (Rose and Hesketh, 1976); the potent immunogenicity of parasite lines that have an absence or depletion of the terminal two schizont generations, e.g. *E. maxima* (McDonald *et al*., 1986b; Shirley and Bellatti, 1988;) and *E. tenella* (McDonald *et al*., 1986a); protection against challenge following oral vaccination with oocysts subjected to a level of gamma irradiation that allows sporozoites to invade host cells and begin development but not to undergo schizogony, e.g. *E. tenella* (Jenkins *et al*., 1993) and *E. acervulina* (Jenkins *et al*., 1991) and the technically simple and elegant demonstration that drug-arrested sporozoites of *E. tenella* induce substantial protection against the body-weight-depressing effects of a challenge infection with a drug-resistant challenge strain (Jeffers and Long, 1985). Thus, immunoprotective antigens capable of inducing complete protection against reinfection appear to be presented by, or within, the sporozoite-infected host cell. The identity of these antigens is not known, but a focus on the early events in the life cycle of the parasites now underpins much of the work being done in the search for antigens that could be included in future generations of subunit or recombinant vaccines.

Infection in the intestine is initiated by sporozoites, which are liberated from sporocysts in the lumen of the upper intestine and which migrate to their preferred sites of development where they invade villus enterocytes. *E. brunetti* and *E. praecox* undergo the whole of the endogenous phase of their life cycles within enterocytes of the villi whereas the remaining five species begin intracellular development within enterocytes of the crypts and usually locate to more superficial enterocytes with successive phases of schizogony. The mechanism by which sporozoites arrive at the crypts is not known precisely, but it is believed that they are transported from the villus within host intraepithelial lymphocytes (Fernando *et al*., 1987), most probably CD8 + T cells, and possibly macrophages (Trout and Lillehoj, 1993, 1995). It is not clear, however, whether sporozoite transport within host cells is essential, since free sporozoites of a reference strain of *E. tenella* not examined by Fernando *et al*. (1987) have also been visualised in the tissue spaces (Vervelde and Jeurissen, 1995; Vervelde *et al*., 1995). Whatever the means by which sporozoites reach the enterocytes in

which they develop, soon after the ingestion of oocysts it is possible to transmit infection to naïve hosts using blood, spleen and liver from the infected host, even for *E. brunetti* and *E. praecox*, which suggests that a brief extra-intestinal phase is common for all species, (e.g. Fernando *et al*., 1987; Perry and Long, 1987; Riley and Fernando, 1988).

Thus, in the early part of the life cycle, when immunity to reinfection is induced most potently, the sporozoite may invade a number of different host cells (villus enterocyte, intraepithelial lymphocyte/macrophage, crypt enterocyte) and any or all of these host–parasite interactions could be critical for the induction of protective immune responses.

The role of the sexual stages in the induction of immunity during natural infection is almost certainly of lesser importance, but they have also been shown to be the target of immune inhibition (Rose and Hesketh, 1976). Moreover, the only vaccine against avian coccidiosis that does not rely on the administration of live oocysts is based on the delivery of sexual stage antigens into laying hens and the attendant generation and transfer of antibody *via* the egg to protect the hatchlings, (e.g. Wallach, 1997), and see below.

2. IMMUNITY TO *EIMERIA* SPP.

Host immunity is pivotal in determining both the outcome of primary infection and any subsequent reinfection with *Eimeria* parasites, and the mechanisms that operate to limit parasite replication differ in the naïve and immune hosts. Several articles have recently reviewed comprehensively the immune responses to *Eimeria* (e.g. Lillehoj and Trout, 1996; Smith and Hayday, 1998; Allen and Fetterer, 2002) and we will limit our discussion here to some of the broader issues that come within the context of vaccination strategies.

2.1. The Immunological Relationship between the Host and *Eimeria* spp.

The immunological relationship between the host and *Eimeria* spp. is complex and changes with factors such as host genetic background,

parasite species/strain and the infection history of the host (e.g. Rose and Long, 1962; Joyner and Norton, 1976; Smith *et al*., 2002). While all *Eimeria* spp. are immunogenic, there is a hierarchy with *E. maxima*, *E. brunetti* and *E. praecox* regarded as the most immunogenic species (Rose, 1976, 1987) and *E. tenella* and *E. necatrix* as the least. This variation is a factor in the composition of live vaccines and the numbers of oocysts of each species that are included may vary considerably.

Many parasite products are recognised by the infected host, with substantial cross-reactivity in both the B- and T-cell responses. Many immunoglobulin responses are specific to *Eimeria* antigens but react to antigen preparations from heterologous species as indicated by ELISA and Western blotting (e.g. Uchida *et al*., 1994; Danforth *et al*., 1994), and lymphocytes from *Eimeria*-immunised animals included in proliferation assays may be stimulated with complex preparations of oocyst antigens (Prowse, 1991). When set against the species-specificity of protective immunity, these data collectively suggest that the adaptive immune response recognises many parasite antigens and that in general the species-cross-reactive epitopes are non-protective.

The number of parasite antigens that are protective is unknown. As the 55 Mb *Eimeria* genome could encode 5–8000+ gene products (Shirley *et al*., 2004) and no predictive assays have yet been developed, the identification or isolation of 'protective' antigens expressed during natural infection has proved elusive. While some attempts have been made to utilise *in vitro* assays that might be predictive of function *in vivo* (Bumstead *et al*., 1995) even work with the most immunogenic species (*E. maxima*) has been disappointing, because the assays were limited to parasite proteins that were accessible from extracellular stages and because of an absence of any protection from those proteins that were identified as being potentially useful (Bumstead *et al*., 1995). However, such problems are not restricted to infections with *Eimeria* spp. as the antigenic complexity of many other pathogens, including large viruses, bacteria, parasitic protozoa and helminths, has similarly hindered the development of subunit vaccines. Work currently underway in our laboratory with *E. maxima* is directed to the rational identification of loci within the genome of

the parasite that encode immunoprotective antigens (Blake *et al.*, 2004). The strategy uses a novel combination of parasite genetics, DNA fingerprinting, drug-resistance and strain-specific immunity and centres on two strains of *E. maxima* that each induce a lethal (to the parasite), strain-specific, protective immune response in the host and show a differential response to chemotherapy. Through classical mating studies with these strains the loci responsible for strain-specific protective immunity or resistance to the anticoccidial drug robenidine have been found to segregate independently. Most interestingly, passage of recombinant parasites in the face of killing in the immune host was accompanied by the elimination of some polymorphic DNA markers that defined the parent strain used to immunise the host, i.e. markers that may reside close to the genes that encode protective antigens. Moreover, consideration of the numbers of parasites recombinant for the two traits suggested that very few loci encode protective antigens.

2.2. Protective Immune Responses

Since vaccination requires the generation of immunological memory against subsequent challenge infection it is appropriate to consider the mechanisms by which the host kills the eimerian parasite. It is now becoming apparent that the host responses that are most critically important in terms of protective immunity may differ between *Eimeria* spp. and reflect subtle variations in the overall host–parasite relationship (e.g. Rose *et al.*, 1991, 1992; Schito *et al.*, 1996). While our understanding of immune function in the chicken has improved considerably in the past few years, many recent data derive from the use of murine models. In this section we will give a broad overview of the types of protective mechanisms that operate against *Eimeria* spp., drawing examples from both murine and avian systems.

Innate natural killer (NK) cell, macrophage and granulocyte responses, as well as adaptive, antigen-specific T- and B-cell activation, have all been described during infections with *Eimeria* spp. (Rose *et al.*, 1990, 1996; Lillehoj and Choi, 1998). The precise timings of the responses by the host differ according to host genetics and the species

of *Eimeria* used (Rose *et al*., 1984, 1990; Rothwell *et al*., 1995, 2000; Yun *et al*., 2000), with the most consistent finding being that the more resistant hosts mount more rapid responses to infection than do the more susceptible hosts. Since the eimerian life cycle progresses serially in the absence of indefinite recycling of asexual or sexual stages, the host will be totally clear of parasites within ~14 days after infection.

Infection of mice with targeted gene disruption (TGD) of T-cell receptor genes (Roberts *et al*., 1996; Smith and Hayday, 1998) has shown that TCR$\alpha\beta$+ T cells are essential for immune-mediated resistance to primary infection and immunity to subsequent reinfection with *E. vermiformis*. Similar studies in other T cell-deficient models have also demonstrated the importance of T cells during primary infection (most *Eimeria* spp.) and secondary infection (all *Eimeria* spp.) (Rose and Hesketh, 1979; Rose *et al*., 1984; Trout and Lillehoj, 1996; Schito *et al*., 1996). Although specific immunoglobulin (IgM, IgG and IgA) is induced by infection, studies with *E. vermiformis* in B cell-deficient mice (Smith and Hayday, 1998) and *E. maxima* in bursectomised chickens (Rose and Hesketh, 1979) suggest that B cells play a minor role during primary infection and are not essential for expression of complete immunity to re-challenge infection.

Depletion of specific T-cell subsets and adoptive transfer strategies showed that CD4+ but not CD8+ cells were essential for resistance to primary infection with *E. vermiformis* (Rose *et al*., 1988, 1992). Similarly, studies using TGD mice that are deficient in presentation of peptide *via* MHC class I or class II pathways also indicated the overriding importance of MHC class II-restricted T cells (presumably CD4+) (Smith and Hayday, 1998, 2000). Anti-CD4 depletion studies in chickens demonstrated a role for CD4+ T cells in primary infection with *E. tenella* but not *E. acervulina* (Trout and Lillehoj, 1996). With *E. papillata*, primary infection is limited by the activity of NK cells (Schito *et al*., 1996) rather than the CD4+ T cells responsible for control of *E. vermiformis* (Smith and Hayday, 1998). Mechanistically, the major mediator of immune-mediated resistance to primary infection with both *E. vermiformis* and *E. papillata* is interferon gamma (IFNγ) although the source of effective IFNγ differs between these two eimerian parasites (Rose *et al*., 1989; Schito and Barta, 1997; Smith and Hayday, 2000).

Recent advances in avian immunology have been driven by the availability of the chicken genome sequence (Hillier *et al.*, 2004) and related tools such as more expression sequence tag (EST) libraries (Min *et al.*, 2005), which allows the identification of many orthologues of mammalian immune genes of known function. Most studies have relied upon the quantitative analysis of mRNA encoded by restricted sets of genes (reviewed in Dalloul and Lillehoj, 2005) although a 400-cDNA microarray was applied to infections with *E. maxima* and *E. acervulina* (Min *et al.*, 2003). The microarray analysis indicated that a broad range of mRNA was upregulated during infection, which is not surprising bearing in mind the large-scale changes in immune cell populations and activity in the gut. Comparison of the cytokine levels in genetically resistant and susceptible lines of chicken during primary infection with *E. maxima* revealed a correlation between high resting levels of IL-10 and susceptibility to infection (Rothwell *et al.*, 2004). Taken together with the knowledge that the Th1-cytokine, IFNγ is a major effector molecule in killing *Eimeria* spp., that IFNγ levels increase during infection and that IL-10 is a negative regulator of Th1 responses the IL-10 findings provide an attractive basis for genetic resistance to *E. maxima* in the chicken.

The essential requirements for immunity to secondary infection appear to be less stringent than those required for immune-mediated resistance to primary infection. For example, although mice that lacked TCR$\alpha\beta+$ T cells remained completely susceptible to secondary infection with *E. vermiformis* those that were deficient in antigen processing to the two major TCR$\alpha\beta+$ T-cell subsets were either completely protected (class I-deficient/CD8+ T cells) or 95% protected (MHC class II-deficient/CD4+ T cells) (Smith and Hayday, 1998, 2000). Similarly, depletion of CD4+ or CD8+ T-cell subsets at the time of reinfection had no, or very little, effect upon the expression of immunity to secondary infection (Rose *et al.*, 1992). Depletion with anti-TCR$\alpha\beta_1$ or anti-CD8 exacerbated secondary infection with either *E. tenella* or *E. acervulina* infections in chickens (Trout and Lillehoj, 1996).

Although IFNγ is an essential component of immune-mediated resistance to primary infections, depletion of IFNγ or TGD of the IFNγ gene did not affect the complete immunity seen during

secondary infection with *E. vermiformis* (Caron *et al*., 1997; Smith and Hayday, 1998, 2000). A wide range of mice with TGD in other 'effector' loci, including those encoding other cytokines and the cellular cytolytic pathways, were also completely immune to secondary infection with *E. vermiformis* (Smith and Hayday, 1998, 2000). In the cases where a primary infection does not engender complete immunity to reinfection there may be less redundancy in the requirement for cell subsets or effector mechanisms during secondary infections (Schito and Barta, 1997; Schito *et al*., 1998). Collectively these data indicate that both TCR$\alpha\beta$ CD4+ and TCR$\alpha\beta$ CD8+ cells can be targeted by vaccination and that multiple immune effector mechanisms operate in the immunised animal.

3. VACCINE STRATEGIES

3.1. Live Vaccines

These vaccines consist of formulations of sporulated oocysts of several or all of the avian species of *Eimeria*. Administration is a key factor in the success of live vaccines and the ideal outcome is that each bird (or the vast majority of birds) in a flock is infected concurrently with just small numbers of oocysts of each component species.

3.2. Wild-type Strains of *Eimeria* spp.

Immunity induced by natural infections with *Eimeria* spp. is so effective at protecting against challenge with homologous parasites that live vaccines, based on wild-type parasites, have been available to the poultry industry for around 50 years. They continue to be successful commercial products and are now used extensively within all of the major poultry-producing regions of the world including the USA and Canada, Brazil and other countries of South America, Europe and many countries in Asia and Africa, (reviewed in Shirley, 1989). Sales of Coccivac® (Schering-Plough) and Immucox® (Vetech Laboratories) vaccines during 2001–2004 were close to 2500 million doses

(Marcelo Lang, Schering-Plough Animal Health and Eng-Hong Lee, Vetech Laboratories, personal communications). Live wild-type vaccines are also available for the control of coccidiosis in turkeys. Although sales of these are small in comparison to the chicken vaccines, their use is increasing such that around 50% of the turkeys reared in Ontario, Canada are now vaccinated (Eng-Hong Lee, Vetech Labs., pers. comm.).

Vaccines based on wild-type strains continue to be introduced, and some of the latest have included ADVENT® (Novus International) and Nobilis®COX ATM (Intervet; Schetters *et al*., 1999). ADVENT® is a microbiologically sterile product approved by the USDA for hatchling spray cabinet or application *via* feed and is differentiated from other live vaccines through a pre-sales DNA staining assay that is used to ensure inclusion of the correct numbers of viable oocysts for each species. Nobilis®COX ATM comprises ionophore-resistant strains of *E. acervulina*, *E. maxima* and *E. tenella* isolated from the field, and thus it can be used alongside ionophore treatment, which may offer advantages for protecting birds in the early period when immunity to *Eimeria* species is developing and could also be valuable if ionophores are required to control *Clostridium perfringens*, a pathogen that causes necrotic enteritis. While this particular concept is new, the use of anticoccidial drugs in combination with a live vaccine ('Vac M', Elanco Products Company) was described earlier (Bafundo and Jeffers, 1988). Vac M, which was not widely used and is no longer available, contained oocysts of an ionophore-sensitive strain of *E. maxima*. It was used specifically to control ionophore-resistant strains of *E. maxima* that were causing disease outbreaks in ionophore-medicated flocks on the east coast of the USA. The vaccine was given by mouth to newly hatched chicks about 2–4 h before they had access to food medicated with ionophores. In this way the drug-sensitive parasites entered epithelial cells, began intracellular development and initiated the protective immune response before the consumption of anticoccidial drugs killed the vaccinal oocysts and provided protection against subsequent natural infection with the other species.

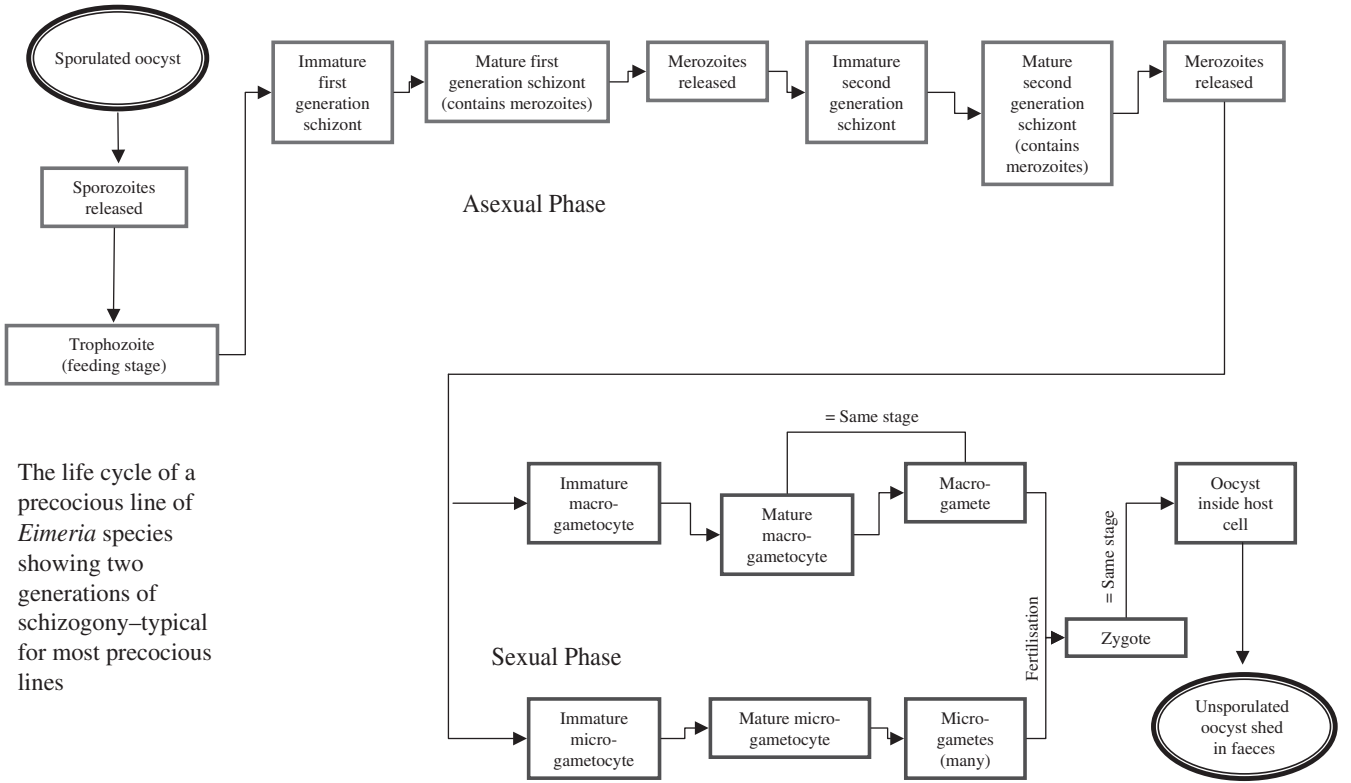
A crucial aspect for the success of vaccines composed of wild-type strains is the method by which they are delivered. Any uneven uptake

of vaccinal oocysts by individual birds within a large flock can lead to a series of asynchronous infections during the first three to five weeks of the rearing period. A failure to infect all, or certainly most, birds at the same time with wild-type parasites can lead to outbreaks of disease when the progeny of the vaccinal oocysts are ingested subsequently in large numbers by birds that were not immunised in the initial vaccination. The use of vaccines that contain wild-type parasites is thus associated with the greatest risk of disease as a direct consequence of vaccination, but such risks may be minimised by the administration of oocysts in ways that best ensure all birds are exposed concurrently to vaccinal oocysts (see below).

3.3. Live-Attenuated Vaccines

Most live-attenuated vaccines contain novel populations of *Eimeria* that were derived in the laboratory by controlled passage through chickens with repeated selection for the first oocysts to be produced during infection. These 'precocious' lines of parasites (Jeffers, 1974) are characterised by abbreviated endogenous life cycles in which the terminal generations of schizogony are either deleted or depleted (e.g. McDougald and Jeffers 1976; McDonald *et al.*, 1982, 1986b, c; McDonald and Shirley, 1984; Shirley and Bellatti, 1984, 1988; Shirley *et al.*, 1984, 1986 and Figure 2 and compare with Figure 1). This loss of one or two generations of schizogony has a profound effect on the numbers of gametes produced, and hence the numbers of oocysts formed, during an infection (Table 1).

Thus, in comparison to wild-type strains, each precocious line has a significantly lower reproductive capacity (e.g. Shirley, 1989), which, in turn, confers a marked attenuation of virulence in comparison to the parent parasite (Shirley, 1989; Montes *et al.*, 1998 and Figure 3). Whereas dramatic losses of weight and high rates of mortality of the host may result from infections with wild-type strains of some species, there are few pathological consequences from infections with precocious lines, even those derived from highly pathogenic species such as *E. necatrix* (Shirley and Bellatti, 1984). Despite such dramatic changes to the terminal phases of asexual reproduction, the immunogenicity



The life cycle of a precocious line of *Eimeria* species showing two generations of schizogony–typical for most precocious lines

Figure 2 Life cycle of a typical precocious line derived from a species of *Eimeria* with four generations of schizogony

Table 1 Some coccidiosis vaccines that are in use or being registered for use in chickens

Vaccine	Manufacturer	Principal recipients	Parasites, species, route	Country of derivation
Coccivac® D	Schering Plough Animal Health	Breeders/layers	Wild type, seven species, oral	USA
Coccivac® B	Schering Plough Animal Health	Broilers	Wild type, four species, oral	USA
Immucox®	Vetech Laboratories	Breeders/layers	Wild type, five species, oral	Canada
Immucox®	Vetech Laboratories	Broilers	Wild type, four species, oral	Canada
ADVENT®	Novus International	Broilers	Wild type, three species, oral	USA
Nobilis® COX-ATM	Intervet International	Broilers	Wild type, ionophore resistant, three species, oral	Netherlands
Livacox® Q	Biopharm	Breeders/layers	Attenuated, four species, oral	Czech Republic
Livacox® T	Biopharm	Broilers	Attenuated, three species, oral	Czech Republic
Paracox®	Schering Plough Animal Health	Breeders/layers	Attenuated, seven species, oral	UK
Paracox® 5	Schering Plough Animal Health	Broilers	Attenuated, four species, oral	UK
Eimervax® 4m	Bioproperties Pty	Breeders/layers/broilers	Attenuated, four species, oral	Australia
Eimerivac® Plus	Guangdong Academy of Agricultural Sciences	Breeders/layers/broilers	Attenuated, three species, oral	China
Inmuner® Gel-Coc	Vacunas Inmuner	Breeders/layers/broilers	Wild type and attenuated, three species, oral	Argentina
CoxAbic®	Abic	Breeders (to protect hatchlings)	Killed antigen, one species, intramuscular injection	Israel
Inovocox	Embrex	Broilers	Wild type, three species, *in ovo* injection	USA

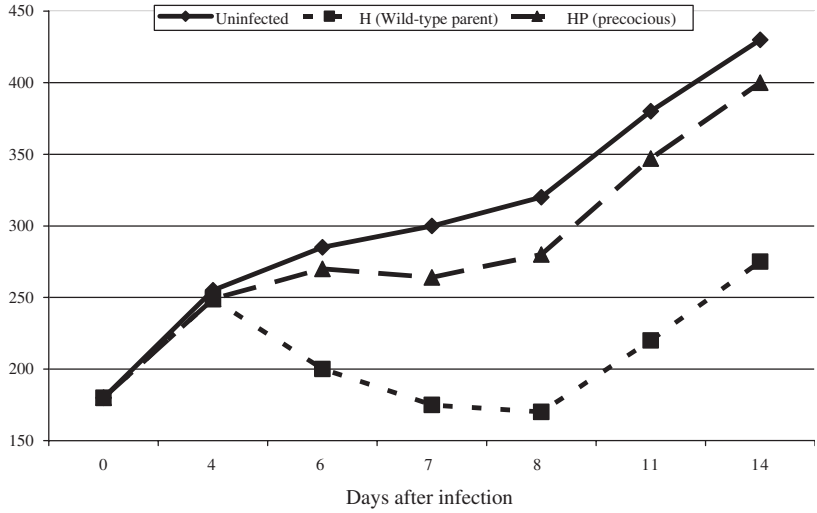


Figure 3 Pathogenicity of wild-type parent strain of *E. necatrix* and a precocious line derived from it

of the precocious line generally remains similar to that of their wild-type parents (e.g. McDonald *et al*., 1986c; Shirley and Millard 1986; Shirley *et al*., 1986 and see below).

The trait of precocious development from wild-type strains is a dynamic process, such that the pre-patent time, which reflects the extent of schizogony and time of transition from an asexual to a sexual phase of the life cycle, decreases progressively as more rounds of selection are applied. At the Institute of Animal Health (IAH), none of the precocious lines destined for inclusion within Paracox® vaccine could be selected during the course of a single passage for faster completion of the life cycle. Instead it proved necessary to passage each line many times with increasing selection before parasites could be recovered with the required combination of reproduction, attenuation and immunogenicity. While increasing selection for precocious development yields lines with greater attenuation of virulence, two other important factors must be considered. Firstly, the low rates of reproduction associated with elimination of asexual stages make the production of vaccinal oocysts more difficult and, secondly, loss of early life cycle stages increases the risk that the lines will be less immunogenic (e.g. McDonald *et al*., 1986b). In practice, it

has proved relatively straightforward to derive precocious lines with the desired combinations of traits so that they are effective as vaccines and can be produced on a large cost-effective scale.

The genetic stability of attenuated lines has to be taken into account by vaccine manufacturers. Once precocious lines with the desired traits had been selected at the IAH as candidates for inclusion within Paracox® vaccine, sublines were established from infections with single oocysts as a step towards minimising the risks of reversion to wild type. Ideally, clonal populations each derived from a single sporocyst or sporozoite (e.g. Shirley and Millard, 1976, 1989) were sought, but the comparatively poor reproduction of the precocious lines mitigated against this. Instead, each population of parasites included within Paracox® vaccine was re-established from infection with a single oocyst once the precocious line with the desired characteristics had been selected and each of these new populations was subsequently shown to meet the genetic stability required (Shirley and Bellatti, 1988).

To date, precocious lines of *Eimeria* have provided the major source of attenuated parasites and are now incorporated into most of the live-attenuated vaccines (Table 2). No other attenuation strategy has offered such a successfully broad utility for work with *Eimeria* spp., although an attenuated egg-adapted line of *E. tenella* has proven efficacy with Livacox® vaccines (Shirley and Bedrnik, 1997).

3.4. Use and Efficacy of Live Vaccines

Although the composition of individual live vaccines varies, all include oocysts of *E. acervulina*, *E. maxima* and *E. tenella* as there is an absolute need to control these species (see above, Table 2). Vaccines intended for use in broiler chickens that are reared for only 6–7 weeks are likely to contain only these three species, whereas vaccines for laying birds are inevitably more comprehensive and may contain all seven species of *Eimeria*. A critical inclusion for laying birds is *E. necatrix*, which can be a major cause of coccidiosis around the time when egg laying commences (McDougald *et al*., 1990). The final formulation of any live vaccine is ultimately a matter of judgement for

Table 2 Reproduction of precocious lines in comparison to that of wild-type parents

Species	Strain	Outputs of oocysts (millions)	Percentage output compared to relevant parent strain
E. acervulina	HP	1.1	7.7
	H (parent)	14.3	100
E. brunetti	HP	3.5	8.4
	H (parent)	41.8	100
E. maxima	CP	1.6	4.1
	C (parent)	39.3	100
E. maxima	MFP	1.9	8.8
	MF (parent)	21.6	100
E. mitis	HP	0.7	2.6
	H (parent)	27.0	100
E. necatrix	HP	2.1	24.7
	H (parent)	6.5	100
E. praecox	HP	5.2	8.5
	H (parent)	61.1	100
E. tenella	HP	28.2	27.9
	H (parent)	101.1	100

Note: Doses of oocysts given were 2×10^2 for all species except *E. brunetti* (1×10^2) and *E. tenella* (5×10^2). Codes 'H'; 'MF' and 'C' refer to parent, virulent strains and addition of 'P' suffix refers to precocious lines.

the manufacturer and factors taken into account may include the costs of producing oocysts, any knowledge (or perception) of the epidemiology of *Eimeria* spp. in different systems of poultry husbandry in different regions of the world, and requirements for product registration, etc. The dose of oocysts intended for each bird varies between vaccine formulations but generally is around 50–100 oocysts for highly immunogenic species such as *E. maxima* and 1000 oocysts for species such as *E. acervulina* and *E. tenella*. As discussed in more detail below, antigenic diversity characterises populations of *E. maxima* encountered in the field and the incorporation of two immunologically distinct strains of this species within a vaccine provides a greater spectrum of protection.

Live-attenuated vaccines have had a major impact on the egg-laying and breeding sectors since their introduction about 15 years ago and in countries such as the UK virtually all breeding and laying flocks are now vaccinated. Unfortunately, few data are available on

the efficacy of vaccines in laying birds as the poultry industry tends not to publish the outcomes of vaccination programmes and, moreover, birds are usually moved between farms around the point of lay which makes difficult the recovery of specific information on coccidiosis control. Not withstanding this general paucity of data, some trials with flocks destined for broiler (Williams *et al*., 1999; Williams and Gobbi 2002) or egg production have been monitored (Shirley *et al*., 1995). A clear feature of live vaccines is that they prevent disease very effectively but they do not eliminate subsequent infections *per se* (Shirley, 1989). Throughout the rearing period of poultry, oocysts may accumulate in the litter in comparatively large numbers (up to 50 000 per gram litter), but absolute numbers are generally smaller than those associated with control flocks medicated with anticoccidial drugs (Williams *et al*., 1999). Thus the occurrence of litter oocysts after successful vaccination contrasts dramatically with the expectations of chemotherapy. In this case effective drugs significantly limit the numbers of oocysts to ~1000 per gram litter (Long *et al*., 1975; Long and Millard, 1978) whereas drug resistance and potentially poor control of *Eimeria* spp. is associated with up to 60 000 oocysts per gram of litter.

An interesting parasitological benefit of using live vaccines that contain drug-sensitive parasites is a reduction in, or even replacement of, the resident drug-resistant parasites within a poultry house. The principle was first established by Jeffers (1976) who showed that a massive introduction of a drug-sensitive precocious line of *E. tenella* into birds reared in a pen heavily contaminated with a drug-resistant strain produced a marked reduction in the proportion of drug-resistant oocysts in the litter. Whilst the study by Jeffers (1976) made use of unrealistic numbers of vaccinal oocysts, subsequent studies have been done with conditions more relevant to current husbandry practices. Chapman (1994) found that control of *Eimeria* spp. by the anticoccidial drug monensin was improved substantially after just a single interval of vaccination with Coccivac® vaccine that comprises drug-sensitive strains. No further improvement in the efficacy of the drug was noted after use of the vaccine in five successive flocks of chickens. Replacement of the resident parasites with drug-sensitive parasites might be expected to occur less rapidly with precocious lines

in view of their poorer replication, but the sexual phase of the parasite life cycle and the opportunity that it presents for genetic exchange may ensure that parasites with wild-type life cycles rapidly gain genes for drug sensitivity. The re-introduction in to the field of genes for drug sensitivity within *Eimeria* spp. appears to be a unique outcome anywhere within the area of microbiology.

Little mention will be made of the methods by which live vaccines may be delivered to chickens as this specialist area of coccidiosis vaccinology has been considered in detailed elsewhere (Chapman, 2000; Williams, 2002). Many different approaches are used to administer coccidiosis vaccines, but the ultimate consideration is that oocysts must be ingested with high efficiency. This is usually achieved over a relatively short period of time (hours) to fit in with vaccination practice and, for practical reasons, most live vaccines are given once only, most commonly on the feed, e.g. by spraying oocysts onto food or placing oocysts within gels that can be pecked (Danforth *et al*., 1997; Danforth, 1998) or in the water with a viscous agent that serves to keep the oocysts in suspension for longer (e.g. Williams *et al*., 1999). Alternative routes for administering live vaccines include spraying onto the bodies of chickens within a few hours after hatching so that oocysts are ingested during preening (Andrews *et al*., 2001) or *via* eye-drop inoculation at the hatchery (Chapman, 1996). The efficiency of administration should be as high as possible, both to avoid wastage of oocysts and to ensure a broad coverage of individual birds within a flock. Uniform coverage is especially important for live wild-type vaccines in order to ensure that no birds remain fully susceptible to a heavy challenge infection around one week later with the progeny of the virulent vaccinal oocysts.

Much of the poultry industry (especially in the USA) makes use of *in ovo* injection around the 18th day of egg incubation to deliver some major viral vaccines. Such an approach is attractive for the mass administration of an *Eimeria* vaccine (Watkins *et al*., 1995) but the need to introduce complex, sterile, cysts (oocysts or sporocysts) as a prelude to the (presumed) release of sporozoites in the intestine of the foetus clearly could pose special technical problems. Nevertheless, proof of principle of this approach was achieved, initially using oocysts, sporocysts and sporozoites of *E. tenella* (Weber and Evans,

2003) and more recently with oocysts of *E. acervulina*, *E. maxima*, *E. mitis*, *E. praecox* and *E. brunetti* (Weber *et al.*, 2004). Interestingly in the latter study it was found that infectivity of sporozoites for 18-day-old chick embryos varied depending on the species of *Eimeria* and on the diluent used to suspend the parasites. Attempts to develop a practical *in ovo* delivery system for *Eimeria* spp. based upon the Embrex Inovoject® have been ongoing for some time and the product Inovocox™, which comprises wild-type oocysts of *E. acervulina*, *E. maxima* and *E. tenella* is currently at the 'pre-launch' stage (www.embrex.com).

Experimentally, a highly effective method to confer protection efficiently is to administer a daily delivery of a small number of oocysts (i.e. 'trickle infections' (Joyner and Norton, 1976). Such infections confer greater levels of immunity than infection with the same total number of parasites administered on a single occasion. A trickle strategy may also improve the efficacy of vaccination with the less immunogenic species where 'complete' immunity develops only after multiple exposures. However, the costs and inconvenience associated with multiple administrations of a coccidiosis vaccine mitigate against the incorporation of this strategy.

3.5. Antigenic Diversity

It is axiomatic that, to be effective, any coccidiosis vaccine should protect against a wide range of field strains that are defined by potentially very different characteristics. Thus the marked immunological diversity found between strains of *E. maxima* (e.g. Long, 1974; Norton and Hein, 1976; Long and Millard, 1979; Martin *et al.*, 1997; Barta *et al.*, 1998; Smith *et al.*, 2002) poses one of the biggest practical concerns about the use of coccidiosis vaccines. Experimentally, a spectrum of immunological relatedness has been demonstrated, with cross-protection between individual strains ranging from as little as ~10–15 to ~70% based on such parameters such as the outputs of oocysts and lesion scores in challenged chickens (e.g. Long, 1974; Norton and Hein, 1976; Shirley and Bellatti, 1988; Martin *et al.*, 1997). Smith *et al.* (2002) most recently showed a complete absence of

cross-protection between the Houghton (H) and Weybridge (W) reference strains in some inbred lines of chickens. Against such a background of immunological diversity it is therefore not surprising that chickens vaccinated with one strain of *E. maxima* may not be adequately protected if they encounter an immunologically divergent strain of *E. maxima*. This type of problem has indeed been reported (Lee, 1993) and resolved in an *ad hoc* manner whereby small numbers of oocysts of the immunologically variant strain of *E. maxima* were added to the vaccine. However, such a 'local solution' may not be compatible with the worldwide use of a vaccine, and is not approved within the EU as part of the registration process. The most effective solution so far to the problem of immunological diversity in *E. maxima* is based upon the finding that chickens given a mixture of up to six different strains of *E. maxima* were almost completely protected against subsequent challenge with each of the strains given separately (Long and Millard, 1979). While the notion of including many strains of *E. maxima* within a live vaccine has merit, the production of such a highly complex vaccine would be prohibitively expensive. In fact, the inclusion of only two populations of *E. maxima* is needed if they are chosen to represent extremes of immunological diversity. Shirley and Bellatti (1988), for example, found that the C strain was only poorly controlled by vaccination with the MF precocious line and Paracox® was formulated to include precocious lines derived from both the MF and C strains. Two immunologically diverse strains of *E. maxima* are also used in Nobilis® COX ATM (Table 3).

The type of immunological diversity that characterises populations of *E. maxima* is different from the rapid and dynamic form of antigenic variation that occurs within individual parasites of other protozoan such as *Plasmodium* (e.g. Brown and Brown, 1965; Biggs *et al*., 1991; Newbold, 1999) and trypanosomes (e.g. Gray, 1965; Donelson and Rice-Ficht, 1985; Rudenko, 1999) during the course of infection. It is reasonable to speculate that this difference reflects the types of survival strategies that are required by a parasite that has a short 'in:all-out' life cycle in the host but persists as a transmission stage (oocyst) in the external environment (*Eimeria*), or a far greater dependency upon staying longer in the host (*Plasmodium* and *Trypanosoma*). While immunological diversity in the avian *Eimeria* spp.

Table 3 Composition of some live vaccines

	Paracox®		Livacox®		Nobilis® Cox ATM	Coccivac®		Immucox®		Eimeriavax®	ADVENT®, Gelcox®, Eimerivac®, Inovocox®
	8	5	Q++	T+		D	B	Breeders	Broilers		
E. acervulina	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
E. brunetti	Yes					Yes		Yes			
E. maxima (1)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
E. maxima (2)	Yes	Yes			Yes						
E. mitis	Yes	Yes				Yes[a]	Yes[a]				
E. praecox	Yes					Yes					
E. necatrix	Yes		Yes			Yes		Yes	Yes	Yes	
E. tenella	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

[a]Listed as containing *E. mivati* and *E. hagani*, but *E. mitis* is the most likely species in view of the uncertain taxonomy of the two former species (Shirley *et al*., 1983).

is almost certainly greatest with *E. maxima*, detailed intra-specific comparisons within the other species have not been done.

3.6. Maternal Immunisation—Transmission Blocking Immunity

CoxAbic® (Wallach, 1997) is produced to confer maternal immunisation and comprises a preparation of purified, killed *E. maxima* gametocytes, which is administered intramuscularly to the breeding (laying) hen. Antibodies produced are passed into the egg yolk and subsequently to the hatchlings, which are passively protected against a challenge infection (e.g. Pugatsch *et al*., 1989; Wallach *et al*., 1990, 1995). Substantial data are not available on the identity of the endogenous stages of a challenge infection of *Eimeria* killed, although it is probable that sexual life cycle stages are targeted. Interestingly, immunisation of laying hens with gametocyte antigens from *E. maxima* protects chickens against challenge with other *Eimeria* species (Wallach, 1997) and increases the usefulness of the vaccination concept.

Floor pen and field trials involving several thousand vaccinated breeder hens and their offspring have been undertaken to assess the practical and commercial feasibility of the maternal vaccination approach. Some of these trials showed that peak litter oocyst counts in broiler chickens (hatched from the vaccinated hens) were reduced by about 60–70% (Wallach and Waldenstedt, 1999) compared to unvaccinated control groups. The vaccine is now registered for use in several countries including Argentina, Israel, South Africa and Thailand, and was introduced in to the marketplace formally in June 2002.

Few other data are available on the potency of protection induced by the maternal route of immunisation. However, from the available studies it seems probable that this vaccination approach will enable young chickens to control low-grade infections effectively during the early period of rearing, although some oocysts will probably complete their life cycles and induce active immunisation, which will boost that gained passively. A great attraction of the maternal immunisation strategy is that each breeding hen lays around 130 eggs

during its lifetime, so the successful vaccination of a single layer would protect a large number of young birds without any additional effort or cost.

4. PROSPECTS FOR THE DEVELOPMENT OF NOVEL VACCINES

In the much longer term, the availability of coccidiosis vaccines that are simpler to manufacture, but are of equivalent potency to live vaccines will be needed. New vaccines will possibly be based on the small subset(s) of protective antigens that characterise *Eimeria* spp. and, ideally, the manufacture of the new generation of vaccines should not require the use of chickens. Clearly this scenario raises many scientific issues that need to be addressed, not least being the development of novel approaches to identify the crucial protective antigens and the construction of robust and effective delivery systems to immunise the chickens. These are just some of the probable challenges that will have to be met if sustainable vaccine strategies are to be available far in to the future.

4.1. Recent Advances that have Relevance to Vaccine Development

To date a small number of parasite antigens have been studied in terms of their ability to induce 'protective' immune responses (see below) and these represent a tiny proportion of the potential antigens encoded by the parasite genome (Shirley *et al.*, 2004). Work on the assembly and annotation of the genome of *E. tenella* is expected to continue until 2007 and all the data are made freely available to the public through the *Eimeria* genome consortium (all participants in the genome sequencing projects can be contacted through http://www.sanger.ac.uk/Projects/E_tenella/consortium.shtml). As annotation goes forward, gene predictions and associated information are stored in a specialised, curated GeneDB database, which is regularly updated and again is freely available to the public (http://www.genedb.org/genedb/etenella/).

The availability of a full genome sequence is dramatically increasing our knowledge of the parasite and projects are already in progress using high-throughput global approaches for protein identification (Bromley *et al*., 2003; deVenevelles *et al*., 2004) concentrating initially on the sporozoite stage of *E. tenella*.

As discussed in Section 1.2, the early part of the *Eimeria* life cycle can induce potent protective immunity against challenge and considerable efforts are ongoing to characterise parasite molecules that mediate early interactions with the host. *Eimeria* spp. share many features with other apicomplexan parasites, including a specialised mode of invasion, which is distinct from the entry of other intracellular pathogens (Sibley and Andrews, 2000). Two types of parasite molecules are implicated in invasion: GPI-linked surface antigens (SAGs) and proteins secreted from the microneme, rhoptry and dense-granule organelles during invasion of the host cell (reviewed by Tomley and Soldati, 2001; Soldati *et al*., 2001; Carruthers, 2002).

Until recently, little was known about GPI-linked SAGs in *Eimeria*, but examination of ESTs from sporozoites and second-generation merozoites revealed two families of transcripts encoding potential GPI-linked variant surface proteins (Tabares *et al*., 2004). Using bioinformatics and wet-lab work 23 genes were shown to be differentially expressed during the parasite life cycle and confirmed to be surface-localised and GPI-anchored (Tabares *et al*., 2004). Genomic analysis indicates that the parasite encodes >60 intact *SAG* genes plus a considerable number of pseudogenes (Fiona Tomley and colleagues, unpublished). The stage-specific expression of repertoires of variant SAGs in *Eimeria* spp. is perhaps surprising since factors that drive SAG diversity in other parasites, such as persistence in the face of host immunity or infection of a wide range of hosts/cell types do not apply in *Eimeria*. Although the biological function of the SAGs is not yet defined, there are clear implications for any vaccine strategy that targets the parasite surface since it is most likely that many gene products may be required to induce full protection.

The use of subcellular fractionation to isolate microneme and rhoptry organelles (Kawazoe *et al*., 1992; Tomley, 1997) combined with molecular cloning and sequencing has made a major contribution to understanding the role of *Eimeria* proteins during invasion (Tomley

et al., 1991, 1996, 2001; Brown *et al.*, 2000, 2001, 2003; Labbe *et al.*, 2005; Periz *et al.*, 2005). Micronemes are the smallest of the secretory organelles and they contain complexes of transmembrane and soluble protein (MICs) that are dischared from the apical tip early in invasion (Carruthers and Sibley, 1999; Bumstead and Tomley, 2000; Rabenau *et al.*, 2001). MICs encode adhesive motifs such as thrombospondin type I domains, epidermal growth factor-like domains, Apple domains and integrin type A domains (Tomley and Soldati, 2001) and during invasion they bind the parasite surface, becoming concentrated at the point of invasion (Tomley *et al.*, 1996) and eventually released from the posterior end. Work in other apicomplexans has shown that this release is mediated by specific proteolytic cleavage of the transmembrane MICs that occurs within their conserved membrane-spanning domains (Opitz *et al.*, 2002). MICs are essential for parasite motility and compounds that interfere with their secretion are extremely effective at blocking parasite attachment and invasion *in vitro* and parasite infectivity *in vivo* (Wiersma *et al.*, 2004), making them attractive targets for therapy. The discovery that the cytosolic tail domain of a malarial MIC protein, *Pf*TRAP, is essential for parasite motility (Kappe *et al.*, 1999) suggested that transmembrane MICs may provide a crucial bridge between the parasite/host adhesive interface and the parasite actinomyosin motor. Proof of this came with independent demonstrations that the cytoplasmic domains of TRAP and of the *T. gondii* microneme protein MIC2 each bind a protein complex that includes the glycolytic protein aldolase, a tetrameric cytoplasmic enzyme that cross-links actin filaments, and moreover that the C-domain/aldolase complexes can recruit actin (Buscaglia *et al.*, 2003; Jewett and Sibley, 2004).

In *E. tenella*, five MIC proteins have been described in detail so far and many more, together with rhoptry and dense-granule proteins are emerging from proteomics studies (Bromley *et al.*, 2003, unpublished). EtMIC1 (Tomley *et al.*, 1991) and EtMIC4 (Tomley *et al.*, 2001) belong to the transmembrane TRAP family; EtMIC2 (Tomley *et al.*, 1996) is a soluble protein that forms an 'escorter' complex with EtMIC1 (Rabenau *et al.*, 2001), which is functionally analogous to the TgMIC2/TgM2AP complex of *T. gondii* (Huynh *et al.*, 2004). EtMIC3, first described alongside EtMIC2 as a regulated secretory

protein (Bumstead and Tomley, 2000), is a soluble MIC that consists of seven tandem repeats of a core TSP-1 motif and several other conserved residues (Tomley and Soldati, 2001; Labbe *et al*., 2005). EtMIC5 (Brown *et al*., 2001) is a soluble protein that contains eleven tandem apple domains, the structure of which closely resembles that of other members of the PAN superfamily (Brown *et al*., 2003). The transcription of these five *EtMIC* genes is co-ordinately regulated (Ryan *et al*., 2000).

4.2. Testing of Recombinant-Expressed Antigens as Vaccines

During the 1980s and 1990s there were many attempts to develop new coccidial vaccines based on the delivery to chickens of a handful of recombinant-expressed parasite antigens derived from the surface of invasive sporozoite or merozoites stages (e.g. Brothers *et al*., 1988; Jenkins *et al*., 1988, 1989), from the secretory apical organelles (e.g. Danforth *et al*., 1989) or from the internal refractile body organelles (e.g. Miller *et al*., 1989; Crane *et al*., 1991). This work was undertaken with great optimism due to early promising results that demonstrated partial protection against coccidial challenge after parenteral vaccination with a mixture of proteins extracted from parasites (Murray *et al*., 1986). Antigens were expressed in a variety of different prokaryotic and eukaryotic vectors, including *Salmonella typhimurium* (Pogonka *et al.,* 2003), fowlpoxvirus and herpes virus of turkeys and delivered either as killed, recombinant protein or as live, replicating vector vaccines (reviewed by Vermeulen, 1998; Vermeulen *et al.,* 2001; Jenkins, 1998, 2001; Lillehoj *et al*., 2000). Nevertheless these studies were universally disappointing in that immunisation only ever elicited partial protection against oocyst challenge and in most cases the results obtained were highly variable.

In the past few years, there has been a small resurgence of activity surrounding 'vaccinology' work on coccidia, due largely to the availability of genomic and cDNA sequences of several chicken cytokines and to progress in understanding how components of the host immune response can be modulated by cytokines, nucleic acids and other

immunomodulatory substances such as probiotics and vitamins (reviewed by Dalloul and Lillehoj, 2005). This has led to further attempts to vaccinate chickens with a small number of target antigens, either as recombinant-expressed proteins or *via* DNA immunisation, in some cases accompanied by cytokines. Although no marked improvements in overall protection against coccidiosis have emerged so far, there has been some success in demonstrating the effective delivery of recombinant proteins to chicks by *in ovo* vaccination (into the amniotic sac), with a dose-dependent partial protection using a recombinant surface protein from *E. acervulina* designated 3-1E (Ding *et al.*, 2004). Co-injection *in ovo* of plasmid DNAs encoding various chicken cytokines had some modulatory effects on the (partially) protective immune response to vaccination with 3-1E protein but there was little consistency between the measured parameters of oocysts outputs, body weight gains and circulation IgG antibody titres, making it difficult to draw firm conclusions. A subsequent study, this time using the secretory MIC2 protein from *E. tenella* to vaccinate chicks *in ovo*, alongside an evaluation of the adjuvant effect of CpG oligodeoxynucleotides, (Dalloul *et al.*, 2005) also conferred some partial protection of the hatched chicks to oocysts challenge.

4.3. DNA Vaccination

Work on DNA vaccines against *Eimeria* spp. is in its early stages, but a comparative account of the efficacy of different immunising forms of the same antigen reported that naked DNA vaccination with a construct from the gene encoding a refractile body antigen (designated SO7) protected chickens against a challenge infection with *E. tenella* (as measured by lesion scores and growth rates), whereas no protection accrued from administration of the native or recombinant antigen (Kopko *et al.*, 2000). However, significant protection against challenge was obtained only following injection of 25 μg of naked DNA and not from other doses of DNA (from 12.5 to 100 μg). Similar variability in protection from coccidial challenge following naked DNA vaccination was seen in studies examining the immunoprotective properties of the SAGs TA4 (Wu *et al.*, 2004) and 3-1E

(Song *et al*., 2001), the latter being extensively tested alongside the delivery of a battery of avian cytokines (Lillehoj *et al*., 2005; Min *et al*., 2002), and of the secreted microneme protein MIC2 (Ding *et al*., 2005). Despite these variations, which suggest that protection by this method may be difficult to achieve uniformly, this clutch of papers does serve to demonstrate a 'proof-of-principle' that DNA vaccination could be an effective strategy, provided that the right antigens can be identified. Most recently, the concept of using *S. typhimurium* as a live replicating vector, to deliver to the chicken gut a pcDNA plasmid-based vaccine against coccidiosis, was tested (Du and Wang, 2005) using the C-terminal region of the microneme secreted protein EtMIC4 (also called 5401, Danforth *et al*., 1989; Tomley *et al*., 2001). The use of bacteria as carrier vehicles for DNA vaccination was developed to overcome the low efficiency and high DNA concentrations that are required in naked DNA vaccination (Donnelly *et al*., 1997) and the results for the 5401 antigen look promising with good vector stability, the induction of consistent, dose-dependent immune responses and induction of dose-dependent and consistent partial protection following challenge (Du and Wang, 2005).

5. CONCLUSIONS

Future directions for the development of coccidiosis vaccines remain a paradox. On the one hand, some new products will be introduced, but on the other the level of investment in new strategies is uncertain and may depend upon the role to be played by anticoccidial drugs. Debates for and against the use of coccidiosis vaccines and/or anticoccidial drugs will continue and be wide ranging, but the specificity of immunoprophylaxis as compared to the additional (and very important) antibacterial effects of the ionophorous anticoccidial drugs is an important practical issue.

The ease with which precocious (attenuated) lines can be selected in the laboratory and live oocyst vaccines assembled *per se*, both point towards the introduction of further vaccines that work empirically. The market for vaccines is likely to expand considerably further and many opportunities may exist both for live vaccines manufactured a

large scale and those produced on a small and a local scale ('bijoux' vaccine plants). It is most likely that some live coccidiosis vaccines will command worldwide sales but even those produced on large scales will only be able to supply a small fraction of the total potential market. The manufacture of high-quality live vaccines is not a trivial undertaking and can probably only be achieved at costs that are greater than those associated with the manufacture of existing anticoccidial drugs.

Maternal immunisation offers a potentially cheaper means of vaccination and its utility will become clearer with the availability of more field data. However, complex manufacturing facilities that incorporate infections of chickens are also required both to produce gametocyte antigens—although the development of a recombinant form of the vaccine may be feasible and perhaps the most realistic genetically engineered vaccine approach in the short term.

In the much longer term, one objective will remain the availability of simpler vaccines that require fewer or no chickens for their manufacture. With the exception of the strategy described above, all other possibilities, including the development of subunit, live-vector or DNA vaccines, will require considerable research.

One crucially important element is missing from any evaluation of candidate vaccine molecules, viz. a method of administration that has been proven to be appropriate. Thus the present scenario for the identification of putative immunogenic molecules of *Eimeria* represents a classic 'Catch 22' situation in which protective antigens may not be identified until a suitable method of delivery has been identified and a suitable method of delivery may not be recognised until protective antigens have been isolated. If the (obviously very potent) molecules responsible for the induction of protective immunity during natural infection with the different *Eimeria* spp. could be identified, one logical step would be the derivation of an attenuated line of *Eimeria* that contains and expresses a portfolio of genes encoding protective antigens from each of the major species of *Eimeria*. Such a step is becoming closer with recent developments in *Eimeria*-transfection technology (Kelleher and Tomley, 1998, unpublished). A more desirable product would be the delivery of a cocktail of antigens without the need for a live *Eimeria* vector, but the complexity of the host–parasite relationship may mitigate against this in the short term.

In summary, the coccidia cannot go about their life style of parasitism without the host declaring immunological war. Understanding, and then utilising, the rapid and effective immune responses of the host should therefore always provide a real and predictive means by which to control coccidial parasites, even in hosts that are the most intensively farmed livestock on the planet.

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