

# Combating wildlife crime

Linzi Wilson-Wilde

Published online: 22 July 2010  
© Springer Science+Business Media, LLC 2010

It is with great pleasure that I introduce this special edition dedicated to wildlife crime. Wildlife crime is an important area of law enforcement that I have a strong commitment to. It involves the illegal trade in animals, plants and their derivatives and can result in the depletion of natural resources, invasion of pest species and the transmission of diseases. For the first time an international journal has dedicated an entire edition to the issue of wildlife crime, bringing together submissions from numerous global experts regarding their work in this area. The aim of this initiative is to generate attention to this significant criminal activity.

The current global situation is summarized and discussed in the commentary by Wilson-Wilde [1]. In a positive move, international action is becoming more coordinated and an overview of the 2009 INTERPOL Wildlife Crime Group meeting in Brazil is presented in the commentary by Neme [2].

At the international level there are 175 signatories to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [3]. CITES provides a system of control to inhibit the exploitation of animals and plants and prevent trade from threatening the extinction of endangered species. Fauna and flora are listed on one of the three Appendices of CITES. Appendix I lists species where international trade is prohibited (exceptions are made for non-commercial purposes, such as scientific research), Appendix II lists species where international trade is regulated in circumstances where the trade does not endanger

the survival of the species and Appendix III lists species where international trade is regulated at the request of a particular country (for example Uruguay has listed the eleven banded armadillo). Paramount to the enforcement of CITES and the subsequent prosecution of offenders is the ability to identify the species in question. Alacs et al. [4] provide an excellent review of genetic DNA analysis methods used in the forensic investigation of wildlife crime, covering various available techniques that can be applied and techniques that have potential for future application. Tobe and Linacre take this a step further investigating the use of DNA techniques in mixed samples from more than one species [5] and Spencer et al. [6] extend DNA techniques to the analysis of historical and degraded samples with much success.

Offenders of wildlife crime can be categorized into three main groups; minor offenders, organized illegal trading and serious major criminal activity [7]. Minor offenders generally relate to abuses against conditions in permits and are more opportunistic types of crime. These offenders are usually tracked through inadequate record keeping and generally involve exchanges between wildlife collectors. Organized illegal trading moves into the realms of deliberate clandestine poaching with intent to make gain and meet the needs of the market. It requires planning and can threaten wildlife, with no consideration of their habitat, for monetary gain in selling specimens on the black market. Serious major criminal activity differs from the latter in that it is highly organized involving major criminal groups who are professional, financially backed and specifically market products. These offenders may also be involved in major fraud and drug shipping [7]. Therefore combating criminal activity requires a well-equipped forensic facility to provide cutting edge technology, maximizing evidentiary outputs. Setting up such a laboratory is not easy and

---

L. Wilson-Wilde (✉)  
ANZPAA National Institute of Forensic Science,  
Melbourne, VIC, Australia  
e-mail: linzi.wilson-wilde.nifs@anzpaa.org.au

Ogden clearly highlights some of the issues and provides an insight into how this might be achieved [8].

It is difficult to ascertain specifically what drives demand in particular wildlife trade; however it is thought that a number of factors such as fashion, rarity of the species, trends in alternative remedies and medicine and criminal elements each play a part. Fashion can have a major impact and is highly variable. Particularly endangered species cost more and can therefore be in higher demand by collectors due to higher profits compared to the risks and penalties incurred. Simply placing a species on the CITES list, Appendix I can make a species more appealing. Yates et al. [9] look at the identification of hairs from elephant and giraffe used in traditional style jewelry (presumably bound for the tourist trade) using light microscopy.

A number of very interesting case studies are included in this edition to highlight the impact wildlife crime has on the animals involved and the type of forensic analysis that must be undertaken to assist the investigation. Byard et al. [10] present a case study on unusual upper aerodigestive tract obstructions in wild dolphins causing death. Byard et al. [11] also discuss a case study on unexpected deaths in captive fur seals and Carapetis et al. [12] present a case illustrating the consequences of ingesting foreign material by seabirds. In the article by Johnson two interesting case studies are discussed regarding the illegal importation of live bird eggs and the illegal possession of shark fins [13].

Wildlife crime also includes offences involving domesticated species, such as animal cruelty cases and where an animal may be used to link an individual to the commission of an offence (for example dog hairs on a suspect). El-Sayed et al. [14] investigate the use of DNA analysis in domesticated species and Clarke and Vandenberg look at the application of canine DNA profiling in forensic casework [15]. Wilson-Wilde et al. [16] look at species identification in the context of a laboratory conducting standard DNA analysis and implications and recommendations for implementing a species identification method. Two book reviews are also included in this special edition, *Forensic Science in Wildlife Investigations*, edited by Linacre and *Introduction to Veterinary and Comparative Forensic Medicine* by Cooper and Cooper.

We hope that the various concepts, research and issues discussed in this edition are thought provoking and provide an insight into this significant global issue.

## References

1. Wilson-Wilde L. Wildlife crime-a global problem. *Forensic Sci Med Pathol.* 2010;6:221–2.
2. Neme L. INTERPOL's Wildlife Crime Working Group Meeting. *Forensic Sci Med Pathol.* 2010;6:223–4.
3. CITES 2010. <http://www.cites.org/eng/disc/what.shtml>. Accessed 10 June 2010.
4. Alacs EA, Georges A, FitzSimmons NN, Robertson J. DNA Detective: A review of molecular approaches to wildlife forensics. *Forensic Sci Med Pathol.* 2010;6:180–94.
5. Tobe SS, Linacre A. DNA typing in wildlife crime: recent developments in species identification. *Forensic Sci Med Pathol.* 2010;6:195–206.
6. Spencer PD, Schmidt D, Hummel S. Identification of historical specimens and wildlife seizures originating from highly degraded sources of kangaroos. *Forensic Sci Med Pathol.* 2010;6:225–32.
7. McDowell D. Wildlife crime policy and the law. Canberra: Australian Government Publishing Service; 1997.
8. Ogden R. Forensic science, genetics and wildlife biology: getting the right mix for a wildlife DNA forensics lab. *Forensic Sci Med Pathol.* 2010;6:172–9.
9. Yates BC, Espinoza EO, Baker BW. Forensic species identification of elephant (Elephantidae) and giraffe (Giraffidae) tail hair using cross section analysis and light microscopy. *Forensic Sci Med Pathol.* 2010;6:165–71.
10. Byard RW, Tomo I, Kemper CM, Gibbs SE, Bossley M, Machado A, Hill M. Unusual causes of fatal upper aerodigestive tract obstruction in wild bottlenose dolphins (*Tursiops aduncus*). *Forensic Sci Med Pathol.* 2010;6:207–10.
11. Byard RW, Machado A, Braun K, Solomon LB, Boardman W. Mechanisms of deaths in captive juvenile New Zealand fur seals (*Arctocephalus forsteri*). *Forensic Sci Med Pathol.* 2010;6:217–20.
12. Carapetis E, Machado AJ, Byard RW. Lethal consequences of ingested foreign material in seabirds. *Forensic Sci Med Pathol.* 2010;6:242–3.
13. Johnson R. The use of DNA identification in prosecuting wildlife-traffickers in Australia. Do the penalties fit the crimes? *Forensic Sci Med Pathol.* 2010;6:211–6.
14. El-Sayed Y, Mohamed O, Ashry K, El-Rahman SA. Using species-specific repeat and PCR-RFLP in typing of DNA derived from blood of human and animal species. *Forensic Sci Med Pathol.* 2010;6:158–64.
15. Clarke M, Vandenberg N. Dog attack: the application of canine DNA profiling in forensic casework. *Forensic Sci Med Pathol.* 2010;6:151–7.
16. Wilson-Wilde L, Norman J, Robertson J, Sarre S, Georges A. Current issues in species identification for forensic science and the validity of using the cytochrome oxidase I (COI) gene. *Forensic Sci Med Pathol.* 2010;6:233–41.

# Dog attack: the application of canine DNA profiling in forensic casework

Melanie Clarke · Nicholas Vandenberg

Accepted: 3 October 2009 / Published online: 30 October 2009  
© Humana Press 2009

**Abstract** More than 100,000 dog attacks occur each year in Australia and many go unsolved. Dog attacks are not only a cause of human injury but may also involve injury and death to family pets, prized livestock and wildlife. Canine biological evidence can often be left behind on a victim or at the scene of an attack. Our laboratory provides canine DNA profiling for forensic investigations, utilising an in-house panel of 11 canine-specific autosomal short tandem repeat markers previously validated for use in casework. Case studies will be presented that outline methods for sampling of suspected canine biological evidence, profiling of canine DNA, statistical analysis, case outcomes and challenges for investigators.

**Keywords** Forensic science · Dog attack · Canine DNA · Short tandem repeat · Canine database · Canine genotyping

## Introduction

Australia has a canine population of over 4 million [1]. It has been estimated that more than 100,000 dog attacks occur every year in Australia [1], causing injuries with varying degrees of severity to people, pets, livestock and wildlife. Many of these attacks go unsolved. Dog attack cases potentially involve non-human DNA evidence, mute suspect/s and victim/s, and an absence of human witnesses, especially when the scene of a crime is a remote rural

location; all of these pose challenges to the investigator. However, an understanding of the relevance of canine DNA evidence can assist investigators such as police officers, local council rangers and animal welfare officers. Canine biological evidence can often be left behind on a victim or at the scene of an attack. Evidence can include dog saliva from bite marks on skin or clothing, dog blood, hair and faeces. The techniques used today to analyse such evidence are well established, sensitive and highly discriminating.

Canine DNA analysis of STR markers has become an effective tool in forensic investigations for verifying parentage and identifying individual animals [2]. Highly polymorphic tetranucleotide repeats have been reported in dogs since 1996 [3] and the variability of canine microsatellites within and between different dog breeds has been well characterised [4, 5]. Population structure and mutation rates of canine microsatellites also have been well studied [6, 7]. The statistical significance of a DNA match using canine microsatellites has been examined and a number of ways of providing reliable, conservative estimates have been developed, such as probability match rates or the use of likelihood ratios and the inclusion of theta estimates to account for population substructure [2, 7]. Salivary DNA evidence obtained from attack wounds on animals has previously been used to identify the species and individual identity of the predator [8, 9].

Our laboratory provides National Association of Testing Authorities (NATA) accredited canine DNA profiling for forensic investigations. An established in-house panel of 11 canine-specific autosomal Short Tandem Repeat (STR) markers has previously been validated by our laboratory for use in forensic casework. Here, we present three regional case studies of dog attacks to further demonstrate the forensic application and value of canine DNA profiling.

---

M. Clarke (✉) · N. Vandenberg  
Genetic Technologies Limited, 60-66 Hanover Street, Fitzroy,  
VIC 3065, Australia  
e-mail: melanie.clarke@gtg.com.au; melanie.marty@gtg.com.au

N. Vandenberg  
e-mail: nick.vandenberg@gtg.com.au

## Materials and methods

### Canine DNA analysis

DNA was extracted from samples using the QIAamp® DNA Micro kit (Qiagen). Canine STR analysis was performed using 11 canine-specific autosomal STR markers (Table 1). The FHC markers are public domain markers identified by the Fred Hutchinson Cancer Research Center [3]. The PEZ markers are described in Halverston et al. [10].

These 11 canine-specific STR markers have been incorporated into a fluorescently labelled in-house multiplex PCR panel. This multiplex of canine markers has previously been examined for sensitivity, species-specificity and reproducibility. The results of our own in-house sensitivity studies (data not shown) indicate that input DNA of at least 0.5 ng is required to obtain a full profile, with generally balanced peaks (i.e. > 50% peak height ratios), however partial profiles can be obtained from as little as 0.025 ng input DNA. DNA from different species, including human, equine, bovine, ovine, avian and camel-line does not produce a DNA profile when amplified using the canine multiplex (data not shown). The markers are amplified using the Qiagen Multiplex PCR kit (Qiagen). The canine multiplex was amplified in a 25 µl reaction volume; which has been scaled down by half from the manufacturer's guidelines, which suggest amplification using a 50 µl reaction. Results of in-house reproducibility testing using five known control samples, each tested 20 times, gave concordant results. However, in forensic casework at least two replicates were run for every sample since it is known that allelic dropout can be a problem when working with low quantity DNA samples [11]. Thermal cycling parameters were: 95°C for 15 min; then 35 cycles of 94°C for 30 s; 57°C for 90 s; 72°C for 60 s, followed by a final extension of 60°C for 45 min. Electrophoresis was conducted on an AB3130xl genetic

analyser (Applied Biosystems) using the internal size standard GeneScan™ 500 ROX™ (Applied Biosystems). GeneMapper® version 3.0 software package (Applied Biosystems) was used for the automated genotyping of samples. An example of a typical canine DNA profile is shown (Fig. 1). Fragment sizing was conducted by comparison to a positive control, which assists with determining run to run precision and reproducibility. Relative Fluorescent Unit (RFU) thresholds for forensic casework were set at 50 for heterozygotes and 250 for homozygotes, in accordance with the results of our sensitivity studies. Values that fall below these thresholds are considered to be at risk of allelic dropout and are not reported. Crime scene samples are typically processed prior to reference canine samples, to reduce the risk of cross contamination, along with reagent blanks at each laboratory processing step. Examination of items, DNA extraction, PCR set-up and post-PCR analysis are all performed in separate rooms using dedicated equipment and reagents.

### Statistical analysis

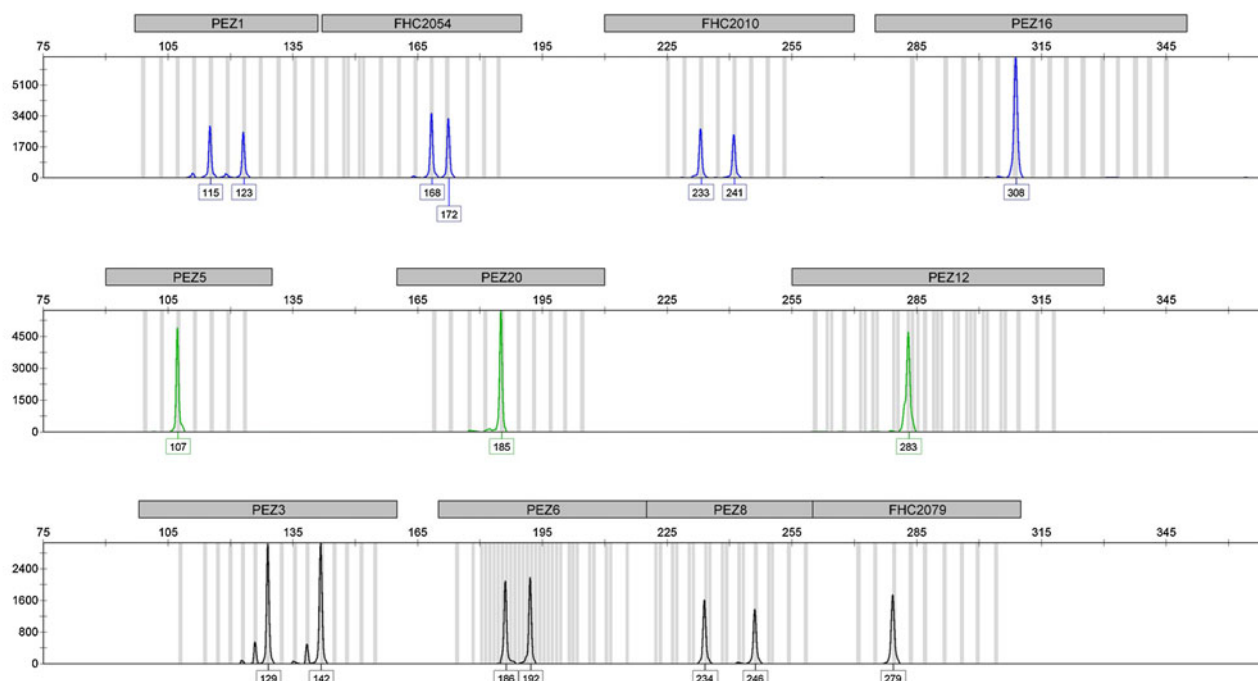
To date, using our multiplex of 11 canine-specific DNA markers, our laboratory has determined DNA profiles for over 30,000 individual dogs. A subset of these profiles was used to compile our standard forensic canine population database, which is a general dog database of unrelated dogs, consisting of the DNA profiles of one dog from 114 different breeds. Promega Power Stats v12 [12] was used to compile allele frequencies for each marker in the canine panel. Population substructure was examined using Genetic Data Analysis (GDA) software [13]. The sample population used to generate the standard forensic canine population database did not exhibit statistically significant association of genotypes at the  $P \leq 0.05$  probability level, indicating that individual genotype frequencies for each of the 11 markers in our canine panel can be multiplied together to obtain a profile frequency. An excel spreadsheet

**Table 1** Information on 11 STR canine loci used in this study

Locus	Repeat type	Allelic range (bp)	Dye label	Canine map location <sup>a</sup>
PEZ01	Tetra	99–139	6FAM	CFA7
FHC2054	Tetra	143–184	6FAM	CFA12
FHC2010	Tetra	225–253	6FAM	CFA24
PEZ16	Tetra	284–345	6FAM	CFA27
PEZ05	Tetra	99–123	VIC	CFA12
PEZ20	Tetra	169–204	VIC	Unmapped <sup>b</sup>
PEZ12	Tetra	261–318	VIC	CFA3
PEZ03	Tri	108–155	NED	CFA19
PEZ06	Tetra	174–215	NED	CFA27
PEZ08	Tetra	222–258	NED	CFA17

<sup>a</sup> [http://research.nhgri.nih.gov/dog\\_genome/breen2001/index.shtml](http://research.nhgri.nih.gov/dog_genome/breen2001/index.shtml)

<sup>b</sup> was not included in the mapping study referenced



**Fig. 1** GeneMapper® plot of a typical canine DNA profile showing results for each of the 11 STR markers. Individual markers are shown in the grey bars above the peaks. The X axis represents base pair size

and the Y axis represents Relative Fluorescent Units (RFU). Each allele is represented as a peak and each peak is labelled in the box below with the allele call in base pair size

is used to calculate likelihood ratios and confidence intervals for canine DNA profiles, based on our forensic canine population database. Genotype frequencies in the excel spreadsheet are calculated by the method of Balding and Nicholls [14]. These calculations are described in the second NRC report and follow the published procedure of Chakraborty et al. [15]. Despite the lack of observed linkage disequilibrium in our standard forensic canine population database set, it is known that purebred dogs do not mate randomly and may exhibit population substructure. Accordingly, a conservative theta value (i.e. 0.15) is applied to genotype frequency and resulting likelihood calculations in casework, to compensate for association of alleles within and between loci. Given the high level of inbreeding in dog populations, studies have shown estimates of theta in dog populations of approximately 0.1, and recommend applying a theta value of between 0.09 and 0.15 in casework [7, 16]. This is about 10 times more conservative than estimates from human populations.

#### Case study 1: attack on livestock

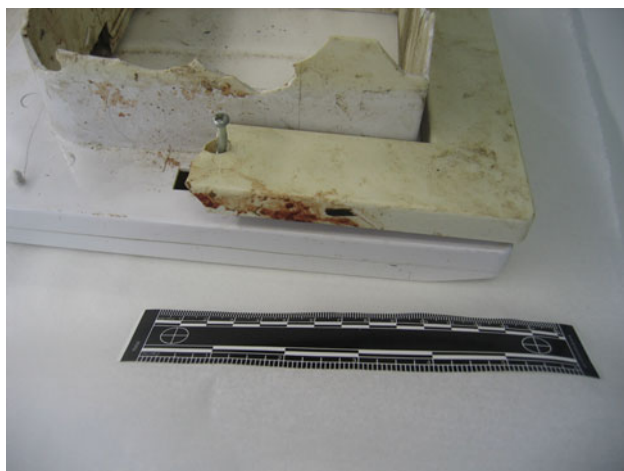
In September 2007, five dead and three seriously injured sheep were found on farmland in a North Eastern Shire of

Victoria, allegedly mauled by a neighbour's German Shepherd. A sample of wool was collected from around a bite mark from one of the deceased sheep. The suspect German Shepherd was seized by local council rangers and a buccal swab was collected from the dog to be used as a reference sample. The wool sample and the reference buccal swab were submitted to our laboratory for examination. The wool was visually examined and was found to be heavily blood stained, evidently from the extensive wounds inflicted on the sheep. A sub-sample was cut from an area that had the least amount of blood staining from the deceased sheep. Blood staining can potentially mask any canine biological evidence and possibly cause PCR inhibition [17]. On separate occasions, the sub-sample of wool and the reference sample were subjected to canine DNA analysis.

#### Case study 2: attack on family pet

In April 2009, in a Shire on Melbourne's eastern fringe, two dogs allegedly broke into a house through a cat door, then attacked and killed a pet cat. The two dogs, one an American Staffordshire Terrier and the other an English Staffordshire Terrier, were seen roaming the nearby streets and were seized by local rangers. Reference buccal swabs



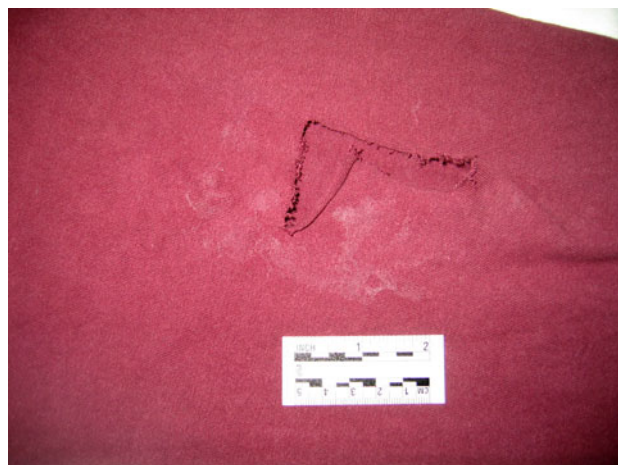


**Fig. 2** A section of the cat door showing apparent blood staining from which a canine DNA profile was obtained in case study 2

were taken from these suspect dogs. The rangers noted that one of the dogs had a bleeding head wound. An external section of the cat door with apparent blood staining was submitted to our laboratory for examination (Fig. 2). It was thought that the apparent blood on the cat door originated from one or both of the suspect dogs, possibly from an injury sustained while breaking into the house. An area of apparent blood staining on the cat door was sampled at our laboratory using a sterile moistened swab and this sample was subjected to canine DNA analysis. On a separate occasion, the two reference samples were also subjected to canine DNA analysis.

#### Case study 3: attack on person

In October 2008, a woman was attacked by a stray dog in her local park. She sustained bruising and abrasions during the attack. The long-sleeved T-shirt that the victim was wearing at the time was submitted to our laboratory for examination (Fig. 3). An American Staffordshire Terrier cross-breed dog was suspected of the attack and was seized by local council rangers and reference buccal swabs were collected. The T-shirt was visually examined and found to have areas of damage with apparent white staining on the right sleeve and the lower left side. The T-shirt was also examined under blue light and the areas of white staining on the right sleeve and the lower left side were found to fluoresce. Fluorescence can indicate the presence of biological material, such as a dried saliva stain [18]. Sterile adhesive tape was used to collect a sample (tape lift) from the right sleeve where the fluorescence and staining were strongest. This tape lift was then subjected to canine DNA analysis. On a separate occasion, the reference sample was also subjected to canine DNA analysis.



**Fig. 3** A section of the right sleeve of the victims T-shirt showing apparent white staining from which a canine DNA profile was obtained in case study 3

## Results

#### Case study 1: attack on livestock

A partial canine DNA profile, comprising 14 out of a possible 22 alleles, was obtained from the wool sub-sample. This partial DNA profile matched the canine DNA profile obtained from the reference dog sample. Statistical analysis using our in-house canine database estimated the DNA match was approximately 3,000 times more likely if the canine DNA profile on the wool sub-sample originated from the reference dog sample than if it originated from another unrelated dog chosen at random from the general canine population. This case resulted in a successful court prosecution with costs awarded against the dog's owner, and the offending dog was destroyed.

#### Case study 2: attack on family pet

A full single-source canine DNA profile was obtained from the apparent blood stain on the cat door. The canine DNA profile obtained from the cat door matched the canine DNA profile from the suspect English Staffordshire Terrier (Table 2). Statistical analysis estimated the DNA match was over a billion times more likely if the canine DNA profile on the cat door originated from the English Staffordshire Terrier than if it originated from another unrelated dog chosen at random from the general canine population. The other suspect dog, an American Staffordshire Terrier, was excluded as a contributor to the blood stain at 9 out of 11 DNA markers (Table 2).

**Table 2** Canine STR profiles obtained from the suspect dogs and crime scene sample in case study 2

Locus	Crime scene sample from the cat door	Reference sample from suspect English Staffordshire terrier	Reference sample from suspect American Staffordshire terrier
PEZ1	<b>119, 119</b>	<b>119, 119</b>	123, 123*
FHC2054	<b>164, 168</b>	<b>164, 168</b>	156, 172*
FHC2010	<b>233, 233</b>	<b>233, 233</b>	233, 233
PEZ16	<b>300, 304</b>	<b>300, 304</b>	300, 304
PEZ5	<b>111, 111</b>	<b>111, 111</b>	107, 107*
PEZ20	<b>185, 189</b>	<b>185, 189</b>	177, 181*
PEZ12	<b>275, 275</b>	<b>275, 275</b>	279, 279*
PEZ3	<b>132, 138</b>	<b>132, 138</b>	135, 138*
PEZ6	<b>191, 191</b>	<b>191, 191</b>	190, 194*
PEZ8	<b>242, 246</b>	<b>242, 246</b>	234, 238*
FHC2079	<b>275, 279</b>	<b>275, 279</b>	283, 283*

Allele sizes shown in base pairs.

Matching profiles shown in bold

\* Exclusionary loci

### Case study 3: attack on person

Full canine DNA profiles were obtained from the tape lift from the victim's T-shirt, and from the reference sample. The full canine DNA profile obtained from the tape lift sample matched the canine DNA profile from the suspect American Staffordshire Terrier cross-breed. Statistical analysis estimated that the DNA match was approximately 10 billion times more likely if the canine DNA profile on the T-shirt originated from the suspect American Staffordshire Terrier cross-breed than if it originated from another unrelated dog chosen at random from the general canine population. This case resulted in a successful court prosecution with costs awarded against the dog's owner, and the offending dog was destroyed.

### Discussion

Our laboratory uses its own forensic canine DNA profiling panel of STR markers in canine investigations, employing established methodology for both the screening and sampling of canine biological material, and the extraction and profiling of DNA. Despite the often small amounts of available biological material and degraded nature of samples, our current method is sufficiently sensitive and robust to reliably determine canine DNA genotypes from typical forensic samples such as hair, blood, faeces, saliva etc. Canine profiles obtained from actual casework samples have been shown to be informative for the purposes of both identity and parentage testing.

From a practical standpoint, investigators in canine forensic cases should attempt to obtain accurate and detailed case histories and consider which samples will be of most probative value so as to prioritise these for analysis. For example, samples heavily stained with victim's

blood may not be suitable if examining for canine saliva from an offending dog. Investigators should be familiar with appropriate areas to sample from victims of dog attacks. For example, the likely areas that canine saliva may be present are around bite marks or wounds which can include the hands and clothing that may be covering the arms and lower legs of a human victim or the face, neck and genitalia of livestock or other animals. Investigators should also have an appreciation for the importance of sample continuity, as the chain of custody can be contested in legal proceedings. However, in the author's experience, the failure to find and collect relevant evidence is primarily what impedes successful case outcomes. This is surprising, as the costs involved in the collection of samples is usually minimal, whether or not canine DNA testing of any or all the samples is ultimately undertaken.

Dogs are not only perpetrators of attacks but can also be victims or witnesses to other crimes. Due to the large number of dogs in Australia and the fact that dogs are one of the most common domestic animals, the presence of canine biological evidence may be found at a crime scene in other criminal matters. Transfer of DNA from canine hair, saliva, blood, or faeces can occur during the commission of a crime, for example onto a suspect or victim. Cases presented to our laboratory for canine DNA analysis have also included animal cruelty cases, theft of an animal, and the linking of suspects with both a crime scene and a victim though canine DNA evidence.

In all of our casework scenarios to date, the results obtained from the panel of 11 markers have proven to be sufficiently statistically discriminating, even when a conservative allowance for population sub-structure is made. It is anticipated that a range of mixed-breed and purebred canine databases will be available in the near future; enabling more precise estimates of appropriate theta values to be used when calculating statistics for canine forensic

DNA profiles. Statistical estimates derived from a breed-specific canine database may be useful to the court in some casework scenarios where the breed of the offending dog is known.

Forensic scientists employed in laboratories that only perform traditional forensic human DNA profiling may still have a role to play in canine forensic cases. In our experience, and in that of others [19], blood and other biological material from human victims can sometimes be found on a suspect dog after an attack, such as on a dog's collar or clothing.

Dogs can also exert predation pressure on native fauna [20]. Feral dogs are a known pest species in Australia [21] and can pose a threat to many native species. Pet dogs can be a serious problem to native fauna if uncontrolled. Even dogs that are heavily reliant on humans for food may still hunt native fauna in nearby bushland and nature reserves [21]. More recently our laboratory has received items for canine DNA analysis from attacks on native wildlife such as wallabies and penguins. Another application for canine DNA profiling may include the ability to monitor feral dog populations by tracking the movements of individual dogs using DNA methods. This may be of interest to some wildlife managers.

Analysis of canine DNA evidence recovered at the scene of an attack has been shown to be a powerful tool in assisting local councils, police officers and state government departments to solve dog attacks by identifying the offending dogs alleged to have been involved or by exonerating the innocent.

### Key points

1. A large number of dog attacks occur every year in Australia and many go unsolved. They may involve injury or death to pets, livestock and wildlife as well as humans. Canine biological material left behind on the victim or at the scene of a dog attack can be used to obtain canine DNA evidence.
2. Our laboratory uses an in-house forensic canine DNA profiling panel of STR markers for canine investigations, which is sensitive and robust enough to reliably determine canine DNA genotypes from typical forensic casework samples.
3. A number of breed-specific population databases have been developed in-house, however our standard forensic canine population database is a general dog database of unrelated dogs and has proven to be sufficiently statistically discriminating, even when only partial profiles are obtained and a conservative allowance for population sub-structure is made.

4. Dogs can also be victims or witnesses to crimes. Considering the extent of human/canine contact, an increase in awareness amongst investigators to the value of canine DNA evidence and a willingness to collect and analyse it, in all manner of crimes, should lead to improved case outcomes.

### References

1. Australian Companion Animal Council. Dogs in society position paper—Review of dog bite prevention programs in Australia, 2007.
2. DeNise S, Johnston E, Halverston J, Marshall K, Rosenfield D, McKenna S, et al. Power of exclusion for parentage verification and probability of match for identity in American kennel club breeds using 17 canine microsatellite markers. *Anim Genet*. 2003;35:14–7.
3. Francisco LV, Langston AA, Mellersh CS, Neal CL, Ostrander EA. A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. *Mamm Genome*. 1996;7:359–62.
4. Zajc I, Mellersh CS, Sampson J. Variability of canine microsatellites within and between different dog breeds. *Mamm Genome*. 1997;8(3):182–5.
5. Morera L, Barba CJ, Garrido JJ, Barbancho M, de Andres DF. Genetic variation detected by microsatellites in five Spanish dog breeds. *J Hered*. 1999;90(6):654–6.
6. Irion DN, Schaffer AL, Famula TR, Eggleston ML, Hughes SS, Pedersen NC. Analysis of genetic variation in 28 dog breed populations with 100 microsatellite markers. *J Hered*. 2003;94(1): 81–7.
7. Halverston J, Basten C. A PCR Multiplex and Database for Forensic DNA Identification of Dogs. *J Forensic Sci*. 2005;50(2): 352–63.
8. Blejwas K, Williams C, Shin G, McCullough D, Jaeger M. Salivary DNA evidence convicts male coyotes of killing sheep. *J Wildl Manag*. 2006;70:1087–93.
9. Sundqvist A, Ellegren H, Vila C. Wolf or dog? Genetic identification of predators from saliva collected around bite wounds on prey. *Conserv Genet*. 2008;9:1275–9.
10. Halverston J, Dvorak J, Stevenson T. Microsatellite sequences for canine genotyping. US Patent 05874217, 1995.
11. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, et al. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res*. 1996;24(16): 3189–94.
12. Tereba A. Tools for analysis of population statistics. *Profiles in DNA* 1999;2(3).
13. Lewis PO, Zaykin D. Genetic data analysis: portable version 1.1 for Linux, 1996 (Software available at <http://lewis.eeb.uconn.edu/lewishome/software.html>).
14. Balding DJ, Nicholls RA. DNA profile match probability calculation: how to allow for population stratification, relatedness, database selection and single bands. *Forensic Sci Int*. 1994;64: 125–40.
15. Chakraborty R, Srinivasan MR, Daiger SP. Evaluation of standard error and confidence interval of estimated multilocus genotype probabilities, and their implications in DNA forensics. *Am J Hum Genet*. 1993;52:60–70.
16. Kanthaswamy S, Tom BK, Mattila A, Johnston E, Dayton M, Kinaga Erickson BJA, et al. Canine population data generated



- from a multiplex STR kit for use in forensic casework J. Forensic Sci. 2009;54(4):829–40.
17. Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci. 2004;39:362–72.
  18. Vandenberg N, van Oorschot RAH. The use of polilight in the detection of seminal fluid, saliva and bloodstains and comparison to conventional chemical based screening tests. J Forensic Sci. 2006;51:361–70.
  19. Brauner P, Reshef A, Gorski A. DNA profiling of trace evidence—mitigating evidence in a dog biting case. J Forensic Sci. 2001;46(5):1232–4.
  20. Mitchell BD, Banks PB. Do wild dogs exclude foxes? evidence for competition from dietary and spatial overlaps. Austral Ecol. 2005;30:581–91.
  21. NSW Scientific Committee—final determination. Predation and Hybridisation by Feral Dogs (*Canis lupus familiaris*)—key threatening process listing, July 2009. [www.environment.nsw.gov.au/determinations/feraldogsFD.htm](http://www.environment.nsw.gov.au/determinations/feraldogsFD.htm).

# Using species-specific repeat and PCR–RFLP in typing of DNA derived from blood of human and animal species

Yasser Said El-Sayed · Omnia Ismaeil Mohamed ·  
Khaled Mohamed Ashry · Salah M. Abd El-Rahman

Accepted: 15 October 2009 / Published online: 28 November 2009  
© Humana Press 2009

**Abstract** Species determination of tissue specimens, including blood, is an important component of forensic analysis to distinguish human from animal remains. DNA markers based on a method of species-specific PCR and amplifying the 359-base pair (bp) fragment of the mitochondrially encoded cytochrome-*b* gene and then digestion with the *TaqI* restriction enzyme were developed for detection and discrimination of human, cattle, buffalo, horse, sheep, pig, dog, cat and chicken blood samples. The results reveal that PCR-amplification of the gene encoding the species-specific repeat (SSR) region generated 603 bp in cattle and buffalo, 221 bp in horse, 374 bp in sheep,  $\leq 100$  bp in pig, 808 bp in dog, 672 bp in cat and 50 bp in chicken. Restriction analysis of the amplified 359-bp portion of the cytochrome-*b* gene using the *TaqI* restriction enzyme results in species-specific restriction fragment length polymorphism (RFLP) between buffalo, cattle and human. Two different bands were generated in buffalo (191 and 168 bp) and human (209 and 150 bp), with no digestion in cattle (359 bp). Cytochrome-*b* is a highly conserved region and consequently a good molecular marker for diagnostic studies. Therefore, the two complementary techniques, SSR-PCR and PCR–RFLP, could be used successfully as routine methods in forensics for sensitive,

rapid, simple and inexpensive identification of the species in bloodstains.

**Keywords** Human identification · Animal identification · Blood sample · SSR · RFLP · Cytochrome-*b* gene

## Abbreviations

bp	Base pairs
cyt- <i>b</i>	Cytochrome- <i>b</i>
D-loop	Displacement loop
mtDNA	Mitochondrial DNA
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNAs
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
SSR	Species-specific repeat
STR	Short tandem repeat

## Introduction

Identifying the species of origin from traces of source materials, such as blood, can sometimes be a difficult, yet important task in forensic DNA analysis. For instance, insurance claims that involve car accidents with animals require authentication [1, 2]. In addition, species identification could be particularly useful in resolving criminal and civil cases, kinship analysis, paternity testing when a female has been exposed to multiple males, determining parentage when an animal switches offspring with another dam, extended lineage reconstruction, estimating inbreeding, identification in breed registries, speciation, poaching, illegal trade, and for the sake of protecting endangered

Y. S. El-Sayed (✉) · O. I. Mohamed · K. M. Ashry  
Department of Veterinary Forensic Medicine and Toxicology,  
Faculty of Veterinary Medicine, Alexandria University,  
Edfina, Rossetta-line, Behera, Egypt  
e-mail: yasser\_tf@yahoo.com

S. M. Abd El-Rahman  
Nucleic Acids Research Division, Genetic Engineering and  
Biotechnology Research Institute, Mubarak City for Scientific  
Research and Technology Applications, New Borg El-Arab City,  
Alexandria, Egypt

animal life [3, 4]. Thus, one of the stages of dealing with biological materials submitted to forensic laboratories is species identification of the biological evidence. However, the problems of distinguishing closely related species can be a serious issue, e.g., identification of cattle versus buffalo remains.

The conventional molecular methods for species identification have been mainly based on protein markers, immunological testing, electrophoresis, high-performance liquid chromatography analysis and DNA hybridization [5–11]. Application of such protocols has, however, failed to successfully differentiate closely related species, highlighting the need for a method with higher specificity and sensitivity. Currently, analysis of DNA via polymerase chain reaction (PCR) is the most commonly used forensic technique that potentially provides definitive information regarding animal species identification [2, 12]. There are certain known DNA sequences in many species, even closely related ones, which are species-specific [13, 14]. Consequently, species-specific primers were designed to only produce a PCR product with the species for which they were designed. It is completely unique for each species [13].

Some PCR approaches (nuclear or mitochondrial) are PCR-based species-specific repeat (SSR), random amplified polymorphic DNAs (RAPD-PCR) fingerprints [15], species-specific short tandem repeat (STR) [16], multiplex PCR [17], quantitative intra-short interspersed element PCR [18], microsatellites analysis [19], and Real-time PCR [20]. Others are focused on sequencing of specific mitochondrial genes such as (12S, 16S and 18S) rRNA [21–25], the Hypervariable Displacement Loop (D-loop) region [21, 25], and the cytochrome-*b* gene (*cyt-b*) [25, 26]. Several features of the mitochondrial DNA (*mtDNA*) genes explain their extensive use as a powerful tool for forensic species identification. They exhibit a high degree of sequence variability between species and have several thousand copies per cell in addition to maternal inheritance [27, 28]. Thus, amplification of a *mtDNA* segment is a relatively sensitive procedure, and can identify even closely related species.

In forensic investigations, the PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of mitochondrial *cyt-b* gene is the most common promising molecular genetic approach to species identification [26, 29]. The nucleotide sequences of human and animal *cyt-b* genes are known to be species-specific [30–32]. Moreover, they have sufficient genetic variability for differences to be found even in very closely related animal species [33]. Amplification of the *cyt-b* gene with one universal PCR-primer flanking a portion of the sequence and digestion of the resulting amplicon with a certain restriction enzyme (RE) can be used to produce species-specific RFLP of various lengths [31, 34]. Hence, the result can be a basis for animal

species identification with a high degree of certainty. In this investigation, we report on the use of quick and simple PCR methods to identify the presence of some animal species and human blood based on species-specific repeat (SSR) and RFLP for *cyt-b* gene. Amplification of the *cyt-b* gene using primer pairs targeting highly conserved regions was followed by PCR-RFLP analysis using the *TaqI* enzyme to perform related animal species and human identification. Confirmation of the human origin of samples is likely to be useful in routine forensic cases.

## Materials and methods

### Blood samples

Blood samples were collected from human (*Homo sapiens*) and commonly domesticated animals including: domestic cattle (*Bos taurus*), domestic water buffalo (*Bubalus bubalis*), domestic dog (*Canis familiaris*), domestic cat (*Felis catus*), horse (*Equus caballus*), domestic pig (*Sus scrofa domestica*), domestic sheep (*Ovis aries*) and chicken (*Gallus gallus*) (at the Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt) into sterilized test tubes containing disodium EDTA as an anticoagulant. The samples were stored at  $-20^{\circ}\text{C}$  until needed for DNA extraction.

### DNA extraction

Genomic-DNA including *mtDNA* was extracted from whole blood samples according to Sharma et al. [35]. To an aliquot of 50  $\mu\text{l}$  of blood (after thawing), 700  $\mu\text{l}$  of lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 1.0 mM EDTA, pH 8.0, 0.5% SDS) and 60  $\mu\text{g}$  of proteinase K (20 mg/ml) were added. The mixture was vortexed and allowed to digest at  $37^{\circ}\text{C}$  overnight. DNA was successively extracted by equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), respectively. DNA was precipitated by adding double volumes of chilled ethanol (95%) in the presence of a high concentration of salt (10% 3 M sodium acetate). The DNA pellets were washed with 70% ice-cold ethanol, air-dried and subsequently dissolved in 50  $\mu\text{l}$  double distilled water for 2 h, and stored at  $-20^{\circ}\text{C}$  until used.

### PCR amplification

Amplification of SSR and *mtDNA cyt-b* genes were performed following the described procedures using primer sequences (NewLab BioQuality, Germany) shown in Table 1. All reactions were carried out in a final volume of 25  $\mu\text{l}$  containing 50 ng genomic DNA as a template,

**Table 1** Primer sequences of species-specific repeat and cytochrome-*b* gene' their annealing temperatures and length of PCR product

Species	Primer sequence 5' → 3'	Length (bp)	Annealing temperature (°C)	Length of PCR product (bp)	Literature reference
Domestic buffalo and cattle	AAG CTT GTG ACA GAT AGA ACG AT CAA GCT GTC TAG AAT TCA GGG A	23 22	60	603	[36]
Domestic dog	GGA GTA TGC TTG ATT CTA CAG AGA AGT GGA ATG AAT GCC	21 18	52	808	[37]
Domestic cat	CTC ATT CAT CGA TCT ACC CA GTG AGT GTT AAA ACT AGT ACT AGA AGA	20 27	52	672	[37]
Horse	TTC TGC TCT GGG TGT GCT ACT CTA CTT CAG CCA GAT CAG GC	21 20	56	221	[38]
Domestic pig	GGA GCG TGG CCC AAT GCA AAT GAA TCC ACT GCA TTC AAT C	18 22	57	≤100	[39]
Domestic sheep	GTT AGG TGT AAT TAG CCT CGC GAG AA AAG CAT GAC ATT GCT GCT AAG TTC	26 24	61	374	[40]
Chicken	GCG TTT TCT CTT CGC AAA TCC ACG CGT GAT TTT CGC TTA AAT G	21 22	55	50	[41]
Buffalo's, cattle's and human's cytochrome- <i>b</i> gene	CCA TCC AAC ATC TCA GCA TGA TGA AA GCC CCT CAG AAT GAT ATT TGT CCT CA	26 26	57	359	[42]

25 pmol dNTPs, 25 pmol of the primer, 1.0 U *Taq* DNA polymerase and reaction buffer (Finnzymes). Amplification reactions were done in a DNA thermal cycler (Eppendorf AG 22331, Gradient, Hambourg, Germany). It was programmed as follows: an initial denaturation step at 94°C for 4 min followed by 34 cycles of 94°C for 1 min for DNA denaturation, annealing temperatures as mentioned with each primer (Table 1), extension at 72°C for 1 min and final extension at 72°C for 10 min. The samples were held at 4°C.

### PCR–RFLP

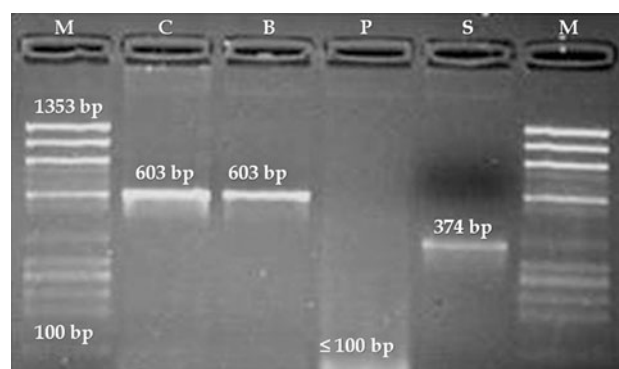
The PCR product generated from the amplified *mtDNA* *cyt-b* gene was digested with 10 U of *TaqI* endonuclease at 65°C for 1 h. The restriction enzyme *TaqI* was used to yield specific restriction profiles that allowed a direct identification of buffalo, cattle and human molecular samples.

### Gel electrophoresis and documentation

The amplified and digested DNA fragments were separated on 3% agarose gel (Boehringer Mannheim, Germany) in IX TBE buffer, and stained with ethidium bromide (Amresco, USA). Appropriate DNA molecular weight markers of  $\Phi$ X174 DNA-*HaeIII* (SibEnzyme, Russia) (contains 10 fragments ranging in size from 1,353 to 72 bp) and 100 bp DNA (Bioron, Germany) (contains 11 DNA fragments ranging in size from 1,500 to 100 bp) were used to estimate the exact size of obtained fragments. The amplified pattern was visualized on a UV transilluminator (BioAmerica Inc., USA) and photographed by Gel Documentation System (Alpha Imager TM1220, Documentation and Analysis system, Canada).

## Results and discussion

The aim of this study was to develop a simple, rapid and reliable PCR assay to discriminate biological samples of common domestic animals and human. For this purpose, the genomic DNA including *mtDNA* was extracted from human and animal blood samples to amplify SSR and *cyt-b* genes. Therefore, this technique has been used for identification of buffalo, cattle, sheep, pig, dog, cat, horse and chicken meat [31, 44–46], buffalo, cattle and sheep milk [43], and buffalo, dog and cat blood [31]. Primers used showed specific fingerprint patterns for each species studied. PCR amplification of the SSR DNA sequence yielded: 603 bp lengths in cattle and buffalo,  $\leq 100$  bp in pig, 374 bp in sheep (Fig. 1, Table 2) [43, 44], 672 bp in cat, 808 bp in dog, 221 bp in horse (Fig. 2, Table 2) and 50 bp



**Fig. 1** Species identification in blood sample, PCR products (603,  $<100$  and 374 bp) generated by species-specific oligonucleotide primers. Lane C, cattle; Lane B, buffalo; Lane P, pig; Lane S, sheep; Lane M, molecular weight marker ( $\Phi$ X174 DNA-*HaeIII* Digest)

**Table 2** Lengths of fragments generated by species-specific repeat, 359 bp mitochondrial *cyt-b* and digestion of 359 bp mitochondrial *cyt-b* products with *TaqI* restriction enzyme

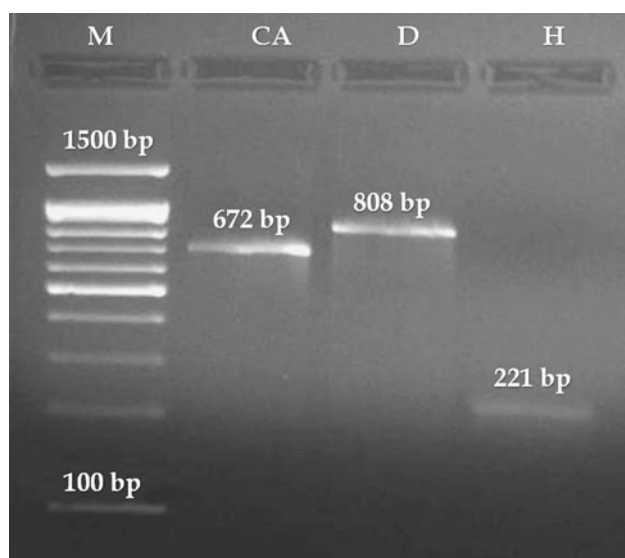
Species	Length of generated PCR fragment (bp)		
	SSR	Cyt- <i>b</i>	Cyt- <i>b</i> / <i>TaqI</i>
Human	na	359	209, 150
Domestic cattle	603	359	359
Domestic water buffalo	603	359	191, 168
Domestic dog	808	na	na
Domestic cat	672	na	na
Horse	221	na	na
Domestic pig	$<100$	na	na
Domestic sheep	374	na	na
Chicken	50	na	na

SSR Species-specific repeat, Cyt-*b* Mitochondrial cytochrome-*b*, na not applicable

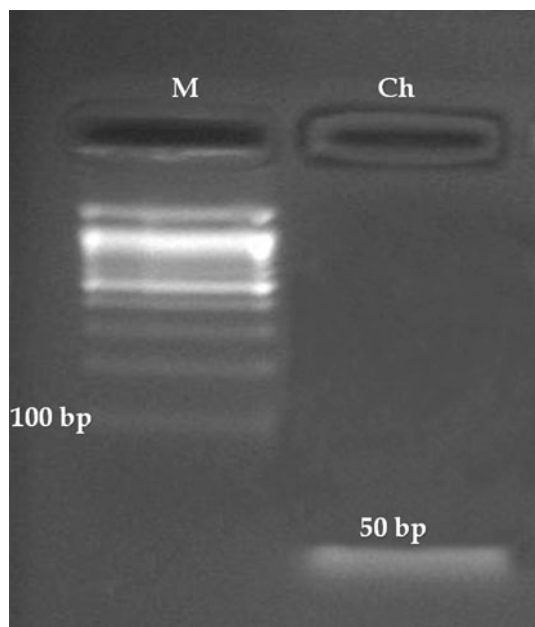
in chicken (Fig. 3, Table 2). With these patterns we could discriminate between eight animal species. It, therefore, has been demonstrated that SSR-PCR analysis enables direct identification of various animal species even of different breeds in field samples by the differences in size and site of patterns.

Figure 1 shows that the size and position of the SSR-PCR generated fragment (603 bp) in cattle and buffalo are exactly the same as the fragment of molecular weight marker ( $\Phi$ X174 DNA-*HaeIII* ladder). Therefore, the SSR method does not differentiate between different bovine species. For discrimination, we recommend the PCR–RFLP technique for the *mtDNA* region containing genes for *cyt-b* for which a universal primer amplifying in several mammalian species is available [30]. A single fragment with a size of 359 bp resulted from PCR amplification of the *cyt-b* gene not only in cattle and buffalo, but also in human blood samples. The *TaqI* endonuclease digested the



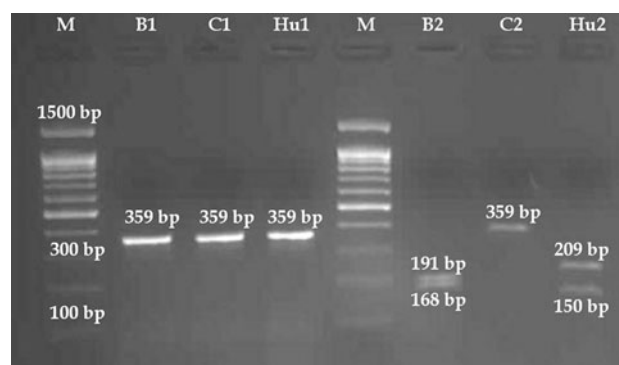


**Fig. 2** Species identification in blood sample, PCR products (672, 808 and 221 bp) generated by species-specific oligonucleotide primers. Lane CA, cat; Lane D, dog; Lane H, horse; Lane M, molecular weight marker (100 bp DNA)



**Fig. 3** Species identification in blood sample, PCR products (50 bp) generated by species-specific oligonucleotide primers. Lane CH, chicken; Lane M, molecular weight marker (100 bp DNA)

359 bp amplicons of buffalo's *mtDNA* *cyt-b* into 168–191 bp fragments in meat and milk [26, 44, 46], allowing simple identification of cattle and buffalo. Digestion of the amplified fragment of *cyto-b* genes with the restriction enzyme *TaqI* endonuclease generated two different bands of sizes 191 and 168 bp with buffalo, and 209 and 150 bp with human, while it was not digested (359 bp) in cattle



**Fig. 4** Mitochondrial PCR–RFLP analysis of purified DNA derived from cattle, buffalo and human blood samples. Agarose gel electrophoresis of amplified cytochrome-*b* gene (Lane Hu1, human; Lane C1, cattle; Lane B1, buffalo) after restriction with *TaqI* endonuclease generated two adjacent fragments with a size of 191 and 168 bp in buffalo (Lane B2), faint band of the same size (359 bp) in cattle (Lane C2) and another two fragments with a size of 209 and 150 bp in human (Lane Hu2). Lane M is a molecular weight marker (100 bp DNA)

(Fig. 4, Table 2) allowing simple identification of cattle, buffalo [43, 44], and human samples.

The data clearly illustrates that PCR amplification of species-specific genes from given blood samples can effectively identify the species origin of the sample. Moreover, this technique is versatile, as it can detect species origin using any biological materials containing DNA, such as meat or milk [31, 43–46]. However, the major limitation of the assay is its inability to discriminate between related animal species, namely cattle and buffalo. Hence, a buffalo or cattle specific primer and/or probe must be designed to differentiate cattle and buffalo blood. However, it is possible to identify related animal species and distinguish between human and non-human molecular evidence using PCR–RFLP and certain restriction enzymes. Therefore, the two complementary assays, SSR-PCR and PCR–RFLP, could be used successfully as routine methods in forensics, being sensitive, rapid, simple and inexpensive for identifying species in fresh bloodstains and for differentiating between human and nonhuman samples. Our results support the application of these methods as a useful molecular analytical method for identification of different mammals and control measures for blood-based evidence.

### Key points

1. The gene encoding species-specific region of DNA could be amplified using SSR-PCR to identify blood samples obtained from human and different animal species.
2. Buffalo or cattle specific primer and/or probe must be designed to differentiate their blood.

3. It is possible to identify related animal species and distinguish between human and non-human molecular evidence using PCR-RFLP and certain restriction enzyme.
4. The SSR-PCR and PCR-RFLP could be applied as a useful molecular analytical method for identification and authentication of different mammals and control measures for any molecular-based evidence.
5. As required by law, it would suggest that while these results demonstrate proof of concept, a great deal more research is required before these tests were validated to a standard sufficient for forensic purposes.

## References

1. DeForest PR, Gaensslen RE, Lee HC. Forensic science: an introduction to criminalistics. New York: McGraw-Hill; 1983.
2. Miller-Coyle H. International forensic science and investigation series, nonhuman DNA typing: theory and casework application. Boca Raton: CRC Press; 2008.
3. Bartlett SE, Davidson WS. FINS (Forensically Informative Nucleotide Sequencing): a procedure for identifying the animal origin of biological specimens. *BioTechniques*. 1992;12:82–92.
4. Wetton JH, Higgs JE, Spriggs AC, Roney CA, Tsang CSF, Foster AP. Mitochondrial profiling of dog hairs. *Forensic Sci Int*. 2003;133:235–41.
5. Andrasko J, Rosen B. Sensitive identification of hemoglobin in bloodstains from different species by high performance liquid chromatography with combined UV and fluorescence detection. *J Forensic Sci*. 1994;39:1018–25.
6. Hsieh YH, Sheu SC, Bridgman RC. Development of a monoclonal antibody specific to cooked mammalian meats. *J Food Prot*. 1998;61:476–81.
7. Skarpeid HJ, Kvaal K, Hildrum KI. Identification of animal species in ground meat mixtures by multivariate analysis of isoelectric-focusing protein profiles. *Electrophoresis*. 1998;19:3103–9.
8. Espinoza EO, Lindley NC, Gordon KM, Ekhoft JA, Kirms MA. Electrospray ionization mass spectrometric analysis of blood for differentiation of species. *Anal Biochem*. 1999;268:252–61.
9. Czesny S, Dabrowski K, Christensen JE, Eenennaam JV, Doroshov S. Discrimination of wild and domestic origin of sturgeon ova based on lipids and fatty acid analysis. *Aquaculture*. 2000;189:145–53.
10. Lowenstein JM, Reuther JD, Hood DG, Scheuenstuhl G, Gerlach SC, Ubelaker DH. Identification of animal species by protein radioimmunoassay of bone fragments and bloodstained stone tools. *Forensic Sci Int*. 2006;159:182–8.
11. Chou CC, Lin SP, Lee KM, Hsu CT, Vickroy TW, Zen JM. Fast differentiation of meats from fifteen animal species by liquid chromatography with electrochemical detection using copper nanoparticle plated electrodes. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;846:230–9.
12. Jobin R, Patterson DK, Stang C. Forensic DNA typing in several big game animals in the province of Alberta. *Can Soc Forens Sci J*. 2003;36:56.
13. Guglich EA, Wilson PJ, White BN. Forensic application of repetitive DNA markers to the species identification of animal tissues. *J Forensic Sci*. 1994;39:353–61.
14. Murray BW, McClymont RA, Strobeck C. Forensic identification of ungulate species using restriction digests of PCR-amplified mitochondrial DNA. *J Forensic Sci*. 1995;40:943–51.
15. Lee JC, Chang JG. Random amplified polymorphic DNA polymerase chain reaction (RAPD PCR) fingerprints in forensic species identification. *Forensic Sci Int*. 1994;67:103–7.
16. Coomber N, David VA, O'Brien SJ, Menotti-Raymond M. Validation of a short tandem repeat multiplex typing system for genetic individualization of domestic cat samples. *Croat Med J*. 2007;48:547–55.
17. Jain S, Brahmabhai MN, Rank DN, Joshi CG, Solanki JV. Use of cytochrome b gene variability in detecting meat species by multiplex PCR assay. *Indian J Anim Sci*. 2007;77:880–1.
18. Walker JA, Hughes DA, Anders BA, Shewale J, Sinha SK, Batzer MA. Quantitative intra-short interspersed element PCR for species-specific DNA identification. *Anal Biochem*. 2003;316:259–69.
19. Lorenzini R. DNA forensics and the poaching of wildlife in Italy: a case study. *Forensic Sci Int*. 2005;153:218–21.
20. Jonker KM, Tilburg JJ, Hagele GH, de Boer E. Species identification in meat products using real-time PCR. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2008;25:527–33.
21. Bellis C, Ashton KJ, Freney L, Blair B, Griffiths LR. A molecular genetic approach for forensic animal species identification. *Forensic Sci Int*. 2003;134:99–108.
22. Rastogi G, Dharne M, Bharde A, Pandav VS, Ghumatkar SV, Krishnamurthy R, et al. Species determination and authentication of meat samples by mitochondrial 12S rRNA sequence analysis and conformation-sensitive gel electrophoresis. *Curr Sci*. 2004;87:1278–81.
23. Martín I, García T, Fajardo V, Rojas M, Hernández PE, González I, et al. Technical note: Detection of cat, dog, and rat or mouse tissues in food and animal feed using species-specific polymerase chain reaction. *J Anim Sci*. 2007;85:2734–9.
24. Melton T, Holland C. Routine forensic use of the mitochondrial 12S ribosomal RNA gene for species identification. *J Forensic Sci*. 2007;52:1305–7.
25. Pereira F, Carneiro J, Amorim A. Identification of species with DNA-based technology: current progress and challenges. *Recent Pat DNA Gene Seq*. 2008;2:187–99.
26. Bellagamba F, Moretti VM, Comincini S, Valfrè F. Identification of species in animal feedstuffs by polymerase chain reaction-restriction fragment length polymorphism analysis of mitochondrial DNA. *J Agric Food Chem*. 2001;49:3775–81.
27. Pesole G, Gissi C, De Chirico A, Saccone C. Nucleotide substitution rate of mammalian mitochondrial genomes. *J Mol Evol*. 1999;48:427–34.
28. Rastogi G, Dharne M, Walujkar S, Kumar A, Patole MS, Shouche YS. Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Sci*. 2007;76:666–74.
29. Pfeiffer I, Burger J, Brenig B. Diagnostic polymorphisms in the mitochondrial cytochrome-b gene allow discrimination between cattle, sheep, goat, roe buck and deer by PCR-RFLP. *BMC Genet*. 2004;5:30.
30. Parson W, Pegoraro K, Niederstätter H, Föger M, Steinlechner M. Species identification by means of the cytochrome-b gene. *Int J Legal Med*. 2000;114:23–8.
31. Partis L, Croan D, Guo Z, Clark R, Coldham T, Murby J. Evaluation of a DNA fingerprinting method for determining the species origin of meats. *Meat Sci*. 2000;54:369–76.
32. Hsieh HM, Chiang HL, Tsai LC, Lai SY, Huang NE, Linacre A, et al. Cytochrome-b gene for species identification of the conservation animals. *Forensic Sci Int*. 2001;122:7–18.

33. Wolf C, Rentsch J, Hübner P. PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. *J Agric Food Chem*. 1999;47:1350–5.
34. Bravi CM, Liron JP, Mirol PM, Ripoli MV, Peral-Garcia P, Giovambattista G. A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome-*b* gene. *Leg Med*. 2004;6:246–51.
35. Sharma D, Appa Rao KB, Totey SM. Measurement of within and between population genetic variability in quails. *Br Poult Sci*. 2000;41:29–32.
36. Jobse C, Buntjer JB, Haagsma N, Breukelman HJ, Beintema JJ, Lenstra JA. Evolution and recombination of bovine DNA repeats. *J Mol Evol*. 1995;41:277–83.
37. Abdulmawjood A, Schönenbrücher H, Bülte M. Development of a polymerase chain reaction system for the detection of dog and cat meat in meat mixtures and animal feed. *J Food Sci*. 2003;68:1757–61.
38. Wijers ER, Zijlstra C, Lenstra JA. Rapid evolution of horse satellite DNA. *Genomics*. 1993;18:113–7.
39. Jantsch M, Hamilton B, Mayr B, Schweizer D. Meiotic chromosome behaviour reflects levels of sequence divergence in *Sus scrofa domestica* satellite DNA. *Chromosoma*. 1990;99:330–5.
40. Chikuni K, Tabata T, Kosugiyama M, Monma M, Saito M. Polymerase chain reaction assay for detection of sheep and goat meats. *Meat Sci*. 1994;37:337–45.
41. Matzke MA, Varga F, Berger H, Schernthaner J, Schweizer D, Mayr B, et al. A 41–42 bp tandemly repeated sequence isolated from nuclear envelopes of chicken erythrocytes is located predominantly on microchromosomes. *Chromosoma*. 1990;99:131–7.
42. Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proc Natl Acad Sci USA*. 1989;86:6196–200.
43. Abdel-Rahman SM, Ahmed MM. Rapid and sensitive identification of buffalo's, cattle's and sheep's milk using species-specific PCR and PCR-RFLP techniques. *Food Control*. 2007;18:1246–9.
44. Ahmed MM, Abd El-Rahman MS, El-Hanafy AA. Application of species-specific polymerase chain reaction and cytochrome-*b* gene for different meat species authentication. *Biotechnology*. 2007;6:426–30.
45. İlhak Oİ, Arslan A. Identification of meat species by polymerase chain reaction (PCR) technique. *Turk J Vet Anim Sci*. 2007;31:159–63.
46. Al-Sanjary RA. Identification of beef using restriction fragment length polymorphism-polymerase chain reaction. *Iraqi J Vet Sci*. 2009;23:43–6.

# Forensic species identification of elephant (Elephantidae) and giraffe (Giraffidae) tail hair using light microscopy

Bonnie C. Yates · Edgard O. Espinoza ·  
Barry W. Baker

Accepted: 14 May 2010 / Published online: 13 June 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** Here we present methods for distinguishing tail hairs of African elephants (*Loxodonta africana*), Asian elephants (*Elephas maximus*), and giraffes (*Giraffa camelopardalis*) from forensic contexts. Such hairs are commonly used to manufacture jewelry artifacts that are often sold illegally in the international wildlife trade. Tail hairs from these three species are easily confused macroscopically, and morphological methods for distinguishing African and Asian tail hairs have not been published. We used cross section analysis and light microscopy to analyze the tail hair morphology of 18 individual African elephants, 18 Asian elephants, and 40 giraffes. We found that cross-sectional shape, pigment placement, and pigment density are useful morphological features for distinguishing the three species. These observations provide wildlife forensic scientists with an important analytical tool for enforcing legislation and international treaties regulating the trade in elephant parts.

**Keywords** *Loxodonta africana* · *Elephas maximus* · *Giraffa camelopardalis* · Hair identification · Forensic mammalogy · Wildlife trade · Light microscopy

“Having pull’d out of an Elephants-tayl a black Hair, and cut transversly from it a thin scale, I exposed it to my Microscope, which represented in the thick of that Hair about an hundred little specks somewhat whitish, and in each speck a black point, and in some few

of those black points, a little hole; and this hair consisted withal of united Globuls, which yet I thought I should have found bigger in this thick hair of so bulky a Beast, than indeed they were. This Scale I keep still by me because of its curious and elegant appearance, not unlike (excepting the Colours) a Peacocks-tayl.”

Anthony van Leeuwenhoek (1674)  
Philosophical Transactions

“It is an elephant’s hair, probably from the tail. But, as you see, it is a compound hair; virtually a group of hairs agglutinated into a single stem.”

Dr. Thorndyke (fictional detective)  
From “The Trail of Behemoth” (1929),  
by R. Austin Freeman (reprinted, 2004)

## Introduction

Artifacts constructed from elephant and giraffe tail hairs are often encountered in the international wildlife trade (Fig. 1). The most common examples include tail hair bracelets, necklaces, rings and flywhisks used by indigenous peoples and sold to tourists throughout Africa and parts of Southeast Asia [1]. Rigorous methods for identifying elephant and giraffe hairs are of interest to law enforcement officials, who monitor the illegal trade in endangered and threatened species. Tail hairs from all three species are known to be used in artifacts and international trade [1]. Similar artifacts are often manufactured from various plastics and botanical fibers (e.g., elephant grass [*Pennisetum purpureum*]), thus forensic analysis may be required to determine if such items contain parts of

B. C. Yates · E. O. Espinoza · B. W. Baker (✉)  
US National Fish and Wildlife Forensics Laboratory, United  
States Fish & Wildlife Service, 1490 East Main Street,  
Ashland, OR 97520-1310, USA  
e-mail: barry\_baker@fws.gov



**Fig. 1** Examples of elephant hair artifacts, including bracelets, rings and earrings

protected species. While wildlife conservation efforts have focused extensively on the ivory trade, all parts and products manufactured from elephants fall under strict regulatory control and forensic methods for their identification are critical to conservation efforts.

Asian elephants (*Elephas maximus*) are protected under Appendix I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), while populations of African elephants (*Loxodonta africana*) are variously protected under Appendix I and II. The Asian elephant is also listed as endangered on the US Endangered Species Act (ESA), while the African elephant is listed as threatened. In contrast, giraffes (*Giraffa camelopardalis*) are not protected under CITES or the ESA. Overall, commercial trade in elephant parts is highly regulated, and forensic methods for distinguishing elephant and giraffe tail hairs are critical to wildlife enforcement efforts.

The thick, coarse, black tail hairs of elephants (African and Asian) and giraffes are easily confused macroscopically. While elephant tail hairs are generally thicker than those of giraffe, the size of an individual hair alone cannot be used to distinguish these species. Previous research [1] has reported spectroscopic and chemical differences between the tail hairs of elephants and giraffes, and here we present methods for distinguishing them using cross section morphology and light microscopy. The technique provides a robust means of identifying these fibers with inexpensive, standard laboratory equipment.

### Previous microscopic studies

The earliest microscopic study of elephant tail hair was reported by none other than Anthony van Leeuwenhoek (1674) in a letter to the Royal Society of London [2]. Van Leeuwenhoek's microscopes functioned at up to 266X [3],

thus allowing him to describe what he saw in the eloquent language of his time as quoted above. Shoshani [4] described, but did not illustrate, the cross section of an elephant tail hair as "...oval in shape..." and possessing "...a number of small unpigmented areas (white as opposed to the dark color elsewhere in the section)." Microscopic observations on elephant tail hairs have even appeared in a crime novel. Freeman's fictional detective Dr. Thorndyke opined on the identification of a suspected elephant hair recovered from a crime scene, noting "...it is a compound hair; virtually a group of hairs agglutinated into a single stem." [5]. Giraffe tail hairs were described by Lochte [6] as possessing multiple strands of medulla. While several subsequent or earlier studies mention the morphology of elephant and giraffe body hairs, to our knowledge no comparative studies of elephant and giraffe tail hair cross sections have been published in an attempt to distinguish these species.

Among early researchers, Smith [7] illustrated cross sections of skin and body hairs of the elephant, and Neuville [8] noted the variable medullation in elephant hairs, but we are unsure if his study included tail hairs. Hausman [9] included the cuticular structures of elephant, mammoth, and giraffe body hairs. Lochte [10] published what may be the earliest photomicrographs of cross sections and cuticular casts of elephant and giraffe body hairs, which for an Indian (=Asian) elephant are described as fine and thin hair, with no explicit detail. The giraffe hairs were taken from the back near the mane and described as transparent, having no medulla.

Body hairs of these mega mammals and others, such as rhino and hippo, have been described and/or illustrated in several reports [11–16]. Ryder [17] described the follicular mammoth body hairs in detail, but he only illustrated a tracing of a group of hairs from the leg of the Liakhov mammoth. Valente [18] compared mammoth hair from the corpse of a frozen specimen found in 1977 to those of zoo elephants—both *Elephas* and *Loxodonta*. He states that the "cortex of *Mammuthus* hair had tiny dark brown pigment granules and the density of pigmentation decreased from the central axis of the hair to the periphery" [18]. The *Elephas* hairs were "non-medullated and circular in cross-section...[and] the cortex may or may not contain pigment granules which, if present, are sparse towards the periphery" [18]. By contrast, the body hairs of *Loxodonta* were "heavily pigmented throughout...except for the region immediately underlying the cuticle" [18].

Rasmussen and Munger [19] show the cross section of a vibrissa in micrographs of skin from the trunk of an Asian elephant, although their paper concerned the sensory innervation of that appendage and not the hairs themselves. A low magnification photomicrograph (50X) of one of these hairs [19] shows an unmedullated, pigmented center



surrounded by concentric rings of connective tissue that is separated from an outer capsule of connective tissue by an amorphous ring of “vascular sinus spaces.” While these previous studies focused on body hair morphology, here we concentrate our analysis on the structural features of elephant and giraffe tail hairs (cross-sectional shape, pigment placement, and density) and the use of these morphological features for identifying the species source of tail hairs in forensic contexts.

## Methods

Vouchered tail hairs of known species origin were examined from the collection of the US National Fish and Wildlife Forensics Laboratory (United States Fish & Wildlife Service). Samples consisted of 18 individual African elephants (*Loxodonta africana*), 18 Asian elephants (*Elephas maximus*), and 40 giraffes (*Giraffa camelopardalis*) from captive zoo settings. Background data on these vouchered samples were previously published [1]. While the forest elephant is recognized as a full species (*Loxodonta cyclotis*) by some researchers [20], here we follow CITES taxonomy and other researchers who treat *Loxodonta* from Africa as a single species (*Loxodonta africana*) [21].

Hairs were cross-sectioned, examined with light microscopy, and evaluated for species diagnostic characters. Cross section analysis has long been used to evaluate mammal hair morphology and facilitate species identification [22]. Hairs were sonicated in test tubes with tap water for 10 min to loosen and remove detritus. Water was decanted, and hairs were subsequently sonicated in isopropyl alcohol to remove non-water-soluble particles. Hairs were allowed to air dry. To facilitate cross-sectioning, individual hairs were placed in centrifuge tubes and filled with epoxy to serve as a stabilizing agent (EPO-Kwik resin and hardener by Buehler, Lake Bluff, IL). The filled tubes were allowed to dry/set for 24 h. Once set, centrifuge tubes were placed in a mechanical microtome (Model 860, American Optical Co., Buffalo, NY) and were cross-sectioned ( $\approx 26 \mu$  thick). Samples were mounted onto permanent slides using Shandon E-Z Mount (Thermo Fisher Scientific Inc., Waltham, MA) and were examined and analyzed unstained at 100, 200, and 400 $\times$  with a Leica dual-stage transmitted light microscope. Images were produced with LAS software (Leica Microsystems, Inc., Bannockburn, IL).

## Results

Our cross sections reveal that tail hairs of elephants and giraffes differ significantly from body hairs, and suggest

that they are more like rhinoceros horn or horse hoof keratins [23, 24]. To describe them as hairs, one would need to qualify that they appear to have multiple medullae on the interior and generally lack cuticular scales on the exterior. They are more like small, cylindrical segments of thicker keratinous outgrowths of epidermis, comparable to that seen in horn or hoof. As Pollitt described for horses' hooves, “...the keratinocytes of individual hoof wall tubules are arranged around a central hollow medulla in non-pigmented concentric layers” [24]. In a single elephant hair, therefore, these tubules appear to be imbedded in a keratinized cellular matrix [23] confined by the diameter of the tail “hair.”

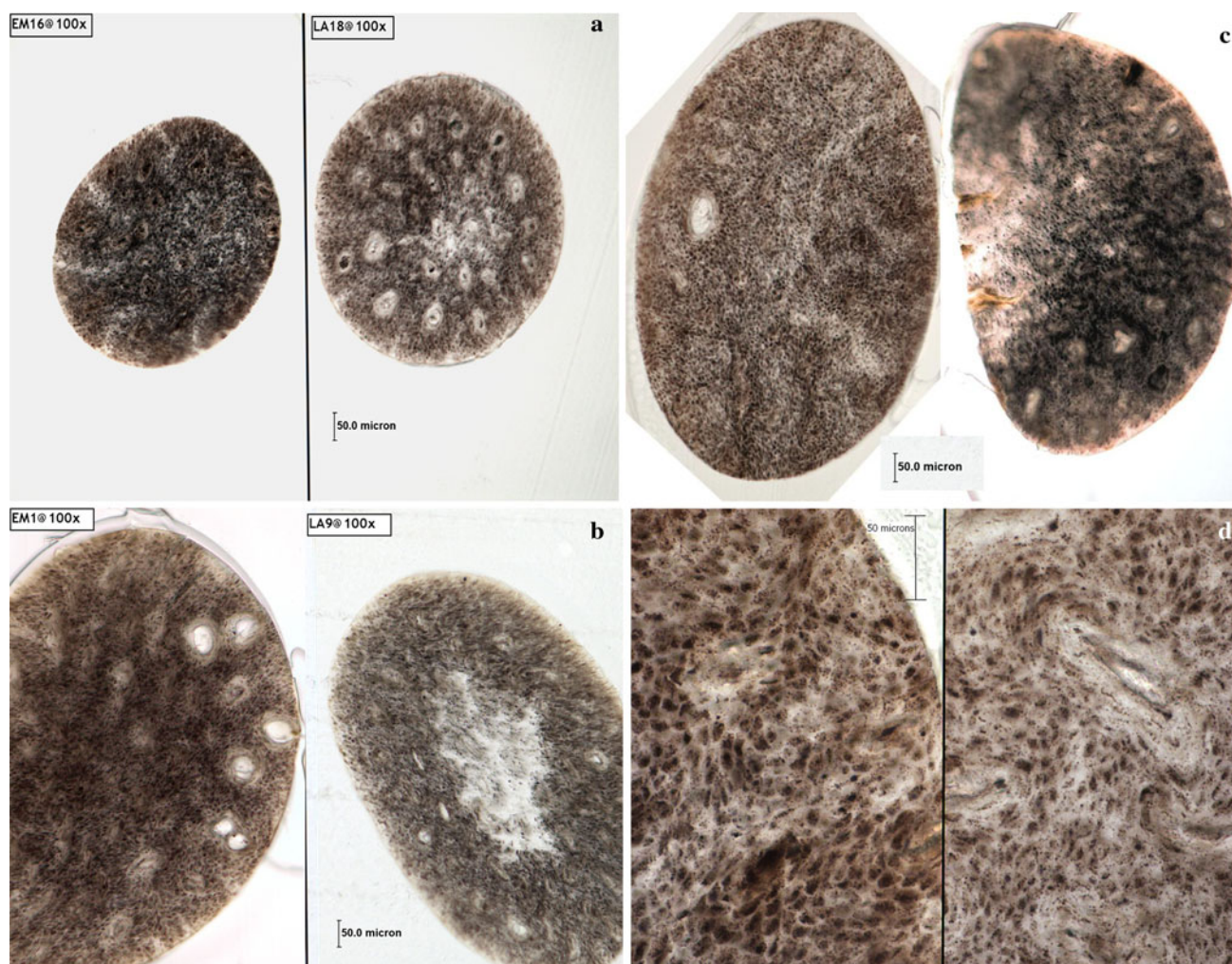
In their expansive SEM study of animal hairs, Chernova and Tselikova [13] published micrographs of cross sections of both mammoth and African elephant hairs. Unlike the fine Asian elephant hair pictured in the early publication by Lochte [10], their electron micrographs of the mammoth and *Loxodonta* hairs show the same tubule-like features at relatively low magnification that we see in our tail hair specimens.

The dispersion of pigment granules in the matrix is affected by the location of the tubules. The resulting pattern that the granules form defines the characterization that distinguishes each species. Thus, it is both the arrangement of the tubules and the dispersion of the pigment that creates the diagnostic traits identifiable in tail hairs of elephants and giraffe (Figs. 2, 3).

Similar tubules observed in hairs and other keratins have variously been referred to as sinuses [19], vacuoles [25], cortical cells with cavities [26], compressed tubules [27], or tubules [24], which is the preferred term here. Chernova [28] stated there is “impelling need for consistent detailed description of cellular structure” for microscopic features of both human and animal hair. In our study, we found some of these tubules to be empty cavities, and, indeed, in a scanning electron micrograph generated on one of our



**Fig. 2** Cross sections of giraffe tail hairs at 100 $\times$



**Fig. 3** **a** Cross section of Asian elephant (*left*) and African elephant (*right*) tail hairs at 100 $\times$ . **b** Cross section of Asian elephant (*left*) and African elephant (*right*) tail hairs at 100 $\times$ . **c** Cross section of Asian

elephant (*left*) and African elephant (*right*) tail hairs at 100 $\times$ . **d** Cross section of Asian elephant (*left*) and African elephant (*right*) tail hairs at 400 $\times$

specimens (Fig. 4), a remnant of the matrix got caught during sectioning in one of the holes near the periphery.

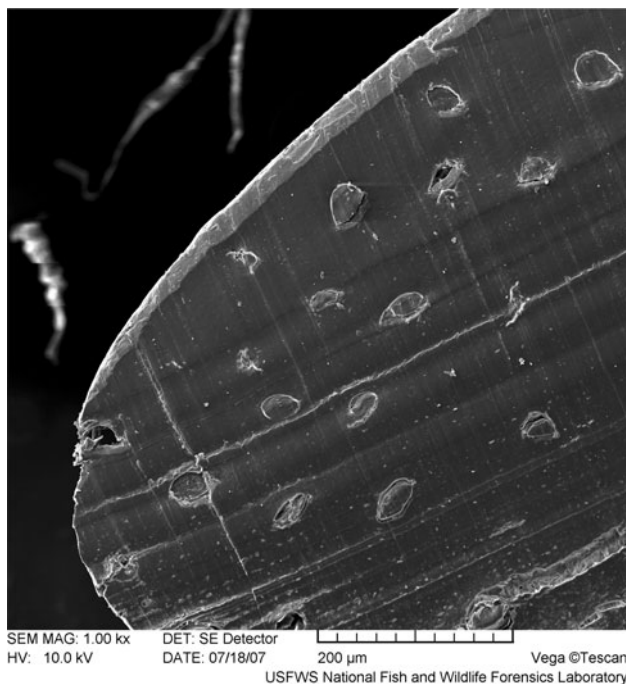
In other tubules shown in Fig. 4, they are in-filled, either with what appears to be a yellowish matter also visible in the transmitted light micrographs (Fig. 5), or by differentiated keratinous cells retained by the elongating tubule during its growth phase. As these tubules migrate during growth toward the periphery of the hair, the vacuous ones reach the edge of the tail hair and form grooves, which in cross section look like a scallop-shaped edge (Fig. 5). These grooves can be seen in stereomicrographs running the length of the tail hair. We speculate that the different expressions of the tubules (compressed, in-filled, or vacuous,) are related to the morphogenesis and development of keratinocytes that eventually mature and migrate away from the tubule to form the matrix.

## Distinguishing traits

### Giraffe vs. elephant

The character trait that distinguishes giraffe from elephant (both African and Asian) is the distinct radial arrangement (in giraffe) of the tubules toward the periphery of the hair shaft (Fig. 2; Table 1). They appear as oval or flattened tubules arranged in a ring positioned more than two-thirds the radius of the tail hair's diameter from its center. The pigment is usually densest in-between the tubules and appears to flow away from the un-pigmented keratin cells that surround the formed or forming tubules. The effect is a pattern of undulating un-pigmented rays on a dark mottled ground, emanating from the center of the cross section and forming regularly spaced, dark-centered tubules surrounded





**Fig. 4** SEM of Asian elephant tail hair at 1,000×



**Fig. 5** Cross section of African elephant tail hair at 100×

by un-pigmented concentric wavy rings, which are the maturing keratinocytes of the tubules' walls.

As noted previously, the structure of these hairs is reminiscent of horse hoof keratin [24], but on a much smaller scale. Unlike horse's hoof, the tubules in tail hair are weaker and, therefore, appear less organized and more variable in shape, and because they comprise hair, the generation of the tubules is centered in the cylinder and grows outward from there. In proximal sections taken from

giraffe tail hairs, the tubules have not begun to migrate toward the periphery, but still appear to be organized in the less pigmented center, trending toward the outside wall where the pigment granules are darkest, but where fingers of un-pigmented space have intruded and are beginning to follow the progression of the tubule and its attendant un-pigmented cells toward the periphery (Fig. 2).

#### African vs. Asian elephant

The character traits that distinguish African elephant (*Loxodonta*) from Asian elephant (*Elephas*) are the density and appearance of the pigment assemblages, and the arrangement of the tubules (Table 1). The two elephant species can be distinguished by the larger, darker pigment granules and denser assemblages in the keratin matrix of *Elephas*, and by the more diffuse, blurry-appearing assemblages in *Loxodonta* (Fig. 3d). While the large granules are about the same size in both elephants, there appears to be fewer large granules in *Loxodonta*. In both elephants' tail hairs, rings of undifferentiated keratinocytes form around the tubules, which may be vacuous or infilled or compressed, but in *Loxodonta*, the rings conform to tubules that are more polygonal in shape, numbering up to 50 in some cross sections. By contrast, the rings around the tubules in *Elephas* are more rounded in shape and do not influence the arrangement of nearby pigment granules as seen in *Loxodonta*. In *Elephas*, there are fewer tubules, and the rings are less distinct and fainter in appearance than those of *Loxodonta*.

Finally, there is a noticeable absence of pigmentation that is expressed differently in each species. For example, cross sections of *Loxodonta* tail hairs frequently are almost void of pigmentation in the center, while *Elephas* is usually heavily pigmented throughout. In addition, *Elephas* exhibits the ray-like features more or less absent of pigment granules that are common to *Giraffa*, though fewer in number, but like *Giraffa* tend to radiate from the center.

#### Conclusions

Our research into thickened tail hairs of elephants and giraffes was driven by the occurrence of these animal parts in the international wildlife trade. To date, morphological cross-section comparison of the tail hairs of these species was lacking. Our light microscopy analysis of tail hairs shows that cross-sectional shape, pigment placement, and pigment density are useful morphological features for distinguishing these three species. While we do not yet know of a functional explanation for these differences, we have demonstrated that these species can be distinguished using inexpensive and standard laboratory equipment.

**Table 1** Summary of microscopic traits that distinguish cross sections of *Elephas*, *Loxodonta*, and *Giraffa* tail hairs

	Giraffe	Asian elephant	African elephant
Pigment	Dark & dense	Dark and dense	Few dark aggregates Blurry fine aggregates
Tubules*	Usually infilled Equidistant when near periphery	May be infilled or empty Scattered unevenly	May be infilled or empty Rarely found in center, which also lacks pigment aggr.
“Rays”**	Trail from tubules back to center	Few in number, but present	Rare

\* Vacuities in the field of keratin that are ovoid or compressed

\*\* Undulating streaks devoid of pigment aggregates

These results compliment previous research demonstrating chemical differences between elephant and giraffe tail hairs [1]. These methods should prove useful for enforcing legislation and international treaties regulating the trade in elephant parts.

### Key points

1. Light microscopy is a useful tool for identifying the species origin of elephant and giraffe tail hairs in forensic contexts.
2. This study describes the first reported morphological differences between the tail hairs of African elephants and Asian elephants.
3. Tail hairs from the three species under investigation can be identified based on differences in cross-sectional shape, pigment placement, and pigment density.
4. This study compliments previous research documenting chemical differences between elephant and giraffe tail hairs.

**Acknowledgments** Doina Voin provided German and Russian translations of relevant literature and helped acquire the tail hairs used in this study. Laura Blount prepared the hair cross sections and mounted the hairs used in this study. Darby Morrell produced Fig. 1 and Michael Scanlan produced Fig. 4. The Sterling Evans Library at Texas A&M University was instrumental in obtaining literature. We thank Linzi Wilson-Wilde for inviting us to submit this manuscript. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

### References

1. Espinoza EO, Baker BW, Moores TD, Voin D. Forensic identification of elephant and giraffe hair artifacts using HATR FTIR spectroscopy and discriminant analysis. *Endangered Species Res.* 2008;9:239–46.
2. Leeuwenhoek Mr. Microscopical observations from Mr. Leeuwenhoek, about blood, milk, bones, the brain, spittle, cuticula, sweat, fatt, teares; communicated in two letters to the publisher. *Phil Trans* 1674;(1665–1678)9:121–31.
3. Van Zuylen J. The microscopes of Antoni van Leeuwenhoek. *J Microsc.* 1981;121:309–28.
4. Shoshani J, et al. On the dissection of a female Asian elephant (*Elephas maximus maximus* Linnaeus, 1758) and data from other elephants. *Elephant.* 1982;2:3–93.
5. Freeman RA. The famous cases of Dr. Thorndyke: thirty-seven of his criminal investigations, Part 2. Kessinger Publishing; 2004.
6. Lochte T. Das mikroskopische bild des giraffen hares. *Zool Garten.* 1952;9:204–6.
7. Smith F. The histology of the skin of the elephant. *J Anat Physiol.* 1890;24:493–503.
8. Neuville MH. Du tégument des proboscidiens. *Bull Mus Hist Nat Paris.* 1917;23:374–87.
9. Hausman LA. Structural characteristics of the hair of mammals. *Am Nat.* 1920;54:496–523.
10. Lochte T. Atlas der Menschlichen und Tierischen Haare. Germany: Leipzig; 1938.
11. Buys D, Hillary JK. Notes on the microstructure of hair of the Orycteropodidae, Elephantidae, Equidae, Suidae and Giraffidae. *S Afr J Wildl Res.* 1984;14:111–9.
12. Cave AJE. Hairs and vibrissae in the Rhinocerotidae. *J Zool.* 1968;157:247–57.
13. Chernova OF, Tselikova TN. An atlas of mammalian hair: fine structure of overhair and hair using scanning electron microscopy. Moscow: KMK Sci Press; 2004. In Russian.
14. Dimond RL, Montagna W. The skin of the giraffe. *Anat Rec.* 1976;185:63–75.
15. Hammond EC, Jones C. An examination of a strand of 30,000 and 70,000 year old mammoth hair. In: Bailey GW, Bentley J, Small JA, editors. Proceedings of the 50th Annual Meeting of the Electron Microscopy Society of America, San Francisco, CA: Electron Microscopy Soc; 1992, vol 50, no 2, pp. 1100–1101.
16. Kranz KR. A note on the structure of tail hairs from a pygmy hippopotamus (*Choeropsis liberiensis*). *Zoo Biol.* 1982;1:237–41.
17. Ryder ML. Hair of the mammoth. *Nature.* 1974;249:190–2.
18. Valente A. Hair structure of the woolly mammoth, *Mammuthus primigenius* and the modern elephants, *Elephas maximus* and *Loxodonta africana*. *J Zool.* 1983;199:271–4.
19. Rasmussen LEL, Munger BL. The sensorineural specializations of the trunk tip (finger) of the Asian elephant, *Elephas maximus*. *Anat Rec.* 1996;246:127–34.
20. Roca AL, Georgiadis N, O'Brien SJ. Cyto-nuclear genomic dissociation and the African elephant species question. *Q Int.* 2007;169:70:4–16.
21. Johnson MB, Clifford SL, Goossens B, Nyakaana S, Curran B, White LJ, Wickings EJ, Bruford MW. Complex phylogeographic history of central African forest elephants and its implications for taxonomy. *BMC Evol Biol.* 2007;7:244.
22. Williams CS. A simple method for sectioning mammalian hairs for identification purposes. *J Mammal.* 1934;15:251–2.

23. Hieronymus TL, Witmer LM, Ridgely RC. Structure of white rhinoceros (*Ceratotherium simum*) horn investigated by x-ray computed tomography and histology with implications for growth and external form. *J Morphol*. 2006;267:1172–6.
24. Pollitt C. Anatomy and physiology of the inner hoof wall. *Clin Tech Equine Prac*. 2004;3:3–21.
25. Clement JL, Hagege R, Le Pareaux A, Carteaude JP. Ultrastructural study of the medulla of mammalian hairs. *Scan Electron Microsc*. 1981;3:377–82.
26. Sato H, Miyasaka S, Yoshino M, Seta S. Morphological comparison of the cross section of the human and animal hair shafts by scanning electron microscopy. *Scan Electron Microsc*. 1982;1:115–25.
27. Blakey PR, Lockwood P. The environment of calcified components in keratins. *Calcif Tissue Res*. 1968;2:361–9.
28. Chernova OF. Architectonics of the medulla of guard hair and its importance for identification of taxa. *Doklady Biol Sci*. 2001;376:81–5.



# Forensic science, genetics and wildlife biology: getting the right mix for a wildlife DNA forensics lab

Rob Ogden

Accepted: 17 June 2010 / Published online: 1 July 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** Wildlife DNA forensics is receiving increasing coverage in the popular press and has begun to appear in the scientific literature in relation to several different fields. Recognized as an applied subject, it rests on top of very diverse scientific pillars ranging from biochemistry through to evolutionary genetics, all embedded within the context of modern forensic science. This breadth of scope, combined with typically limited resources, has often left wildlife DNA forensics hanging precariously between human DNA forensics and academics keen to seek novel applications for biological research. How best to bridge this gap is a matter for regular debate among the relatively few full-time practitioners in the field. The decisions involved in establishing forensic genetic services to investigate wildlife crime can be complex, particularly where crimes involve a wide range of species and evidential questions. This paper examines some of the issues relevant to setting up a wildlife DNA forensics laboratory based on experiences of working in this area over the past 7 years. It includes a discussion of various models for operating individual laboratories as well as options for organizing forensic testing at higher national and international levels.

**Keywords** Wildlife DNA · Laboratory · Forensic identification · Validation · Illegal trade

## Introduction

Wildlife DNA forensics is becoming established as an applied scientific field incorporating disciplines ranging from biochemistry to evolutionary genetics to support the development and application of forensic analytical methods. Despite tracing its origins back to the late 1980's, researchers and practitioners have only recently begun to draw together the multiple strands of work underway around the world, under the umbrella of organisations such as the Society for Wildlife Forensic Science (est. 2009) and the TRACE Wildlife Forensics Network (est. 2006). One of the many issues to emerge from such endeavours is the collective need to share experiences and discuss best practice with respect to capacity building. The aim of this paper is to examine one area of particular interest: setting up a laboratory facility for wildlife DNA forensics.

The application of DNA forensic techniques to wildlife crime investigation is complicated by a number of factors. The potential range of target species for which methods require development, validation and reference data is large; the frequency with which any single analysis is employed may be low and the priority given to resourcing wildlife forensic work is often lower still, resulting in wildlife DNA forensic services being viewed as expensive to maintain. With notable exceptions, the majority of wildlife DNA forensic work has traditionally taken place in academic institutions where scientists with particular expertise undertake forensic analysis on an ad-hoc basis. The involvement of academic scientists is essential to drive the development of new genetic identification techniques and generate comparative data, however, the performance of forensic analysis by scientists who may lack forensic training and operate in research-grade laboratories can

---

R. Ogden (✉)  
TRACE Wildlife Forensics Network, Royal Zoological Society  
of Scotland, Edinburgh EH12 6TS, UK  
e-mail: rob.ogden@tracenetwork.org

R. Ogden  
LGC Forensics, Queen's Road, Teddington, Middlesex TW11  
0LY, UK

seriously compromise the integrity of analytical evidence [1].

This paper briefly describes how the field has developed and assesses the different laboratory models under which wildlife DNA forensics has been performed, highlighting examples of good practice and situations to avoid. It then explores the practical options available to authorities or organizations wishing to develop capacity in this area.

## History

As with most scientific applications, wildlife DNA forensics began with the discovery of a technique, followed by some thoughts on what could be done with it. The two core analytical approaches, DNA sequencing and fragment analysis, were developed in the mid to late 1980's and their potential applications to sample identification and legal enforcement were rapidly demonstrated. The first publication describing hypervariable minisatellite markers in 1985 [2] led to their recognition as human forensic markers [3], then as non-human markers [4], then as wildlife forensic markers [5], in the space of 4 years. At the same time, the use of DNA sequencing for species identification and its subsequent application to wildlife crime investigations was also underway [6].

With the support of government resources, the field of human DNA forensics expanded rapidly and techniques were transferred to dedicated, accredited forensic laboratory facilities, while wildlife DNA forensics remained a very specialist field, practiced by few scientists. The relative lack of support for wildlife applications, together with the breadth of biological identification issues that need to be addressed have been key constraints to the transfer of casework from research laboratories to the type of human DNA forensic facilities found in many countries today. Despite steadily increasing awareness of how DNA methods can provide intelligence to support wildlife crime investigations, for example through work on the illegal trade in whale meat [7], caviar [8] and ivory [9], wildlife DNA forensics remained a niche subject and this has often dictated the laboratory environment in which the work takes place.

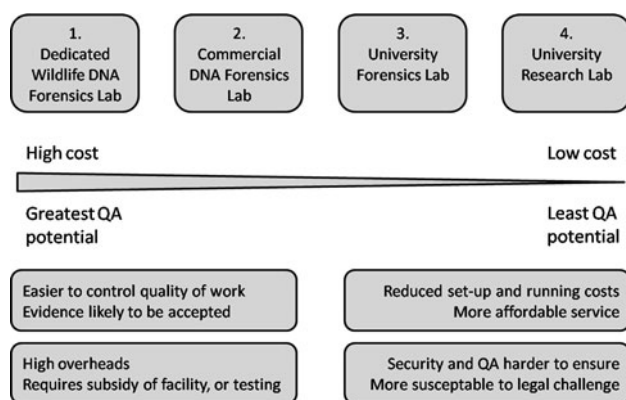
Over the past 5 years, interest in wildlife DNA forensics has increased. Public and scientific concern over the loss of biodiversity has prompted governments to strengthen enforcement of legislation regulating hunting and trade in wildlife products and derivatives. At the same time the growing suspicion that organized crime is involved in illegal trade has helped to raise the status of wildlife crime among enforcement agencies. The potential for forensic genetic approaches to investigate wildlife crime is gradually being realized, resulting in steadily increasing demand for wildlife DNA forensic services.

The effects of these developments are that more countries are seeking to establish capacity in this area while at the same time the performance of forensic identification is coming under increasing scrutiny. These are both welcome developments to the field of wildlife DNA forensics, but they also raise a number of challenges to new and existing practitioners alike. From an analytical perspective, it is important to define the end-use of data and clearly distinguish between forensic casework and intelligence applications. While the genetic techniques used and the results generated by a research laboratory will invariably be identical to those of a forensics lab, they fundamentally differ in the process used to achieve those results. One key consideration when aiming to develop a true forensic analytical service is the type of laboratory facility involved. The optimum solution will almost certainly vary on a case-by-case basis; an assessment of the key options and some lesson learnt is presented here.

## Models for laboratory services

It is important to recognize the distinction between the laboratory facilities used to undertake research and development and those used for casework. The research underpinning wildlife DNA forensic applications almost always includes work undertaken in a non-forensic laboratory environment; this is perfectly acceptable and should be encouraged in order to increase the number of identification methods available. Validation studies, essential to demonstrate that a method is fit for the purpose, can also theoretically be undertaken in a non-forensic environment, however a well-planned study will be based on considerable experience of quality assurance issues and their performance and outputs are usually of little interest to academic scientists, resulting in validation work typically being carried out by forensic scientists. The level of validation required for wildlife DNA forensic methods has been the subject of some debate [10, 11] and is worthy of further discussion. The focus of this paper, however, is on the laboratory facilities used to undertake casework in order to provide legal evidence in a criminal investigation.

Laboratory facilities used for wildlife DNA forensic work range from non-forensic research labs to specialist wildlife DNA forensics facilities operating under externally accredited quality assurance (QA) schemes (Fig. 1). In reality most labs fall somewhere between these two extremes and attempt to balance the need for forensic rigour with the fundamental limit of service cost. The two key issues affecting this balance are: what level of QA is required to produce evidential data, and how is the analysis being funded?



**Fig. 1** Summary of four possible laboratory models for undertaking wildlife DNA forensic analysis, describing their key characteristics

## Quality

The purpose of QA is to prevent errors in data production, to ensure that any errors are identified prior to data release and to feedback improvements into the analytical process. To achieve this, laboratories can implement and be accredited under a number of quality systems, such as ISO17025 or Good Laboratory Practice (GLP). These systems will address issues such as continuity of evidence, equipment calibration and maintenance, correct use of analytical controls, staff training, data storage and reporting procedures. While clearly seen as a gold standard, and often a requirement for human forensic labs, the cost of accreditation for a wildlife DNA forensic laboratory may be prohibitively expensive, particularly given the range of tests performed. Nevertheless, the principles of QA schemes such as ISO17025 are fundamental to producing accurate, reproducible and secure results and therefore any laboratory undertaking wildlife DNA forensic analysis must adhere to a QA system, regardless of whether or not formal accreditation has been gained. The capacity for a laboratory to implement a QA scheme and the commitment of its staff and management to follow it, are vital considerations when establishing a forensic laboratory service.

## Funding

The second consideration when balancing forensic quality requirements with service cost is the way in which the analysis is funded. Any analytical service, whether commercial or governmental, must operate within a budget and there are a number of ways to achieve this. A government or university facility may receive core funding to subsidize a service, enabling end-users to have samples analysed at no charge, or for a fraction of the real cost. In many respects, this is an ideal system as it enables the laboratory to maintain a range of genetic analysis techniques that

individually may be used too infrequently to justify providing commercially. However, such government subsidies are not available in many countries, where insufficient funds are available for wildlife forensics, or where there is an ethos of full cost recovery within non-commercial institutions.

The second option is for a laboratory to operate either commercially, or on the basis of full cost recovery and charge a commercial rate. This has been the situation in the UK for the past 10 years and provides a useful example of this approach. Following a decision not to use university research laboratories for forensic DNA testing, the only alternative for wildlife crime enforcers was to submit samples to the UK Forensic Science Service (FSS). However, at the time, the FSS was being converted from a government laboratory service into a government company operating full cost recovery. The fee charged for techniques such as DNA species identification was prohibitively expensive (~3,000 GBP per sample), owing to the occasional nature of the work and the cost model applied. This effectively resulted in no casework being processed. In 2003 a commercial operation began offering forensic services at a much reduced rate. While this enabled some investigations to use forensic DNA analysis, many UK enforcement agencies still struggled to find sufficient money to pay for it. This prompted the UK government and several non-governmental organisations to establish a forensic analysis fund to provide 50% match funding to the cost of any wildlife forensic test required by the police or customs authorities. This initiative has further increased the use of wildlife DNA forensics by UK enforcement agencies, but ultimately still limits both the number and range of tests that can be undertaken.

The method of funding wildlife DNA forensic testing affects the feasibility of the laboratory model. The UK experience serves to demonstrate that wildlife DNA forensic testing, unlike its human equivalent, is not a commercially viable service. Some form of government subsidy is essential; the level of support dictates the type of service that can be provided (Fig. 1).

## Comparative laboratory systems

For the purposes of broad comparison, the following section describes the typical properties of four laboratory models: (1) A government-funded dedicated wildlife DNA forensic facility; (2) a private forensic genetic facility offering wildlife DNA services; (3) a university or institutional research facility incorporating dedicated forensic laboratory space; (4) a multi-use research laboratory (Fig. 1). It is stressed that there will always be exceptions to such categorization and support or criticism of individual laboratories is not implied.

### *Government-funded dedicated wildlife DNA forensics facility*

A core facility for processing all casework together with the capacity to undertake method specific validation studies represents an ideal scenario in most respects. Such a system should enable all enforcement agencies to access services for a nominal cost, which in turn will help promote the use of forensic genetic techniques in wildlife crime enforcement. This positive feedback is maintained by the economies of scale associated with a high throughput of casework. The implementation of a rigorous QA system is not compromised by the demand for profit, or alternate uses of the same laboratory space. A pool of forensic expertise can be developed able to address a very wide range of questions.

The major drawback of this system is cost. Most individual countries cannot justify the cost of developing and maintaining a facility based on the limited number of samples processed per year. Associated with this issue is the length of time it may take to establish a dedicated facility. While the conversion of an existing laboratory, staff training and internal validation may be possible within 12 months, the decision process and construction of a new laboratory is likely take many more years. Regional core facilities servicing multiple countries under an international agreement offer a potential solution to these issues, but such models are difficult to implement, as discussed in subsequent sections.

### *Private forensic genetic facility offering wildlife DNA services*

Organisations operating for profit (or at least full cost recovery) may be interested in providing services, however financial controls will limit the forensic methods supported to those used most frequently, restricting the skill base and reducing the range of services available. The laboratory facility is likely to offer quality assured analysis, however, the recovery of cost in relation to the QA system is likely to make the per sample analysis fee very high. In most countries, such a model will only function if the enforcement agencies receive specific additional support to fund forensic analysis.

It should be noted that there may be other drawbacks to using private forensic services that have been experienced in the UK. Without the necessary security clearance, it may be impossible for enforcement officers to fully discuss ongoing casework with the forensic scientist. This hampers an investigation by increasing the risk of undertaking inappropriate analysis, or missing important avenues of investigation.

### *University or institutional research facility with dedicated forensic laboratory space*

This model encompasses a broad range of institutional laboratory facilities, from correctly designed, properly controlled, professional forensic units, to academic experts in their field with little understanding of forensic processes who are, for a variety of reasons, offering a forensic service. There is no doubt that at one extreme, a number of universities have dedicated sufficient laboratory space, staff and resources to provide first class forensic genetic services. However, if a department has funded the facility itself, it will be looking for a return on investment, increasing the service price. A department making a smaller investment may rely on other sources of income to support the facility, reducing the service price but potentially limiting the level of quality assurance. Direct government support of a university-run facility can be an economic way to deliver wildlife DNA forensic services, although a university-based service may be limited by the skill base and time commitments of the resident academics involved in running the unit.

### *Multi-use research laboratory*

Research laboratories typically operate with extremely broad access to buildings, rooms, storage units and computer systems. Pre- and post-PCR liquid handling often occurs at the same bench, equipment is rarely fully calibrated, quality systems are not in place, and neither staff nor students have undergone any forensic training. The routine use of research laboratories to provide forensic evidence in wildlife crime investigations should therefore not occur. Nevertheless, it does and it is very important that the enforcement agencies and scientists involved are made aware of the potential failings of such systems and are encouraged to either transfer the work elsewhere, or move towards the creation of a dedicated forensic unit within the laboratory facility, as described in the third model, above.

As a rare exception to the general rule preventing forensic work from taking place in research laboratories, it is recognized that the breadth of potential investigative questions may sometimes require expertise not available within an existing forensic laboratory. In these circumstances it may be necessary to approach a research expert for assistance with a case. Such work is fraught with difficulties from a forensic perspective, including issues of laboratory security, analytical QA, data interpretation and reporting. The technique involved is unlikely to be validated which may prevent acceptance of evidence in court, limiting the information gained to the level of investigative intelligence. Despite these problems, it is possible to generate forensic genetic data from a research laboratory, but



work should be well-planned, undertaken in isolation, fully recorded at the time and under the direct and constant observation of a forensic scientist who is able to witness and confirm every stage of the work.

Comparing these four model systems it is easy to conclude that the fully-funded government model is the ideal and that a research laboratory model is not acceptable. In reality many countries already using wildlife DNA forensic analysis, or that are seeking to develop capacity, are restricted to operating under the second and third models. In order for these models to work, it is essential to have the correct resources in place, not only in terms of laboratory facilities, but also in relation to personnel. The following section will briefly discuss options for developing expertise in wildlife DNA forensics.

### Building expertise

What does it take to become a wildlife DNA forensic scientist? Who should a laboratory try to recruit? There are currently no academic or vocational qualifications that will train people specifically to work in this field and staff will probably require training in post. The primary recruitment decision is often between a human DNA forensic candidate who will require training in wildlife genetics and a wildlife geneticist who will require training in forensics. In a large laboratory with multiple staff, a blend of backgrounds is probably preferable, allowing for knowledge transfer in a common environment. However, most countries building capacity in this area appear to begin the process by nominating a single individual per facility to be responsible for wildlife DNA forensics.

The main consideration in choosing between an established forensic scientist and a wildlife geneticist should be the duration and level of training required to obtain the other's skills. From personal experience based on training scientists from a dozen different countries, it is generally easier to teach a wildlife geneticist to do forensic casework than it is to convert a human forensic DNA specialist into a wildlife DNA forensic scientist. The reason for this is primarily that the consistency of approach employed in forensic analysis allows general rules and guidelines to be applied to a wide range of laboratory processes and genetic markers, addressing species, geographic and individual identification questions. The reverse is not the case; a human forensic scientist attempting to learn the range of scientific techniques and underlying biological assumptions involved in different wildlife identification enquiries is faced with a very large, diverse body of knowledge to attain.

The recruitment and training of staff is vital to establishing a viable wildlife DNA forensic laboratory. Although

the selection of candidates may be limited for many different reasons, an experienced wildlife geneticist with an interest in forensic methods and an appreciation of the need for quality assurance will often prove to be the best choice. The scarcity of such expertise becomes an important consideration when building capacity in countries without existing wildlife DNA forensic laboratories. The next section considers the possible approaches for delivering services within such regions.

### Developing capacity in new countries

The challenges and compromises in choosing how to establish an individual wildlife DNA forensics laboratory have been discussed. Where insufficient capacity, resources or demand are present to create laboratory facilities within national regions, or entire countries, coordinating resources at a national or international level may provide the most effective solution. While a collaborative approach often makes sense in principle, in reality it can be very hard to achieve. This section will briefly consider the issues restricting the development of single national wildlife DNA forensics labs, before examining several models for international collaboration based on current and past experience.

#### Establishing a single national facility

Many countries that are beginning to examine their national capacity for undertaking wildlife DNA forensic analysis find that services are currently provided by a number of different universities or institutions with a variable degree of forensic quality assurance. Casework is typically assigned to an expert working in the same taxonomic field as the species under investigation and in some cases the laboratory may develop a routine service for a specific investigative issue, for example, bird parentage testing. Where a country is seeking to formalize its wildlife DNA forensic work, it often makes sense to bring casework together under one roof, via one of the first three models discussed earlier. The advantages to such a strategy are obvious: increased economies of scale, the opportunity for a single forensic-grade service and a single point of contact for enforcement authorities, among others. However, such a move requires the transfer of expertise, data and probably reference samples from a personal research environment to a shared national resource and there can be reluctance on the part of the researcher to engage and commit time to this process, when they often receive no benefit.

A second consideration is that additional validation studies will always need to be undertaken when transferring techniques to a single central facility. While this

should ideally be limited to an internal validation exercise, it is likely that a degree of additional developmental validation would also be preferable [12]. This requires time and money that must be factored into plans to rationalize wildlife DNA forensic services.

While neither of these issues should necessarily prevent the development of a single national facility, awareness of them is important to prevent stagnation of projects that are aiming to improve the coordination and delivery of wildlife DNA forensic services.

### International collaborations

Many of the arguments for and against a single national facility can be extended up to an international level. In many parts of the world, it simply does not appear to make sense to spend time and money to train staff and build capacity in every country, where this might result in over-capacity and over-stretched resources within individual states. The European Union (EU) provides a good example of this situation. There are currently 27 member states within the EU; the entire area is treated as a single customs zone in terms of implementing CITES regulations (i.e. there is free trade across borders) and legislation relating to national wildlife protection is broadly similar. The results of a recent survey of member states indicated that approximately half have no current forensic DNA capacity for wildlife crime investigation, while the other half use a range of services from university research labs

through to accredited national forensic facilities. Enforcement officers collaborate at a European level and the demand for forensic services is reasonably high. In many respects, the best solution would be to create a single European facility for validation and casework analysis, but where would such a service be based and who would run it?

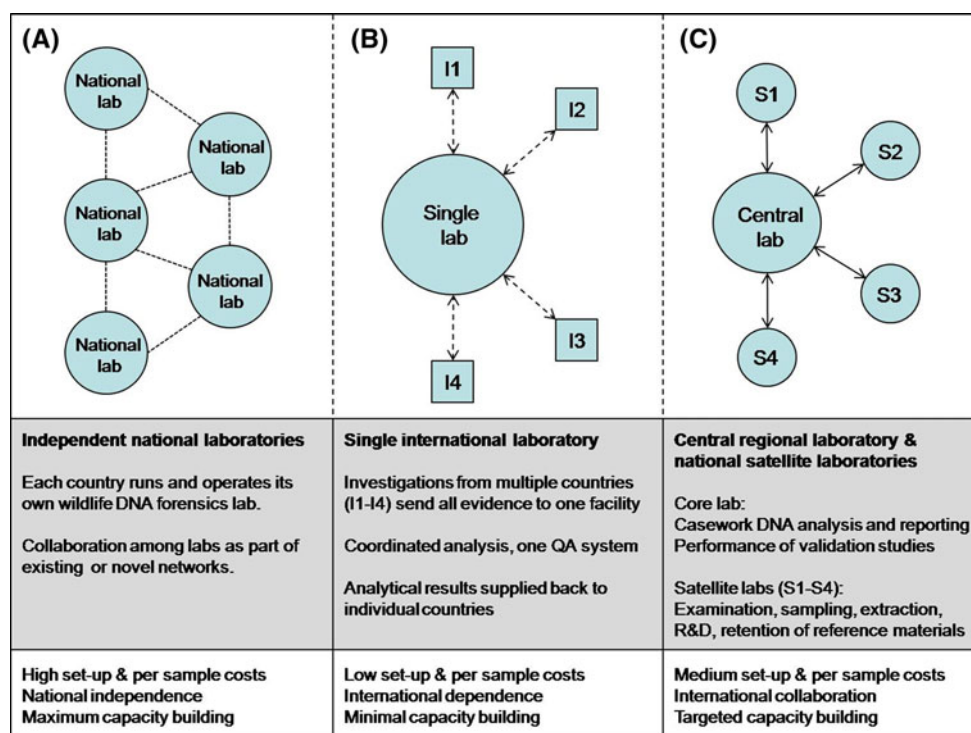
Three possible models for an international collaborative approach are considered here: (a) A network of independent national laboratories, (b) A single international laboratory, and (c) A central regional laboratory with national satellite laboratories (Fig. 2).

### A network of independent national laboratories

The development of independent laboratories in every country within a region has disadvantages, principally relating to overall cost, efficiency of analysis and the practicalities of training sufficient wildlife DNA forensic scientists. However in some regions, it is the only politically viable option and does bring benefits relating to capacity building and national ownership within each country (Fig. 2a). In such cases it is worth considering how to optimize the development of services.

As an example, the UK government is currently funding a wildlife forensics network in the ASEAN region (Association of Southeast Asian Nations; Box 1). The first aim of the project is to offer training and support to scientists in the areas of research and development, laboratory organization, forensic protocols and casework approaches.

**Fig. 2** Three models for providing wildlife DNA forensic laboratory services across a number of regions or countries. *Model A* describes a loosely linked system of independent laboratories, *Model B* describes a single laboratory system and *Model C* describes an intermediate solution, incorporating one central laboratory and multiple satellite laboratories



**Box 1** Developing wildlife forensic capacity for ASEAN biodiversity conservation—a Darwin initiative

Countries in the ASEAN region have been coordinating action against wildlife crime since 2005 through the ASEAN Wildlife Enforcement Network. As part of this approach, individual nations recognized the need to increase their capacity for undertaking forensic analysis to support wildlife crime investigations. The regional laboratory facilities for undertaking forensic DNA work vary widely, as does the experience of scientists in each country. The Darwin project began in 2009 with an evaluation of existing capacity and an assessment of wildlife DNA forensic needs at a national level. This included identifying key scientists and wildlife enforcement managers with responsibility and enthusiasm for developing laboratory services

The project has since proceeded on the basis of selecting the most appropriate laboratory model for each country, based on local facilities, expertise, administrative structures and enforcement systems. At an international level, training workshops have been run to address common issues in laboratory set-up and operation, as well as to develop a network of practitioners facing similar wildlife DNA forensic challenges

In the ASEAN region at this time, the independent laboratory network model is considered to be the only viable political option. However it is hoped that ongoing communications will lead to regional collaborations among institutions and wildlife DNA forensic scientists, enabling laboratories to exchange and implement best practice

The project is managed by TRACE Wildlife Forensics Network in partnership with TRAFFIC Southeast Asia and ASEAN-WEN

The second aim is link together scientists and enforcement authorities within and among countries in order to provide a platform for exchanging techniques, data and common enforcement issues.

Working with governments and scientists in the ASEAN region takes time, but there are clear advantages to engaging at this level, which ensures that the laboratory facilities and processes that are developed are tailored to meet national needs. By stressing the benefits of international collaboration and providing a forum for communication, it is hoped that scientists will have regional access to advice and facilities as necessary.

*A single international laboratory*

The opposite approach to a system of national laboratories is to have a single, quality assured, internationally recognized laboratory offering affordable services to all nations (Fig. 2b). This is what the US Fish and Wildlife Service's Forensic Laboratory (USFWS-FL) set out to provide when it offered forensic analysis of wildlife crime samples relating to CITES enforcement free of charge, over 10 years ago. The laboratory is not restricted to DNA analysis and offers a wide range of modern forensic services. The single laboratory model should be the most cost-effective solution, providing analysis to a very high standard; however this type of system requires effective international collaboration and is not suitable for individual nations that wish to build capacity for solving wildlife crime issues themselves.

The service provided by the USFWS-FL has resulted in many notable successes, including for example, the investigation of illegal ivory shipments from Africa to east Asia. In many cases, the capacity to undertake the analysis in the range state simply did not exist, demonstrating the value of such a model. Despite the existence and global promotion of this free service, however, it has been observed that the opportunity to access forensic analysis is

often not taken. Certain countries may be unwilling to engage with such a service due to issues of data exchange, investigative process or higher political disagreement. Others may not want to rely on an external agency at the perceived expense of developing their own capacity. Until this situation changes, the single international laboratory model, however attractive, is not a total solution to the provision of wildlife DNA forensic laboratory analysis.

*A central regional laboratory with national satellite laboratories*

In regions where international collaboration is highly developed and legislation allows, a third model is proposed that seeks to rationalize the use of resources while maintaining national involvement in casework and ownership of wildlife DNA forensic research (Fig. 2c). Under this model, each country is responsible for coordinating the forensic analysis of their own samples, including submission to a 'satellite' laboratory, examination of the item, recovery of evidential samples and, as appropriate, extraction of DNA. In addition to these processes, countries would be encouraged to undertake research and development to support new wildlife forensic techniques. The satellite laboratory would then submit the sample to a single central wildlife DNA forensic laboratory that maintains a full range analytical tests operating under an accredited quality system. The central laboratory would be fully equipped and staffed by wildlife forensic scientists experienced in performing casework analysis and the developmental validation of methods developed by the research community.

The advantages and disadvantages of this model relate to those already discussed for the national laboratory network and the single international laboratory models. However the intention is to mitigate the disadvantages and retain the advantages of each. It is envisaged that this approach may be suitable at a European level, or even within a single large country.

## Summary

The issues involved in setting up a wildlife DNA forensics laboratory are complex. The opposing influences of restrictive budgets and the need for forensic rigour are complicated by the breath of analytical techniques, variable casework demand and access to expertise. While ideal and unacceptable models for a laboratory facility are moderately simple to define, intermediate solutions will inevitably be required, necessitating careful consideration of how to ensure the implementation of quality systems while preventing analytical costs from becoming prohibitively high.

Although the engagement of individual researchers and links to academia will always be an integral part of this applied field, it is essential that correct forensic laboratory practices are followed, not only with respect to individual cases, but also to maintain the reputation of wildlife DNA analysis in the wider forensic community. Where solutions at a local level fail to offer a suitable environment for undertaking wildlife DNA forensic analysis, opportunities should be sought through collaboration and cooperation at a wider national or international level.

Different approaches will be appropriate in different regions and the question of how to establish a wildlife forensic DNA laboratory will always be answered on a case by case basis. However as an overall strategy, it is recommended to explore to what extent it is feasible to collaborate and coordinate resources from the local to the national to the international level. Placing a laboratory as far along this path as is practicable should help to provide the most cost-effective, quality-assured, broad range of wildlife DNA forensic services possible.

## Key points

1. Forensic genetic techniques are increasingly used to identify non-human evidence in wildlife crime investigations.
2. Wildlife DNA forensic applications require a synthesis of wildlife genetics and forensic expertise which is rarely available at a single facility.
3. The development of new facilities can follow several alternative models, all of which are based upon performing DNA analysis to rigorous forensic standards.

4. The casework requirements and available resources to support wildlife DNA forensic services may favour solutions at a national or international level, where the correct levels of expertise and quality assurance can be most effectively combined.

**Acknowledgments** The author is grateful to Ross McEwing for numerous discussions of these issues over many years, as well as to Ed Espinoza and Linzi Wilson-Wilde for their comments on an earlier version of this manuscript. RO is part-funded by the UK Darwin Initiative.

**Conflict of interest statement** None.

## References

1. Ogden R, Dawnay N, McEwing R. Wildlife DNA forensics—bridging the gap between conservation genetics and law enforcement. *Endangered Species Res.* 2009;9:179–95.
2. Jeffreys AJ, Wilson V, Thein SL. Hypervariable minisatellite regions in human DNA. *Nature.* 1985;314:67–73.
3. Gill P, Jeffreys AJ, Werrett DJ. Forensic applications of DNA ‘fingerprints’. *Nature.* 1985;318:577–9.
4. Burke T, Bruford MW. DNA fingerprinting in birds. *Nature.* 1987;327:149–52.
5. Thommasen HV, Thomson MJ, Shutler GG, Kirby LT. Development of DNA fingerprints for use in wildlife forensic science. *Wildl Soc Bull.* 1989;17:321–6.
6. Cronin MA, Palmisciano DA, Vyse ER, Cameron DG. Mitochondrial-DNA in wildlife forensic-science—species identification of tissues. *Wildl Soc Bull.* 1991;19:94–105.
7. Baker CS, Palumbi SR. Which whales are hunted?—a molecular genetic approach to monitoring whaling. *Science.* 1994;265:1538–9.
8. DeSalle R, Birstein VJ. PCR identification of black caviar. *Nature.* 1996;381:197–8.
9. Wasser SK, Shedlock AM, Comstock K, et al. Assigning African elephant DNA to geographic region of origin: applications to the ivory trade. *Proc Natl Acad Sci USA.* 2004;101:14847–52.
10. Budowle B, Garofano P, Hellman A, et al. Recommendations for animal DNA forensic and identity testing. *Int J Legal Med.* 2005;119:295–302.
11. Dawnay N, Ogden R, Thorpe RS, et al. A forensic STR profiling system for the Eurasian badger: a framework for developing profiling systems for wildlife species. *Forensic Sci Int: Genetics.* 2008;2:47–53.
12. Butler JM. Debunking some urban legends surrounding validation within the forensic DNA community. *Profiles DNA.* 2006;9:3–6.



# DNA detective: a review of molecular approaches to wildlife forensics

E. A. Alacs · A. Georges · N. N. FitzSimmons ·  
J. Robertson

Accepted: 15 November 2009 / Published online: 16 December 2009  
© Springer Science+Business Media, LLC 2009

**Abstract** Illegal trade of wildlife is growing internationally and is worth more than USD\$20 billion per year. DNA technologies are well suited to detect and provide evidence for cases of illicit wildlife trade yet many of the methods have not been verified for forensic applications and the diverse range of methods employed can be confusing for forensic practitioners. In this review, we describe the various genetic techniques used to provide evidence for wildlife cases and thereby exhibit the diversity of forensic questions that can be addressed using currently available genetic technologies. We emphasise that the genetic technologies to provide evidence for wildlife cases are already available, but that the research underpinning their use in forensics is lacking. Finally we advocate and encourage greater collaboration of forensic scientists with conservation geneticists to develop research programs for phylogenetic, phylogeography and population genetics studies to jointly benefit conservation and management of traded species and to provide a scientific basis for the development of forensic methods for the regulation and policing of wildlife trade.

**Keywords** Wildlife crime · Species identification · Population assignment · Sexing · Individual identification · Wildlife trade

## International wildlife trade and forensic genetics

According to Interpol (International Policing Organisation), the illegal trade of plants, animals and their by-products is a growing global black market commerce estimated to be worth more than USD \$20 billion per year [1]. Organised international criminal networks have been linked to the trafficking of wildlife using their established drug smuggling routes to illegally transport wildlife across international borders [2, 3]. In Brazil, recent estimates suggest that at least 40% of all illegal drugs shipments are combined with wildlife [4]. Similarly, one-third of all cocaine seized in 1993 was reported by the United States Fisheries and Wildlife Service (USFWS) to be associated with wildlife imports. The illicit wildlife trade is attractive to criminals because weight-for-weight wildlife is equally or more profitable than drugs or arms and with less associated risk. The rate of detection is lower and the penalties, if offenders are caught and convicted, are typically far more lenient for wildlife crimes than for drugs or arms trafficking. Gaol sentences for wildlife smuggling are often minimal and fines disproportionately less than the commodity values of the goods on the black market [5–8]. With little disincentive for criminal activity, the black market in wildlife continues to flourish, and places ever-increasing pressures on endangered species.

The illegal wildlife trade pose serious threats, both direct and indirect, to global biodiversity. Species sought for trade are directly impacted by over-exploitation. Over-exploitation is fuelled by the black market placing

---

E. A. Alacs (✉) · A. Georges  
Institute for Applied Ecology and National Centre for Forensic  
Studies, University of Canberra, Canberra, ACT 2601, Australia  
e-mail: alacs@aerg.canberra.edu.au

N. N. FitzSimmons  
Institute for Applied Ecology, University of Canberra, Canberra,  
ACT 2601, Australia

J. Robertson  
Australian Federal Police, PO Box 401, Canberra, ACT 2601,  
Australia

exaggerated values on rarer species. As a species becomes rarer from exploitation, its value on the black market escalates making it even more desirable despite the greater effort required to collect individuals from declining populations [9]. Over-exploitation of wild populations can rapidly cause local extinctions and, if harvesting is extensive across the range of the species, can cause global extinction. Widespread extinctions have occurred in taxonomic groups that are particularly vulnerable to the effects of over-exploitation because of their life history characteristics such as longevity, high natural juvenile mortality, and low reproductive outputs. For example, turtles worldwide are in peril with 3% extinct or extinct in the wild, 9% critically endangered, 18% endangered, and 21% vulnerable [10]. In Asia, the situation is even more dire with 1% extinct or extinct in the wild, 20% critically endangered, 31% endangered, and 25% vulnerable [10]. Over-exploitation of wild populations for meat, pets, and the use of the shells in traditional medicines are the major cause of declines in turtles worldwide, especially in Asia [11]. Turtles are just one of many examples of taxa that are threatened globally from over-exploitation for trade. The list of species directly threatened by wildlife trade is extensive, encompassing all major taxonomic groups across all biomes. It includes many ‘keystone’ species (e.g. African horn bills, sea otters, grizzly bears, sea stars, elephants, orangutans, beavers, truffles and oysters), so named because they are ‘key’ to the functioning of the ecosystem and their loss causes widespread declines in many other species [12–14]. Direct exploitation for hunting, trade, and collection has been identified by the World Conservation Union (IUCN) as the second greatest driver (surpassed only by habitat destruction) of declines in endangered animals, impacting 33%, 30% and 6% of threatened mammals, birds and amphibians, respectively [15]. Wildlife trade also provides avenues for the introduction of exotics with the potential to spread disease to native species [16–20] or to become invasive [21–24].

Monitoring trade in wildlife requires firstly the identification of the species traded, then assessment of whether they are derived from legal or illegal trade. Diagnostic morphological traits have traditionally been used as markers, but they are not suitable when traded products are degraded or highly processed as the morphological traits may not remain discernable. Molecular markers are ideal for species identification because unlike morphological markers they do not require intact specimens. DNA can be readily extracted from highly processed and degraded products commonly encountered in wildlife trade markets such as cooked and dried meats [25], claws left on tanned hides [26], dried shark fins [27], egg shells [28], animal hairs [29], bone [30], ivory [31, 32], rhinoceros horns [33], turtle shell [34], feathers [35] and fish scales [36].

Molecular technologies have great utility for wildlife forensics. Assigning geographic origins of trade products can also be achieved using molecular methods, a task that is often impossible using morphological traits alone. Knowledge of geographic origin can be used to distinguish between legal and illegal products, to assist in the repatriation of seized animals back to their source population, and to identify which populations are most intensively harvested for trade. At a finer resolution, individuals themselves can be marked and tracked using unique DNA profiles to characterise them. Additional information such as sex and parentage can also be ascertained which is especially useful for monitoring the compliance of registered breeders to wildlife regulations, such as to detect whether breeding stock has been supplemented or restocked with illegally caught wild stock.

In this review we detail the various contributions of genetics to wildlife forensics. The techniques employed for species identification, determination of geographic origin, individual identification and sexing will be briefly explained. Considerations for the application of these techniques to wildlife forensics will be discussed and illustrated with published case studies. To conclude, we will describe new technologies on the horizon for wildlife forensics and the future role of genetics to combat the growing global black market dealing in wildlife.

### Species identification methods

Several approaches have been adopted for identification of wildlife species distinguished by the DNA target (mitochondrial or nuclear) and the technique applied to develop the genetic marker (Table 1). Some techniques, such as sequencing, can be applied to investigate both types of DNA, while other techniques are specific to nuclear DNA (nDNA).

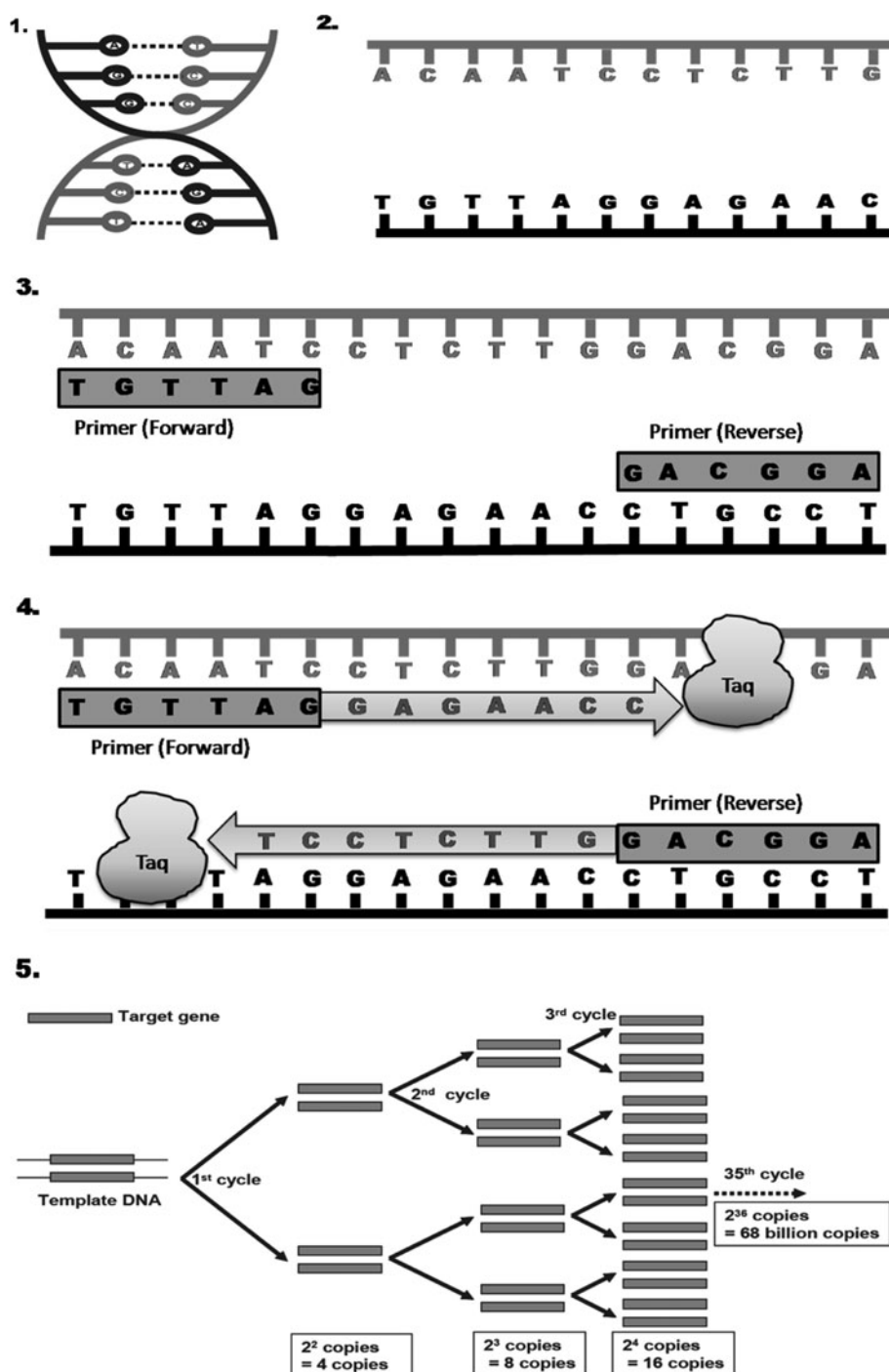
Mitochondrial DNA (mtDNA) is often favoured as a genetic marker over nDNA for species identification of wildlife because mtDNA is easier to type from highly processed and degraded tissue. This is because mitochondria are present in multiple copies per cell compared to one copy of nDNA from each parent [37]. Method development time is typically substantially less for mtDNA markers compared to nDNA markers because universal mtDNA primers are available, which are used to amplify an informative segment of mtDNA across a wide range of taxa [38]. Amplification is done using the polymerase chain reaction (PCR, Fig. 1) [39]. Universal mtDNA markers have been successfully applied in the identification of wildlife for forensic cases. The most commonly used universal markers for species identification are the mitochondrial cytochrome b (*Cyt b*) and the cytochrome oxidase 1 (*COI*) genes.

**Table 1** Comparison of genetic markers used for forensic applications

Species ID	Regional ID	Population ID	Individual ID	Parentage	Limitations for forensics	Advantages for forensics	Applications to generate baseline genetic data
Mitochondrial gene (mtDNA) sequencing	✓✓	✓	×	✓✓ maternity × paternity	Heteroplasmy  Nuclear paralogs Maternal inheritance Single linked genome hence effectively is one single marker	Suitable for trace and degraded DNA  Universal primers available	Phylogenetics  Phylogeography Population genetics
Nuclear gene (nDNA) sequencing	✓✓	×	×	×	Not suitable for trace or degraded DNA  Universal primers not available for most species	Recommended for use in combination with mtDNA for species Identification  Can detect hybrid individuals	Phylogenetics
Pyrosequencing	✓✓	✓ not assessed	✓✓ for SNP genotyping	✓✓ for SNP genotyping	Only short fragments of 10 to 500 bp can be sequenced	Enables very rapid high throughput genotyping of short fragments or SNPs	Population genetics for SNP genotyping
Amplified fragment length polymorphism (AFLP)	✓	✓	✓	✓	Dominant marker, therefore less informative for all applications	No prior genetic knowledge of the organism required	Phylogenetics, phylogeography population genetics. Limited use because of their dominance
Species-specific priming	✓✓	×	×	×	Not suitable for trace or degraded DNA Knowledge of species boundaries required	Rapid screening once developed Cost effective	None
Short tandem repeat (STR). Also called simple tandem repeat (SSR) or microsatellite	×	✓✓	✓✓	✓✓	Allelic dropout can occur when trace or degraded DNA is used	Highly informative marker for many applications	Most commonly used marker for population genetics because of its high information content
Single nucleotide polymorphism	✓✓	✓✓	✓	✓	Development time is substantial Approx five times more loci required compared to STRs	Techniques have already been validated for human forensics Highly reproducible	Use of this marker for phylogenetics, phylogeography and population genetics is still in its infancy
					Currently not available for many species	Rapid screening of samples	

✓✓ is highly informative, ✓ informative, × not informative

**Fig. 1** The polymerase chain reaction (PCR) is a thermocycling reaction used to amplify targeted regions of DNA. Extracted genomic DNA (Step 1) is denatured by heating to 94–98°C for 20–40 s to separate the two complementary strands of DNA (Step 2). Primers of 15–25 nucleotides in length anneal to the DNA template strand and flank the gene (or region) of interest at a temperature of 50–65°C for 20–40 s (Step 3). A DNA polymerase enzyme, such as *Taq* polymerase, with an optimal temperature of 75–80°C, recognizes the primers and synthesizes a new DNA strand complementary to the template DNA strand. As a standard rule of thumb, at its optimum temperature, *Taq* polymerase can synthesize 1,000 bases per minute (Step 4). Steps 2 to 4 are repeated to produce a PCR cycle. Each PCR cycle produces an exponential increase in the number of copies of the gene (or region) of interest with approximately 68 billion copies after 35 cycles (Step 5). An entire PCR of 35 cycles is completed within 2–4 h in a thermal cycler machine



Discrimination of species using a fragment of the *Cyt b* or *COI* genes can be based directly on DNA sequence differences between species [26, 36, 40–43] or by DNA profiling (discussed later). The *Cyt b* gene is an informative marker used in the identification of many vertebrate species from trade products including sharks [28], snakes [44], marine turtles [36], seals [45], birds [46], and tigers [30, 43, 47, 48]. Sequencing of a 600 base pair (bp) portion of the *COI* gene has been proposed to be an efficient, fast,

and inexpensive way to characterise species and an international effort is underway to use this gene to catalogue all vertebrate biodiversity on earth ([www.barcodinglife.org](http://www.barcodinglife.org)). Pyrosequencing is an alternative method for direct sequencing of DNA templates that uses a series of enzymatic reactions to detect visible light emitted during the synthesis of DNA and enables more rapid screening of samples compared to conventional sequencing methods [48]. Only short fragments of 10–500 bp of DNA can be



sequenced with pyrosequencing methods, which can limit its application in forensics unless highly variable and informative regions are targeted [48]. Karlsson and Holmlund (2007) [49] used pyrosequencing to develop a highly sensitive assay to identify 28 species of European mammals based on short fragments of the mitochondrial 12S rRNA and 16S rRNA regions (17–18 and 15–25 bases, respectively).

While mtDNA can be effective for species identification, it does have limitations that need to be considered and overcome before it can be used for forensic application. The matrilineal mode of inheritance of mtDNA may not reflect the patterns of nuclear genetic relationships between species, particularly if there is strong sex-biased dispersal [50, 51]. Inheritance of the mtDNA genome can be complicated when paternal leakage results in heteroplasmy, that is, the coexistence of two or more different mtDNA genomes in the organism [52–56]. Nuclear paralogs (also called nuclear pseudogenes) of mtDNA genes occur when segments or the entire mtDNA genome inserts into the nucleus. These inserts can be subsequently subject to duplications, rearrangements or recombination and they may experience a different rate of mutation from that of their mtDNA parent [57–59]. Nuclear pseudogenes can be amplified simultaneously or even preferentially with the mtDNA gene in the polymerase chain reaction and the resultant mixture of genes with different modes of inheritance and mutation rate obscures any inferences of ancestry and evolutionary relationships of species [20, 59–61]. However, techniques such as sequencing of the whole mitochondrial genome can be used to test for the presence of pseudogenes and once they are accounted for they may even be phylogenetically informative [60, 62]. Owing to a lack of recombination (exceptions do occur, see [54, 63]) the mtDNA genome represents a single gene history and the evolutionary history of what is effectively a single gene may not accurately reflect the species history. Multiple genes, preferably both mitochondrial and nuclear genes, are recommended for species delimitation [64–67]. Currently, markers derived from nuclear genes are not available for the majority of wildlife and consequently mtDNA approaches dominate systems for species identification. However, the advent of whole genome sequencing of non-model organisms is expected to increase the availability of nuclear genes for wildlife in the next few years. If species have been delimited then mitochondrial data can serve, and has been used, as a robust tool for species identification to provide evidence for forensic cases [30, 68–71]. Mitochondrial techniques for species identification have undergone preliminary validations studies for use in forensic application [30, 41].

DNA profiles can also be generated using the technique of PCR-restriction fragment length polymorphism (PCR-RFLP) to target specific areas of genetic variation among

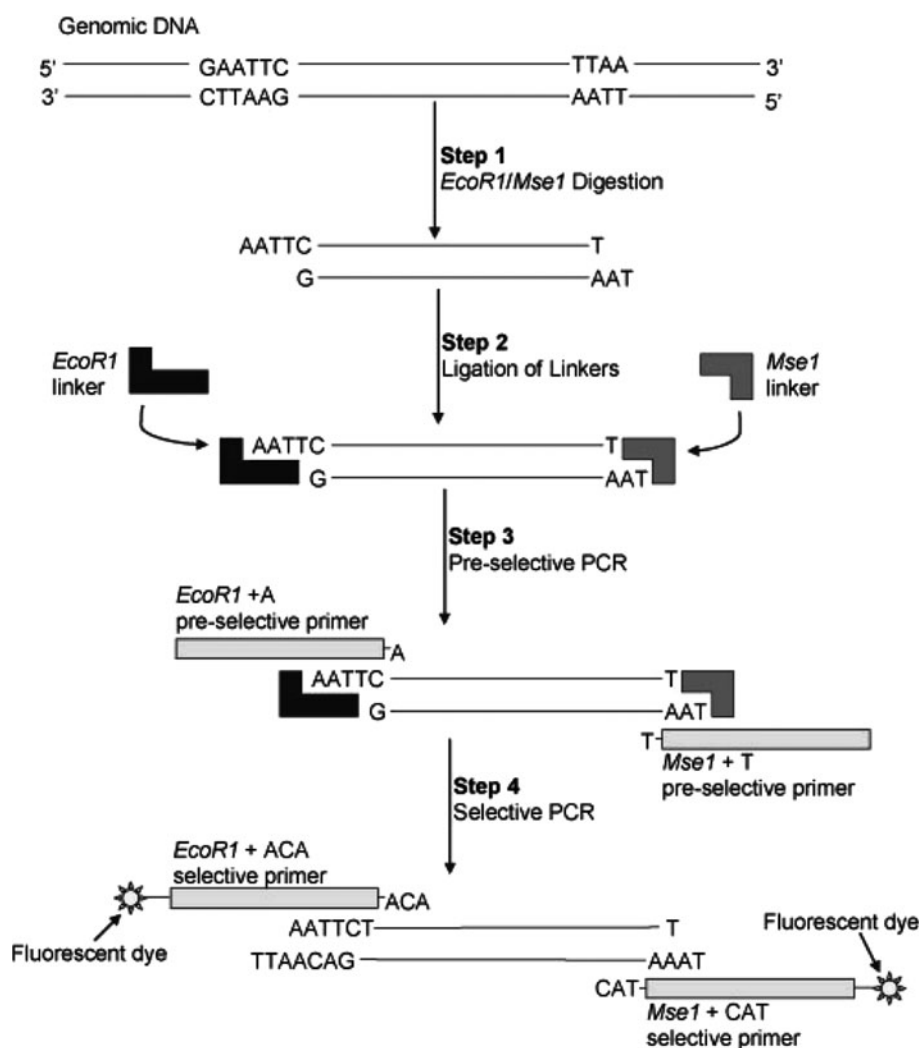
samples. Initially, the DNA segment of interest is amplified using PCR to generate billions of copies of the gene, and then subjected to digestion by restriction enzymes. These enzymes recognise specific base pair sequence motifs (that are often mirror images such as ATTA, GATTAG, etc.) and cut the amplified fragment at these sites. Species that differ in base composition at the restriction enzyme recognition sites will differ in whether or not the enzyme cuts the DNA. This generates DNA fragments of differing lengths (i.e. polymorphic fragments), in which the number and size of the fragments depends on the number of cutting sites in the DNA fragment of interest [72]. Electrophoresis of samples through an agarose or polyacrylamide gel separates fragments based on size and the different taxa will have characteristic banding patterns. Selection of restriction enzymes for PCR-RFLP analysis must ensure that the variability between species is appropriately represented and consequently that all species tested can be accurately discriminated from each other by their unique banding pattern, termed a RFLP profile. This technique has been successfully applied to a case in Argentina of a theft of livestock [73] and for species identification of marine turtles [29]. RFLP approaches are cheaper than direct sequencing and are suitable for forensic applications but they do not provide the baseline information that is required for the interpretation of forensic data such as the delineation of species boundaries.

For both direct sequencing and profiling approaches, phylogenetic studies (preferably with support from morphological, behavioural or physiological-based taxonomy) are necessary to form the foundation for accurate molecular species identification of wildlife. Phylogenetic studies estimate the evolutionary relationships of genes by inferring their common history and representing these relationships in the form of a phylogenetic tree. More closely related genes are in closer proximity to each other on the gene tree compared to more distant relatives, with rooting of the tree at their implied most recent common ancestor [74]. These gene trees are used to infer the phylogenetic species, although this does require careful consideration because genes can evolve in an independent manner to the evolution of the species [65]. It is also important to choose a gene with the appropriate mutation rate for phylogenetic analyses of species. A highly conserved gene will not be informative at the species level but will be better suited to the resolution of deep taxonomic relationships. Conversely, a gene that is evolving at a high rate will be informative at the level of the population or individual, but become saturated at the species level owing to homoplasy [75]. Homoplasy arises when certain nucleotide sites are subject to repeated mutation over time, and mutations back to the original state occur, or when the same mutation occurs independently in different lineages. As a result two individuals can share a derived

diagnostic base pair by chance rather than by descent [76]. When the appropriate gene region is chosen based on its rate of mutation, the levels of genetic diversity within and among species need to be sufficiently characterised before the gene can be applied to species identification of wildlife for forensic applications. This is to ensure that cryptic species—species that are morphologically indistinguishable but reproductively isolated—are represented [77]. In addition, the biological characteristics of the species also need to be considered such as their propensity to hybridize. Hybridization between species is common for many groups of taxa [78, 79]. Owing to the predominantly maternal mode of inheritance of mtDNA, these phenomena might not be reliably detected in forensic samples with mtDNA markers alone particularly if there are sex-biased hybrid compatibilities [50, 80–82]. A combination of mtDNA and nuclear markers (with their bi-parental inheritance) are recommended when hybridisation between species is suspected [80, 83–85].

Arbitrary fragments of genomic DNA (both mitochondrial and nuclear) have been used for species identification of wildlife with the major advantage that no prior genetic knowledge of the organism is required. One technique is termed Amplified Fragment Length Polymorphism (AFLP) and it generates segments of DNA of varying lengths that differ among individuals and species and can be visualised on polyacrylamide gels as series of bands. To use the AFLP technique, genomic DNA is cut at specific sites using two restriction enzymes that target common sites and produce hundreds of fragments. A subset of these is specifically selected for PCR amplification and tagged with a fluorescent dye (Fig. 2) [86]. This allows their detection by a laser by gel electrophoresis on a polyacrylamide gel. AFLP profiling has been used to discriminate between illicitly cultivated marijuana and hemp [87–90], identification of illegal hallucinogenic fungi [91–93], and for the identification of species of legally protected owls and their hybrids [94].

**Fig. 2** The amplified fragment length polymorphism (AFLP) procedure is used to generate informative DNA fingerprints by (1) digestion of genomic DNA with a combination of two restriction enzymes, commonly *EcoR1* and *Mse1* for animals; (2) ligation of the double-stranded *EcoR1*- (black) and *Mse1*- (grey) specific linkers to the fragment ends; (3) a pre-selective application step using primers that anneal to the linker ends of the fragments and have one selective nucleotide at their 3' end used to amplify *EcoR1*/*Mse1* templates; (4) a final selective PCR in which additional nucleotides are added at the end of the *EcoR1* and *Mse1* selective primers; (5) Separation of fragments by migration through a polyacrylamide gel (not shown). Fluorescent dyes enable pool-plexing of AFLP reactions and are detected by a laser as the fragments migrate through the gel



Compared to mitochondrial DNA, AFLP is better suited to the detection of hybrid individuals because of its biparental mode of inheritance [95, 96]. However, AFLP is not well suited for trace samples or highly degraded samples that are commonly encountered in forensics because it requires at least 50–100 ng of high molecular weight DNA [97]. Main sources of genotyping error for AFLP are differences in the peak intensities of loci between individual runs but error rates can be minimized by genotyping replicates for 5–10% of the samples and normalizing the peak height for loci against their average intensities [98, 99]. A format for databasing and comparing AFLP profiles has been developed by Hong and Chuah (2003) in a user friendly software package which minimizes sources of genotyping error and shows great promise for use in the validation of the AFLP technique for forensic applications [99].

Sequence information from either mtDNA or nDNA can be used to develop species-specific PCR primers based on nucleotide differences between the aligned homologous sequences of the target species with other closely related taxa. These primers amplify DNA regions of the target species exclusively or have a PCR product of a characteristic size, and have been used to identify taxa of commercial interest or those commonly encountered in markets. Development of species-specific primer techniques first requires sequence data from all species likely to be encountered, for the design of putative primers. It is imperative that mixed genomic DNA samples are included in PCR tests of the development phase, to ensure that the primers are specific for the target species. Once developed, species-specific primer tests are a very rapid, sensitive and cost effective screening method to detect the presence of the target species in market products or from a mixture of genomic DNA. Species-specific primers that amplify the nuclear *ITS2* region, and the mitochondrial *Cyt b* have been used to develop assays for the identification of various shark species from dried fins or meat [28, 100–103]. Where there is no prior sequence information available for the target species, RAPD (Randomly Amplified Polymorphic DNA—where arbitrary primers are used to amplify random segments of DNA) or AFLP profiling can be used to generate species-specific primers [104]. Bands specific to the target species are identified from the DNA fingerprint, extracted and sequenced. The sequence is used to design primers that will specifically amplify the species-specific region, termed a sequence characterized amplified region (SCAR). The SCAR method has the advantage of being highly reproducible: a shortcoming of the RAPD technique that has caused its redundancy in population genetic and forensic applications [105]. Genotyping individuals with species-specific primers developed using the SCAR technique is considerably cheaper than sequencing or AFLP approaches, and has been successfully used to identify deer [106], snake [44], fish [107] and fly species [108].

Regardless of how species-specific primers are developed, results that are more robust are achieved using multiplex PCR where several primers are added to a PCR to simultaneously amplify different DNA regions of the target species in a single PCR reaction. To reduce the incidence of false negatives, universal primers that amplify across all potential taxa are included in the multiplex assay. If the universal primer amplifies but the species-specific primers fails to amplify, the absence of the target species in the sample is confirmed. If both the universal and species-specific regions fail to amplify, the PCR reaction is deemed not successful and the result is inconclusive. Multiplex PCR reactions may also be used to identify several different species in a single assay. For example a multiplex PCR with six different species-specific primers and two universal shark primers for positive controls has been used to identify six species of sharks commonly encountered in North Atlantic fisheries [103]. Species-specific primers are ideal for forensic applications because they are cost effective and can be used for large scale screening of samples. However, a considerable amount of development time is required and once developed it can be difficult to incorporate additional species in the multiplex assays. Furthermore, a comprehensive understanding of species boundaries is required prior to the development of species-specific primers to ensure that all species likely to be encountered, including cryptic species, are included in the assay and can be reliably distinguished from each other.

### Identification of a forensic specimen to its geographic origin

In many wildlife forensic cases, such as where commercial trade is established, the identification of the traded product or 'specimen' to the species level may not be sufficient and the geographic origin needs to be determined. Geographic origins of an individual can be identified if there is known genetic structure within the region of interest using phylogeography or population assignment methods. Phylogeographic studies assess the geographic distribution of genealogical lineages where specific mtDNA haplotypes are associated with broad geographic regions [109]. For example, phylogeographic data for four species of seahorse (*Hippocampus barbouri*, *H. spinosissimus*, *H. trimaculatus*, and *H. ingens*) were used successfully to determine the broad geographic origins of seahorses that were found for sale in traditional medicine and curio shops in California [110].

More subtle genetic differences can often be detected using population assignment methods in comparison to phylogeographic analyses. Population assignment methods are based on allelic differences at hypervariable nDNA

genetic markers between groups of individuals, also loosely called ‘populations’. Assignment tests are used to estimate the probability of an individual belonging to each of these putative populations, and the forensic specimen is ‘assigned’ to its most probable population of origin. Conversely, exclusion tests can be used to reject the hypothesis that a specimen originated from a particular population [111–114]. The hypervariable markers most often used for population assignment or exclusion are AFLPs and microsatellites (also called short tandem repeats or STRs). Microsatellites are short sequence motifs typically 1–6 nucleotides in length (e.g., ATATATAT) that have a high mutation rate predominantly due to slippage of the polymerase during DNA replication (although other mutation mechanisms have been proposed, see [115]) resulting in lengthening or shortening of the number of repeat units. Microsatellites are codominant markers in which the gene variants (or alleles) inherited from both parents are amplified in a PCR reaction and visualised on a polyacrylamide gel or CE instrument. Homozygote individuals have the same sized STR repeats (e.g., [AT]<sub>6</sub>, [AT]<sub>6</sub>) whereas heterozygote individuals have different sized repeats (e.g., [AT]<sub>3</sub>, [AT]<sub>6</sub>). In contrast AFLPs are dominant markers where an allele (or fingerprint band) is either present in the individual or absent. The heterozygosity of an individual cannot be determined directly from an AFLP band (or locus) and hence these dominant markers have far less resolving power per locus to determine the population origins of an individual in comparison to the codominant microsatellite markers [97]. Typically, at least eight microsatellite loci or 50 AFLP loci are recommended for population assignment studies [116]. The AFLP technique requires high quality DNA and hence is less versatile for degraded or trace samples in comparison to microsatellites [116]. The genotyping errors associated with AFLPs have been found to be greater than for microsatellites mainly because of differences in peak height intensity [98].

A suite of statistical analyses for assignment methods are currently used to identify the origins of individuals based on their AFLP or microsatellite profile, with the most suitable method depending on the scenario [111, 114, 116–122]. Assignment tests are highly accurate when all potential source populations have been sampled, populations boundaries are well defined, sampling is random, and populations are in Hardy–Weinberg equilibrium (i.e. there is a balance between mutation and genetic drift, no inbreeding and random mating). However, these assumptions are not realistic for many populations such as when populations are small, population boundaries are not clear or the genetic divergences between populations are low [119]. For populations with ill-defined boundaries, clustering methods perform well because they can determine the number of populations (i.e. clusters) present based on

the multilocus genotypes of individuals rather than on predetermined boundaries. They then assign individuals to these identified populations, including to populations that have not been sampled [123]. Programs such as Geneland [124] can map the probabilities of an individual belonging to a ‘cluster’ or ‘population’ onto the landscape in an easily interpretable visual format ideal for the presentation of evidence to a jury in a court of law. Other methods such as spatial smoothing are most effective when the organism has a continuous distribution across the landscape and a spatial structure is not imposed (see [114] for review of assignment methods).

For example, spatial smoothing assignment has been successfully used to monitor the African elephant ivory trade by characterisation of the allele frequencies of 16 STR loci across the entire African elephants’ range. Geographic-specific alleles were shown to be effective in the inference of the geographic origin of individual DNA samples with 50% identified to within 500 km of their source, and 80% to within 932 km of their source [34]. This study was later applied to a forensic case involving the largest seizure of contraband ivory since the 1989 ban on the ivory trade. A total of 532 ivory tusks, and 42,210 “hankos” which are ivory cylinders cut from the solid portion of the tusk, were found in a container shipped via South Africa to Singapore in June 2002. Assignment tests using the 16 STR loci indicated that the ivory was entirely from savannah rather than forest elephants and most likely originated from a narrow strip of southern Africa that centred on Zambia [33]. This information is invaluable for wildlife enforcement agencies to identify current poaching “hot spots” and to identify whether legally declared government stockpiles are being illegally traded and replenished [33].

Assignment tests have also been used to relocate seized animals of unknown origin back to their original population. European pond turtles (*Emys orbicularis*) are highly sought after for pets and hence are often subject to illegal collection. Specimens seized by wildlife authorities are sent to recovery centres or zoos where they rapidly accumulate in large numbers. When the turtles become too numerous to maintain in these facilities they are sacrificed or re-located to their supposed region of origin. Characterisation of three turtle populations at seven microsatellite loci assigned 22 of 36 turtles in recovery centres to their population of origin [125]. Releasing turtles that have been genotypically assigned to the population reduces the risk of outbreeding depression, which is a reduction in reproductive output and fitness that can result when two genetically distinct populations interbreed. Such targeted releases also minimize the possibility of corruption of the evolutionary processes leading to divergence among geographic isolates, an important precursor to speciation.



In a similar manner to population assignment tests, exclusion tests can be used to exclude individuals as belonging to a given population based on their allelic or genotype frequencies. An example of the use of exclusion tests to provide evidence for a wildlife related crime is the 2004 case of a suspected illegal translocation of four red deer (*Cervus elaphus*) into a hunting area in Luxembourg. Exclusion tests based on allelic frequencies for 13 microsatellite loci verified that the Luxembourg red deer were not founded from migrants from the adjacent populations of France, Belgium and Germany. Instead, they were most likely sourced from deer farms and had been illegally translocated into the area for recreational hunting [126]. Genotype exclusion tests, based on ten microsatellite markers, have also been used to successfully discriminate between hatchery-raised versus wild stocks of the commercially important marine fish red drum, *Sciaenops ocellatus*, of the south eastern United States [127].

### Individual identification, sexing, and parentage

Identification of an individual based on their unique genetic profile can be used to monitor the number of animals entering commercial markets, even if they are sold as meat or highly processed products. Baker et al. (2007) [128] combined market surveys with DNA profiling to estimate the numbers of North Pacific minke whales (*Balaenoptera acutorostrata* spp.) sold in 12 markets in the Republic of (South) Korea from 1999 to 2003. A 464 bp fragment of the mtDNA control region and eight STRs were used to develop a 'DNA profile' for each market product. The DNA profiles were evaluated for matches with other profiles and the numbers of unique DNA profiles were assumed to be minimum number of individual whales sold on the market, with matching DNA profiles representing replicates from the same individual. The total number of individual whales sold over a 5 year period was estimated to be 827, almost double the officially recorded by-catch of 458 whales during this period, suggesting that illegal trade of North Pacific minke whales in South Korea is rampant [128]. Characterising individuals with unique DNA profiles is an accurate method of monitoring markets to determine what species are present, and the numbers of individuals of each species sold.

To distinguish between legally and illegally obtained specimens, a DNA register can be established where each legal specimen is DNA profiled in a certified laboratory and the profiles are lodged in a database. When there is a confiscated specimen, the DNA database can be interrogated to rapidly identify unregistered (and presumably illegally obtained) specimens. In Norway, a DNA register for minke whale has been established containing 2676

individual genetic profiles. The genetic profiles are generated using information from the mitochondrial control region, two sex determination markers and 10 microsatellite loci. The Norwegian minke whale DNA register has proven to be effective in verifying legal specimens by consistently matching 20 specimens of minke whales obtained from Norwegian markets to reference samples in the register [129]. An effective DNA register requires all legal specimens to be lodged and genotyped using highly sensitive hypervariable markers, such as microsatellites, that have the resolution to differentiate between individuals. Wildlife DNA registers can also be used to monitor the compliance of breeders to ensure that captive bred stock are not being replenished or supplemented from illegally caught wild stock. The offspring of captive breeding stock can also be verified by assessing the parentage using a suite of hypervariable microsatellite markers, similar to methods used for human paternity analyses [69].

Determining the sex of an animal can be difficult for some taxa where differences between the sexes are not obvious or the illegally killed carcass is decomposed. Determining the gender of the Asian elephant is important because tusks are only present in males and drastic declines in the numbers of males from hunting for their ivory can result in unbalanced sex ratios in the population. It can be difficult or impossible to determine the sex of Asian elephants when the carcass is decomposed, but a simple and inexpensive test based on the SRY gene on the Y chromosome has been developed specifically for identifying male Asian elephants from poached carcasses [130].

In some countries, qualified hunters are restricted to hunting only one sex and monitoring the trade requires determination of the sex of the animals hunted. In Korea the hunting of female pheasant is illegal and sex-specific markers have been used to identify illegal hunting. In one case (February 2004), five pheasant carcasses were found in a suspect's refrigerator. Using two avian sex-specific markers, one marker on the Z chromosome and one on the W chromosome, gender could be determined because avian males are homozygotes (ZZ), whereas females are heterozygotes (ZW). Two of the five pheasant carcasses were female and the suspect was subsequently prosecuted for illegal hunting based on the DNA evidence [68].

### Method validation

Genetic techniques need to be validated for use in forensic applications. This is not a trivial matter either at a scientific level or legal level. The use of DNA markers for wildlife forensic application need to be tested against what has become the 'gold standard' for forensic science, the validation of human DNA. Butler (2005) defines validation as

the process of demonstrating that a laboratory procedure is robust, reliable, and reproducible in the hands of the personnel performing the test [131]. A robust method is one in which successful results are obtained in a high percentage of tests at first testing. A reliable method is one in which the results are accurate and correctly reflect the sample being tested, and a reproducible method is one in which the same result is obtained each time a sample is tested. All three properties are important for techniques performed in forensic laboratories [131]. To meet these exacting standards, forensic scientists need to document (a) full details of the tests used to validate new techniques; (b) the technical procedures and policies to instil confidence in the laboratory processes and policies; and (c) the policies relating to the interpretation of data.

In the forensic world, most DNA analysis is conducted using commercially available technologies, reagents and 'kits'. The *development validation* of the latter will have been carried out by the commercial entity prior to product release. Hence, the forensic laboratory is required to carry out more limited *internal validation* (also called verification) aimed at showing the laboratory can meet accepted validation requirements [131].

Development validation is an exacting process and several organisations at an international level have defined the standards for forensic application. The most commonly used standards are these developed by SWGDAM (Scientific Working group on DNA Analysis Methods) and the ISO17025 or IEC17025 laboratory accreditation. This group and laboratory accreditation was first established in the late 1980s under FBI sponsorship to aid forensic scientists as DNA applications in forensics first emerged. A detailed discussion of these and similar guidelines is beyond the scope of this paper, but they include testing the technology for consistency and reproducibility against standard samples, samples in more complex matrices, mixed samples and samples exposed to a variety of environmental conditions. These criteria are aimed at ensuring the technologies are robust in producing reliable results with real life forensic samples. SWGDAM also makes recommendations with regards to population studies and data interpretation [132].

It can readily be appreciated that forensic validation studies are onerous and time consuming. There have been few comprehensive attempts to validate non human DNA tests for forensic applications. One recent major study validated DNA markers for Cannabis plant samples [133] and this work gives a useful insight to the challenges which will face scientists seeking to cross the bridge between research and development to professional application in forensics.

The admissibility of evidence in the legal system is governed by different rules which also need to be met.

These vary according to the legal frameworks and systems in different countries. In much of the United States, and also followed by many other countries, scientific evidence must meet the Daubert standards [134] under which scientific techniques must have:

- been tested before;
- been subject to peer review and publication;
- standards which can verify the reliability of the technique;
- known potential error notes; and
- gained widespread acceptance in the scientific community.

Scientists seeking to bring wildlife forensics into the court system need to be aware of the legal framework and rules, and the role and expectations for the expert witness within their jurisdiction. These are not trivial matters.

### Future directions of genetic markers in wildlife forensics

The advent of whole genome sequencing of non-model organisms will greatly increase the markers that are currently available for forensic genetics of wildlife. Universal nuclear primers that can amplify informative regions over a broad range of taxa will become more readily available, and will complement current initiatives such as the mtDNA barcoding of life project ([www.barcodinglife.org](http://www.barcodinglife.org)). In addition, the availability of single nucleotide polymorphisms (SNPs) that are informative for species identification, population assignment, and individual identification of wildlife will increase considerably. SNP techniques target multiple regions of the genome where single base pair mutations have occurred and they have the major advantage compared to other forensic genetic methods of being easily amplifiable from highly degraded material and are highly reproducible across different laboratories [135–138]. Furthermore, SNPs are amenable to multiplexing of up to 50 loci on a microarray platform enabling rapid and high throughput screening of forensic samples [139]. SNPs have already proven to be effective for forensic identification of the population of origin for Chinook salmon [140] and show great promise as a genetic marker to contribute to existing forensic genetic technologies.

Whilst emerging technologies will add to the forensic genetic toolbox, current genetic technologies are capable of addressing most forensic questions as evidenced by the suite of methods discussed in this review which have been successfully applied to wildlife forensic cases. The choice of genetic marker will depend on the forensic question to be addressed and the ecology, biology and genetic knowledge of the species. Each genetic technique has it

advantages and limitations for forensic applications and these must be carefully evaluated when choosing a marker (Table 1). To overcome the limitations of the genetic techniques the most powerful approach is to use a combination of complimentary markers with the appropriate resolution to address the forensic outcomes.

Ecological, biological, and genetic knowledge of wildlife has traditionally been covered in the disciplines of wildlife ecology, physiology and conservation genetics and it is this research that forms the foundation for the interpretation of genetic data for forensic applications. We argue that for forensic science to advance in the field of wildlife biology, cross-disciplinary collaborations with ecologists, biologists and conservation geneticists are essential.

Phylogenetic, phylogeographic and population genetic studies are required for species, population and individual identification of wildlife, respectively. The objectives of conservation research are often complimentary with forensic outcomes. For example, phylogenetics can be used to delineate species boundaries and this is important for the enforcement of wildlife legislation, which recognises and protects groups that are classed as ‘species’ or ‘subspecies’. In addition, the markers developed for phylogenetics can also be used, or modified for use, for forensic species identification (Table 1). Phylogeography and population genetic studies not only provide baseline data that is required for assignment of individuals to their geographic source of origin, but are also used to identify populations that are most vulnerable to extinction from overexploitation. The markers used for these same studies can be applied to elucidate the source of traded specimens and thereby identify “hotspots” for illegal collection where enforcement efforts can be directed.

Forensic scientists can greatly benefit from liaison with conservation geneticists to collaboratively develop genetic technologies that will benefit the conservation and management of traded species, and to extend these technologies for use in a forensic context to monitor trade activities and provide DNA evidence that can be presented in court for cases of illicit trade of wildlife. Cross-disciplinary collaboration in the initial planning phase of the research programmes will foster the development of new technologies that have greater versatility with applications for both conservation and forensics.

### Key points

1. The illegal trade of wildlife is worth more than USD \$20 billion per year and poses a major threat to biodiversity.
2. DNA technologies are well suited to detect and provide evidence for cases of illegal wildlife trade

because they can identify the species, geographic origin, individual identity, parentage, and sex of the confiscated specimen.

3. Current DNA technologies for wildlife are capable of addressing most forensic questions but further research is required to validate their routine use for forensic application and admissibility as evidence.
4. Studies of phylogenetics, phylogeography, and population genetics are complementary, and in many cases required, for the development of DNA-based forensic identification tools for wildlife.

**Acknowledgments** We are grateful to the Australian Federal Police and the University of Canberra for funding this project.

### References

1. Interpol. Wildlife crime. In: Interpol. 2007. <http://www.interpol.int/Public/EnvironmentCrime/Wildlife/Default.asp>. Accessed 28 Mar 2008.
2. Cook D, Roberts M, Lowther J. The international wildlife trade and organised crime: a review of the evidence and the role of the UK. Gdalming: WWF-UK; 2002.
3. Warchol GL. The transnational illegal wildlife trade. *Crim Justice Stud.* 2004;1:57–73.
4. Faiola A. Animal smugglers sucking life from Amazon. In: The Washington Post. Sunday 9 December 2001. <http://www.latinamericanstudies.org/brazil/smuggling.htm>. Accessed 25 Mar 2008.
5. Alacs EA, Georges A. Wildlife across our borders: a review of the illegal trade in Australia. *Aust J Forensic Sci.* 2008;40:107–23.
6. Claridge G, Chea-Leth V, Chhoan IV. The effectiveness of law enforcement against forest and wildlife crime. A study of enforcement disincentives and other relevant factors in South-western Cambodia. Report prepared for East–West Management Institute, Conservation International and USAid. 2005. [http://pdf.usaid.gov/pdf\\_docs/pnadf439.pdf](http://pdf.usaid.gov/pdf_docs/pnadf439.pdf). Accessed 21 Mar 2008.
7. Leader-Williams N, Milner-Gulland EJ. Policies for the enforcement of wildlife laws: the balance between detection and penalties in Luangwa Valley, Zambia. *Conserv Biol.* 1993;7: 611–7.
8. Li YM, Gao ZX, Li XH, Wang S, Niemela J. Illegal wildlife trade in the Himalayan region of China. *Biodiv Conserv.* 2000;9:901–18.
9. Courchamp F, Angulo E, Rivalan P, Hall RJ, Signoret L, Bull L, et al. Rarity value and species extinction: the anthropogenic allee effect. *Plos Biol.* 2006;4:2405–10.
10. Turtle Conservation Fund. A global action plan for conservation of tortoises and freshwater turtles. Strategy and funding prospectus 2002–2007. Conservation International and Chelonian Research Foundation. Washington DC;2002.
11. Van Dijk PP, Stuart BL, Rhodin AGJ. Asian turtle trade: proceedings of a workshop on conservation and trade of freshwater turtles and tortoises in Asia. In: Chelonian Research Monographs Chelonian Research Foundation, Lunenburg, MA; 2000.
12. Davic RD. Linking keystone species and functional groups: a new operational definition of the keystone species concept—response. *Conserv Ecol.* 2003;7:r11.

13. Kotliar NB. Application of the new keystone-species concept to prairie dogs: how well does it work? *Conserv Biol.* 2000;14:1715–21.
14. Mills LS, Soule ME, Doak DF. The keystone species in ecology and conservation. *Bioscience.* 1993;43:219–24.
15. IUCN. 2004 IUCN red list of threatened species. A global species assessment. IUCN, Gland, Switzerland and Cambridge;2004.
16. Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, et al. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc Nat Acad Sci USA.* 2006;103:3165–70.
17. Pedersen AB, Jones KE, Nunn CL, Altizer S. Infectious diseases and extinction risk in wild mammals. *Conserv Biol.* 2007;21:1269–79.
18. Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, et al. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth.* 2007;4:125–34.
19. Smith KF, Sax DF, Lafferty KD. Evidence for the role of infectious disease in species extinction and endangerment. *Conserv Biol.* 2006;20:1349–57.
20. Spinks PQ, Shaffer HB. Conservation phylogenetics of the Asian box turtles (Geomydidae, Cuora): mitochondrial introgression, numts, and inferences from multiple nuclear loci. *Conserv Genet.* 2007;8:641–57.
21. Keller RP, Lodge DM. Species invasions from commerce in live aquatic organisms: problems and possible solutions. *Bioscience.* 2007;57:428–36.
22. Normile D. Invasive species—expanding trade with China creates ecological backlash. *Science.* 2004;306:968–9.
23. Reed RN. An ecological risk assessment of non-native boas and pythons as potentially invasive species in the United States. *Risk Anal.* 2005;25:753–66.
24. Weigle SM, Smith LD, Carlton JT, Pederson J. Assessing the risk of introducing exotic species via the live marine species trade. *Conserv Biol.* 2005;19:213–23.
25. Wong K-L, Wang J, But PP-H, Shaw P-C. Application of *cytochrome b* DNA sequences for the authentication of endangered snake species. *Forensic Sci Int.* 2004;139:49–55.
26. Hedmark E, Ellegren H. Microsatellite genotyping of DNA isolated from claws left on tanned carnivore hides. *Int J of Legal Med.* 2005;119:370–3.
27. Chapman DD, Abercrombie DL, Douady CJ, Pikitch EK, Stanhope MJ, Shivji MS. A streamlined, bi-organelle, multiplex PCR approach to species identification: application to global conservation and trade monitoring of the great white shark, *Carcharodon carcharias*. *Conserv Genet.* 2003;4:415–25.
28. Moore MK, Bemiss JA, Rice SM, Quattro JM, Woodley CM. Use of restriction fragment length polymorphisms to identify sea turtle eggs and cooked meats to species. *Conserv Genet.* 2003;4:95–103.
29. Branicki W, Kupiec T, Pawlowski R. Validation of *cytochrome b* sequence analysis as a method of species identification. *J Forensic Sci.* 2003;48:83–7.
30. Prado M, Franco C, Fente C, Cepeda A, Vázquez B, Barros-Velázquez. Comparison of extraction methods for the recovery amplification and species-specific analysis of DNA from bone and bone meals. *Electrophoresis.* 2002;23:1005–12.
31. Wasser SK, Mailand C, Booth R, Mutayoba B, Kisamo E, Clark B, et al. Using DNA to track the origin of the largest ivory seizure since the 1989 trade ban. *Proc Nat Acad Sci USA.* 2007;104:4228–33.
32. Wasser SK, Shedlock AM, Comstock K, Ostrander EA, Mutayoba B, Stephens M. Assigning African elephant DNA to geographic region of origin: applications to the ivory trade. *Proc Nat Acad Sci USA.* 2004;101:14847–52.
33. Hsieh HM, Huang LH, Tsai LC, Kuo YC, Meng HH, Linacre A, et al. Species identification of rhinoceros horns using the *cytochrome b* gene. *For Sci Int.* 2003;136:1–11.
34. Lo CF, Lin YR, Chang HC, Lin JH. Identification of turtle shell and its preparations by PCR-DNA sequencing method. *J Food Drug Anal.* 2006;14:153–8.
35. Rudnick JA, Katzner TE, Bragin EA, DeWoody JA. Species identification of birds through genetic analysis of naturally shed feathers. *Mol Ecol Notes.* 2007;7:752–62.
36. Kumar R, Singh PJ, Nagpure NS, Kushwaha B, Srivastava SK, Lakra WS. A non-invasive technique for rapid extraction of DNA from fish scales. *Indian J Exp Biol.* 2007;45:992–7.
37. Randi E. Mitochondrial DNA. In: Baker AJ, editor. *Molecular methods in ecology.* Malden: Blackwell Science; 2000.
38. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc Nat Acad Sci USA.* 1989;86:6196–200.
39. Mullis KB, Ferré F, Gibbs RA. The polymerase chain reaction. Birkhäuser: Boston; 1994.
40. Alacs E, Alpers D, de Tores P, Dillon M, Spencer PBS. Identifying the presence of quokkas (*Setonix brachyurus*) and other macropods using *cytochrome b* analyses from faeces. *Wildlife Res.* 2003;30:41–7.
41. Dawnay N, Ogden R, McEwing R, Carvalho GR, Thorpe RS. Validation of the barcoding gene CO1 for use in forensic genetic species identification. *For Sci Int.* 2007;173:1–6.
42. Hsieh HM, Chiang HL, Tsai LC, Lai SY, Huang NE, Linacre A, et al. *Cytochrome b* gene for species identification of the conservation animals. *For Sci Int.* 2001;122:7–18.
43. Verma SK, Singh L. Novel universal primers establish identity of an enormous number of animal species for forensic application. *Mol Ecol Notes.* 2003;3:28–31.
44. Yau FCF, Wong KL, Shaw PC, But PPH, Wang J. Authentication of snakes used in Chinese medicine by sequence characterized amplified region (SCAR). *Biodiv Conserv.* 2002;11:1653–62.
45. Malik S, Wilson PJ, Smith RJ, Lavigne DM, White BN. Pinniped penises in trade: a molecular-genetic investigation. *Conserv Biol.* 1997;11:1365–74.
46. Lee JC, Tsai L-C, Huang M-T, Jhuang J-A, Yao C-T, Chin S-C, et al. A novel strategy for avian species identification by *cytochrome b* gene. *Electrophoresis.* 2008;29:2413–8.
47. Wan QH, Fang SG. Application of species-specific polymerase chain reaction in the forensic identification of tiger species. *For Sci Int.* 2003;131:75–8.
48. Ronaghi M, Uhlen M, Nyren P. A sequencing method based on real-time pyrophosphate. *Science.* 1998;281:363.
49. Karlsson AO, Holmlund G. Identification of mammal species using species-specific DNA pyrosequencing. *Forensic Sci Int.* 2007;173:16–20.
50. Ballard JWO, Whitlock MC. The incomplete natural history of mitochondria. *Mol Ecol.* 2004;13:729–44.
51. Durand JD, Collet A, Chow S, Guinand B, Borsa P. Nuclear and mitochondrial DNA markers indicate unidirectional gene flow of Indo-Pacific to Atlantic bigeye tuna (*Thunnus obesus*) populations, and their admixture off southern Africa. *Mar Biol.* 2005;147:313–22.
52. Breton S, Beaupre HD, Stewart DT, Hoeh WR, Blier PU. The unusual system of doubly uniparental inheritance of mtDNA: isn't one enough? *Trends Genet.* 2007;23:465–74.
53. Kvist L, Martens J, Nazarenko AA, Orell M. Paternal leakage of mitochondrial DNA in the great tit (*Parus major*). *Mol Biol Evol.* 2003;20:243–7.
54. Rokas A, Ladoukakis, Zouros E. Animal mitochondrial DNA recombination revisited. *Trends Ecol Evol.* 2003;18:411–7.



55. Sherengul W, Kondo R, Matsuura ET. Analysis of paternal transmission of mitochondrial DNA in *Drosophila*. *Genes Genetic Syst.* 2006;81:399–404.
56. Ujvari B, Dowton M, Madsen T. Mitochondrial DNA recombination in a free-ranging Australian lizard. *Biol Lett.* 2007; 3:189–92.
57. Arctander P. Comparison of a mitochondrial gene and a corresponding nuclear pseudogene. *Proc R Soc London Ser B.* 1995; 262:13–9.
58. Parr RL, Maki J, Reguly B, Dakubo GD, Aguirre A, Wittrock R, et al. The pseudo-mitochondrial genome influences mistakes in heteroplasmy interpretation. *BMC Genomics.* 2006;7:185.
59. Thalmann O, Hebler J, Poinar HN, Paabo S, Vigilant L. Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other great apes. *Mol Ecol.* 2004; 13:321–35.
60. Behura SK. Analysis of nuclear copies of mitochondrial sequences in honeybee (*Apis mellifera*) genome. *Mol Biol Evol.* 2007;24:1492–505.
61. Podnar M, Haring E, Pinsker W, Mayer W. Unusual origin of a nuclear pseudogene in the Italian wall lizard: intergenomic and interspecific transfer of a large section of the mitochondrial genome in the genus *Podarcis* (*Lacertidae*). *J Mol Evol.* 2007; 64:308–20.
62. Xu JP, Zhang QQ, Xu XF, Wang ZG, Qi J. Intragenomic variability and pseudogenes of ribosomal DNA in stone flounder *Kareius bicoloratus*. *Mol Phylogenet Evol.* 2009;52:157–66.
63. Saville BJ, Kohli Y, Anderson JB. mtDNA recombination in a natural population. *Proc Nat Acad Sci USA.* 1998;95:1331–5.
64. Brower AVZ, DeSalle R, Vogler A. Gene trees, species trees, and systematics: a cladistic perspective. *Ann Rev Ecol Syst.* 1996;27:423–50.
65. Maddison WP. Gene trees in species trees. *Syst Biol.* 1997; 46:523–36.
66. Page RDM. Extracting species trees from complex gene trees: reconciled trees and vertebrate phylogeny. *Mol Phylogenet Evol.* 2000;14:89–106.
67. Sites JW, Davis SK, Guerra T, Iverson JB, Snell HL. Character congruence and phylogenetic signal in molecular and morphological data sets: a case study in the living iguanas (*Squamata*, *Iguanidae*). *Mol Biol Evol.* 1996;13:1087–105.
68. An J, Lee MY, Min MS, Lee MH, Lee H. A molecular genetic approach for species identification of mammals and sex determination of birds in a forensic case of poaching from South Korea. *For Sci Int.* 2007;167:59–61.
69. Cassidy BG, Gonzales RA. DNA testing in animal forensics. *J Wildl Manage.* 2005;69:1454–62.
70. Ebach MC, Holdrege C. DNA barcoding is no substitute for taxonomy. *Nature.* 2005;434:697.
71. Fernandes CA, Ginja C, Pereira I, Tenreiro R, Bruford MW, Santos-Reis M. Species-specific mitochondrial DNA markers for identification of non-invasive samples from sympatric carnivores in the Iberian Peninsula. *Conserv Genet.* 2008;9:681–90.
72. Upholt WB. Estimation of DNA-sequence divergence from comparison of restriction endonuclease digests. *Nucleic Acids Res.* 1977;4:1257–65.
73. Bravi CM, Liron JP, Rippoli MV, Peral-Gracia P, Giovambattista G. A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome *b* gene. *Legal Med.* 2004;6:246–51.
74. Vandamme A. Basic concepts of molecular evolution. In: Salemi M, Vandamme A, editors. *The phylogenetic handbook. A practical approach to DNA and protein phylogeny.* New York: Cambridge University Press; 2003.
75. Rosenberg NA. The probability of topological concordance of gene trees and species trees. *Theor Pop Biol.* 2002;61:225–47.
76. Sanderson MJ, Shaffer HB. Troubleshooting molecular phylogenetic analyses. *Ann Rev Ecol Syst.* 2002;33:49–72.
77. Bickford D, Lohman DJ, Sodhi NS, Ng PK, Meier R, Winker K, et al. Cryptic species as a window on diversity and conservation. *Trends Ecol Evol.* 2007;22:148–55.
78. Arnold ML. Natural hybridization as an evolutionary process. *Ann Rev Ecol Syst.* 1992;23:237–61.
79. Dowling TE, Secor CL the role of hybridization and introgression in the diversification of animals. *Ann Rev Ecol Syst.* 1997;28:593–619.
80. Crochet PA, Chen JJZ, Pons JM, Lebreton J-D, Hebert PDN, Bonhomme F. Genetic differentiation at nuclear and mitochondrial loci among large white-headed gulls: sex-biased interspecific gene flow? *Evolution.* 2003;57:2865–78.
81. Wang RX, Zhao YL. Differential barrier strength and allele frequencies in hybrid zones maintained by sex-biased hybrid incompatibilities. *Heredity.* 2008;100:326–36.
82. Whitworth TL, Dawson RD, Magalon H, Baudry E. DNA barcoding cannot reliably identify species of the blowfly genus *Protophormia* (Diptera: Calliphoridae). *Proc R Soc London Ser B.* 2007;274:1731–9.
83. Saetre GP, Borge T, Lindell J, Moum T, Primmer CR, Sheldon BC, et al. Speciation, introgressive hybridization and nonlinear rate of molecular evolution in flycatchers. *Mol Ecol.* 2001;10: 737–49.
84. Tegelstrom H, Gelter HP. Haldane rule and sex biased gene flow between two hybridizing flycatcher species (*Ficedula albicollis* and *F. hypoleuca*, Aves, Muscicapidae). *Evolution.* 1990;44:2012–21.
85. Tosi AJ, Morales JC, Melnick DJ. Paternal, maternal, and biparental molecular markers provide unique windows onto the evolutionary history of macaque monkeys. *Evolution.* 2003; 57:1419–35.
86. Vos P, Hogers R, Bleeker M, Reijmans M, van de Lee T, Hornes M. AFLP—a new technique for DNA fingerprinting. *Nucleic Acids Res.* 1995;23:4407–14.
87. Alghanim HJ, Almirall JR. Development of microsatellite markers in *Cannabis sativa* for DNA typing and genetic relatedness analyses. *Anal Bioanal Chem.* 2003;376:1225–33.
88. Coyle HM, Palmbach T, Juliano N, Ladd C, Lee HC. An overview of DNA methods for the identification and individualization of marijuana. *Croat Med J.* 2003;44:315–21.
89. Datwyler SL, Weiblen GD. Genetic variation in hemp and marijuana (*Cannabis sativa* L.) according to amplified fragment length polymorphisms. *J For Sci.* 2006;51:371–5.
90. Hakki EE, Uz E, Sag A, Atasoy S, Akkaya MS. DNA fingerprinting of *Cannabis sativa* L. accessions using RAPD and AFLP markers. *For Sci Int.* 2003;136:31.
91. Coyle HM, Ladd C, Palmbach T, Lee HC. The green revolution: botanical contributions to forensics and drug enforcement. *Croat Med J.* 2001;42:340–5.
92. Lee JCI, Cole M, Linacre A. Identification of hallucinogenic fungi from the genera *Psilocybe* and *Panaeolus* by amplified fragment length polymorphism. *Electrophoresis.* 2000;21:1484–7.
93. Linacre A, Cole M, Lee JCI. Identifying the presence of ‘magic mushrooms’ by DNA profiling. *Sci Justice.* 2002;42:50–4.
94. Haig SM, Mullins TD, Forsman ED, Trail PW, Wennerberg L. Genetic identification of spotted owls, barred owls, and their hybrids: legal implications of hybrid identity. *Conserv Biol.* 2004;18:1347–57.
95. Congiu L, Dupanloup I, Patanello T, Fontana F, Rossi R, Arlati G, et al. Identification of interspecific hybrids by amplified fragment length polymorphism: the case of sturgeon. *Mol Ecol.* 2001;10:2355–9.
96. Nijman IJ, Otsen M, Verkaar ELC, de Ruijter C, Hanekamp E, Ochieng JW, et al. Hybridization of banteng (*Bos javanicus*) and



- zebu (*Bos indicus*) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites. *Heredity*. 2003;90:10–6.
97. Bensch S, Akesson M. Ten years of AFLP in ecology and evolution: why so few animals? *Mol Ecol*. 2005;14:2899–914.
  98. Bonin A, Bellemain E, Eidesen PB, Pompanon F, Brochmann C, Taberlet P. How to track and assess genotyping errors in population genetics studies. *Mol Ecol*. 2004;13:3261–73.
  99. Hong Y, Chuah A. A format for databasing and comparison of AFLP fingerprint profiles. *BMC Bioinf*. 2003;4:7.
  100. Clarke SC, Magnussen JE, Abercrombie DL, McAllister MK, Shivji MS. Identification of shark species composition and proportion in the Hong Kong shark fin market based on molecular genetics and trade records. *Conserv Biol*. 2006;20:201–11.
  101. Magnussen JE, Pikitch EK, Clarke SC, Nicholson C, Hoelzel AR, Shivji MS. Genetic tracking of basking shark products in international trade. *Animal Conserv*. 2007;10:199–207.
  102. Pank M, Stanhope M, Natanson L, Kohler N, Shivji M. Rapid and simultaneous identification of body parts from the morphologically similar sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (Carcharhinidae) using multiplex PCR. *Marine Biotechnol*. 2001;3:231–40.
  103. Shivji MS, Chapman DD, Pikitch EK, Raymond PW. Genetic profiling reveals illegal international trade in fins of the great white shark, *Carcharodon carcharias*. *Conserv Genet*. 2005;6:1035–9.
  104. Negi MS, Devic M, Delseny M, Lakshmikumar M. Identification of AFLP fragments linked to seed coat colour in *Brassica juncea* and conversion to a SCAR marker for rapid selection. *Theor Appl Genet*. 2000;101:146–52.
  105. Perez T, Albornoz J, Dominguez A. An evaluation of RAPD fragment reproducibility and nature. *Mol Ecol*. 1998;7:1347–57.
  106. Wu XB, Liu H, Jiang ZG. Identification primers for sika deer (*Cervus nippon*) from a sequence-characterised amplified region (SCAR). *NZ J Zool*. 2006;33:65–71.
  107. Zhang JB, Cai ZP. Differentiation of the rainbow trout (*Oncorhynchus mykiss*) from Atlantic salmon (*Salmon salar*) by the AFLP-derived SCAR. *Eur Food Res Tech*. 2006;223:413–7.
  108. He L, Wang SB, Miao XX, Wu H, Huang YP. Identification of necrophagous fly species using ISSR and SCAR markers. *For Sci Int*. 2007;168:148–53.
  109. Avise JC, Arnold J, Martin Ball R, Bermingham E, Lamb T, Neigel JE, et al. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Ann Rev Ecol Syst*. 1987;18:489–522.
  110. Sanders JG, Cribbs JE, Fienberg HG, Hulburd GC, Katz LS, Palumbi SR. The tip of the tail: molecular identification of seahorses for sale in apothecary shops and curio stores in California. *Conserv Genet*. 2008;9:65–71.
  111. Cornuet JM, Piry S, Luikart G, Estoup A, Solignac M. New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics*. 1999;153:1989–2000.
  112. DeYoung RW, Demarais S, Honeycutt RL, Gonzales RA, Gee KL, Anderson JD. Evaluation of a DNA microsatellite panel useful for genetic exclusion studies in white-tailed deer. *Wildlife Soc Bull*. 2003;31:220–32.
  113. Gomez-Diaz E, Gonzalez-Solis J. Geographic assignment of seabirds to their origin: combining morphologic, genetic, and biogeochemical analyses. *Ecol Appl*. 2007;17:1484–98.
  114. Manel S, Gaggiotti OE, Waples RS. Assignment methods: matching biological questions techniques with appropriate. *Trends Ecol Evol*. 2005;20:136–42.
  115. Ellegren H. Microsatellites: simple sequences with complex evolution. *Nat Rev Genet*. 2004;5:435–45.
  116. Campbell D, Duchesne P, Bernatchez L. AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. *Mol Ecol*. 2003;12:1979–91.
  117. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol*. 2005;14:2611–20.
  118. Manel S, Berthoud F, Bellemain E, Gaudet M, Luikart G, Swenson JE, et al. Intra-biodiv consortium. A new individual-based spatial approach for identifying genetic discontinuities in natural populations. *Mol Ecol*. 2007;16:2031–43.
  119. Maudet C, Miller C, Bassano B, Breitenmoser-Würsten C, Gauthier D, Obexer-Ruff G, et al. Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in *Alpine ibex Capra ibex* (ibex). *Mol Ecol*. 2002;11:421–36.
  120. Paetkau D, Slade R, Burden M, Estoup A. Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Mol Ecol*. 2004;13:55–65.
  121. Piry S, Alapetite A, Cornuet JM, Paetkau D, Baudouin L, Estoup A. GENECLASS2: a software for genetic assignment and first-generation migrant detection. *J Hered*. 2004;95:536–9.
  122. Waples RS, Gaggiotti O. What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. *Mol Ecol*. 2006;15:1419–39.
  123. Mank JE, Avise JC. Individual organisms as units of analysis: Bayesian-clustering alternatives in population genetics. *Genetical Res*. 2004;84:135–43.
  124. Guillot G, Mortier F, Estoup A. GENELAND: a computer package for landscape genetics. *Mol Ecol Notes*. 2005;5:712–5.
  125. Velo-Anton G, Godinho R, Ayres C, Ferrand N, Rivera AC. Assignment tests applied to relocate individuals of unknown origin in a threatened species, the European pond turtle (*Emys orbicularis*). *Amphibia-Reptilia*. 2007;28:475–84.
  126. Frantz AC, Pourtois JT, Heuertz M, Schley L, Flamand MC, Krier A, et al. Genetic structure and assignment tests demonstrate illegal translocation of red deer (*Cervus elaphus*) into a continuous population. *Mol Ecol*. 2006;15:3191–203.
  127. Renshaw MA, Saillant E, Broughton RE, Gold JR. Application of hypervariable genetic markers to forensic identification of ‘wild’ from hatchery-raised red drum, *Sciaenops ocellatus*. *For Sci Int*. 2006;156:9–15.
  128. Baker CS, Cooke JG, Lavery S, Dalebout ML, Yu M, Funashi N, et al. Estimating the number of whales entering trade using DNA profiling and capture-recapture analysis of market products. *Mol Ecol*. 2007;16:2617–26.
  129. Palsboll PJ, Berube M, Skaug HJ, Raymakers C. DNA registers of legally obtained wildlife and derived products as means to identify illegal takes. *Conserv Biol*. 2006;20:1284–93.
  130. Gupta SK, Thangaraj K, Singh L. A simple and inexpensive molecular method for sexing and identification of the forensic samples of elephant origin. *J For Sci*. 2006;51:805–7.
  131. Butler JM. Forensic DNA typing. Amsterdam: Elsevier; 2005.
  132. Scientific Working group on DNA Analysis Methods. Forensic Science Communications. <http://www.fbi.gov/hq/lab/fsc/current/backissu.htm>; 2004. Accessed 25 Mar 2008.
  133. Howard C, Gilmore S, Robertson J, Peakall R. Application of new DNA markers for forensic examination of *Cannabis sativa* Seizures—developmental validation of protocols and a genetic database, NDLERF monograph series, no. 29. Hobart: National Drug Law Enforcement Research Fund; 2008.
  134. Girard JE. Criminalistic, forensic science and crime. Boston: Jones and Bartlett Publishers; 2008.
  135. Amorim A, Pereira L. Pros and cons in the use of SNPs in forensic kinship investigation: a comparative analysis with STRs. *For Sci Int*. 2005;150:17–21.

136. Budowle B. SNP typing strategies. *For Sci Int.* 2004;146:S139–42.
137. Chakraborty R, Stivers DN, Su B, Zhong YX, Budowle B. The utility of short tandem repeat loci beyond human identification: implications for development of new DNA typing systems. *Electrophoresis.* 1999;20:1682–96.
138. Sarkar N, Kashyap VK. SNP markers in forensic testing—a preliminary study. *For Sci Int.* 2003;136:52–3.
139. Divne AM, Allen M. A DNA microarray system for forensic SNP analysis. *For Sci Int.* 2005;154:111–21.
140. Schwenke PL, Rhydderch JG, Ford MJ, Marshall AR, Park LK. Forensic identification of endangered Chinook Salmon (*Oncorhynchus tshawytscha*) using a multilocus SNP assay. *Conserv Genet.* 2006;7:983–9.

# DNA typing in wildlife crime: recent developments in species identification

Shanan S. Tobe · Adrian Linacre

Accepted: 13 May 2010 / Published online: 5 June 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** Species identification has become a tool in the investigation of acts of alleged wildlife crimes. This review details the steps required in DNA testing in wildlife crime investigations and highlights recent developments where not only can individual species be identified within a mixture of species but multiple species can be identified simultaneously. ‘*What species is this?*’ is a question asked frequently in wildlife crime investigations. Depending on the material being examined, DNA analysis may offer the best opportunity to answer this question. Species testing requires the comparison of the DNA type from the unknown sample to DNA types on a database. The areas of DNA tested are on the mitochondria and include predominantly the cytochrome *b* gene and the cytochrome oxidase I gene. Standard analysis requires the sequencing of part of one of these genes and comparing the sequence to that held on a repository of DNA sequences such as the GenBank database. Much of the DNA sequence of either of these two genes is conserved with only parts being variable. A recent development is to target areas of those sequences that are specific to a species; this can increase the sensitivity of the test with no loss of specificity. The benefit of targeting species specific sequences is that within a mixture of two or more species, the individual species within the mixture can be identified. This identification would not be possible

using standard sequencing. These new developments can lead to a greater number of samples being tested in alleged wildlife crimes.

**Keywords** Species identification · Non-human DNA · Cytochrome *b* · Cytochrome oxidase I · DNA mixtures

## Introduction

Forensic science may be called to assist in a range of alleged wildlife crimes. The types of offences include: the trade in internationally protected species; the possession of control species; the identification of a species as part of an alleged cruelty incident or illegal poaching; and the identification of a species as part of associative evidence in a criminal case. In all the above investigations, it is the identification of a species that is required. Depending on the type of legislation, it is common for species to be listed within the legislation. For instance, in the UK there are many separate acts for different species including deer [1, 2], seal [3, 4] and badgers [5]. These laws set out if and when hunting is permitted and there are similar laws in other countries, therefore there is a need to perform a test to determine if the species in question is present or not.

Legislation that governs wildlife is both national and international. Currently 175 countries are signatories to the Convention on the International Trade in Flora and Fauna (CITES) [6]. Forensic science methods can be used in the enforcement of national and international legislation. Although this review will focus mainly on the identification of mammals, the techniques are the same for other organisms including other vertebrates, invertebrates and even botanical species, and are used on different genes than those discussed.

---

S. S. Tobe  
Centre for Forensic Science, Strathclyde University,  
WestCHEM, 204 George Street, Glasgow, UK

A. Linacre (✉)  
School of Biological Sciences, Flinders University,  
Adelaide 5001, Australia  
e-mail: adrian.linacre@flinders.edu.au

The need to identify a species requires that the species is defined. The use of morphology and more recently genetics has provided a systematic identification of species. The classification works in that voucher specimens are used for comparison to any unknown sample. A problem arises with sub-species and hybrids where there is little genetic variation between the different sub-species. For example, there are five recognized sub-species of tiger: *Panthera tigris tigris* (Bengali), *P. tigris altaica* (Siberian), *P. tigris corbetti* (Indo Chinese), *P. tigris amoyensis* (Amoy), and *P. tigris sumatrae* (Sumatran). The number of tiger sub-species has recently been evaluated based on morphological, biochemical and molecular genetic studies resulting in diverse conclusions [7–9]. The five currently recognised sub-species of tiger are generally classified on their phenotypic appearance, particularly on skull morphology [7]. There is much debate about tiger sub-species with some [9] wanting to add new sub-species while others [8] think the number should be reduced. Further, the different sub-species can interbreed and produce viable offspring, but these hybrid individuals are not classified and may not be protected.

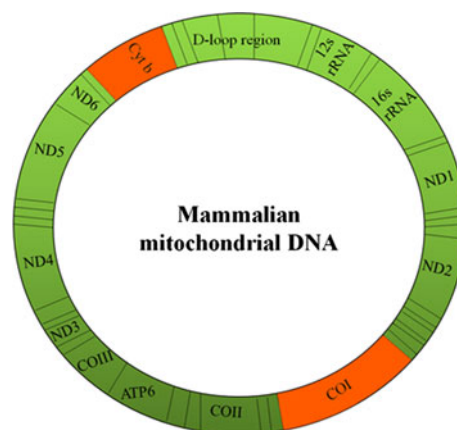
There are many ways in which species can be identified. The first and foremost is through morphological characteristics, which if present may simply require identification by microscopy or osteology so that no further examination is needed for species identification [10–16]. However identifying characteristics may be intentionally removed [17] making morphological methods unsuitable [18] and therefore other methods of evaluation are needed [12]. This generally falls to molecular analyses, usually through serology or DNA. Serological analysis takes the form of antigen–antibody reactions to identify species [19–21] and are indeed still used in many laboratories today [22]. A recent application is in the detection of bear bile used in traditional medicines where the World Society for the Protection of Animals (WSPA) developed an immunoassay to detect bear protein in products alleged to contain biological material from a bear species [23, 24]. There are several problems with this method of testing including the production and isolation of the antibody, and a large amount of sample generally being required [19, 20, 25]. Additionally, many proteins lose their biological activity soon after death [17]. Hybridization methods are therefore generally used to identify if a sample is human or non-human and will rarely identify an exact species [25].

Species identification is therefore usually accomplished through the analysis of DNA, generally areas that are specific to a particular species. Early whole genome applications include Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP), which have been applied to identification of animal components [26–37]. These methods

are now generally considered obsolete as they have poor reproducibility and interpretation of mixture results may be difficult due to overlapping restriction patterns which may be generated [26], although there is still recent reference and use of these techniques in the literature [38–44].

It is preferable then to use known genetic loci which exhibit either interspecies or intraspecies variation. This information is often provided from studies compiling phylogenetic trees [45–52]. The sequences are then deposited on databases such as GenBank ([www.genbank.nih.gov](http://www.genbank.nih.gov)) or EMBL-Bank (<http://www.ebi.ac.uk/embl/>) and can be incorporated into forensic analyses.

Two genetic loci have emerged as the most commonly analysed in species identification, both of which are on the mitochondrial genome: the cytochrome *b* gene and the cytochrome oxidase I gene [53] (Fig. 1), however other genes such as 16S rRNA [26, 29, 30, 54, 55], 12S rRNA [26, 56–59] and even the control region (HVI, HVII and HVIII found within the D-loop) [60–65] can be used. The cytochrome *b* (cyt *b*) gene is 1,140 bases in length and lies between bases 14,747 and 15,887 in the human mitochondrial genome [66]. It encodes a protein, 380 amino acids in length [10], involved in the oxidative phosphorylation pathway within mitochondria [67]. The entire gene sequence has been decoded for thousands of species, ranging from mammalian to invertebrate species. Within taxonomic Orders, such as Mammalia or Aves, there is much conservation of the gene sequences between species [68, 69]. Alignment of the DNA sequences has shown that there are areas of higher homology for members of the same Order, with areas of lesser homology [69, 70]. The entire gene is long and often not present in degraded samples and therefore parts of the gene locus are used in species identification [63, 71–77]. The typical size of the



**Fig. 1** The Mammalian mitochondrial genome. The gene order is the same in all mammalian species, but can be different in other Classes. The cyt *b* and COI genes have been highlighted. This image is adapted from [127] and courtesy of T. Kitpipit

amplification fragment using these primers sets is ~400 bp [69].

The cytochrome oxidase I (COI) gene lies between bases 5,904–7,445 of the human mitochondrial genome [66]. This gene, being 1,541 bp in length and slightly larger than the *cyt b* gene, also produces a protein involved in the process of respiration within the mitochondria [67]. Alignment of the DNA sequence from the COI gene for many species shows a similar scenario to that of the *cyt b* gene where there are areas of high homology for species of the same Order interspersed with areas of less conservation of sequence. A section of the COI gene locus is used by the Barcode for Life consortium where a section of over 600 bp is amplified and sequenced [69, 78].

Crucially for genetic loci used in species testing, there is variation of the DNA sequence between closely related species and relatively little variation within members of the same species [10, 77, 79]. All members of the same species, including members of sub-species, should be sufficiently similar to each other that they cannot be misidentified as a member of a different species. With the exception of a few anomalies, both *cyt b* and COI meet the criteria for species identification, although for some Orders *cyt b* has marginally fewer anomalies [53, 70].

### DNA v amino acid sequence conservation

Both *cyt b* and COI have domains of amino acid sequences that are conserved throughout a Class such as Mammalia. These conserved domains are a result of selection pressure on areas of function within the protein [80]. Where there are differences at the amino acid level many of these changes are between amino acids of the same chemical variety; i.e. polar, non-polar, acid and basic. Where there is an alteration of the amino acid within the protein for different species, there will be a difference in the corresponding DNA sequence. Even when there are matching amino acid sequences between two species, there is a possibility that the DNA will be different. The three base codon for some amino acids is variable, particularly at the third base (Table 1). A change in this third base may result in no change to the amino acid sequence. Examples are shown in Fig. 2.

Mutations at the DNA level that cause no change to the amino acids sequence are called synonymous mutations (see Fig. 2a). A non-synonymous mutation will change the amino acid encoded (see Fig. 2b). If there is a mutational event at the second base of a codon it will invariably alter the amino acid encoded. There are examples where mutational events at the first base will result in a synonymous event, although these are less common than changing an amino acid. A mutational event at the third base of a codon

**Table 1** The genetic code showing redundancy predominantly at the third base and at the first base to a lesser extent but that any change in the second base of the codon will result in a change in the amino acid encoded

First position	Second position				Third position
	U(T)	C	A	G	
U(T)	Phe	Ser	Tyr	Cys	U(T)
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	Trp	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U(T)
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Met	U(T)
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Stop	A
	Met	Thr	Lys	Stop	G
G	Val	Ala	Asp	Gly	U(T)
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G
Codon	Universal code		Human mitochondrial code		
UGA	Stop		Trp		
AGA	Arg		Stop		
AGG	Arg		Stop		
AUA	Ile		Met		

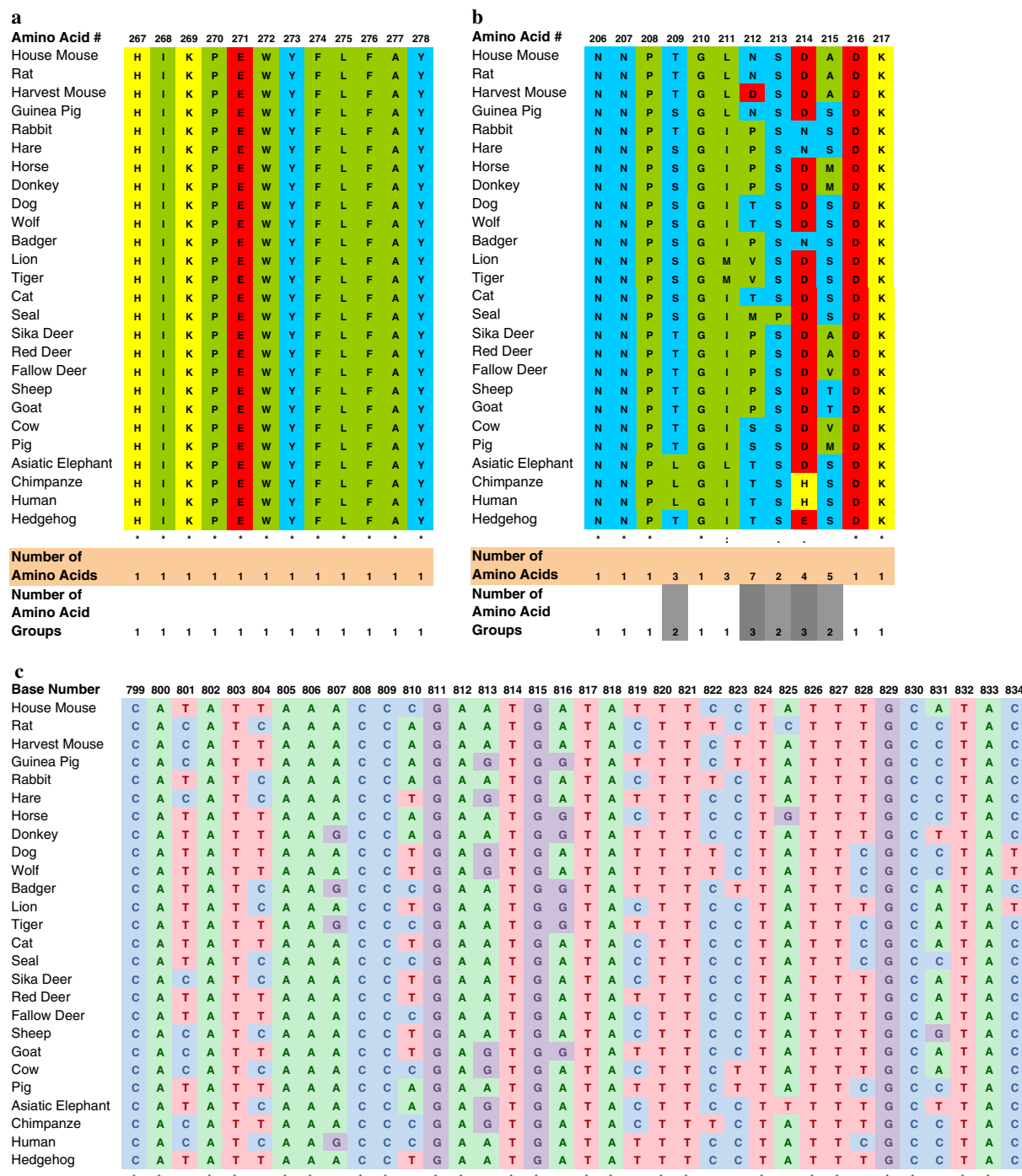
The last 4 rows illustrate the difference between the code in the nucleus and in the mitochondria

<b>a</b>						
DNA Sequence:	GGG	CC	T	GCA	CTC	TTC
Amino Acid	Gly	Pro	Ala	Leu	Phe	
DNA Sequence:	GGG	CC	C	GCA	CTC	TTC
Amino Acid	Gly	Pro	Ala	Leu	Phe	
<b>b</b>						
DNA Sequence:	GGG	CT	T	GCA	CTC	TTC
Amino Acid	Gly	Pro	Ala	Leu	Phe	
DNA Sequence:	GGG	C	T	GCA	CTC	TTC
Amino Acid	Gly	Leu	Ala	Leu	Phe	

**Fig. 2 a** An example of how mutational events can affect the amino acid encoded by showing 12 DNA bases encoding 4 amino acids. A transition mutation at the third base of a codon for proline results in a synonymous mutation, i.e. no change in the amino acid encoded. **b** an example showing 12 DNA bases encoding 4 amino acids. A transition mutation at the second base of a codon results in a change in the amino acid encoded

has the greatest chance of not altering the amino acid encoded. An example of an alignment of mammalian *cyt b* amino acid sequence is shown in Fig. 3.





**Fig. 3** The effect of homology or variation within amino acid sequences corresponds to homology and variation within DNA sequences. Two different parts of the *cyt b* gene are shown. In **a** there is a stretch of 12 amino acids that is invariant for all 26 mammalian species listed. This does not mean that the DNA sequence is necessarily the same as there may be silent mutations, however it is the region where the DNA sequences found in all mammals are most likely to be similar. **b** shows a different part of the *cyt b* gene where there is variation at the amino acid level and therefore at the DNA

level. These regions offer the prospect of locating species-specific DNA regions. **c** shows the corresponding sequences in DNA to the amino acids in **(a)**. Notice that variation occurs mainly at the third base of a codon. One exception is at base 823, amino acid position 275 (L), where there is variation at the first base position of the codon. This is one of the few examples where variation at the first position results in a silent mutation. **d** The corresponding sequences in DNA to the amino acids in **(b)**. Notice the large areas of variation corresponding to the variation in amino acids

d

Base Number	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651
House Mouse	A	A	C	A	A	C	C	C	A	A	C	A	G	G	A	T	T	A	A	A	C	T	C	A	G	A	T	G	C	A	G	A	T	A	A	A
Rat	A	A	T	A	A	C	C	C	A	A	C	A	G	G	C	C	T	A	A	A	C	T	C	C	G	A	C	G	C	A	G	A	C	A	A	A
Harvest Mouse	A	A	C	A	A	C	C	C	C	A	C	A	G	G	C	C	T	C	A	A	C	T	C	A	G	A	T	G	C	A	G	A	T	A	A	A
Guinea Pig	A	A	C	A	A	C	C	C	A	T	C	A	G	G	A	C	T	A	A	A	C	T	C	A	G	A	C	T	C	C	G	A	C	A	A	A
Rabbit	A	A	C	A	A	C	C	C	A	C	A	G	G	A	A	T	T	C	C	C	T	T	C	A	A	A	C	T	C	A	G	A	T	A	A	A
Hare	A	A	T	A	A	C	C	C	A	T	C	A	G	G	C	A	T	C	C	C	A	T	C	A	A	A	C	T	C	T	G	A	T	A	A	A
Horse	A	A	T	A	A	C	C	C	C	T	C	A	G	G	A	A	T	C	C	C	A	T	C	C	G	A	T	A	T	G	G	A	C	A	A	A
Donkey	A	A	C	A	A	C	C	C	C	T	C	A	G	G	A	A	T	C	C	C	A	T	C	T	G	A	C	A	T	A	G	A	C	A	A	A
Dog	A	A	C	A	A	C	C	C	T	T	C	A	G	G	A	A	T	C	A	C	A	T	C	A	G	A	C	T	C	A	G	A	C	A	A	A
Wolf	A	A	C	A	A	C	C	C	T	T	C	A	G	G	A	A	T	C	A	C	A	T	C	A	G	A	C	T	C	A	G	A	C	A	A	A
Badger	A	A	T	A	A	C	C	C	C	T	C	T	G	G	A	A	T	C	C	C	C	T	C	C	A	A	T	T	C	T	G	A	T	A	A	A
Lion	A	A	T	A	A	C	C	C	C	T	C	A	G	G	A	A	T	G	G	T	A	T	C	T	G	A	C	T	C	A	G	A	T	A	A	A
Tiger	A	A	T	A	A	C	C	C	C	T	C	A	G	G	A	A	T	G	G	T	A	T	C	C	G	A	C	T	C	A	G	A	C	A	A	A
Cat	A	A	C	A	A	C	C	C	C	T	C	A	G	G	A	A	T	T	A	C	A	T	C	C	G	A	T	T	C	A	G	A	C	A	A	A
Seal	A	A	C	A	A	C	C	C	C	T	C	C	G	G	A	A	T	C	A	T	A	C	C	C	G	A	C	T	C	A	G	A	C	A	A	A
Sika Deer	A	A	C	A	A	C	C	C	A	A	C	A	G	A	A	A	T	C	C	C	A	T	C	G	A	C	G	C	A	G	A	C	A	A	A	A
Red Deer	A	A	T	A	A	C	C	C	A	A	C	A	G	G	A	A	T	C	C	C	A	T	C	A	G	A	C	G	C	A	G	A	C	A	A	A
Fallow Deer	A	A	T	A	A	C	C	C	A	A	C	A	G	G	A	A	T	C	C	C	A	T	C	A	G	A	T	G	T	A	G	A	T	A	A	A
Sheep	A	A	C	A	A	C	C	C	C	A	C	A	G	G	A	A	T	T	C	C	A	T	C	G	A	C	A	C	A	G	A	T	A	A	A	A
Goat	A	A	C	A	A	C	C	C	C	A	C	A	G	G	A	A	T	T	C	C	A	T	C	A	G	A	C	A	C	A	G	A	T	A	A	A
Cow	A	A	C	A	A	C	C	C	A	A	C	A	G	G	A	A	T	T	T	C	C	T	C	A	G	A	C	G	T	A	G	A	C	A	A	A
Pig	A	A	C	A	A	C	C	C	T	A	C	C	G	G	A	A	T	C	T	C	A	T	C	A	G	A	C	A	T	A	G	A	C	A	A	A
Asiatic Elephant	A	A	C	A	A	C	C	C	A	C	T	A	G	G	T	C	T	C	A	C	T	T	C	C	A	A	C	T	C	A	G	A	C	A	A	A
Chimpanze	A	A	T	A	A	C	C	C	C	T	A	G	G	A	A	T	C	A	C	C	T	C	C	C	A	C	T	C	C	C	A	G	A	C	A	A
Human	A	A	C	A	A	C	C	C	C	T	A	G	G	A	A	T	C	A	C	C	T	C	C	C	A	T	T	C	C	G	A	T	A	A	A	A
Hedgehog	A	A	T	A	A	T	C	C	C	A	C	A	G	G	A	A	T	T	A	C	T	T	C	A	G	A	G	T	C	T	G	A	C	A	A	A

Fig. 3 continued

This variation in the amino acid sequence indicates that there will be corresponding variation in the DNA sequences. The alignment of DNA sequences for these sections of a gene can illustrate where there is a base, or series of bases, that are specific to a species (Fig. 3c, d). It is necessary to ensure that any variation of a single DNA base is shared by other members of the same species but is different to any other species. Such variation is termed a single nucleotide polymorphism (SNP) and is the foundation of species testing without the need for DNA sequencing. Most SNP loci are biallelic, as mutations at a single base are predominantly transitions, e.g. a purine for a purine or a pyrimidine for a pyrimidine. Less common are variations from a purine to a pyrimidine, e.g. A or G to C or T. SNPs occurring on the mitochondrial DNA result in individuals carrying only one type (called a haplotype). For instance a SNP at the third base of a codon for proline may result in some individuals carrying CCT and others CCC or CCA (as shown in Fig. 3).

### Species identification: gene sequence comparison

The process of species identification using either *cyt b*, COI or any other gene is the same. Large areas of both the *cyt b* and COI genes show areas of high homology [81]. This allows universal primer sequences to be designed such that the DNA primer is a compliment to all members of the same Order. There is a range of universal primer sets for

mammalian species, such that a sample from any mammalian species will generate the same size product after amplification using the polymerase chain reaction (PCR) [62, 63, 71, 79, 81–83]. The PCR product is then generally used in a sequencing reaction to determine the full sequence [29, 30, 64, 72, 75–77, 84–90]. This sequence is compared to those registered on a DNA database such as GenBank. The resulting sequence alignments should show a 100% match with a registered sequence (Fig. 4).

For the case of highly degraded DNA, shorter amplicons can be developed [91, 92]. An example is the amplification from ivory [91] where smaller and smaller amplicons are used depending on the quality of the DNA isolated. It should be noted that with smaller and smaller sequences for comparison, the chance of closely related species sharing the same sequence, or having high homology scores, will increase.

### Single nucleotide polymorphisms

Single Nucleotide Polymorphism, or SNP, testing has a number of advantages when compared to direct sequencing of a locus. These are: (a) that there is no need to sequence DNA data if much is non-informative being shared by many other species and; (b) current DNA sequencing technology cannot differentiate between mixtures [79]. This last potential advantage is not applicable when examining a pelt, skull or shell. However it is particularly

Tiger 1	TCATTAATTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Tiger 2	TCATTAATTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Tiger 3	TCATTAATTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Jaguar 1	TCCTAACTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Jaguar 2	TCCTAACTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Lion 1	TCCTAACTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Lion 2	TCCTAACTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Leopard	TCCTAACTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Snow Leopard	TCCTAACTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Clouded Leopard	TCCTAACTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Unknown 1	TCATTAATTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA
Unknown 2	TCATTAATTATCCCTATTCTTCTCGCCGTAGCCTTCCTAACCCCTAGTTGAACGTAAGTA

\*\*\* \*\*

**Fig. 4** An example of an alignment of two unknown sequences thought to originate from tiger obtained from hair samples compared to sequences registered on GenBank belonging to tiger and closely related species. Unknown samples 1 & 2 match each other and the

samples registered as being from tiger as opposed to other felid species. Bases which do not match have been shaded. The figure only shows 60 bases but normally around 400 bases are used

useful in cases of traditional medicines where there may be numerous species in the same concoction.

Identification of SNP loci can be undertaken in a number of ways but there are two common methods used in species identification: through a method called SNaPshot or through species-specific primers.

A process called SNaPshot<sup>®</sup> is a variant on standard sequencing except that the only trinucleotides are the dideoxy form and this process has been used in forensic science for human identification [93–101] and in non-human identification [102–108]. This modified base terminates the chain, thus not allowing any additional bases to be added. A single primer is used that will bind to the sequence of interest up to, but not including, the SNP to be tested. If the next base in the sequence is the SNP to be tested and it is either a C or a T type, then the DNA polymerase will add either a dideoxy G or A depending on the SNP. The chain produced will be one base longer than the primer, and ends in either a C or T (Fig. 5). If the dideoxy bases are fluorescently tagged then it can be deduced as to which SNP was present. There are many tests which utilise this technique [107–109], however SNP direct testing still cannot separate mixtures.

A second version of SNP testing is to make use of the SNP variations that are species specific to design species-specific primers. This has the advantage of being slightly faster than tests such as SNaPshot as there is no second PCR step. These can be coupled with universal binding sites used in sequencing reactions (e.g. [62, 63, 71, 79, 81–83, 89]). This species-specific primer will normally incorporate a few SNPs and have the variation focused at the 3' end of the primer, as this is the region that most affects the binding of the primer to the DNA template [11, 26, 79, 110–120]. The benefit of this type of test is that the species-specific primers can be staggered such that several can be multiplexed together and can be identified even if found in a mixture [79].

An example of this is in a test designed for the identification of 18 European mammals, even if found in a mixture [79]. Three different universal primer sites were identified within the *cyt b* gene. Downstream primers were developed that would bind to one of 18 mammalian species. An example using fox specific primers is shown in Fig. 6. If fox is present then two products specific to fox are produced (Fig. 6).

The main benefit of this method is that with items where there is a mix of human and another mammal, such as sheep, dog, badger, or fox, the two or more species can be identified as being present. Examples where this test is useful include blood from an animal mixed with human blood [121], or taking samples from the bite mark within a carcass [122, 123] (Fig. 7). In the case of taking a swab from a carcass, biological material from the skin of the carcass will be collected and also from the animal that inflicted the bite mark. If there is in addition a human contamination then this will not interfere with the test, rather it will be detected in its own right [121]. This is depicted in Fig. 7 where a badger carcass was found with bite marks. In order to determine if the bite marks were from a fox (which could be natural scavenging) or from a dog (which could indicate badger baiting) the described test was performed. In this instance the bite marks were found to have originated from a dog, indicating the possibility of badger baiting, an illegal act in the UK.

The main disadvantage of this technique is that the test needs to include within it a specific DNA primer to each species and if the unknown sample is not one that is incorporated into the test, then it will not be detected. Therefore the multiple species test has to incorporate all the potential species that are most likely to be present. These tests are ideal for a defined group of species.

In order for a multiple SNP test to work effectively all SNP primers need to work at similar temperatures at which they have similar binding affinities. In the test described

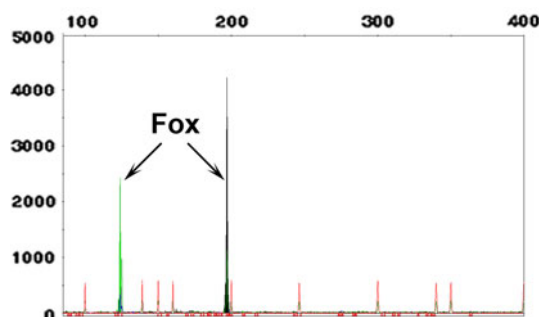
**a**  
ACGTACCTGGATACCGTAACACAGACATTAG**C**ACATAGACAT  
TGCATGGACCTATGGCATTGTGTCTGTAATC**G**TGTATCTGTA

**b**  
**Primer**  
TGGACCTATGGCATTCTGTCTGTAATC

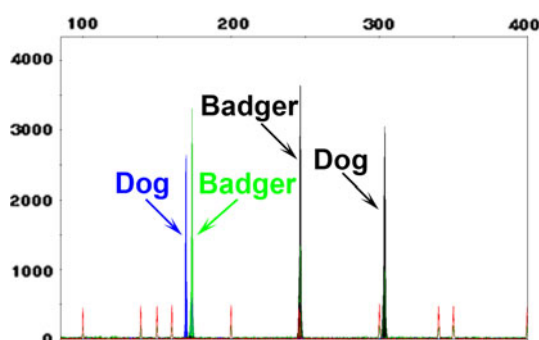
**c**  
TGGACCTATGGCATTCTGTCTGTAATC**G**  
ACGTACCTGGATACCGTAACACAGACATTAG**C**ACATAGACAT

**d**  
TGGACCTATGGCATTCTGTCTGTAATC**G**

**Fig. 5** An illustration of the basis behind SNaPshot® testing. **a** Double stranded DNA is heated to denature the two strands. The base of interest is shown highlighted and in bold. **b** A primer is introduced that is the complement and ends one base 3' of the SNP. **c** The DNA polymerase can only add dideoxy modified bases, and in this case a ddGTP. The DNA polymerase can add no further bases and the enzymatic reaction halts. **d** The DNA is denatured to release a fragment one base longer than the primer and with a G at the 3' terminus

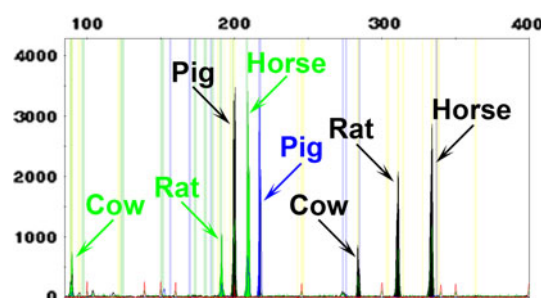


**Fig. 6** An example of the test developed by the authors [79] showing the results obtained when universal primers are coupled with fox specific reverse primers



**Fig. 7** The results of the described test [79] on the bite marks from a badger carcass showing a clear mixture of badger and dog. This could indicate that badger baiting has taken place, an illegal act within the UK

above there were 3 universal primers and 35 species-specific primers [79]. In order to take steps towards validating the method, multiple combinations of samples from species within the test were used. An example of one of the



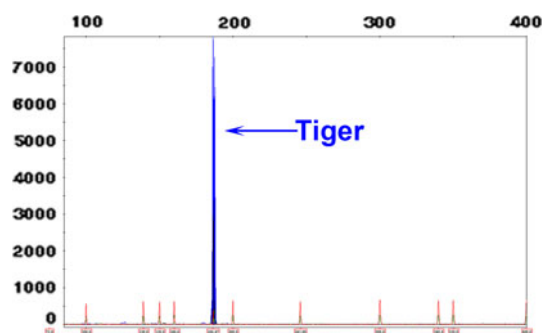
**Fig. 8** An example of a mixture of four species separated and identified using species-specific primers. The species are cow, rat, pig and horse. An example of 10 species separated using the same test can be found in [79]

validation results from four species is shown in Fig. 8. Although this is an unlikely combination to encounter in forensic science, it illustrates the potential for the test.

### Examples of SNP testing

Different SNP tests are required for different purposes. For example, in traditional medicines favoured in parts of South East Asia, products exist that claim to contain one of, or a combination of, CITES listed species such as leopard species (*Panthera pardus*), tiger (*Panthera tigris*), bear species (*Ursus* spp.) and/or deer species (*Cervus* sp. or *Moschus* sp.). It is likely that if there is DNA present from any of these species, it will be at trace levels. Analysis of small fragments within the mitochondrial DNA, including partial sections of either the *cyt b* gene or COI gene offers the best opportunity for successful identification. Amplification of the 400 bp or 600 bp fragment typically used in species testing for the *cyt b* and COI gene respectively is unlikely to be successful as the DNA fragments present will be smaller than this. Either amplification with new 'universal' primers to produce short amplification products is needed or SNP testing can be used. SNP testing has been shown to work on fragmented and highly degraded DNA [91, 120] and can be applied to traditional medicines where there is potential for degraded DNA from CITES listed species.

SNP based testing has already been developed for such species as bear [124] and tiger [125–127] and research is currently underway to create multiplex tests able to identify multiple CITES listed species simultaneously [120]. The difficulty with testing traditional medicines is that often species are substituted that are not on the CITES list or given international protection. This is due to the illegal nature of some of the species as well as the cost associated with having, for example, genuine tiger bone as opposed to cow bone.



**Fig. 9** An example of a peak specific for tiger from [120]. This peak will only appear if tiger is present, even if found in a mixture with other species, and will not cross react with other closely related species such as leopard, lion or domestic cat

Additionally, the CITES listed species for which the genetic test is developed may be very similar at the DNA level to other species not listed currently on any of the three appendices of CITES. It is necessary to ensure that the SNP loci chosen do not cross react under the conditions used with any other species for which they were not developed. An example of a test specific to tiger is demonstrated in Fig. 9.

### Future prospects

One future direction is in the use of STRs for individual identification. STRs are being developed to individually identify different species [122, 128–136] and should only react with the species for which they are designed although some cross reaction has been observed with human STR tests [137–141]. The main roadblock in the development of these tests is that the process of identifying suitable STRs, and the subsequent multiplexing and validation are expensive and time consuming. There also needs to be knowledge of the species present before attempting STR testing. However, in the future a combination of species identification testing using SNPs followed by STR testing for the relevant or desired species can be used for greater discrimination.

Additionally, DNA sequencing is getting faster and cheaper, allowing for whole genome sequencing of mitochondrial as well as nuclear genomes. There is a rapidly advancing push [142] to develop technology that will be able to sequence whole nuclear genomes for under \$1,000. This could lead to an unprecedented amount of data for different species as well as multiple members of the same species.

With the possibility of more data held within databases such as GenBank, there is greater potential to identify DNA bases that are species-specific for a much wider range of species. Targeting such bases not only within *cyt b* and

COI but throughout the mitochondrial genome will increase the confidence of species identification even between closely related species, using a battery of SNPs.

Currently the SNP fragments are separated on a column by capillary electrophoresis. This process can be a bottleneck and also requires the amplification of the DNA to be detected. Newer methods are on the horizon that allow for smaller scale rapid testing with greater sensitivity [143–145].

### Key Points

1. The use of a mitochondrial gene locus is now a standard method in the identification of mammalian species in a forensic science investigation.
2. The two main loci used for mammalian species identification are the cytochrome b and the cytochrome oxidase 1 genes where a section of the DNA sequence of either gene is compared to those on a DNA database.
3. This standard approach works on single source material but does not work on a mixture of two or more species. In such instances it is necessary to design species-specific primers to target species of particular interest.
4. The development of a range of species-specific primers now allows for the identification of mammalian species within a complex mixture. This approach can be used on a range of circumstances including the identification of species within traditional medicines or in the identification of species from bite wounds.

### References

1. Deer Act 1991 Stat. 1991 c54 (1991).
2. Deer (Scotland) Act 1996 Stat. 1996 c58 (1996).
3. Conservation of Seals Act 1970, Stat. 1970 c30 (1970).
4. Conservation of Seals (Scotland) Order 2004, Stat. 2004 No. 283 (2004).
5. Protection of Badgers Act 1992 Stat. 1992 c51 (1992).
6. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). What is CITES. [29 March 2010]; Available from: <http://www.cites.org/eng/disc/what.shtml>.
7. Mazák JH. On the sexual dimorphism in the skull of the tiger (*Panthera tigris*). *Mamm Biol.* 2004;69(6):392–400.
8. Kitchener AC. Tiger distribution, phenotypic variation and conservation issues. In: Seidensticker J, Christie S, Jackson P, editors. *Riding the tiger, tiger conservation in human-dominated landscapes*. Cambridge: Cambridge University Press; 1999. p. 19–39.
9. Luo S-J, Kim J-H, Johnson WE, Walt JVD, Martenson J, Yuhki N, et al. Phylogeography and Genetic Ancestry of Tigers (*Panthera tigris*). *PLoS Biol.* 2004;2(12):e442.
10. Linacre A. Application of mitochondrial DNA technologies in wildlife investigations—species identification. *Forensic Sci Rev.* 2006;18(1):1–8.



11. Berry O, Sarre SD. Gel-free species identification using melt-curve analysis. *Mol Ecol Notes*. 2006;Primer Notes:1–4.
12. Hillier ML, Bell LS. Differentiating human bone from animal bone: a review of histological methods. *J Forensic Sci*. 2007; 52(2):249–63.
13. Martiniakova M, Grosskopf B, Omelka R, Vondrakova M, Bauerova M. Differences among species in compact bone tissue microstructure of mammalian skeleton: use of a discriminant function analysis for species identification. *J Forensic Sci*. 2006;51(6):1235–9.
14. Suchentrunk F, Flux JEC, Flux MM, Ben Slimen H. Multivariate discrimination between East African cape hares (*Lepus capensis*) and savanna hares (*L. victorae*) based on occipital bone shape. *Mamm Biol*. 2007;72(6):372–83.
15. Moore JE. A key for the identification of animal hairs. *J Forensic Sci Soc*. 1988;28(5–6):335–9.
16. Petraco N, Kubic T. Chapter 6: animal hair identification. *Color atlas and manual of microscopy for criminalists, chemists and conservators*. London: CRC Press; 2004. p. 69–76.
17. Bartlett SE, Davidson WS. FINS (Forensically Informative Nucleotide Sequencing)—a procedure for identifying the animal origin of biological specimens. *BioTechniques*. 1992;12(3):408–11.
18. Verma SK, Prasad K, Nagesh N, Sultana M, Singh L. Was elusive carnivore a panther? DNA typing of faeces reveals the mystery. *Forensic Sci Int*. 2003;137(1):16–20.
19. Saferstein R. Chapter 7: identification and grouping of bloodstains. *Forensic science handbook*. New Jersey: Prentice Hall, Inc; 1982. p. 267–96.
20. Balitzki-Korte B, Anslinger K, Bartsch C, Rolf B. Species identification by means of pyrosequencing the mitochondrial 12S rRNA gene. *Int J Leg Med*. 2005;119(5):291–4.
21. Prakash PS, Ghumatkar MS, Nandode SV, Yogesh SK, Shouche YS. Mitochondrial 12S rRNA sequence analysis in wildlife forensics. *Curr Sci*. 2000;78(10):1239–41.
22. Macedo-Silva A, Barbosa SFC, Alkmin MGA, Vaz AJ, Shimokomaki M, Tenuta-Filho A. Hamburger meat identification by dot-ELISA. *Meat Sci*. 2000;56(2):189–92.
23. World Society for the Protection of Animals. Bear detection kits a forensic system for controlling the illegal trade in bear products: WSPA; 2006.
24. Peppin L, McEwing R, Webster S, Rogers A, Nicholls D, Ogden R. Development of a field test for the detection of illegal bear products. *Endanger Species Res* 2008 September 18. 2008;9(3): 263–70.
25. Parson W, Pegoraro K, Niederstatter H, Foger M, Steinlechner M. Species identification by means of the cytochrome *b* gene. *Int J Leg Med*. 2000;114(1):23–8.
26. Bottero MT, Civera T, Nucera D, Rosati S, Sacchi P, Turi RM. A multiplex polymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. *Int Dairy J*. 2003;13(4):277–82.
27. Bravi CM, Liron JP, Mirol PM, Ripoli MV, Peral-Garcia P, Giovambattista G. A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome *b* gene. *Leg Med*. 2004;6(4):246–51.
28. Burton RS. Molecular tools in marine ecology. *J Exp Mar Biol Ecol*. 1996;200(1–2):85–101.
29. de los Angeles Barriga-Sosa I, Perez-Ramirez MY, Soto-Aguirre F, Castillo-Rivera M, Arredondo-Figueroa JL. Inter-specific variation of the mitochondrial r16S gene among silversides, "Peces Blancos", (Atherinopsidae: Menidiinae) and its utilization for species identification. *Aquaculture*. 2005;250(3–4):637–51.
30. Guha S, Kashyap VK. Molecular identification of lizard by RAPD & FINS of mitochondrial 16S rRNA gene. *Leg Med*. 2006;8(1):5–10.
31. Partis L, Croan D, Guo Z, Clark R, Coldham T, Murby J. Evaluation of a DNA fingerprinting method for determining the species origin of meats. *Meat Sci*. 2000;54(4):369–76.
32. Partis L, Wells RJ. Identification of fish species using random amplified polymorphic DNA (RAPD). *Mol Cell Probes*. 1996; 10(6):435–41.
33. Sasazaki S, Itoh K, Arimitsu S, Imada T, Takasuga A, Nagaishi H, et al. Development of breed identification markers derived from AFLP in beef cattle. *Meat Sci*. 2004;67(2):275–80.
34. Sunnucks P. Efficient genetic markers for population biology. *Trends Ecol Evol*. 2000;15(5):199–203.
35. Verkaar ELC, Nijman IJ, Boutaga K, Lenstra JA. Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA. *Meat Sci*. 2002;60(4):365–9.
36. Woolfe M, Primrose S. Food forensics: using DNA technology to combat misdescription and fraud. *Trends Biotechnol*. 2004; 22(5):222–6.
37. Ishizaki S, Yokoyama Y, Oshiro N, Teruya N, Nagashima Y, Shiomi K, et al. Molecular identification of pufferfish species using PCR amplification and restriction analysis of a segment of the 16S rRNA gene. *Comp Biochem Physiol Part D Genomics Proteomics*. 2006;1(1):139–44.
38. Alacs E, Georges A, FitzSimmons N, Robertson J. DNA detective: a review of molecular approaches to wildlife forensics. *Forensic Sci Med Pathol*. 2010;(in press).
39. Abdel-Rahman SM, Ahmed MMM. Rapid and sensitive identification of buffalo's, cattle's and sheep's milk using species-specific PCR and PCR-RFLP techniques. *Food Control*. 2007; 18(10):1246–9.
40. Hsieh H-S, Chai T-J, Hwang D-F. Rapid PCR-RFLP method for the identification of 5 billfish species. *J Food Sci*. 2005;70(4): C246–9.
41. Hsieh H-S, Chai T-J, Hwang D-F. Using the PCR-RFLP method to identify the species of different processed products of billfish meats. *Food Control*. 2007;18(4):369–74.
42. Lin W-F, Hwang D-F. Application of PCR-RFLP analysis on species identification of canned tuna. *Food Control*. 2007;18(9): 1050–7.
43. Rastogi G, Dharne MS, Walujkar S, Kumar A, Patole MS, Shouche YS. Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Sci*. 2007;76(4):666–74.
44. Rea S, Storani G, Mascaro N, Stocchi R, Loschi AR. Species identification in anchovy pastes from the market by PCR-RFLP technique. *Food Control*. 2009;20(5):515–20.
45. Goebel AM, Donnelly JM, Atz ME. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome *b* in *Bufo* and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Mol Phylogenet Evol*. 1999; 11(1):163–99.
46. Kuwayama R, Ozawa T. Phylogenetic relationships among European Red Deer, Wapiti, and Sika Deer inferred from mitochondrial DNA sequences. *Mol Phylogenet Evol*. 2000; 15(1):115–23.
47. Ludt CJ, Schroeder W, Rottmann O, Kuehn R. Mitochondrial DNA phylogeography of red deer (*Cervus elaphus*). *Mol Phylogenet Evol*. 2004;31(3):1064–83.
48. Matthee CA, Robinson TJ. Cytochrome *b* phylogeny of the Family *Bovidae*: resolution within the Alcelaphini, Antilopini, Neotragini, and Tragelaphini. *Mol Phylogenet Evol*. 1999;12(1): 31–46.
49. Rokas A, Holland PWH. Rare genomic changes as a tool for phylogenetics. *Trends Ecol Evol*. 2000;15(11):454–9.
50. Su B, Wang Y-X, Lan H, Wang W, Zhang Y. Phylogenetic study of complete cytochrome *b* genes in musk deer (Genus

- Moschus*) using museum samples. *Mol Phylogenet Evol.* 1999;12(3):241–9.
51. Zhang D-X, Hewitt GM. Nuclear integrations: challenges for mitochondrial DNA markers. *Trends Ecol Evol.* 1996; 11(6):247–51.
  52. Zhang Y-P, Wang X-X, Ryder O, Li H-P, Zhang H-M, Yong Y, et al. Genetic diversity and conservation of endangered animal species. *Pure Appl Chem.* 2002;74(4):575–84.
  53. Tobe SS, Kitchener A, Linacre A. Cytochrome b or cytochrome c oxidase subunit I for mammalian species identification—An answer to the debate. *Forensic Sci Int Genet Sup.* 2009;2(1): 306–7.
  54. Imaizumi K, Akutsu T, Miyasaka S, Yoshino M. Development of species identification tests targeting the 16S ribosomal RNA coding region in mitochondrial DNA. *Int J Leg Med.* 2007; 121(3):184–91.
  55. Vences M, Thomas M, Meijden AVD, Chiari Y, Vieites DR. Comparative performance of the 16S rRNA gene in DNA bar-coding of amphibians. *Front Zool.* 2005;2(5).
  56. Lopez-Calleja I, Gonzalez I, Fajardo V, Martin I, Hernandez PE, Garcia T, et al. Real-time TaqMan PCR for quantitative detection of cows' milk in ewes' milk mixtures. *Int Dairy J.* 2007; 17(7):729–36.
  57. Lopez-Calleja I, Gonzalez I, Fajardo V, Martin I, Hernandez PE, Garcia T, et al. Quantitative detection of goats' milk in sheep's milk by real-time PCR. *Food Control.* 2007;18(11): 1466–73.
  58. Fajardo V, Gonzalez I, Lopez-Calleja I, Martin I, Rojas M, Hernandez PE, et al. Identification of meats from red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) using polymerase chain reaction targeting specific sequences from the mitochondrial 12S rRNA gene. *Meat Sci.* 2007;76(2):234–40.
  59. Che Man YB, Aida AA, Raha AR, Son R. Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for halal verification. *Food Control.* 2007;18(7):885–9.
  60. Pun K-M, Albrecht C, Castella V, Fumagalli L. Species identification in mammals from mixed biological samples based on mitochondrial DNA control region length polymorphism. *Electrophoresis.* 2009;30(6):1008–14.
  61. Gupta SK, Thangaraj K, Singh L. A simple and inexpensive molecular method for sexing and identification of the forensic samples of elephant origin. *J Forensic Sci.* 2006;51(4):805–7.
  62. Kitano T, Umetsu K, Tian W, Osawa M. Two universal primer sets for species identification among vertebrates. *Int J Leg Med.* 2007;121(5):423–7.
  63. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc Natl Acad Sci USA.* 1989;86(16):6196–200.
  64. Nussbaumer C, Korschineck I. Non-human mtDNA helps to exculpate a suspect in a homicide case. *Int Congr Ser.* 2006; 1288:136–8.
  65. Fumagalli L, Cabrita CJ, Castella V. Simultaneous identification of multiple mammalian species from mixed forensic samples based on mtDNA control region length polymorphism. *Forensic Sci Int Genet Sup.* 2009;2(1):302–3.
  66. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. *Nature.* 1981;290(5806):457–65.
  67. Alberts B, Bray D, Johnson A, Lewis J, Raff M, Roberts K. Chapter 13: energy generation in mitochondria and chloroplasts. *Essential cell biology: an introduction to the molecular biology of the cell.* London: Garland Publishing, Inc; 1998. p. 407–45.
  68. Boonseub S, Tobe SS, Linacre AMT. The use of mitochondrial DNA genes to identify closely related avian species. *Forensic Sci Int Genet Sup.* 2009;2(1):275–7.
  69. Tobe SS, Linacre AMT. Species testing using DNA Loci. In: Linacre AMT, editor. *Wildlife forensic investigation.* London: Taylor and Francis; 2009. p. 61–94.
  70. Tobe SS, Kitchener AC, Linacre AMT. Reconstructing mammalian phylogenies: a detailed comparison of the cytochrome b and cytochrome oxidase subunit I mitochondrial genes. *PLoS ONE.* 2010; submitted.
  71. Hsieh H-M, Chiang H-L, Tsai L-C, Lai S-Y, Huang N-E, Linacre A, et al. Cytochrome b gene for species identification of the conservation animals. *Forensic Sci Int.* 2001;122(1):7–18.
  72. Caine L, Lima G, Pontes L, Abrantes D, Pereira M, Pinheiro MF. Species identification by cytochrome b gene: casework samples. *Int Congr Ser.* 2006;1288:145–7.
  73. Colombo F, Marchisio E, Pizzini A, Cantoni C. Identification of the goose species (*Anser anser*) in Italian “Mortara” salami by DNA sequencing and a polymerase chain reaction with an original primer pair. *Meat Sci.* 2002;61(3):291–4.
  74. Hsieh H-M, Huang L-H, Tsai L-C, Kuo Y-C, Meng H-H, Linacre A, et al. Species identification of rhinoceros horns using the cytochrome b gene. *Forensic Sci Int.* 2003;136(1–3):1–11.
  75. Hsieh H-M, Huang L-H, Tsai L-C, Liu C-L, Kuo Y-C, Hsiao C-T, et al. Species identification of *Kachuga tecta* using the cytochrome b gene. *J Forensic Sci.* 2006;51(1):52–6.
  76. Pereira F, Meirinhos J, Amorim A, Pereira L. Analysis of inter-specific mitochondrial DNA diversity for accurate species identification. *Int Congr Ser.* 2006;1288:103–5.
  77. Wetton JH, Braidley GL, Tsang CSF, Roney CA, Powell SL, Spriggs AC. Generation of a species-specific DNA sequence library of british mammals. A study by the forensic science service for the joint nature conservation committee and the environment and heritage service, Northern Ireland; 2002. p. 37.
  78. Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. *Proc R Soc B Biol Sci.* 2003;270(1512):313–21.
  79. Tobe SS, Linacre AMT. A multiplex assay to identify 18 European mammal species from mixtures using the mitochondrial cytochrome b gene. *Electrophoresis.* 2008;29(2):340–7.
  80. Beckstead WA, Ebbert MTW, Rowe MJ, McClellan DA. Evolutionary Pressure on Mitochondrial Cytochrome b Is Consistent with a Role of Cytb17T Affecting Longevity during Caloric Restriction. *PLoS ONE.* 2009;4(6):e5836.
  81. Irwin D, Kocher T, Wilson A. Evolution of the cytochrome b gene of mammals. *J Mol Evol.* 1991;32(2):128–44.
  82. Tobe SS, Linacre A. A method to identify a large number of mammalian species in the UK from trace samples and mixtures without the use of sequencing. *Forensic Sci Int Genet Sup.* 2008;1(1):625–7.
  83. Verma SK, Singh L. Novel universal primers establish identity of an enormous number of animal species for forensic application. *Mol Ecol Notes.* 2003;3(1):28–31.
  84. de Pancorbo MM, Castro A, Fernandez-Fernandez I, Cuevas N. Cytochrome b for identification of animal species in processed food. *Int Congr Ser.* 2004;1261:592–4.
  85. Girish PS, Anjaneyulu ASR, Viswas KN, Anand M, Rajkumar N, Shivakumar BM, et al. Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. *Meat Sci.* 2004; 66(3):551–6.
  86. Gupta SK, Verma SK, Singh L. Molecular insight into a wildlife crime: the case of a peafowl slaughter. *Forensic Sci Int.* 2005;154(2–3):214–7.
  87. Wong K-L, Wang J, But PP-H, Shaw P-C. Application of cytochrome b DNA sequences for the authentication of endangered snake species. *Forensic Sci Int.* 2004;139(1):49–55.

88. Saigusa K, Takamiya M, Aoki Y. Species identification of the forensically important flies in Iwate prefecture, Japan based on mitochondrial cytochrome oxidase gene subunit I (COI) sequences. *Leg Med*. 2005;7(3):175–8.
89. An J, Lee M-Y, Min M-S, Lee M-H, Lee H. A molecular genetic approach for species identification of mammals and sex determination of birds in a forensic case of poaching from South Korea. *Forensic Sci Int*. 2007;167(1):59–61.
90. Meganathan PR, Dubey B, Haque I. Molecular identification of crocodile species using novel primers for forensic analysis. *Conserv Genet*. 2009;10(3):767–70.
91. Lee J, Hsieh H-M, Huang L-H, Kuo Y-C, Wu J-H, Chin S-C, et al. Ivory identification by DNA profiling of cytochrome b gene. *Int J Leg Med*. 2009;123(2):117–21.
92. Lee JC-I, Tsai L-C, Yang C-Y, Liu C-L, Huang L-H, Linacre A, et al. DNA profiling of Shahtoosh. *Electrophoresis*. 2006;27(17):3359–62.
93. Alvarez-Iglesias V, Jaime JC, Carracedo A, Salas A. Coding region mitochondrial DNA SNPs: Targeting East Asian and Native American haplogroups. *Forensic Sci Int Genet*. 2007;1(1):44–55.
94. Sanchez JJ, Børsting C, Balogh K, Berger B, Bogus M, Butler JM, et al. Forensic typing of autosomal SNPs with a 29 SNP-multiplex—Results of a collaborative EDNAP exercise. *Forensic Sci Int Genet*. 2008;2(3):176–83.
95. Völgyi A, Zalán A, Szvetnik E, Pamjav H. Hungarian population data for 11 Y-STR and 49 Y-SNP markers. *Forensic Sci Int Genet*. 2009;3(2):e27–8.
96. Westen AA, Matai AS, Laros JFJ, Meiland HC, Jasper M, de Leeuw WJF, et al. Tri-allelic SNP markers enable analysis of mixed and degraded DNA samples. *Forensic Sci Int Genet*. 2009;3(4):233–41.
97. Mosquera-Miguel A, Álvarez-Iglesias V, Cerezo M, Lareu MV, Carracedo A, Salas A. Testing the performance of mtSNP minisequencing in forensic samples. *Forensic Sci Int Genet*. 2009;3(4):261–4.
98. Grignani P, Turchi C, Achilli A, Peloso G, Alù M, Ricci U, et al. Multiplex mtDNA coding region SNP assays for molecular dissection of haplogroups U/K and J/T. *Forensic Sci Int Genet*. 2009;4(1):21–5.
99. Børsting C, Rockenbauer E, Morling N. Validation of a single nucleotide polymorphism (SNP) typing assay with 49 SNPs for forensic genetic testing in a laboratory accredited according to the ISO 17025 standard. *Forensic Sci Int Genet*. 2009;4(1):34–42.
100. Krjutskov K, Viltrop T, Palta P, Metspalu E, Tamm E, Suvi S, et al. Evaluation of the 124-plex SNP typing microarray for forensic testing. *Forensic Sci Int Genet*. 2009;4(1):43–8.
101. Dixon LA, Murray CM, Archer EJ, Dobbins AE, Koumi P, Gill P. Validation of a 21-locus autosomal SNP multiplex for forensic identification purposes. *Forensic Sci Int*. 2005;154(1):62–77.
102. Heaton MP, Harhay GP, Bennett GL, Stone RT, Grosse WM, Casas E. Selection and use of SNP markers for animal identification and paternity analysis in U.S. beef cattle. *Mamm Genome*. 2002;13(5):272–81.
103. Andreassen R, Hagen-Larsen H, Sánchez-Ramos I, Lunner S, Høyheim B. STR and bi-allelic polymorphisms in Atlantic salmon: Tools for tracing large scale escapees from salmon farms. *Forensic Sci Int Genet Sup*. 2008;1(1):586–8.
104. Sato I, Nakaki S, Murata K, Takeshita H, Mukai T. Forensic hair analysis to identify animal species on a case of pet animal abuse. *Int J Leg Med*. 2010;124(3):249–56.
105. Martinsohn JT, Ogden R. FishPopTrace—Developing SNP-based population genetic assignment methods to investigate illegal fishing. *Forensic Sci Int Genet Sup*. 2009;2(1):294–6.
106. Araujo R, Amorim A, Gusmão L. Microbial forensics: do aspergillus fumigatus strains present local or regional differentiation? *Forensic Sci Int Genet Sup*. 2009;2(1):297–9.
107. Ogden R, McGough HN, Cowan RS, Chua L, Groves M, McEwing R. SNP-based method for the genetic identification of *ramin Gonystylus* spp. timber and products: applied research meeting CITES enforcement needs. *Endanger Species Res*. 2010;9(3):255–61.
108. Nakaki S-I, Hino D, Miyoshi M, Nakayama H, Moriyoshi H, Morikawa T, et al. Study of animal species (human, dog and cat) identification using a multiplex single-base primer extension reaction in the cytochrome b gene. *Forensic Sci Int*. 2007;173(2–3):97–102.
109. La Neve F, Civera T, Mucci N, Bottero MT. Authentication of meat from game and domestic species by SNaPshot minisequencing analysis. *Meat Sci*. 2008;80(2):216–24.
110. Lin W-F, Hwang D-F. A multiplex PCR assay for species identification of raw and cooked bonito. *Food Control*. 2008;19(9):879–85.
111. Dubey B, Meganathan PR, Haque I. Multiplex PCR assay for rapid identification of three endangered snake species of India. *Conserv Genet*. 2009;10(6):1861–4.
112. Bellis C, Ashton KJ, Freney L, Blair B, Griffiths LR. A molecular genetic approach for forensic animal species identification. *Forensic Sci Int*. 2003;134(2–3):99–108.
113. Dalmaso A, Fontanella E, Piatti P, Civera T, Rosati S, Bottero MT. A multiplex PCR assay for the identification of animal species in feedstuffs. *Mol Cell Probes*. 2004;18(2):81–7.
114. Dooley JJ, Paine KE, Garrett SD, Brown HM. Detection of meat species using TaqMan real-time PCR assays. *Meat Sci*. 2004;68(3):431–8.
115. Lahiff S, Glennon M, O'Brien L, Lyng J, Smith T, Maher M, et al. Species-specific PCR for the identification of ovine, porcine and chicken species in meat and bone meal (MBM). *Mol Cell Probes*. 2001;15(1):27–35.
116. Lopez-Andreo M, Lugo L, Garrido-Pertierra A, Prieto MI, Puyet A. Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. *Anal Biochem*. 2005;339(1):73–82.
117. Matsunaga T, Chikuni K, Tanabe R, Muroya S, Shibata K, Yamada J, et al. A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Sci*. 1999;51(2):143–8.
118. Ono K, Satoh M, Yoshida T, Ozawa Y, Kohara A, Takeuchi M, et al. Species identification of animal cells by nested PCR targeted to mitochondrial DNA. *In Vitro Cell Dev Biol Anim*. 2007;43(5):168–75.
119. De-Franco B, Mendonca FF, Hashimoto DT, Porto-Foresti F, Oliveira C, Foresti F. Forensic identification of the guitarfish species *Rhinobatos horkelli*, *R-percellens* and *Zapteryx brevirostris* using multiplex-PCR. *Mol Ecol Resour*. 2010;10(1):197–9.
120. Tobe SS, Linacre A. Identifying endangered species from degraded mixtures at low levels. *Forensic Sci Int Genet Sup*. 2009;2(1):304–5.
121. Tobe S, Linacre A. Species identification of human and deer from mixed biological material. *Forensic Sci Int*. 2007;169(2–3):278–9.
122. Eichmann C, Berger B, Reinhold M, Lutz M, Parson W. Canine-specific STR typing of saliva traces on dog bite wounds. *Int J Leg Med*. 2004;118(6):337–42.
123. Serra A, Pinheiro J, Batista L, Bento AM, Balsa F, Costa HA, et al. Human being eaten by his own dogs: Genetic confirmation through analysis of bones recovered in a dog's stomach content. *Forensic Sci Int Genet Sup*. 2009;2(1):210–2.

124. Peppin L, McEwing R, Carvalho GR, Ogden R. A DNA-Based Approach for the Forensic Identification of Asiatic Black Bear (*Ursus thibetanus*) in a Traditional Asian Medicine. *J Forensic Sci.* 2008;53(6):1358–62.
125. Wetton JH, Tsang CSF, Roney CA, Spriggs AC. An extremely sensitive species-specific ARMs PCR test for the presence of tiger bone DNA. *Forensic Sci Int.* 2004;140(1):139–45.
126. Linacre A, Tobe SS. On the trail of tigers-tracking tiger in Traditional East Asian Medicine. *Forensic Sci Int Genet Sup.* 2008;1(1):603–4.
127. Kitpipit T, Linacre A, Tobe SS. Tiger species identification based on molecular approach. *Forensic Sci Int Genet Sup.* 2009;2(1):310–2.
128. van Asch B, Pinheiro R, Pereira R, Alves C, Pereira V, Pereira F, et al. A framework for the development of STR genotyping in domestic animal species: Characterization and population study of 12 canine X-chromosome loci. *Electrophoresis.* 2010;31(2):303–8.
129. van Asch B, Alves C, Pereira F, Gusmão L, Amorim A. A new autosomal STR multiplex for canine genotyping. *Forensic Sci Int Genet Sup.* 2008;1(1):628–9.
130. Robino C, Menegon S, Caratti S, Sona B, Gino S, Torre C. Forensic application of a multiplex PCR system for the typing of pig STRs. *Forensic Sci Int Genet Sup.* 2008;1(1):614–5.
131. Müller K, Brugger C, Klein R, Miltner E, Reuther F, Wiegand P. STR typing of hairs from domestic cats. *Forensic Sci Int Genet Sup.* 2008;1(1):607–9.
132. Lee JC-I, Tsai L-C, Kuan Y-Y, Chien W-H, Chang K-T, Wu C-H, et al. Racing pigeon identification using STR and chromo-helicase DNA binding gene markers. *Electrophoresis.* 2007;28(23):4274–81.
133. Dawney N, Ogden R, Wetton JH, Thorpe RS, McEwing R. Genetic data from 28 STR loci for forensic individual identification and parentage analyses in 6 bird of prey species. *Forensic Sci Int Genet.* 2009;3(2):e63–9.
134. Dawney N, Ogden R, Thorpe RS, Pope LC, Dawson DA, McEwing R. A forensic STR profiling system for the Eurasian badger: a framework for developing profiling systems for wildlife species. *Forensic Sci Int Genet.* 2008;2(1):47–53.
135. Singh A, Gaur A, Shailaja K, Satyare Bala B, Singh L. A novel microsatellite (STR) marker for forensic identification of big cats in India. *Forensic Sci Int.* 2004;141(2–3):143–7.
136. Eichmann C, Berger B, Steinlechner M, Parson W. Estimating the probability of identity in a random dog population using 15 highly polymorphic canine STR markers. *Forensic Sci Int.* 2005;151(1):37–44.
137. Crouse CA, Schumm JW. Investigation of species specificity using nine PCR-based human STR systems. *J Forensic Sci.* 1995;40(6):952–6.
138. Meyer E, Wiegand P, Rand SP, Kuhlmann D, Brack M, Brinkmann B. Microsatellite polymorphisms reveal phylogenetic relationships in primates. *J Mol Evol.* 1995;41(1):10–4.
139. Minaguchi K, Takenaka O. Structural variations of the VWA locus in humans and comparison with non-human primates. *Forensic Sci Int.* 2000;113(1–3):9–16.
140. Wiegand P, Meyer E, Brinkmann B. Microsatellite structures in the context of human evolution. *Electrophoresis.* 2000;21(5):889–95.
141. Ago K, Orihara Y, Ago M, Nakagawa S, Ogata M. Evaluation of the species specificity for six human short tandem repeat loci CSF1PO, TPOX, TH01, F13A01, FESFPS and vWA, in the Japanese macaque. *Leg Med.* 2004;6(2):102–8.
142. Toward \$1000 Genomes. *Science.* 2010 January 1, 2010;327(5961):11.
143. Käller M, Lundeberg J, Ahmadian A. Arrayed identification of DNA signatures. *Expert Rev Mol Diagn.* 2007;7(1):65–76.
144. Hall TA, Sannes-Lowery KA, McCurdy LD, Fisher C, Anderson T, Henthorne A, et al. Base composition profiling of human mitochondrial DNA using polymerase chain reaction and direct automated electrospray ionization mass spectrometry. *Anal Chem.* 2009;81(18):7515–26.
145. Hu X, Gao Y, Feng C, Liu Q, Wang X, Du Z, et al. Advanced technologies for genomic analysis in farm animals and its application for QTL mapping. *Genetica.* 2009;136(2):371–86.



## Unusual causes of fatal upper aerodigestive tract obstruction in wild bottlenose dolphins (*Tursiops aduncus*)

Roger W. Byard · Ikuko Tomo · Catherine M. Kemper ·  
Susan E. Gibbs · Mike Bossley · Aaron Machado ·  
Mark Hill

Byard RW, Tomo I, Kemper CM, Gibbs SE, Bossley M, Machado A, Hill M. Unusual causes of fatal upper aerodigestive tract obstruction in wild bottlenose dolphins (*Tursiops aduncus*). *Forensic Sci Med Pathol*. 2010 Sep;6(3):207–10.

Accepted: 8 January 2010 / Published online: 18 February 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** Necropsy examination of dolphins living in Gulf St Vincent, Australia is routinely undertaken to enable the evaluation of disease processes and to provide rapid medicolegal assessment of any inflicted and/or accidental injuries. Two Indo-Pacific Bottlenose Dolphins (*Tursiops aduncus*) are reported to demonstrate conditions that may result in unexpected death involving upper airway compromise by quite unusual mechanisms. In the first case an adult male was found with extensive soft tissue trauma suggesting human interaction. At necropsy, death was due instead to upper airway obstruction from an impacted Slender-spined Porcupine Fish (*Diodon nichthemerus*) in the posterior pharynx and upper esophagus. In the second case, an adult male dolphin was found to have died, following several weeks' illness, from upper airway obstruction due to extensive respiratory tract papillomatosis within

the blowhole. Given the infectious etiology of this condition the local population will be monitored for similar lesions. These cases demonstrate rare causes of upper airway obstruction in wild dolphins that were identifiable only after detailed necropsy examination. The possibility of human involvement in the deaths could be excluded.

**Keywords** Forensic science · Wildlife forensics · Unexpected death · Airway obstruction · Papillomatosis · Bottlenose dolphin · Café coronary

### Introduction

Obstructive lesions of the upper aerodigestive tract are well-recognised causes of unexpected death in humans. Obstructions may be due to inhaled or impacted foreign material, or to natural diseases. Lethal foreign material impaction usually occurs at the extremes of life involving young children who aspirate food or toy parts or the elderly who choke on food [1].

Obstructing foreign bodies may impact in the airway or esophagus and may cause immediate or delayed airway compromise [2]. Accidental choking on food in adult humans has been termed the “café coronary” syndrome and has been associated with intellectual impairment from dementia or intoxication, resulting in a failure to appreciate the appropriate size of food bolus to swallow [3]. Natural diseases with the potential for causing critical airway compromise include infection and neoplasia. Infectious causes of upper airway obstruction may be localized, or part of a generalized illness such as infectious mononucleosis [4, 5]. Neoplastic lesions may be benign, as in upper airway hemangiomas, or malignant as in squamous cell carcinoma of the larynx [6]. The essential feature is that the

---

R. W. Byard (✉)  
Discipline of Pathology, Level 3 Medical School North Building,  
The University of Adelaide, Frome Road, Adelaide 5005,  
Australia  
e-mail: roger.byard@sa.gov.au; byard.roger@saugov.sa.gov.au

I. Tomo · C. M. Kemper · S. E. Gibbs  
South Australian Museum, Adelaide, SA, Australia

S. E. Gibbs  
Macquarie University, Sydney, NSW, Australia

M. Bossley  
Australian Dolphin Research Foundation, Adelaide,  
SA, Australia

A. Machado  
Project Dolphin Safe, Adelaide, SA, Australia

M. Hill  
Somerton Park Veterinary Clinic, Adelaide, SA, Australia



tumor masses produce a critical reduction in luminal diameter of the airway.

Two cases of lethal upper aerodigestive tract occlusion in wild dolphins are reported to demonstrate similar deaths from unusual obstructive situations in a non-human species and to discuss forensic issues that arose during and after the necropsies. The case involving blowhole obstruction was particularly important as it provided an explanation for the gradual deterioration in the animal that had been observed in the weeks preceding death.

## Case reports

### Case 1

The carcass of an adult male Indo-Pacific Bottlenose Dolphin was collected in a fresh condition from Port Clinton in upper Gulf St Vincent. The dolphin appeared robust and weighed 136 kg. At necropsy, extensive bruising around the head, throat and abdomen was identified, raising concerns of inflicted or accidental injury. In addition there was soft tissue hemorrhage around the cervical vertebrae, on the dorsal side of the abdomen and around the melon.

The most significant finding was, however, impaction of a 25 cm Slender-spined Porcupine Fish in the back of the mouth and upper esophagus (Figs. 1, 2) resulting in lethal upper airway occlusion. The stomach contents included fish bones, fish otoliths, squid beaks and porcupine fish spines. There was no evidence of other significant trauma or any underlying disease processes that could have caused or contributed to death. The dolphin specimen and associated samples are registered in the South Australian



**Fig. 1** Examination of the mouth of an Indo-Pacific Bottlenose Dolphin (*Tursiops aduncus*) (Case 1) revealed the tail of a Slender-spined Porcupine Fish (arrow)



**Fig. 2** Dissection of the throat of the dolphin in Case 1 revealed an impacted Slender-spined Porcupine Fish. The fish had caused distension of the upper esophagus immediately next to the upper airway



**Fig. 3** Slender-spined Porcupine Fish removed from the throat of Case 1

Museum collections as SAMA 23992, and the Slender spine Porcupine Fish (Fig. 3) as SAMA F12498.

### Case 2

An adult male dolphin known as ‘Blaze’ was a uniquely identified individual in an ongoing study of bottlenose dolphins in the Adelaide region. In the weeks preceding death his behavior was abnormal with loud breathing noted to be “wheezy”, associated with lifting of his head out of the water in an unusual manner with each breath (Fig. 4). He also appeared somewhat emaciated.

At necropsy the body weight was 113 kg (which is below average for an Indo-Pacific Bottlenose Dolphin of 208 cm body length) with several abscesses noted on the external skin surface. The most significant finding was



**Fig. 4** Unusual lifting of the head associated with difficulty breathing in the days prior to death in Case 2



**Fig. 5** Dissected upper airway in Case 2 showing an extensive obstructive papillomatosis lesion

obstruction of the upper airway by an irregular papillomatous tumor mass measuring 60 mm in maximum dimensions (Fig. 5). Histological evaluation revealed hyperkeratosis and papillomatosis with papillae composed of a central core of fibrovascular tissues covered by squamous epithelium. Koilocytosis and nuclear hyperchromasia were characteristic of papilloma virus (Fig. 6). Similar lesions were found in the mouth, anus and urogenital slit. The stomach contained remains of fish in an advanced state of digestion (i.e., disarticulated bones and no flesh). Although detailed electron microscopic and immunohistochemical studies were undertaken without identifying any viral organisms, these investigations may have been affected by post-mortem changes in tissues, and by the use of anti-human, rather than anti-cetacean, papilloma viral reagents. The specimen and associated samples are registered in the South Australian Museum collections as SAMA M23356.



**Fig. 6** Histological sections of the tumour mass from the blowhole in Case 2 revealed characteristic papillomatous proliferation with hyperkeratosis, koilocytosis and nuclear hyperchromasia characteristic of papilloma virus infection (hematoxylin & eosin; wholemount)

## Discussion

Evaluation of the two reported cases provided useful information on the causes and mechanisms of death. In Case 1 the initial suspicions were of significant soft tissue trauma from, for example, entanglement in fishing line or netting. While no characteristic patterned injuries or net marks were observed around the head, soft tissue dissection confirmed the presence of widespread and significant bruising. Examination of the mouth and upper esophagus, however, revealed the cause of death as an impacted Slender-spined Porcupine Fish (*Diodon nichthemerus*) adjacent to the upper airway.

Porcupine fish are common in southern Australian inshore waters [7] but have not previously been identified in the stomach contents of dolphins from South Australia [8]. Fish in the families Diodontidae and Tetradontidae have neurotoxins in their flesh that are toxic to humans, however, as they are preyed upon by a wide variety of marine predators, including birds, bony and cartilaginous fish, turtles and dolphins [9–13], it appears unlikely that neurotoxins contributed to the dolphin's death. One of the defence mechanisms of this group of fish is to inflate the body with water when threatened, thus causing their spines to project. It appeared that this had occurred as the fish was being swallowed by the dolphin, resulting in impaction and penetration of the esophageal wall by the erected spines. Once inflated, the spines would have prevented expulsion of the fish. The presence of digested remains of porcupine fish indicated that the dolphin had recently and successfully consumed this species.

The mechanism of death in Case 1 may have been quite complex, involving occlusion of the nearby airway by direct pressure from the impacted fish with possible vagal inhibition [1]. It is also likely that the protruding fish spines would have caused intense pain resulting in the dolphin moving around violently in an attempt to relieve the obstruction and pain. The marked bruising noted at necropsy probably resulted from this agonal activity. Airway obstruction from feeding has also been reported in another wild Indo-Pacific Bottlenose Dolphin (*Tursiops aduncus*) from near Adelaide, South Australia, that had attempted to ingest a fish that was much larger than prey normally eaten [14]. A similar case involving a Common Bottlenose Dolphin (*Tursiops truncatus*) has occurred in Puerto Rico (Mignucci-Giannoni, personal communication, 2008).

The dolphin in Case 2 was one of the residents of the Adelaide region and had been studied for over 13 years. It was noted that the animal appeared to be having difficulty breathing for least 2 weeks before death. Hyperkeratosis inside the blowhole was extreme, extending into the upper respiratory tract and was histologically identical to upper airway papillomatosis in humans. This condition occurs mainly in children and is due to human papilloma virus infection. Recurrence is a well-recognized problem and sudden death, although rare, has been reported in humans due to acute upper airway occlusion [15]. A concern in this case relates to the probable infectious nature of the lesion and the possibility that other dolphins in the population could be affected. The necropsies of a number of other dolphins from this area have subsequently revealed keratinized epithelium in the oral cavity, and orogenital papillomas have been described in bottlenose dolphins from the south-eastern United States [16].

The reported cases demonstrate the ongoing usefulness of necropsy and forensic examination of wild dolphins [17]. In Case 1 human interaction was initially suspected but excluded after careful examination of all factors. Coincidentally, an unusual and previously unsuspected form of death was revealed. In Case 2 lethal respiratory tract papillomatosis was identified with similar features to such cases in humans. The identification of a potentially infectious agent also means that monitoring of both living and dead animals in the population should be undertaken for similar lesions.

### Key points

1. Necropsy examination of wild dolphins enables evaluation of disease processes and provides medicolegal assessment of any inflicted and/or accidental injuries.
2. Conditions that may result in death from upper airway compromise include impaction of ingested fish or extensive respiratory tract papillomatosis.

3. Given the infectious etiology of the latter condition monitoring of other dolphins for similar lesions is advised.

**Acknowledgments** We would like to thank the following people for their assistance with these cases: Rob Laver and Verity Gibbs, Department for Environment and Heritage South Australia, Maria Bellis, Forensic Science SA, and David Stemmer and Ralph Foster, South Australian Museum, Adelaide, Australia.

### References

1. Byard RW. Mechanisms of unexpected death in infants and young children following foreign body ingestion. *J Forensic Sci.* 1996;41:438–41.
2. Byard RW, Moore L, Bourne AJ. Sudden and unexpected death—a late effect of occult intraesophageal foreign body. *Pediatr Pathol.* 1990;10:837–41.
3. Wick R, Gilbert JD, Byard RW. Café coronary syndrome—fatal choking on food: an autopsy approach. *J Clin Forensic Med.* 2006;13:135–8.
4. Houldsworth G, James RA, Gilbert JD, Byard RW. Unexpected death in association with an occult posterior lingual abscess. *Legal Med.* 2000;4:221–3.
5. Byard RW. Unexpected death due to infectious mononucleosis. *J Forensic Sci.* 2002;47:202–4.
6. Byard RW, Burrows PE, Izakawa T, Silver MM. Diffuse infantile haemangiomas: clinicopathological features and management problems in five fatal cases. *Eur J Pediatr.* 1991;150:224–7.
7. Kuitert R. Coastal fishers of south-eastern Australia. 2nd ed. Smithfield: Gary Allen Pty. Ltd; 2000.
8. Kemper CM, Gibbs SE. Dolphin interactions with tuna feedlots at Port Lincoln, South Australia and recommendations for minimising entanglements. *J Cet Res Manag.* 2001;3:283–92.
9. Manooch CS III, Mason DL, Nelson RS. Food and gastrointestinal parasites of dolphin *Coryphaena hippurus* collected along the southeastern and Gulf coast of the United States. *Bull Jpn Soc Sci Fish.* 1984;50:1511–25.
10. Higgins PJ, Davies JN, editors. Handbook of Australian, New Zealand and Antarctic birds. Volume 3: snipe to pigeons. Melbourne: Oxford University Press; 1996.
11. Limpus CJ, de Villiers DL, de Villiers MA, Limous DJ, Read MA. The loggerhead turtle, *Caretta caretta* in Queensland: observations on feeding ecology in warm temperate waters. *Mem Queens Mus.* 2001;46:631–45.
12. Shibuya A, de Souza Rosa R, Gadig OBF. Stomach contents of *Galeocerdo curieri* and *Carcharhinus plumbeus* (Elasmobranchii: Carcharhinidae) caught off Paraíba State, Brazil. *Arq Cienc Mar.* 2005;38:105–7.
13. Potier M, Marsac F, Cherel Y, Lucas V, Sabatie R, Maury O, et al. Forage fauna in the diet of three large pelagic fishes (lancetfish, swordfish and yellowfin tuna) in the western equatorial Indian Ocean. *Fish Res.* 2007;83:60–72.
14. Byard RW, Gilbert JD, Gibbs SE, Kemper CM. Cetacean café coronary. *J Clin Forensic Med.* 2003;10:85–8.
15. Byard RW. Sudden death in the young. 3rd ed. Cambridge: Cambridge University Press (in press).
16. Bossart GD, Ghim S-J, Rehtanz M, Goldstein J, Varela R, Ewing RY, et al. Orogenital neoplasia in Atlantic bottlenose dolphins *Tursiops truncatus*. *Aquat Mam.* 2005;31:473–80.
17. Byard RW, Gilbert JD, Kemper CM. Dolphin deaths: forensic investigations. *Med J Aust.* 2001;175:623–4.



# The use of DNA identification in prosecuting wildlife-traffickers in Australia: do the penalties fit the crimes?

Rebecca N. Johnson

Accepted: 8 June 2010 / Published online: 17 July 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** The use of genetic identification techniques in wildlife forensic investigations has increased significantly in recent years. The utilization of DNA is especially important when species identification using other methods are inconclusive. Australia has strict laws against illegal importation of wildlife as well as laws to protect its unique biodiversity from pests and diseases of quarantine concern. Two separate case studies in which genetic identification was essential for species identification are presented—the first involved illegally held shark fins, the second illegally imported live bird eggs. In the latter case genetic identification enabled charges to be laid for illegal importation of CITES Appendix I species. Australian laws allow for some of the highest penalties for illegal trade of wildlife compared to other countries, however only a fraction of cases are prosecuted and penalties applied to date have been lower than the maximum permitted. Both of the reported cases resulted in fines, and one in imprisonment of the offender, which provides a persuasive precedent for future prosecutions.

**Keywords** DNA identification · CITES · Wildlife forensics · Species identification · Wildlife trade · Wildlife trafficking · Shark fin · Bird eggs · Bird embryo · Voucher specimens

## Introduction

Wildlife trafficking is an illegal multi-billion dollar industry worldwide [1, 2], with catastrophic consequences for the individual animals involved, as well as threatening the survival of entire species because the rarer a species the more desirable it becomes to collectors. There are also serious environmental consequences associated with the crime of illegal wildlife trafficking, including disruption of ecosystem processes, introduction of exotic pest species and spread of wildlife diseases. Trade in wildlife in Australia is regulated by a suite of legislation, including those which enforce the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) treaty [3] and others that are specific to protecting Australia's unique fauna and flora.

Wildlife forensics is the science that often assists in prosecuting wildlife crime; which is a complex and multifaceted discipline involving both flora and fauna. However, for this paper will focus predominantly on the sub-discipline of genetics and DNA-based identification of fauna (animals or animal parts) to ensure accurate species or individual identification.

DNA-based identification is particularly useful in cases such as:

- (1) The species identification of an animal part or product e.g. shark fin, musk gland, bush meat, animal powder, etc.
- (2) The species identification of a juvenile or immature animal e.g. bird embryo, insect larval or pupae, young primate, etc.
- (3) Identification of the population of origin of an individual e.g. assigning elephant ivory to a geographic region of origin.

---

R. N. Johnson (✉)  
DNA Laboratory, Australian Museum, 6 College Street Sydney,  
New South Wales 2010, Australia  
e-mail: rebecca.johnson@austmus.gov.au

- (4) Identification of the individual of origin e.g. matching meat to a carcass.
- (5) Identification of species that may not be identifiable on morphological criteria.

Australia has a unique biota which it protects through a variety of legislation. There are state penalties for the taking of wildlife without a permit and for crimes of animal cruelty. There are also federal penalties that protect Australia's biodiversity as well as allowing for prosecution for breaches of the CITES treaty. In Australia the CITES treaty is legally enforceable through Part 13A of the Commonwealth Environment Protection and Biodiversity Conservation Act 1999 (the EPBC Act (1999)) [4]. Penalties for breaches of the EPBC Act (1999) include fines of up to AUD\$110,000 for individuals or AUD\$550,000 for corporations, and up to 10 years' imprisonment. In order for charges to be laid under the EPBC Act (1999) or other relevant legislation, species identification is a prerequisite.

There are a growing number of cases where DNA has been the most robust method available to conclusively identify species in instances of wildlife trafficking [5–13]. In the majority of cases involving genetic techniques, species are identified using DNA, most frequently mitochondrial DNA (mtDNA) which is present in very high copy number, and is particularly useful if a specimen is poorly preserved or has been treated in any way [14–16]. There is also a substantial volume of scientific literature demonstrating the utility of mtDNA for phylogenetic studies which specifically exploit the unique species level differences typically present in individual mitochondrial genes [17–19].

In order to conclusively identify a sample from an unknown species, a validated reference dataset is essential for comparison against the DNA sequences obtained from any "unknown" sample. Such high quality reference material for animals is often available in museum collections, for example the Australian Museum natural sciences collections with holdings of more than 16 million specimens [20]. These specimens typically exist in the natural history collection in perpetuity (also known as "vouchered" or "voucher" specimens) and are available for closer inspection by taxonomic experts to confirm their identification if required.

Now that DNA identification is a frequently used tool in wildlife forensics, natural history specimens provide an unrivalled resource; particularly if frozen subsamples exist that can also provide validated reference DNA sequences, such as in the Australian Museum frozen tissue collection as well as the many other tissue collections held at museums and other repositories world-wide.

This report will detail two quite different cases of wildlife forensics analyses carried out in the laboratories of

the Australian Museum, Sydney, which involved presentation of DNA evidence and ultimately resulted in the enforcement of penalties. The first involved a large number of illegally caught shark fins seized within New South Wales (NSW). The second involved a large number of bird embryos seized at the Australian border, which were then irradiated due to their quarantine risk. The circumstances of each case will be outlined including the DNA identifications achieved and the penalties imposed on the offenders.

## Case 1

Eighty-seven shark fins were seized from a Commonwealth registered fishing vessel in September 2006. As part of the rules and regulations in force for Commonwealth endorsed vessels each vessel must hold a valid endorsement authorising the catch of a particular species and a limited amount of bycatch, for certain species. The conditions clearly stipulate that shark fins must be attached to a carcass for certain shark species. In this particular instance, the shark fins located by Australian Fisheries Management Authority (AFMA) officers were not attached to carcasses, thereby being an offence contrary to the provisions of the Fisheries Management Act 1991. Subsequent to this the shark fins were seized as evidence and sent for DNA testing to provide evidence on the species of shark.

Shark fin morphology is quite conservative and once removed from a carcass can be extremely difficult to link to a particular species [21, 22]. Therefore in the reported case DNA evidence was critical for identifying the species.

## Methods

All fins were measured, photographed and allocated to either pectoral (left or right) or dorsal/tail, and were separated by size into putative sets derived from a single shark in order to make a preliminary estimate of the minimum number of individuals (MNI). There were 22 pectoral fins that were from the right hand side (RHS) of the body, based on the shape and orientation of the white underside and pigmented topside. This allowed a conservative estimate that a minimum of 22 sharks were represented in the seizure suggesting that 3–4 fins had been taken from each individual given the total seizure of 87 fins.

A small (–25 mg) tissue biopsy was then taken from the inside of each RHS fin, sampled from >10 mm inside the skin opening, to minimise the chances of contamination from extraneous DNA sources.

Genomic DNA was then extracted from the sample tissue using standard protocols for the DNeasy Animal tissues protocol (manufactured by QIAGEN Pty Ltd).



Sections of two mitochondrial genes cytochrome b (*Cyt b*) and cytochrome oxidase 1 (*COI*), well characterised for species identification [23–27], were then amplified using standard PCR conditions (carried out in 25  $\mu$ L volumes containing 2.5  $\mu$ L of QIAGEN 10 $\times$  PCR buffer, 1.5 mM  $MgCl_2$ , 0.05 mM of each dNTP, 10 pmol of each primer, one unit of QIAGEN Taq DNA polymerase, and 1–100 ng of whole genomic DNA). A negative control (containing no DNA template) is included for each batch of amplifications to exclude the possibility that any results achieved are due to contaminant DNA. Amplifications were performed on a MastercyclerS Gradient (Eppendorf Inc). The PCR thermal cycling profile was 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 52°C for 40 s, 72°C for 1 min and a 5 min final extension at 72°C. Successful amplifications were then purified using the ExoSAP-IT PCR purification (USB Corporation) system then bi-directionally sequenced using Big Dye terminator premix v3.1 (Applied Biosystems) with the same primers used for PCR according to manufacturers' instructions. The samples are purified using ethanol following manufacturers' instructions, and then run on an Applied Biosystems 310 DNA Sequencer.

### Analyses

Forward and reverse strands were combined and sequence quality checked for errors using the program Sequencher (Genecodes). Only samples that provided clear and unambiguous sequences were subject to further analyses. Each protein coding sequence was examined to establish the absence of stop codons, the presence of which could indicate a pseudo-gene or nuclear copy. Sequences were then aligned using ClustalX [28] to include a selection of shark reference sequences derived from validated reference specimens and the genetic identity (%) of each unknown sequence to each reference sequence was determined. The aligned sequences were then subjected to Maximum likelihood and Maximum parsimony analyses using PAUP v4.0 [29]. Sequences with >98% identity to the reference *Cyt b* and *COI* gene sequences and bootstrap values of >95 were considered to be a species level match.

### Results

DNA sequences were successfully obtained from the 22 RHS pectoral fins. The DNA sequence data identified the fins as being from two shark families, Carcharhinidae (four different species), and Lamnidae (one species). Table 1 lists the species and common names along with the number of individuals detected of each species (based on one RHS pectoral fin per animal).

### Findings

These types of sharks are all having coastal-pelagic, warm-temperate distribution [30, 31] and some are commonly caught as by-catch, making it possible that these were opportunistically but illegally caught. The defendants pleaded guilty to charges laid under the Commonwealth Fisheries Management Act 1991 sections—s95(1)d “holder of fishing concession, contravene conditions” and s95(1)f “contravening conditions of fishing concession” and the case was found to be proven by the Commonwealth Director of Public Prosecutions. The defendants were convicted and collectively fined more than AUD\$23,000 and ordered to forfeit the shark fins.

### Case 2

Twenty-three bird eggs were seized from a passenger in August 2006 who had arrived at Sydney International Airport from Thailand. The eggs were concealed in a purpose-fashioned body vest. The eggs were considered to be a suspected quarantine risk for H5N1 (Avian Influenza) country [32] and were therefore ordered to be euthanased and gamma-irradiated by the Australian Quarantine Inspection Service.

None of the eggs had hatched at the time of seizure and were assumed to be in varying states of embryonic development. This would make it virtually impossible to identify the embryos' species using traditional morphological characteristics. Therefore DNA was critical for providing accurate identification.

**Table 1** Sharks species represented in the seized fins as determined by DNA identification

Identification	Common name	Family	Minimum number of individuals	World conservation union (IUCN) red list rating
<i>Carcharhinus brevipinna</i>	Spinner Shark	Carcharhinidae	2	Near threatened (NT)
<i>Carcharhinus obscurus</i>	Dusky Whaler	Carcharhinidae	2	Vulnerable (VU)
<i>Galeocerdo cuvier</i>	Tiger Shark	Carcharhinidae	1	Near threatened (NT)
<i>Prionace glauca</i>	Blue Shark	Carcharhinidae	9	Vulnerable (VU)
<i>Isurus oxyrinchus</i>	Short fin Mako	Lamnidae	8	Near threatened (NT)

## Methods

All eggs were candled (using a specialty light source) by an avian expert from Taronga Zoo, Sydney, to determine the presence of blood vessels, then measured and weighed. Photographs were taken by the Australian Federal Police. A small subsample (–25 mg) of tissue was then taken from the tip of the right wing (if the embryo was developed enough) or from part of the blastocyst or yolk (if only in very early stages of development). Genomic DNA was extracted and sequenced as per the methods outlined for Case 1.

## Analyses

Sequences were checked for quality, assembled, and aligned as above (Case 1). Sequences were then aligned using ClustalX [28] to include a selection of avian reference sequences derived from validated reference specimens, and the genetic identity (%) of each unknown sequence to each reference sequence was determined. The aligned sequences were then subjected to Maximum likelihood and Maximum parsimony analyses using PAUP v4.0 [29]. Sequences with >98% identity to the reference *Cyt b* and *COI* gene sequences and bootstrap values of > 95 were considered to be a species level match.

## Results

Despite the risk of not being able to amplify the target gene regions due to the eggs being gamma-irradiated, DNA sequences were successfully obtained from the 23 eggs. Using DNA sequence data from the two mitochondrial genes they were identified to be from seven bird species from two families: Psittacidae (six different species), and Cacatuidae (single species). Table 2 lists the species and common names along with the number of individuals detected for each species.

## Findings

Almost all parrots are CITES listed. The majority of the species involved in this seizure are in CITES Appendix II

which includes “species not necessarily threatened with extinction, but in which trade must be controlled in order to avoid utilization incompatible with their survival” [3]. However, two individuals seized were Appendix I “species threatened with extinction. Trade in specimens of these species is permitted only in exceptional circumstances” [3]. These birds were bought into Australia illegally without appropriate permits for CITES listed live animals. The defendant in this case pleaded guilty to charges laid for contravening the Commonwealth Environment Protection and Biodiversity Act 1999, the Convention on International Trade in Endangered Species (CITES), the Customs Act 1901 and the Quarantine Act 1908. Evidence was submitted that the birds would have fetched almost \$250,000 on the illegal black market. The defendant was fined AUD\$10,000 and sentenced to 2 years imprisonment [33, 34].

## Discussion

In both of these cases DNA identification was critical for accurate species identification. This was especially true for Case 2 where accurate species identification revealed birds listed in both CITES Appendix I and II allowing more serious charges to be brought under the Australian EPBC Act (1999).

Not all wildlife trade in Australia is illegal. Provided legal requirements under the EPBC Act (1999) are met and permits issued there is a highly regulated import and export trade for both commercial and non-commercial purposes. Illegal wildlife trafficking however is extremely cruel for the individual animals involved who are often taken from the wild and shipped in harmful conditions which leads to the high mortality rates widely reported in seizures of smuggled animals.

The risk illegal wildlife smuggling poses to biosecurity is also significant. For example bird smuggling risks the introduction into Australia of highly infectious diseases including Newcastle Disease, Avian Influenza and the virulent bursal disease. There is also the risk of inadvertent introductions of pest and disease species, for example from some reptile and aquatic species [35–37], which could cost

**Table 2** Bird species represented by the seized eggs as determined by DNA identification

Preliminary DNA species ID	Common name	Family	Number of individuals	CITES appendix
<i>Cacatua moluccensis</i>	Salmon-crested Cockatoo	Cacatuidae	2	I
<i>Aratinga acuticaudata</i>	Blue-crowned Conure	Psittacidae	1	II
<i>Ara ararauna</i>	Blue and yellow Macaw	Psittacidae	8	II
<i>Ara manilata</i>	Red-bellied Macaw	Psittacidae	1	II
<i>Ara severa</i>	Chestnut-fronted Macaw	Psittacidae	2	II
<i>Eclectus roratus</i>	Eclectus Parrot	Psittacidae	1	II
<i>Psittacus erithacus</i>	Grey Parrot	Psittacidae	8	II

Australia millions of dollars in rectifications to the native biota and damaged industry.

Illegal wildlife trafficking is an extremely lucrative crime with relatively low penalties and few prosecutions compared to other illegal activities [34, 38]. There is also evidence that wildlife trafficking is commonly associated with other crimes such as drug smuggling [34, 38, 39]. Australian laws allow for harsher penalties than both United States and United Kingdom but there is a trend to suggest the penalties imposed are less severe than in other countries. In most cases where fines have been imposed they are usually significantly less than the value of the goods on the black market [34].

There is evidence that wildlife crime is increasing in Australia [34], which will presumably continue while there is a willing black market and relatively lenient penalties. Applying precedents such as those established in the two case studies presented will hopefully lead to a higher frequency of prosecutions and more severe penalties—as already available under the EPBC Act (1999). Ideally this would be done through increased monitoring in order to disrupt key markets for this illegal trade [40, 41]. The cases serve to further demonstrate that DNA is becoming an increasingly important tool in the wildlife forensics tool box.

### Key points

- (1) DNA is becoming an extremely powerful tool in the prosecution of illegal wildlife trafficking. Mutilating or euthanasing an animal or plant to avoid prosecution is, therefore, no longer a viable method of avoiding species identification.
- (2) High quality reference samples, such as museum voucher or herbarium specimens, are ideal for comparison against DNA of unknown samples.
- (3) Well established phylogenetic and population genetic techniques and analyses provide a solid foundation for the relatively new discipline of wildlife forensics.
- (4) Illegal wildlife trafficking is an extremely lucrative crime with serious consequences yet relatively low penalties and few prosecutions. Australian laws allow for some of the highest penalties for illegal trade, however severe penalties are rarely applied.

**Acknowledgments** I would like to thank the many different agencies that assisted with the work associated with this article including staff from: Australian Customs Service, Australian Federal Police, Australian Museum, Australian Fisheries Management Authority, NSW Department of Primary Industries, and Taronga Zoo. I would also like to thank Dr Mark Eldridge (AM), Mr Adam Wade (AFMA) and two anonymous reviewers for valuable comments on the manuscript.

### References

1. Interpol. Wildlife crime. In: Interpol. 2007. <http://www.interpol.int/Public/EnvironmentalCrime/Wildlife/Default.asp>. Accessed 5 May 2010.
2. Ferrier P. The economics of agricultural and wildlife smuggling. 2009. Economic research report number 81 September 2009.
3. Convention on International Trade in Endangered Species of Wild Fauna and Flora <http://www.cites.org/>. Accessed 5 May 2010.
4. Commonwealth Environment Protection and Biodiversity Conservation Act 1999 <http://www.environment.gov.au/epbc/index.html>. Accessed 5 May 2010.
5. Clarke SC, Magnussen JE, Abercrombie DL, McAllister MK, Shivji MS. Identification of shark species composition and proportion in the Hong Kong shark fin market based on molecular genetics and trade records. *Conserv Biol*. 2006;20:201–11.
6. Hsieh H-M, Tsai C-C, Chiang H-L, Tsai L-C, Huang N-E, Shih R-T, Linacre A, Lee J-C. Species identification of meat products using the cytochrome b gene. *Forensic Sci J*. 2005;4:29–36.
7. Hsieh H-M, Huang L-H, Tsai L-C, Kuo Y-C, Meng H-H, Linacre A, Lee J-C. Species identification of rhinoceros horns using the cytochrome b gene. *Forensic Sci Int*. 2003;136:1–11.
8. Lee J-C, Hsieh H-M, Huang L-H, Kuo Y-C, Wu J-H, Chin S-C, Lee A-H, Linacre A, Tsai L-C. Ivory identification by DNA profiling of cytochrome b gene. *Int J Legal Med*. 2009;123:117–21.
9. Lee J-C, Tsai L-C, Liao S-P, Linacre A, Hsieh H-M. Species identification using the cytochrome b gene of commercial turtle shells. *Forensic Sci Int Genet*. 2009;3:67–73.
10. Magnussen JE, Pikitch EK, Clarke SC, Nicholson C, Hoelzel AR, Shivji MS. Genetic tracking of basking shark products in international trade. *Animal Conserv*. 2007;10:199–207.
11. Shivji MS, Chapman DD, Pikitch EK, Raymond PW. Genetic profiling reveals illegal international trade in fins of the great white shark, *Carcharodon carcharias*. *Conserv Genet*. 2005;6:1035–9.
12. Wasser SK, Mailand C, Booth R, Mutayoba B, Kisamo E, Clark B, et al. Using DNA to track the origin of the largest ivory seizure since the 1989 trade ban. *Proc Natl Acad Sci USA*. 2007;104:4228–33.
13. Wasser SK, Shedlock AM, Comstock K, Ostrander EA, Mutayoba B, Stephens M. Assigning African elephant DNA to geographic region of origin: applications to the ivory trade. *Proc Natl Acad Sci USA*. 2004;101:14847–52.
14. Alacs E, Alpers D, de Tores P, Dillon M, Spencer PBS. Identifying the presence of quokkas (*Setonix brachyurus*) and other macropods using cytochrome b (Cyt b) analyses from faeces. *Wildl Res*. 2003;30:41–7.
15. El-Sayed YS, Ismaeil O, Khaled M, Ashry M, Salah M, Abd El-Rahman using species-specific repeat and PCR-RFLP in typing of DNA derived from blood of human and animal species. *Forensic Sci Med Pathol*. 2010;6:158–64.
16. Spencer PBS, Schmidt D, Hummel S. Identification of historical specimens and wildlife seizures originating from highly degraded sources of kangaroos and other macropods. *Forensic Sci Med Pathol*. 2010;6:225–32.
17. Irwin D-M, Kocher T-D, Wilson A-C. Evolution of the cytochrome b gene of mammals. *J Mol Evol*. 1991;32:128–44.
18. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc Nat Acad Sci USA*. 1989;86:6196–200.
19. Tougaard C, Delefosse T, Hanni C, Montgelard C. Phylogenetic relationships of the five extant rhinoceros species (*Rhinocerotidae* *Perissodactyla*) based on mitochondrial cytochrome b and 12S rRNA genes. *Mol Phylogenet Evol*. 2001;19:34–44.

20. <http://australianmuseum.net.au/How-big-are-the-Australian-Museum-collections>.
21. Sebastian H, Haye PA, Shivji MS. Characterization of the pelagic shark-fin trade in north-central Chile by genetic identification and trader surveys. *J Fish Biol.* 2008;73:2293–304.
22. Shivji M, Clarke S, Pank M, Natanson L, Kohler N, Stanhope M. Genetic identification of pelagic shark body parts for conservation and trade monitoring. *Conserv Biol.* 2002;16:1036–47.
23. Branicki W, Kupiec T, Pawlowski R. Validation of cytochrome b sequence analysis as a method of species identification. *J Forensic Sci.* 2003;48:83–7.
24. Dawnay N, Ogden R, McEwing R, Carvalho GR, Thorpe RS. Validation of the barcoding gene CO1 for use in forensic genetic species identification. *Forensic Sci Int.* 2007;173:1–6.
25. Lowenstein JH, Amato G, Kolokotronis S-O. The real maccoyii: identifying Tuna Sushi with DNA barcodes—contrasting characteristic attributes and genetic distances. *PLoS ONE.* 2009; 4(11):e7866.
26. Ogden R, Dawnay N, McEwing R. Wildlife DNA forensics—bridging the gap between conservation genetics and law enforcement. *Endanger Species Res.* 2009;9:179–195.
27. Verma SK, Singh L. Novel universal primers establish identity of an enormous number of animal species for forensic application. *Mol Ecol Notes.* 2003;3:28–31.
28. Clustal W, Clustal X version 2.0, Larkin M et al. *Bioinformatics* 2007;23:2947–8.
29. PAUP\*: phylogenetic analysis using parsimony, version 4.0b10. 2003 by D. L. Swofford.
30. ICUN. 2004 ICUN red list of threatened species. A global species assessment. <http://www.iucnredlist.org/>. Accessed 5 May 2010.
31. Compagno L. *Sharks of the world: an annotated and illustrated catalogue of shark species known to date*. Rome, Italy: Food and Agriculture Organization of the United Nations; 2002.
32. World Health Organisation 2010 [http://www.who.int/csr/disease/avian\\_influenza/en/](http://www.who.int/csr/disease/avian_influenza/en/), and [http://www.who.int/csr/don/2005\\_10\\_20a/en/](http://www.who.int/csr/don/2005_10_20a/en/). Accessed 5 May 2010.
33. Two years for bird smuggler. Canberra: Customs Media Releases Jan 20; 2007. <http://www.customs.gov.au/site/content8431.asp>. Accessed 5 May 2010.
34. Alacs E, Georges A. Wildlife across our borders: a review of the illegal trade in Australia. *Aust J Forensic Sci.* 2008;40:147–60.
35. Keller RP, Lodge DM. Species invasions from commerce in live aquatic organisms: problems and possible solutions. *Bioscience.* 2007;57:428–36.
36. Reed RN. An ecological risk assessment of non-native boas and pythons as potentially invasive species in the United States. *Risk Anal.* 2005;25:753–66.
37. Weigle SM, Smith LD, Carlton JT, Pederson J. Assessing the risk of introducing exotic species via the live marine species trade. *Conserv Biol.* 2005;19:213–23.
38. Lowther J, Cook D, Roberts M. Crime and punishment in the wildlife trade. 2002. A WWF Traffic Report May 2002 [http://www.wwf.org.uk/filelibrary/pdf/crime\\_and\\_punishment.pdf](http://www.wwf.org.uk/filelibrary/pdf/crime_and_punishment.pdf). Accessed 5 May 2010.
39. Zimmerman ME. The black market for wildlife: combating transnational organized crime in the illegal wildlife trade. *Vanderbilt J Transnatl Law.* 2003;36:1657–89.
40. Lemieux AM, Clarke RV. The international ban on ivory sales and its effects on elephant poaching in Africa. *Br J Criminol.* 2009;49:451–71.
41. Schneider JL. Reducing the illicit trade in endangered wildlife. The market reduction approach. *J Contemp Crim Justice.* 2008; 24(3):274–95.

# Mechanisms of deaths in captive juvenile New Zealand fur seals (*Arctocephalus forsteri*)

Roger W. Byard · Aaron Machado ·  
Kerry Braun · Lucian B. Solomon ·  
Wayne Boardman

Accepted: 11 May 2010 / Published online: 25 May 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** Juvenile seals are sometimes encountered in waters around South Australia with injuries and/or diseases that require veterinary treatment. Two cases are reported where apparently stable animals died soon after being rescued due to quite disparate conditions. In Case 1 a juvenile male New Zealand fur seal (*Arctocephalus forsteri*) was found unexpectedly dead in its enclosure. A necropsy examination revealed an emaciated juvenile male with no injuries. The intestine was filled throughout its length with melena stool that was due to heavy infestation of the stomach with roundworms with adjacent gastritis. Death was due to shock from upper gastrointestinal blood loss secondary to parasitosis. In Case 2 a second juvenile male New Zealand fur seal (*Arctocephalus forsteri*) also died unexpectedly in its enclosure. It had been

listless with loud respirations since capture. At necropsy there was no blood around the head, neck or mouth, and no acute external injuries were identified. An area of induration was, however, present over the snout with fragmentation of underlying bones. The maxilla was freely mobile and CT scanning revealed multiple comminuted fractures of the adjacent facial skeleton. Examination of the defleshed skull showed fragmentation of the facial skeleton with roughening of bones in keeping with osteomyelitis. Death was attributed to sepsis from osteomyelitis of a comminuted midfacial fracture. These cases demonstrate two unusual and occult conditions that may be present in recently retrieved juvenile fur seals. Failure to establish the correct diagnosis rapidly may result in death soon after capture. The usefulness of imaging techniques such as CT scanning in delineating underlying injuries prior to necropsy is clearly demonstrated.

R. W. Byard (✉) · L. B. Solomon  
Discipline of Anatomy and Pathology, The University of  
Adelaide, Level 3 Medical School North Building, Frome Road,  
Adelaide, SA 5005, Australia  
e-mail: roger.byard@sa.gov.au

R. W. Byard  
Forensic Science SA, 21 Divett Place, Adelaide, SA 5000,  
Australia

A. Machado · K. Braun  
Australian Marine Wildlife Research and Rescue Organization,  
Port Adelaide, SA, Australia

L. B. Solomon  
Discipline of Orthopaedics and Trauma, The University of  
Adelaide, Royal Adelaide Hospital, North Terrace, Adelaide,  
SA 5000, Australia

W. Boardman  
Royal Zoological Society of South Australia, Adelaide,  
SA, Australia

**Keywords** New Zealand fur seal · Death · Parasitosis · Hemorrhage · Osteomyelitis · Fracture · Blunt trauma

## Introduction

Sudden and unexpected death in all species may result from a variety of natural diseases and inflicted or non-inflicted injuries. Unfortunately the symptoms and signs of many of these conditions may be quite nonspecific. A problem, therefore, with recently rescued animals is in establishing the underlying cause of clinical manifestations rapidly, so that appropriate treatment can be initiated. Two cases of sudden death in juvenile male New Zealand fur seals (*Arctocephalus forsteri*) are reported to demonstrate the range of occult conditions that may be present in recently retrieved animals, and the usefulness of imaging



with necropsy assessment in establishing the cause and mechanism of death.

## Case reports

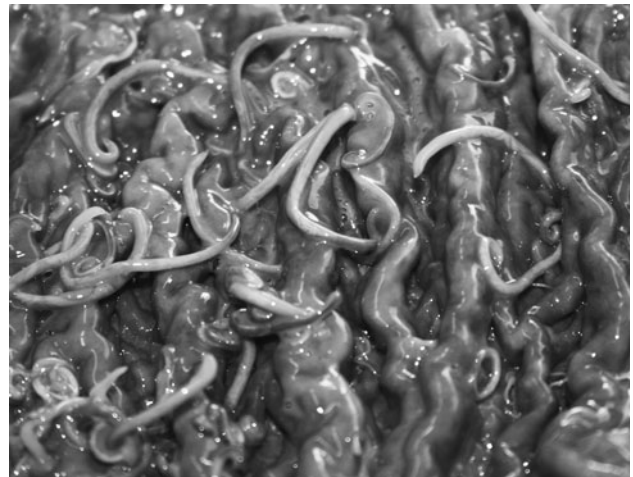
### Case 1

A juvenile male New Zealand fur seal (*Arctocephalus forsteri*) was found in an emaciated condition at a beach south of Adelaide, South Australia. He was taken to the Australian Marine Wildlife Research and Rescue Organization for assessment and treatment. On admission he was dehydrated, weighing 10.5 kg, and was administered 400 mls of subcutaneous normal saline. Around 1,400 h during the second day of captivity he was found unexpectedly dead at the waters edge of his enclosure, having been responsive and feeding that morning. Melena stool was noted.

At necropsy the body was that of a thin juvenile New Zealand fur seal. The only evidence of trauma consisted of superficial lacerations of the webbing of both hind flippers. Melana stool was noted around the anus. Internal examination revealed minimal subcutaneous fat with normal positioning of internal organs. The peritoneal cavity was unremarkable with normal appearing intestines. Specifically there was no ischemic necrosis, internal herniation, volvulus or intussusception. The colon and small intestine were opened in their entirety revealing normal intestinal mucosa with no colitis. Melena stool was present extending from the duodenum to the rectum. The major findings involved the stomach that was filled with hundreds of roundworms (*Anisakis spp.*), many of which were still attached to the gastric mucosa (Fig. 1). The mucosa appeared reddened and there were numerous punctate erosions where worms had detached. Granular material was present within the submucosa and mucosa that represented shed material from the nematodes. Occasional nematodes were also present attached to the lower esophageal mucosa. The chest cavity and organs were unremarkable and there was no evidence of intracerebral hemorrhage. Histology confirmed attachment of worms to the gastric mucosa with adjacent acute gastritis and superficial ulceration (Fig. 2). Death was, therefore, due to upper gastrointestinal hemorrhage complicating gastric parasitosis.

### Case 2

A second juvenile male New Zealand fur seal (*Arctocephalus forsteri*) was found in an emaciated condition in the Port River Estuary, northwest of Adelaide, South Australia. He was taken to the Australian Marine Wildlife Research and Rescue Organization for assessment and

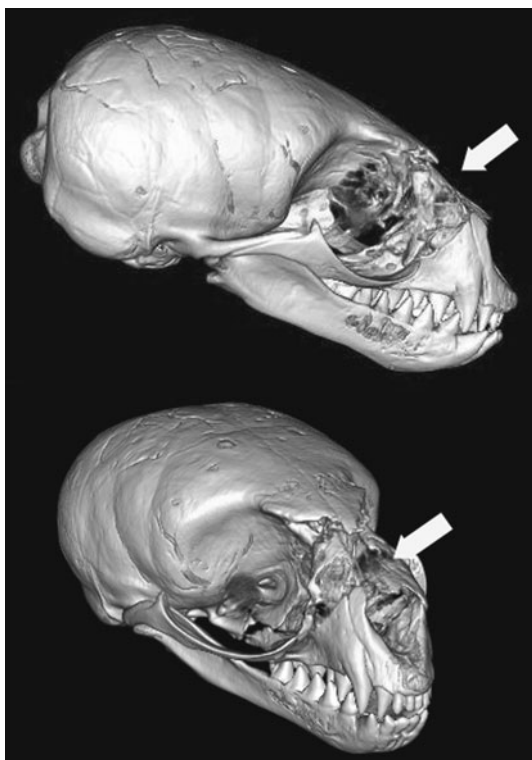


**Fig. 1** Close up view of roundworms in the stomach of a juvenile male New Zealand fur seal (*Arctocephalus forsteri*) associated with significant hemorrhage (case 1)



**Fig. 2** Whole mount view of a single attached roundworm in the stomach in case 1 (a). Histology of the gastric mucosa demonstrating remnants of a roundworm with surrounding ulceration and erosion into superficial blood vessels (b). (Hematoxylin & eosin,  $\times 100$ )

treatment. On admission he weighed 6.1 kg and was administered 400 mls of subcutaneous normal saline. The animal was noted to be breathing heavily through his mouth and making unusual sounds. In addition he



**Fig. 3** Two CT views of the skull from case 2 showing extensive comminuted fracturing of the midfacial skeleton (arrows)

occasionally would bite at the cage and his rear flank. He died soon after admission.

At necropsy the body was that of a thin juvenile New Zealand fur seal with no external evidence of trauma except a possible healed laceration over an indurated and unusually mobile area of the snout. Specifically, the anterior facial skeleton appeared separate from the remainder of the skull. A head CT was performed before necropsy. One mm transverse scans were taken on an Aquilion 64 slice CT scanner (Toshiba Medical System Corporation, Japan), and reconstructions were performed on a Vitrea 4.0 workstation (Minnetonka, Minnesota, USA). The scans demonstrated a comminuted fracture of the facial skeleton involving the nasal bones, the maxilla and zygomas in a cranio-caudal and supero-inferior direction from the face, and crossing the nasal cavities, inferior orbital floors and maxillary sinuses to exit through the palate (Fig. 3). The lower jaw was intact.

Dissection of soft tissues from the facial skeleton confirmed the presence of the facial fractures including fragments of shattered bone without evidence of recent hemorrhage. After defleshing the central facial skeleton collapsed. Irregular areas of reactive bone around the edges of the fractures were in keeping with osteomyelitis (Fig. 4). Death was therefore attributed to sepsis complicating



**Fig. 4** Defleshed skull from case 2 showing loss of the mid facial skeleton due to comminuted fracturing

osteomyelitis arising from an extensive open fracture of the mid facial skeleton following blunt trauma.

## Discussion

Causes of hemorrhage from gastrointestinal lesions in humans vary with age, with diverticulitis and angiodysplasia occurring in older individuals, compared to Meckel diverticulum and intussusception in the young [1]. Parasites are usually not encountered as a cause of significant hemorrhage in human forensic practice, although *Strongyloides stercoralis* has been reported as a cause of life threatening duodenal hemorrhage [2], and the roundworm *Ascaris lumbricoides* has caused massive hematemesis in infancy due to duodenal ulceration and volvulus [3]. While hydatid disease often remains clinically occult, very rare cases have presented with massive lower gastrointestinal hemorrhage from splenic colic fistula [4, 5].

Seals are known to harbour intestinal parasites with the species of nematode varying depending on the species of seal. For example, 60% of Juan Fernandez fur seal pups (*Arctocephalus philippii*) in one study had hookworms (*Uncinaria sp.*) [6], with 100% of grey seals (*Halichoerus grypus*) in another study revealing the small intestinal helminth *Corynosoma strumosum* [7].

In the reported case the stomach was filled with hundreds of attached roundworms with superficial ulceration and acute gastritis. In a study of the gastric mucosa of a seal infested with *Contacaecum osculatum*, *Pseudoterranova decipiens* and *Anisakis simplex* the findings were similar, with congestion and hemorrhage, in addition to cellular infiltration and fibrosis [8]. The fact that the current case was under direct observation at the time of death

enabled the rapid nature of the terminal event to be accurately documented. It appeared that roundworm infestation had resulted in considerable emaciation of the young animal with evidence of continued hemorrhage in the form of melena throughout the small and large intestines. As in humans it is likely that terminal cardiovascular collapse was due to acute cardiac failure complicating acute chronic blood loss.

In the second case, the seal had sustained an injury to the facial skeleton from significant blunt trauma, but had managed to survive for some time, despite breathing and feeding difficulties, and likely considerable pain. The unhealed maxillary fracture would have prevented the animal from effectively biting and chewing and would have explained the emaciated state. It is likely that generalized sepsis from subsequent osteomyelitis initiated the terminal event, as there was no airway obstruction or underlying diseases identified at autopsy. The absence of oronasal hemorrhage, with healing of the skin over the maxillary fractures, in addition to the presence of reactive bone, were all in keeping with prolonged survival following trauma. The odd behaviour of the animal in captivity could be explained by pain associated with such a major injury. The nature of the trauma remains uncertain, however blunt impact would appear likely. If the injury occurred while the animal was in the water, the direction of the fracture and focal nature of the induration suggests that it occurred with the head in an upright position, and thus could have been due to a collision with a boat or the fins of a wind or kite surf. If the injury occurred while the animal was on the beach or in shallow water it could have been the result of impact with a blunt object. The use of CT scanning in this case provided an excellent method for identifying the precise nature of the facial skeletal fracture prior to formal necropsy dissection.

These cases demonstrate a variety of points. In the first case the significant effect that a large gastrointestinal parasite population may have on a juvenile animal was shown. While parasite load varies considerably among studies, the possibility of sudden and unexpected death must be considered when apparently stable but emaciated animals are being assessed. The very early administration of anti-helminth medication may be indicated. The second case demonstrates that juvenile seals may survive significant

facial trauma and still maintain feeding. The absence of evidence of acute trauma in a captured animal does not, therefore, exclude significant occult injury.

### Key points

1. Gastrointestinal parasitosis may cause significant hemorrhage in juvenile New Zealand fur seals (*Arctocephalus forsteri*) and result in sudden death.
2. Juvenile New Zealand fur seals (*Arctocephalus forsteri*) may survive significant facial trauma and still maintain feeding.
3. The absence of evidence of acute trauma in a captured seal does not exclude significant occult injury.
4. CT scanning is an excellent method for the identification of occult skeletal injury prior to necropsy.

### References

1. Byard RW, Simpson A. Sudden death and intussusception in infancy and childhood -autopsy considerations. *Med Sci Law*. 2001;41:41–5.
2. Bhatt BD, Cappell MS, Smilow PC, Das KM. Recurrent massive upper gastrointestinal hemorrhage due to *Strongyloides stercoralis* infection. *Am J Gastroenterol*. 1990;85:1034–6.
3. Sangkhathat S, Patrapinyokul S, Wudhisuthimethawee P, Chedphaopan J, Mitamun W. Massive gastrointestinal bleeding in infants with ascariasis. *J Pediatr Surg*. 2003;38:1696–8.
4. Byard RW. An analysis of possible mechanisms of unexpected death occurring in hydatid disease (echinococcosis). *J Forensic Sci*. 2009;54:919–22.
5. Teke Z, Yagci AB, Atalay AO, Kabay B. Splenic hydatid cyst perforating into the colon manifesting as acute massive lower gastrointestinal bleeding: an unusual presentation of disseminated abdominal echinococcosis. *Singapore Med J*. 2008;49:e113–6.
6. Sepúlveda MS. Hookworms (*Uncinaria* sp.) in Juan Fernandez fur seal pups (*Arctocephalus philippii*) from Alejandro Selkirk Island, Chile. *J Parasitol*. 1998;84:1305–7.
7. O'Neill G, Whelan J. The occurrence of *Corynosoma strumosum* in the grey seal *Halichoerus grypus*, caught off the Atlantic coast of Ireland. *J Helminthol*. 2002;76:231–4.
8. Ito M, Sato T, Shirai W, Kikuchi S. Parasites and related pathological lesions in the gastrointestinal tract of a seal (*Phoca vitulina* Linnaeus). *J Vet Med Sci*. 1998;60:1025–8.

# Wildlife crime: a global problem

Linzi Wilson-Wilde

Accepted: 13 May 2010 / Published online: 29 May 2010  
© Springer Science+Business Media, LLC 2010

Wildlife crime, the illegal trade in animals and animal products, is a growing concern, with estimates of the cost ranging from US\$10 billion up to US\$20 billion globally per year [1–3]. This equates to approximately 5% of the size of the international drug trade [2–4]. However, resources allocated to combating this crime do not compare. While broader attention to this problem is beginning to occur, the application of forensic techniques is predominantly in the research and educational arenas. Currently a number of international networks have been established, including the INTERPOL Wildlife Crime Working Group (<http://www.interpol.int/public/EnvironmentalCrime/Wildlife>) and TRAFFIC (Wildlife Trade Monitoring Network) ([www.traffic.org](http://www.traffic.org)). However these networks have not been significantly funded and instead predominantly rely on public donations and member contributions.

Animals most commonly targeted include birds, reptiles, insects, fish and large game animals. Rare or endangered species are particularly targeted by collectors, or for the pet trade. Some species are chosen for the end product such as Shahtoosh wool from the Tibetan Antelope; which must be killed in order to harvest the wool. Recently the trade in rhinoceros horn and ivory has markedly increased. Traditionally rhinoceros horn is used for Yemeni dagger handles, but recently there has been a significant increase in illegal poaching and trafficking of the horn, on route to the Far East. This is due to the belief in the medicinal benefits of ground up horn in the treatment and prevention of cancer. Biopiracy (illegal, unauthorized use of genetic information taken from wildlife protected under wildlife

law for commercial gain) is also linked to scientists conducting research, such as in the creation of new pharmaceutical agents.

The trade in wildlife is very popular in China where it was found that there are a large number of CITES I and II (the Convention on International Trade in Endangered Species of Wildlife Fauna and Flora) listed species offered for sale on the Chinese-Language internet (in China, Hong Kong and Taiwan); with over 20,000 advertisements in China alone [5]. An important move has been the internet's largest trading venue eBay prohibiting the trade in live animals (except some fish in the United States) and ivory.

Wildlife crime is one portion of a much bigger issue and although there is some active research in the area, there is a lack of a focussed effort that is backed by resources and political support. Offenders exploit this gap to circumvent the basic provisions currently in place for their apprehension. Without strong centralized or collaborative international control this important issue will continue and will increase. It is estimated that in Brazil 40% of organized crime is now associated with wildlife crime [6] and the figure is rising, due in part to the global economic turn-down and disparities between the penalties and financial gains when compared to other organised crime types.

There is a worldwide network of suppliers, distributors, middlemen and traffickers with contacts in habitat countries and involvement of customs officers in others. Offenders are often associated with other trafficking offences such as drugs, guns and humans and flow-on offences such as forgery, with the counterfeiting of CITES documents, export/import permits and documents to reflect fictitious breeding by registered breeders [7]. Offenders are often part of large groups which are organized, use jargon and nicknames to conceal communications and utilise technology such as Skype and the internet [8].

---

L. Wilson-Wilde (✉)  
ANZPAA National Institute of Forensic Science,  
Melbourne, Australia  
e-mail: [linzi.wilson-wilde.nifs@anzpaa.org.au](mailto:linzi.wilson-wilde.nifs@anzpaa.org.au)



Transnational wildlife crime is facilitated by the massive size of some countries with areas difficult to reach and police, inadequately controlled borders, limited economic alternatives for indigenous populations, livelihoods based on illegal activities due to inadequate resources for law enforcement, weak or disinterested governments with ineffective laws and penalties, and corruption. Contraband wildlife and its derivatives are exclusive products often in high demand and provide lucrative markets. Purchasers are often ignorant or uncaring of the mode in which the animals are transported, with up to 90% of live traffic dying in transit and subsequent flow on effects to the environment. Due to the lack of resources allocated to combating wildlife crime, Non-Government Organizations (NGOs) are becoming involved in law enforcement which should be of concern at the international level.

Wildlife crime impacts on the Australian and New Zealand environment due to the unique nature of the region's fauna and flora and its close proximity to south-east Asian criminal networks. The Australia New Zealand Policing Advisory Agency—National Institute of Forensic Science (ANZPAA NIFS) ([www.anzpaa.org.au](http://www.anzpaa.org.au)) facilitates the Australian Wildlife Forensics Network. At a recent INTERPOL meeting of the Wildlife Crime Working Group (Manaus, Brazil, September 2009) numerous international representatives provided examples of seizures of Australian animals illustrating that Australia is being targeted for its unique fauna by organized criminal networks. The INTERPOL Wildlife Crime Working Group is actively involved in conducting targeted operations on organized crime. This involves sharing intelligence, capability building initiatives including training and method development and general information.

There are few capacity building activities currently occurring globally. The Association of Southeast Asian Nations Wildlife Enforcement Network (ASEAN-WEN) initiative has the largest potential to affect Australia. However Australia is not currently involved in the ASEAN-WEN project; TRAFFIC has assisted in this initiative. Additionally, the world's only laboratory dedicated to the forensic analysis of wildlife crime (the US National Fish and Wildlife Forensic Laboratory) recently announced the creation of a Society for Wildlife Forensic Sciences and an associated tri-annual conference dedicated to wildlife forensic sciences (the first meeting was held in Ashland Oregon, 19–23 April 2010).

Partnerships between multiple agencies are very effective in identifying contraband and perpetrators and deterring

other offenders. Effective action requires collaboration between investigators, forensic specialists and Governments of source, transit and recipient countries. It requires the commitment of government and law enforcement agencies, international collaboration and information exchange and importantly the subsequent prosecution of offenders. The prosecution should focus on the theft of a public resource, which is motivated by profit and presents a potential risk to human health in the spread of diseases (such as avian influenza (H5N1) and severe acute respiratory syndrome (SARS) [1]). This effort needs to be in conjunction with wildlife and habitat protection, promotion of eco trade (which can also target former poachers), public outreach campaigns, sustainable agriculture and funding for further research. Eco-tourism communities can earn 2–3 times the money from tourism compared to poaching which ensures a long term funding source for these communities.

Research provides information that underpins the impact of wildlife crime and assists with the development of tools, such as species identification tests used to prosecute offenders. Initiatives such as the dedication of this edition of this journal to wildlife crime will provide attention but much more is needed if this issue is to be thoroughly addressed.

## References

1. Wyler LS, Sheikh PA. CRS report for congress—international illegal trade in wildlife: threats and U.S policy. Hauppauge: Nova Science Publishers; 2008.
2. Brack D. The growth and control of international environmental crime. *Environ Health Perspect.* 2004;112:80–1.
3. Holden J. By hook or by crook a reference manual on illegal wildlife trade and prosecutions in the United Kingdom. The Royal Society for the Protection of Birds; 1998.
4. Ong DM. The convention on international trade in endangered species (CITES 1973): implications of recent developments in international and EC law. *J Environ Law.* 1998;10.
5. Xianlin M. Law Enforcement activities regarding Falcons, Tibetan Antelope and trade on the internet. Presentation at the Interpol Wildlife Crime Working Group Meeting, Manaus, Brazil; 2009.
6. Renctas—national network to fight the trafficking of wild animals. First national report on wild fauna traffic. Brazil 2001; [www.rencta.org.br](http://www.rencta.org.br).
7. Lowther J, Cook D, Roberts M. Crime and punishment in the wildlife trade. A WWF/TRAFFIC Report. 2002; [www.wwf.org.uk](http://www.wwf.org.uk).
8. IFAW (International fund for animal welfare) caught in the web: wildlife trade on the internet. 2005; [http://www.ifaw.org/ifaw/dfiles/file\\_562.pdf](http://www.ifaw.org/ifaw/dfiles/file_562.pdf).



## INTERPOL's wildlife crime working group meeting

Laurel A. Neme

Accepted: 20 May 2010 / Published online: 18 June 2010  
© Springer Science+Business Media, LLC 2010

The INTERPOL Wildlife Crime Working Group held its 21st meeting in Manaus, Brazil from 21 to 25 September, 2009. The aim of this group is to promote coordination between wildlife law enforcement agencies. At this meeting, almost 100 participants from over 30 countries gathered to discuss recent wildlife law enforcement cases and build opportunities for international collaborations.

The INTERPOL Wildlife Crime Working Group meeting included both open and closed sessions with the first 2 days being open to both government and non-government organizations (NGOs). Sessions included: updates on Operations Oxossi and Lora and on the Convention on International Trade in Endangered Species (CITES). NGOs, including the International Fund for Animal Welfare (IFAW), Humane Society International, TRAFFIC, Greenpeace, and The Last Great Ape Organization (LAGA), also shared information about their programs. In addition, attendees participated in a workshop on wildlife forensics with the Netherlands Forensics Institute and Australia's National Institute of Forensic Science which detailed their activities, and the author of *Animal Investigators: How the World's First Wildlife Forensics Labs is Solving Crimes and Saving Endangered Species* (Scribner, 2009) described the work of the US Fish and Wildlife Service Forensic Lab. It closed with a presentation by EBay/Paypal that explained the workings of these systems and their actions to prevent illegal wildlife trade via the Internet.

Operation Oxossi, an investigation into Brazil's largest-ever wildlife trafficking ring, involved over 450 Brazilian Federal Police agents from nine Brazilian states. The

investigation uncovered an illegal trade in live macaws and macaw eggs destined for the pet bird trade in Europe, and was notable not only because of its size—over 70 people were arrested for illegally smuggling \$20 million and 500,000 animals per year—but also because of the international coordination of the law enforcement agencies involved. To illustrate the importance of international coordination, the presentation on Operation Oxossi noted how the undercover operation revealed that Czech nationals were engaged in the illegal trade. As a result, the Czech Environmental Inspectorate assigned an agent to work with the Brazilian Federal Police to transcribe and evaluate evidence. That cooperation, in addition to helping the Brazilian investigation, resulted in Operation Lora, a related Czech investigation into four independent groups of Czech traffickers who smuggled rare birds, such as African grey parrots, palm cockatoos and Lear's macaws, from Brazil, Africa, Australia and Indonesia into Europe. INTERPOL's assistance was vital in both operations to facilitate the coordination between countries and disseminate information such as Red Notices.

The 3-day closed session for government enforcement agencies included a visit to the Brazilian Federal Police's environmental crime training facility, 2 h upstream from Manaus, as well as reports on strategic planning, trade in reptiles, elephants and rhinoceros, wildlife prosecution assistance, shahtoosh training, a wildlife smuggling identification handbook, a checklist of questions for persons implicated in wildlife smuggling, and Ecomessage. Participants included the Lusaka Agreement Task Force, Kenya, the Association of Southeast Asian Nations Wildlife Enforcement Network (ASEAN WEN) and the Community of Portuguese Speaking Countries (CPLC), who also provided reports on their regional activities. Finally, agents discussed their enforcement actions regarding the

---

L. A. Neme (✉)  
93 Butternut Lane, Shelburne, VT 05482, USA  
e-mail: laurel@laurelneme.com

European bird trade, trade in falcons, the status of Tibetan antelopes and trade in shahtoosh and the Internet trade.

As the only international meeting focused on wildlife crime, the INTERPOL Wildlife Crime Working Group Meeting serves a critical function in facilitating coordination between law enforcement agencies and establishing the methods and relationships necessary to share operational intelligence.

As David Higgins of INTERPOL's Environmental Crime Programme said, "Environmental crime is clearly an international problem which calls for international law enforcement co-operation. The success of Operation Oxossi in Brazil demonstrates the results that can be obtained when the fight against wildlife and environmental crime is taken to the frontline as part of concerted international action using global law enforcement networks and resources."

# Identification of historical specimens and wildlife seizures originating from highly degraded sources of kangaroos and other macropods

P. B. S. Spencer · D. Schmidt · S. Hummel

Accepted: 25 August 2009 / Published online: 5 November 2009  
© Humana Press 2009

**Abstract** Forensic investigations are an important area in the regulation of food mis-description, wildlife seizures and the international trade in wildlife and its products. An early, but important stage in dealing with many biological materials that are submitted for forensic scrutiny is species identification. We describe a method and new primers to amplify three small DNA fragments of the *cytochrome b* region of the mitochondrial DNA that are suitable for marsupial species identification from degraded sources, such as wildlife seizures. They were designed as consensus sequences from a comparison of 21 marsupial species. The primers also contained sequences intended specifically not to amplify human DNA, thereby reducing the likelihood of amplifying contaminants. Examples of the utility of these primers are given using a range of conditions that may be applied using such an approach, including (1) field-collected sub-fossil bones, (2) an example of museum mis-identification from a specimen collected in 1930 and (3) a skull collected from Bernier Island, in the harsh mid-west of Western Australia.

**Keywords** Wildlife forensic · Marsupial · Bone · Teeth · Sub-fossil

## Introduction

In Australia, forensic investigations are important in a range of wildlife areas including the regulation of fraudulent description of food products, wildlife seizures and the international trade in wildlife and its products [1]. One such group, the kangaroos are internationally recognized iconic wildlife. The group belong to the superfamily Macropodoidea (or macropods), and contain about 45 living species in Australia [2]. There is currently no specific method or comparative database to identify DNA from highly degraded sources of kangaroo species in Australia. Modern molecular techniques have enabled the extraction and sequencing of DNA from old or museum specimens such as bone, teeth, feathers or skin [3–6]. Access to this degraded DNA provides an invaluable resource for wildlife protection, systematics and conservation research over a large time scale. Museum specimen sequences have already proved crucial to a number of wildlife genetic studies e.g. [5, 7–9]. It is estimated that the world's natural history museums hold in the order of 1.5 billion specimens [10] and these, from a genetic perspective, have hitherto been greatly under-utilised. It is also recognised that the world's mammals are declining rapidly [11]. The advantages of using museum specimens over fresh specimens are well recognised and include the possibility that fresh specimens may be costly to obtain due to the remoteness of habitat, the behaviour of the species or the fact that the species under investigation may exist in politically sensitive regions [10]. Museum collections may also contain specimens from species which are now extinct [10, 12]. Use of museum specimens may therefore be both time and cost saving, and importantly, allow access to information which may no longer be obtainable from living systems.

---

P. B. S. Spencer (✉)  
School of Biological Sciences and Biotechnology,  
Wildlife Forensic Laboratory, Murdoch University, Perth,  
Western Australia 6150, Australia  
e-mail: P.Spencer@murdoch.edu.au

D. Schmidt · S. Hummel  
Institute of Zoology and Anthropology, Historical Anthropology  
and Human Ecology, Georg August-Universität Göttingen,  
Bürgerstrasse 50, 37073 Göttingen, Germany

In Australia there are an increasing number of studies that have utilised old museum specimens for marsupial research e.g. [13, 14] but there remains little general information about this potentially powerful application to marsupial fauna. Future marsupial research may need to rely to a greater extent on museum specimens due to Australia's recognised small mammal decline, high extinction rates, habitat destruction and fragmentation [15] and difficulty in obtaining specimens [10]. For example in the case of endangered species persisting in fragmented habitats, an understanding of the historical changes (using museum or sub-fossil material) is crucial for conservation.

An important consideration in analysing ancient DNA (aDNA) sources is that the material is often highly degraded. For repeatability, the amplification of the targeted sequence information should be from primers that give a PCR product as short as possible (preferably <200 bp [6]). We applied this approach to our sequencing of the *cytochrome b* (cyt b) region of the mitochondrial genome for three marsupial specimens. *Cytochrome b* is a well defined region with adequate variation to discriminate marsupial taxa e.g. [8, 16, 17] and has been very widely used, and commonly is amplified by 'universal primers' [18, 19] or in the identification of forensic samples [20, 21]. Technical difficulties inherent to the analysis of small quantities of DNA generally tend to limit the efficiency of this approach. Two of the limitations with using these universal primers for museum specimens (particularly marsupials) are that the fragments are generally too large for successful amplification when the DNA is highly degraded and by virtue of the fact that they are 'universal', they have not been designed specifically for the taxa under investigation and may amplify non-target DNA.

In order to answer questions on sample identification of marsupial museum specimens and those sourced from

degraded samples, we designed a set of primers to amplify three relatively small amplicons of the *cytochrome b* gene in the marsupial mitochondrion. Using these primers, we demonstrated that it was possible to (1) obtain authentic amplicons from a range of macropod species, (2) obtain such DNA from different types of material (bone and teeth) and (3) identify and assign each specimen to a species.

## Materials and methods

### Samples and source material for degraded-source DNA analysis

A total of seven DNA extracts were isolated from different skeletal components (bone and teeth) from three specimens of marsupials described in Table 1. Each specimen represented a common situation found in our laboratory that has been encountered when dealing with marsupial questions involving degraded DNA, such as from wildlife seizures and international trade questions. These include:

1. *sub-fossil Euro (Macropus robustus)*. This sample was collected as a crushed skull, which included the complete set of upper molar teeth (Sample identification G001; Table 1). The partial bleached skull was collected in the field at Mt. Keith Station, Western Australia. No other bones were found in the vicinity of the skull, suggesting that it had been exposed for a considerable period of time, although we do not have an exact period of exposure. This specimen was intended to represent a 'typical' field sample, where the specimen was obviously in a poor state of preservation and has been exposed to extreme ambient temperatures (0–45°C; Meekathara weather station).

**Table 1** Details of specimens, condition and DNA yield from marsupials

Species	Specimen condition	Sample No.	DNA conc (ng/μl)	DNA yield (ng/mg sample)
<i>Macropus robustus</i> <sup>a</sup>	Bleached skull (partial) collected at Mt. Keith Station, W.A. in 2001. No bones in vicinity of skull. Exposed for >6 months	G001-1 (tooth)	60	21
		G001-2 (tooth)	8	2
		G001-3 (bone)	<1	–
		G001-4 (tooth)	12	1
<i>Notomys</i>	Femur sampled from museum specimen labelled <i>Notomys alexis</i>			
(M1461) <sup>b</sup>	Suspect mis-labelled. Collected in 1930, Canning stock Route, W.A.	G002-1 (bone)	158	23
<i>Lagorchestes hirsutus</i> <sup>a</sup>	Partial bleached skull (crushed) collected from Bernier Island, W.A. in 1989	G003-1 (tooth)	12	1
		G003-2 (bone)	37	4
(M47274) <sup>b</sup>				

<sup>a</sup> Exposed to extreme humidity and ambient temperatures of 0–45°C

<sup>b</sup> Western Australian museum accession number

Duplicate DNA extractions were performed on samples of bone and tooth material (i.e. a total of four extractions were carried out on this specimen). Each extract was carried out separately (details given in Table 1).

2. *mis-labelled museum specimen* (a “Notomys”). A single DNA extraction was carried out on a small section (0.34 g) of the femur from a museum specimen labelled “*Notomys alexis*”, collected in 1930 on the Canning Stock route (Western Australian Museum Accession No. M1461). We suspected that this specimen may have been mis-labelled, as the bone fragment was 92 mm long, only slightly shorter than the 95 mm head-body length of the spinifex hopping mouse it was supposedly from [2]. The sample appeared to be well preserved and in good condition.
3. *Rufous Hare-wallaby or Mala (*Lagorchestes hirsutus*)*. Two DNA extractions were performed from bone (0.45 g) and tooth (0.48 g) material of a partially bleached and crushed skull which included the molar teeth (WA museum accession No. M47274) collected from Bernier Island, Western Australia in 1989. The age of the sample was unknown, but it appeared old and weathered, having been exposed to extreme ambient temperatures and periods of high humidity for an unknown length of time, similar to that described in the first specimen.

#### Preparation of bone and tooth samples (from Hummel 2003)

The outer surface (~0.5 mm) of the bone and teeth samples were removed using a dentist grinding drill (Horico Diamond Instruments, Model EWL K10) to remove possible surface contaminants. A small section of each sample (~0.5 g) was cut out and then pulverised in a mixing mill (Retsch Type MM2). The sample was decalcified by mixing 0.5 g of the powdered bone with 1.0 ml of 0.5 M EDTA (pH 8.3) via constant rotation for 18–24 h at room temperature. Samples were then centrifuged for 5 min at 3,000 rpm to pellet any remaining hard material. The supernatant was used for DNA extraction in an automated nucleic acid extractor (Gene Pure 341; Applied Biosystems) beginning with a Proteinase K (39 U/mg; lyophilised, Applied Biosystems) digestion step at 56°C for 1 h, and then followed by a standard phenol and chloroform extraction.

At the final automated step, samples were mixed with 5 µl silica particle solution (Glassmilk™, QBiogene), resulting in the DNA binding to the glass milk in the presence of iso-propanol (3.3 ml of 100% solution) and sodium acetate (100 µl of 2.0 M NaAc; pH 4.5; Applied

Biosystems). The mixture was gently mixed for 10 min, and the silica particles collected on a filtration membrane (Applied Biosystems). The filtration membranes containing the DNA/silica samples were manually removed from the automated extractor and washed with 80% ethanol then allowed to air dry. The eluted DNA/silica samples were re-suspended in 50 µl of sterile water (Ampuwa™, Fresenius).

The quantity and quality of the DNA extract was determined using a scanning photometer (Secomam, Jouan). In order to remove the silica particles prior to spectrophotometry, the extracts were vortexed and agitated at 56°C for 10 min, then centrifuged for 10 s at 5,000 rpm to pellet the glass milk. DNA extract was used to measure the absorbance from  $\lambda$  200 to 400 nm with DNA quantity determined at 260 nm. The distribution of the curve generated could be used to determine the quality of the extract, following the approach of Hummel [6]. The extracts were ranked as being low, medium or high quality extracts. Low quality had poor DNA yield and associated co-purification of extraneous material and high quality had high DNA yield with little co-purification of other material, such as proteins (Table 1).

#### Samples and source material for non-ancient DNA analysis

In the same laboratory as the ancient DNA extractions were performed, a control DNA extraction (from human cheek cells) was also carried out using a Chelex™ protocol [22]. This ‘contamination control sample’ was used in each PCR to investigate that the PCR-primers were not targeting human DNA (the most likely source of contamination; see below).

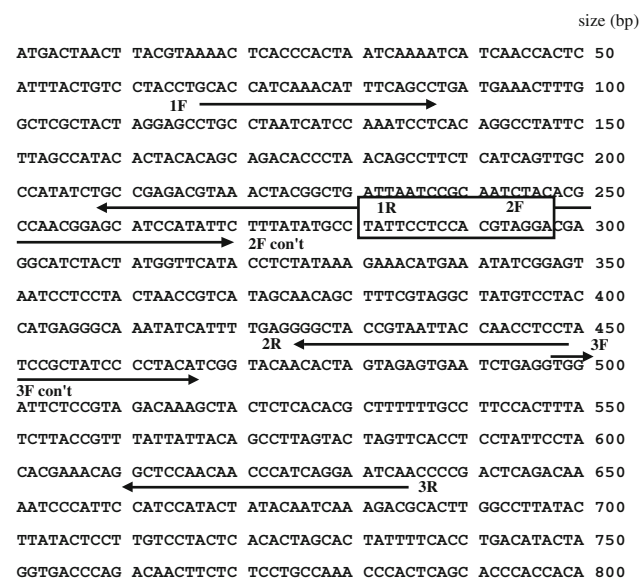
In a separate laboratory, DNA was extracted from a blood sample from the 21 marsupial species used in primer design using a standard salting out procedure [23] for use as reference samples.

#### Primer design

We trialled the commonly used ‘universal’ *cytochrome b* primers [18, 24] on a range of ancient DNA extracts ( $n = 7$ ) following the procedures in Alacs et al. [25]. The success of these trials was very low (see results section) and so to improve amplification, primers were specifically designed to amplify relatively small DNA products, suitable for highly degraded DNA sources [6].

A human mitochondrial sequence was aligned using the ‘PrimerSelect’ module of the Lasergene™ program (DNASTar Inc) with sequences from 21 marsupial taxa (12 are shown in Fig. 1), with the remaining nine taxa being the brush wallaby (*Macropus irma*), western grey





**Fig. 1** A schematic representation of 800 base-pair sequence of the mitochondrial *cytochrome b* gene from the Eastern grey kangaroo (*Macropus giganteus*; GenBank accession number MGU87137 [26]), with the approximate size of fragments generated from the PCR using the three new primer pairs given above the sequence to which they refer

kangaroo (*M. fuliginosus*), quokka (*Setonix brachyurus*), rufous rat kangaroo (*Aepyprymnus rufescens*), red-legged pademelon (*Thylogale stigmatica*), spectacled hare-wallaby (*Lagorchestes conspicillatus*), rufous hare-wallaby (*Lagorchestes hirsutus*), allied rock wallaby (*Petrogale*

*assimilis*) and bridled nailtail wallaby (*Onychogalea fraenata*).

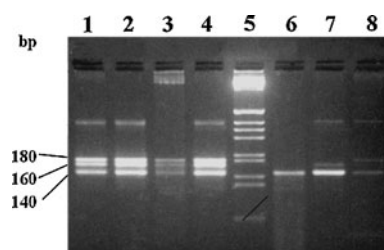
Primers, and where appropriate partially degenerative primers, were designed in conserved regions. Wherever possible, care was taken to incorporate mis-priming at the 3' end with the human sequence in at least one of the primer sequences (Fig. 1). This was done in order to prevent amplification of potential human DNA contaminants. In addition, pentamer free energy plots (kcal/mol) were used to increase the theoretical binding ability, particularly at the 3' end of the primers, as discussed by Hummel [6]. Three pairs of macropod primers were designed (named *Macytb1*, *Macytb2* and *Macytb3*) and the products of these gave predicted fragment sizes of 164, 188 and 202 bp, respectively (primer sequences given in Table. 2 and products shown schematically in Fig. 1). The total of the three fragments encompass a large amount of the region amplified with the single pair of 'universal' primers, the only section not amplified was 17 base pairs between the first and second sets of primers, which corresponded to a highly conserved region in the Macropodidae.

We also designed the primers to amplify the three fragments in a multiplex PCR (with the six primers), giving three distinct products in a single reaction. As the target sequences were of different size they were able to be resolved adequately on agarose (4%) gels (Fig. 2) to determine PCR success. For definitive species identification the three PCR fragments were sequenced.

**Table 2** Primer sequences for the *Macyt3* sequences for 12 of 21 marsupial species used for the design of primers for the *cytochrome b* region in marsupials (the *Macyt1* and *Macyt2* primer alignments are not shown, but the sequences are given at the bottom of the figure)

Reference / sequence taxa	Marsupial <i>cytochrome b</i> No. 3 primer sequence ( <i>Macyt3</i> )	
	Forward (F)	Reverse (R)
<b>primer sequence</b>	<b>GTGGATTCTCCGTAGACAAAG</b>	<b>GTCTTTGATTGTATAGTAKGGATGG</b>
Eastern grey kangaroo	.....	.....C.....
Dibbler	...T...G.A.....	A....A..C.....G...
Common wombat	.....	.....A.CG.....G.A.
Brushtail Possum	.A.....	A....A..G..C.....G...
<i>Antechinus minimus</i>	.C..T..T.GA.....	A....A..G.....G...
Wallaroo	.G.....	A.....G..C..A..A..G...
Koala	...G.....T...	.....A..G.....
Numbat	.C..C..T..A....T...	.....A..G..C....C..G...
Red kangaroo	.....	A.....C..A....G...
Thylacine	.A.....G..G.....	A.....G.....C..G...
Long-tailed dunnart	.A..C..T..A..T..T...	A....A....C.....G...
Tammar	.....A.....	.....G...
Human	.A..C.A...A.....GTC	.....C.....
<i>cytochrome b</i> primer sequence <i>Macyt1</i>	CTGCCTAATCATCCAAATCYT	GGCATATAAAGAATATGGATGCT
<i>cytochrome b</i> primer sequence <i>Macyt2</i>	CGAGGCATCTACTATGGTTCATA	CACCTCAGATTCACCTCTACTAGKGT

All primer sequences are given 5'–3' orientation



**Fig. 2** Agarose gel electrophoresis showing discrimination of the three primer products. Lane 1–4 are samples Gö 01–1–Gö 01–4 (Table 1). Lane 5 is a molecular weight standard (reading from bottom to top are 75, 134, 154, 201, 220, 298, 344, 396 and 506/517 base pairs). Lane 6 shows a single small band of 142 bp from sample Gö03-1, lane 7 shows bands from the same sample showing products at 142 and 162 bp and in lane 8 sample Gö 03-2, showing products at 142 and 180 bp. These varying sized bands were generated by using combinations of the three primer sets

### PCR conditions

All PCRs for the three new ‘ancient’ DNA primer pairs (for fragments Macytb1, 2 and 3; Table 2) were initially carried out in a multiplex PCR of a total volume of 50 µl with 1U of AmpliTaqGold<sup>TM</sup> DNA polymerase (Applied Biosystems), approximately 100 ng of template DNA (which included the silica suspension), 1.0 pmol of each primer, 200 µM dNTPs (solutions of dATP, dCTP, dGTP, dTTP; Sigma–Aldrich), 2 mM MgCl<sub>2</sub>, and 1× polymerase buffer II (100 mM Tris–HCl; pH 8.3, 500 mM KCl) supplied by the manufacturer. The reaction mix was overlaid with Mineral oil (Nujol<sup>TM</sup>; Applied Biosystems), which helped to reduced aerosol contamination when opening tubes. After an initial 10 min denaturation step at 94°C, the reaction tubes were exposed to 45 cycles of the following protocol: 1 min at 94°C; 1 min at 50°C; 1 min at 72°C, with a final extension step of 30 min at 64°C. These products were resolved on a 4% agarose gel (Fig. 2). All PCR amplification procedures included a negative and a non-marsupial (human DNA) control.

In a separate laboratory, the reference *L. hirsutus* sequence was obtained by PCR amplification using the *cytochrome b* universal primer L14724 (5′ GAAGCTT-GATGAAAAACCATCGTTG 3′ [24]) and the new reverse primer Macytb3R (Fig. 2), with a final product size of 662 bp.

### Sequencing conditions

The multiplex PCR products were sequenced directly using *Taq* cycle sequencing. This included a re-amplification reaction for each of the three products. The re-amplifications are carried out separately each using one primer of the respective primer pair as a sequencing primer. One microliter of the initial PCR was used as a template for the cycle sequencing using the Big-Dye terminator<sup>TM</sup> cycle

sequencing chemistry (ABI PRISM, Applied Biosystems). The PCR conditions were: 30 s. 96°, 15 s. 50°C, 4 min. 60°C, 30 cycles.

The cycle sequencing products were electrophoresed using an automated DNA sequencer (model 310; ABI systems, Bethesda, Md.). Other reference sequences were obtained from GenBank (see Table 2), and these were from the complete mitochondrial sequences of the euroo (*Macropus robustus* [26]) and the eastern grey kangaroo (*Macropus giganteus* [26]). All sequences were edited and aligned by eye using SeqEd (Applied Biosystems, Perkin-Elmer).

## Results

### Samples and DNA extractions

Amplifiable DNA was successfully obtained from each bone and tooth sample (Fig. 2), although the quantity of the DNA varied widely, both with repeated extractions of a single specimen and among different specimens (Table 1). In general, bone material gave significantly greater yields of DNA than did tooth material ( $F = 410$ ;  $df = 1$ ;  $P = 0.003$ ).

### PCR primers and sequencing success

#### ‘Universal’ primer trials

PCR amplifications using the widely utilised ‘universal primers’ [18, 21, 24] were attempted for the seven ancient DNA extracts (each sample run twice) and the control samples (negative and human). Only two PCR (662 bp) products were obtained and these were sequenced. One derived from the human DNA control, and the other from one of the Gö01-1 samples, which aligned using the BLAST search (<http://www4.ncbi.nlm.nih.gov/BLAST/>) with *Macropus robustus* (GenBank accession # Y10524). Despite the relatively high DNA concentration (160 µg/µl, Table 1) the fact that only one amplification attempt (from repeated PCRs) of the Gö01-1 sample was successful suggests that very few larger intact fragments were present in the extract. This corresponds to the general experiences in ancient and degraded DNA analysis.

#### New ‘degraded’ DNA primer trials

All of the extracts were amplified using the three newly designed primer sets. Again this was carried out with duplicate PCRs and included the negative and the human controls. In contrast to the amplification attempts using the

**Table 3** Size of sequenced product and similarity to the most closely aligned GenBank sequence of the three new primers for amplifying the *cytochrome b* region in three macropod samples (given in Table 1)

Species	<i>Macropus robustus</i>	“Notomys”	<i>Lagorchestes hirsutus</i>
Sample number	Gö01	Gö02	Gö03
Product length			
Macytb1 primers	144	141	142
Macytb2 primers	180	n/a <sup>b</sup>	190
Macytb3 primers	162	165	162
GenBank alignment	Y10524 ( <i>Macropus robustus</i> )	AY099273 ( <i>Lagorchestes hirsutus</i> )	AY099274 ( <i>Lagorchestes hirsutus</i> )
Sequence similarity <sup>a</sup>	99.9%	99.9%	98.6%

Each sample was amplified and sequencing independently

<sup>a</sup> Sequence similarity to the most closely aligned data from GenBank

<sup>b</sup> No product was obtained, despite repeated attempts

“universal primers” of Kocher et al. [18] the human control sample remained blank with the newly designed primer set. Also in the negative control amplification products were not detectable.

From the sub-fossil Euro (*Macropus robustus*, samples Gö01-1–Gö01-4), the multiplex PCRs each resulted in three distinct-sized PCR products (Fig. 2), giving a total sequence length of 486 bp (Table 3). The duplicate amplifications resulted in identical sequences, and showed 99.98% homology to *M. robustus* from Genbank (accession # Y10524).

For the second specimen (the mis-labelled museum specimen, Gö02-1), only the first (cytb1) and third (cytb3) fragments could be amplified, despite repeated (four) amplification attempts. Nonetheless, for these two successfully amplified fragments, with a total sequence length of 306 bp, the resulting sequences showed 100% sequence identity to the rufous hare-wallaby (*Lagorchestes hirsutus*) reference sequence.

The third specimen, a partial skull from the Rufous hare-wallaby (*Lagorchestes hirsutus*, samples Gö03-1 and Gö03-2) yielded PCR fragments from each of the three amplified regions (total sequence length of 494 bp). However, due to an obviously stronger degree of DNA degradation the fragments were not always obtained simultaneously from each sample but at least the two larger fragments were only amplified randomly (Fig. 2). This phenomenon is commonly described as stochastic amplification. The analysis of the resultant sequences of a total length of 494 bp confirmed the specimen to be near-identical (99.98%) to the *L. hirsutus* reference sequence.

## Discussion

Although the extraction and amplification of degraded DNA is well known, it remains underutilized and described in only a few reports investigating marsupial remains e.g.

[8, 13, 14]. In this study we set out to develop a marsupial-specific approach to the analysis of degraded DNA and have successfully amplified DNA from marsupials that had been preserved or found in a variety of different states of preservation. Specimen sources ranged from appropriately curated museum vouchers to material that had been found in the field. Being able to obtain DNA from a variety of sources are important because an enormous amount of marsupial material are accessible as sub-fossil specimens only, and many of the existing material derives from species that are now extinct.

We described the development and utilisation of a set of three primer pairs for the amplification of *cytochrome b* PCR fragments suitable for amplifying degraded DNA from marsupial samples. The new primer set offers a number of advantages to more conventional primers in the approach to ancient and degraded DNA. Firstly, the simultaneously amplified PCR fragments are relatively short (164, 188 and 202 bp), and are therefore like the characteristics of degraded DNA. Secondly, the simultaneous amplification of different fragments in a multiplex approach saves valuable sample materials. Thirdly, the use of primers where the focus was on species-specificity, avoids the amplification of potentially present contaminants. The experiments showed that only a few mismatches at the 3' ends of the primers are sufficient to discriminate against human DNA which is the most probable contaminant in ancient DNA analysis from field and museum specimens.

The importance of degraded DNA sources for investigating conservation questions with Australian marsupials will increase as more species experience decline. There are already a number of macropods with high conservation importance. To illustrate the use of this technology, there are four species of hare-wallabies of which all but one (*L. conspicillatus*) are either extinct (*L. leporides* and *L. asomatus*) or endangered (*L. hirsutus*). In mainland Australia, populations of the mala (*L. hirsutus*) persisted in

the Great Sandy and Gibson Deserts until the 1950s. By 1960 only two small colonies of mainland populations of the mala remained in the Tanami Desert (about 500 km north-west of Alice Springs). One colony became extinct in late 1987, destroyed by a single fox. The second colony was wiped out by wildfire in 1991. The mala is now classified as endangered, and has been extinct on mainland Australia for over a decade [27]. The species now persists in a single fenced enclosure in central Australia, and on three offshore islands. It should now be possible to exploit the vast amounts of museum and sub-fossil material to investigate historical and conservation questions in these and many other macropod species. An analysis system that combines primers specific for marsupials, small target sizes, and a multiplex PCR amplification approach will clearly be in favour in future studies using marsupial degraded DNA.

### Keypoints

1. Wildlife forensic investigations are important for enforcing legal areas such as ‘truth in labelling’ of food, wildlife seizures and in monitoring the international trade in wildlife and its products.
2. Species identification is an important area of interest in wildlife forensics cases.
3. We describe a method that amplifies three short DNA fragments of the *cytochrome b* region of the mitochondrial DNA in a number of marsupial species.
4. They were designed as consensus sequences from 21 marsupial species. The primers also contained sequence intended to exclude human DNA, reducing the chance of amplifying contaminants.
5. The methods described in this manuscript offer a mechanism to generate data that may help define the past distribution of marsupials and place those distribution patterns in some context to climate regimes based on geological or paleontological studies.

**Acknowledgments** We are very grateful for the comments and improvements made to the original manuscript by the reviewers and editor. This study received financial support from the Australian Academy of Sciences, Department of Environment and Conservation and Murdoch University (SRF and Animal Research Institute).

### References

1. Alacs E, Georges A. Wildlife across our borders: a review of the illegal trade in Australia. *Aust J For Sci*. 2008;40(2):147–60.
2. Van Dyck S, Strahan R. *The mammals of Australia*. Sydney: New Holland Publishers; 2008.
3. Herrmann B, Hummel S. Ancient DNA recovery and analysis of genetic material from paleontological archaeological, museum, medical and forensic specimens. Berlin: Springer; 1993.
4. Lassen C, Hummel S, Herrmann B. Comparison of DNA extraction and amplification from ancient human bone and mummified soft tissue. *Internatl J Legal Med*. 1994;107:152–5.
5. Rosenbaum HC, Egan MG, Clapham PJ, Brownell RL, Malik S, Brown MW, et al. Utility of North Atlantic right whale museum specimens for assessing changes in genetic diversity. *Conserv Biol*. 2000;14:1837–42.
6. Hummel S. Ancient DNA typing methods, strategies and applications. Berlin: Springer; 2003.
7. Janczewski DN, Yukhi N, Gilbert DA, Jefferson GT, O'Brien SJ. Molecular phylogenetic inference from saber-toothed cat fossils of Rancho La Brea. *Proc Natl Acad Sci*. 1992;89:9769–73.
8. Krajewski C, Driskell AC, Baverstock PR, Braun MJ. Phylogenetic relationships of the thylacine (Mammalia: Thylacinidae) among dasyuroid marsupials: evidence from *cytochrome b* DNA sequences. *Proc Roy Soc Lond Series B*. 1992;250:19–27.
9. Wayne RK, Leonard JA, Cooper A. Full of sound and fury: the recent history of ancient DNA. *Ann Rev Ecol Syst*. 1999;30:457–77.
10. Thomas RH. Molecular ecology and evolution: approaches and applications. Berlin: Birkhauser Verlag; 1994.
11. Cardillo M, Mace GM, Gittleman JL, Jones KE, Bielby J, Purvis A. The predictability of extinction: biological and external correlates of decline in mammals. *Proc Roy Soc Lond Series B*. 2008;275:1441–8.
12. Cooper A. DNA from museum specimens. In: Herrmann B, Hummel S, editors. *Ancient DNA*. New York: Springer-Verlag; 1994. p. 149–65.
13. Taylor AC, Sherwin WB, Wayne RK. Genetic variation of microsatellite loci in a bottlenecked species—the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Mol Ecol*. 1994;3:277–90.
14. Krajewski C, Buckley L, Westerman M. DNA phylogeny of the marsupial wolf resolved. *Proc Roy Soc Lond Series B*. 1997;264:911–7.
15. Serena M. Reintroduction biology of Australian and New Zealand fauna. Chipping Norton: Beatty Surrey & Sons; 1995.
16. Armstrong LA, Krajewski C, Westerman M. Phylogeny of the dasyurid marsupial genus *Antechinus* based on cytochrome b, 12S rRNA, and protamine P1 genes. *J Mammal*. 1998;79:1379–89.
17. Krajewski C, Painter J, Driskell AC, Buckley L, Westerman M. Molecular systematics of New Guinean Dasyurids. *Sci New Guinea*. 1993;19:157–66.
18. Kocher TD, Thomas WK, Meyer SV, Edwards SV, Paabo S, Villablanca FX, et al. Dynamics of mtDNA evolution in animals: amplification and sequencing with conserved primers. *Proc Natl Acad Sci*. 1989;86:6196–200.
19. Teletchea F, Bernillon J, Duffrais M, Laudet V, Hanni C. Molecular identification of vertebrate species by oligonucleotide microarray in food and forensic samples. *J Appl Ecol*. 2008;45:967–75.
20. Branicki W, Kupiec T, Pawlowski R. Validation of cytochrome b sequence analysis as a method of species identification. *J For Sci*. 2003;48:1–5.
21. Verma SK, Singh L. Novel universal primers establish identity of an enormous number of animal species for forensic application. *Mol Ecol Notes*. 2003;3:28–31.
22. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques*. 1991;10:506–13.
23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nuc Acids Res*. 1988;16:1215.
24. Meyer A, Kocher TD, Basasibwaki D, Wilson AC. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature*. 1990;347:550–3.
25. Alacs E, Alpers DL, de Tores P, Dillon M, Spencer PBS. Identifying the presence of Quokkas (*Setonix brachyurus*) and other

- macropods using cytochrome *b* analyses from faeces. Wildl Res. 2003;30:40–7.
26. Janke A, Xu X, Arnason U. The complete mitochondrial genome of the wallaroo (*Macropus robustus*) and the phylogenetic relationship among monotremata, marsupialia, and eutheria. Proc Natl Acad Sci. 1997;94:1276–81.
27. Maxwell S, Burbidge A, Morris K. The 1996 action plan for Australian marsupials and monotremes Australasian marsupial and monotreme specialist group. Canberra: IUCN Species survival Commission, Environment Australia; 1996.



# Current issues in species identification for forensic science and the validity of using the cytochrome oxidase I (COI) gene

Linzi Wilson-Wilde · Janette Norman ·  
James Robertson · Stephen Sarre · Arthur Georges

Accepted: 5 June 2010 / Published online: 20 June 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** Species identification techniques commonly utilized in Australian Forensic Science laboratories are gel immunodiffusion antigen antibody reactions and hair comparison analysis. Both of these techniques have significant limitations and should be considered indicative opinion based tests. The Barcode of Life Initiative aims to sequence a section of DNA (~648 base pairs) for the **Cytochrome Oxidase I mitochondrial gene (COI) in all living species on Earth**, with the data generated being uploaded to the Barcode of Life Database (BOLD) which can then be used for species identification. The COI gene therefore offers forensics scientists an opportunity to use the marker to analyze unknown samples and compare sequences generated in BOLD. Once sequences from enough species are on the database, it is anticipated that routine identification of an unknown species may be possible. However, most forensic laboratories are not yet suited to this type of analysis and do not have the expertise

to fully interpret the implications of matches and non matches involving a poorly sampled taxa (for example where there are cryptic species) and in providing the required opinion evidence. Currently, the use of BOLD is limited by the number of relevant species held in the database and the quality assurance and regulation of sequences that are there. In this paper, the COI methodology and BOLD are tested on a selection of introduced and Australian mammals in a forensic environment as the first step necessary in the implementation of this approach in the Australian context. Our data indicates that the COI methodology performs well on distinct species but needs further exploration when identifying more closely related species. It is evident from our study that changes will be required to implement DNA based wildlife forensics using the BOLD approach for forensic applications and recommendations are made for the future adoption of this technology into forensic laboratories.

L. Wilson-Wilde (✉)  
ANZPAA National Institute of Forensic Science, PO Box 415,  
Melbourne, VIC 3005, Australia  
e-mail: linzi.wilson-wilde.nifs@anzpaa.org.au

J. Robertson  
Forensic and Data Centres, Australian Federal Police, Weston,  
ACT, Australia

L. Wilson-Wilde · S. Sarre · A. Georges  
Institute for Applied Ecology, University of Canberra, Canberra,  
ACT, Australia

L. Wilson-Wilde · J. Norman  
Museum Victoria, Carlton Gardens, VIC, Australia

J. Norman  
Department of Genetics, University of Melbourne, Parkville,  
VIC, Australia

**Keywords** COI · Cytochrome oxidase I · DNA analysis · Species identification · Wildlife crime · Forensic science laboratories · Sequencing · Barcode of Life · BOLD · Barcoding

## Introduction

Wildlife crime is a broad ranging offense covering issues such as loss of biodiversity (in the removal of specimens from the gene pool), habitat destruction (during the collection of specimens), undermining of the legal trade, and the potential to increase the transmission of diseases and exposure of the Australian environment to the invasion of pest species (when specimens are illegally imported) [1–4]. It is thought to cost between US\$10 and US\$20 billion

dollars worldwide annually [5–7]. A critical element to solving most wildlife crime is the identification of species through conventional morphological as well as biochemical and molecular approaches. Standard species identification, whilst offered in all major Australian forensic laboratories, is not used in every case, but can be an important tool available in forensic analysis. A species discrimination test has application in criminal investigations through the identification of blood or hairs on suspects, illegal poaching, exportation of Australian native species, and the importation of exotic species [3]. Broader applications include confirmation of species and scat content analysis in ecological studies [8–10], food product analysis [11–13], Chinese medicine content analysis [14] and animal feed analysis for the identification of processed animal proteins [15].

Most investigations conducted by Australian forensic laboratories involve crimes against the person and property crime (domestic crime). In these cases, animal species identification is usually considered an adjunct to forensic biological testing and, when more complex analysis is required, usually relies upon the expertise of museums and universities. Species identification tests more commonly used in broad based Forensic laboratories involve hair comparison analysis [16, 17] and the Ouchterlony test [18–20]. The former requires a database of known hairs and a high level of expertise which can be problematic as there are a limited number of experts in this field. The latter is a gel immuno-diffusion antibody/antigen reaction which, because of the loss of biological activity of the proteins and narrow parameters for optimal detection [18, 20, 21], is limited in its application. In addition, the necessity of raising specific antigens, in laboratory animals, means that the test is available for a very limited number of species (~10) and control blood samples for each species for which a test is available also need to be sourced [20]. Although the Ouchterlony test is currently used as a confirmatory (definitive) test, it has been found to produce cross-reactions in non-target species [22, 23], which could lead to confusing results, or worse miss-identifications. It is therefore the authors opinion that in a forensic environment this test is being applied incorrectly and should be considered only as a presumptive (indicative) test.

Other tests currently available for species identification include molecular techniques commonly involving sequencing of either mitochondrial or nuclear DNA [24–26]. Wildlife crime can involve any species although generally there is a focus on CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) listed species. Species identification can also be complex as the concept of what a species is as an entity is still being debated [27, 28]. Genetic variation exists between and within species and discrete boundaries

between particularly closely related species can be difficult to ascertain. Under these circumstances providing a definitive assessment becomes less likely and may be considered more as opinion evidence based on the underpinning knowledge of the nature of a species (such as dog and wolf [29]).

Defining biological units of concern, whether they be species or populations, or some other variants, is a key issue in wildlife forensics. A species may not always be easily defined by genetic means, particularly if the baseline knowledge of genetic variation within and among species is not well characterized. Moreover, the assignment of provenance in cases where some populations or species are of greater conservation interest is most likely to be based on probabilities, and the ability to determine to which group a given sample can be assigned will be related to the type of marker being used and how well it has been characterised across the group of concern. The DNA analysis of two closely related populations may result in most of the specimens being grouped into one of the two populations, however, there will always be outliers in the 'grey area' between the two populations that cannot be readily assigned to one group or the other [27].

The task of identifying species is underpinned by the discipline of evolutionary biology, particularly the fields of taxonomy (the classification of organisms into groups according to their morphological similarity), phylogeography (the evolutionary relatedness of organisms in space and time), and population genetics (distribution of genetic variation within and among populations) [30]. The latter two of these fields use molecular DNA techniques as their basic tool for defining taxonomic units. For a sample to be accurately placed into a species group these three aspects of underpinning knowledge need to be investigated and applied at a level appropriate to the problem at hand, taking into account the level of variation between two samples. The opinion evidence should therefore be based on experience and judgment using a high of level expertise to evaluate the match [25].

The most commonly applied methods of analysis for species involve the use of mitochondrial DNA, particularly cytochrome b and cytochrome oxidase I [25]. Cytochrome b has traditionally been used in forensic science for species identification [31–34], however, comparison databases are either public access with limited quality assurance or in-house databases which must be created. Whilst both of these can be successful they may not meet accreditation requirements or contain sufficient reference sequences.

The COI marker has been identified as the marker of choice for species discrimination by the Barcode of Life Project as there are a broad range of primers applicable across a wide range of taxa [35], a relatively short sequence and changes to the sequence occur more slowly than Cyt b [36].

The COI gene can be used to accurately place individuals not only into the correct higher levels (phyla and order) but also into the correct species [37, 38] and, through the bar-code of life project ([www.barcodinglife.org](http://www.barcodinglife.org)), is the focus of a world-wide effort to characterize, using molecular techniques, all living animals through a single genetic entity. The project aims to catalog and database a section of DNA sequence (~648 base pairs) for the COI mitochondrial gene [39, 40]. The database can then be challenged with an unknown sequence and compared to all existing sequences held on the database to enable the identification of the questioned sequence. The database is freely available to researchers and scientists can place sequences from vouchered specimens onto the database [41]. The Barcode of Life Database (BOLD) is aiming to obtain three sequences from each species in order to capture the geographical variation of the species, as there will be some variation in the sequence within a species. Additionally, for each sequence the relevant trace files (raw data) must also be submitted. These measures are to ensure the quality of the data submitted, and whilst they are an important distinction to other database systems which do not require this information, this still falls short of the standard forensic validation requirements [42].

The COI gene offers forensic scientists an opportunity to use the marker for species identification where unknown samples can be analyzed and sequences generated compared against the BOLD. It is possible that once sequences from enough species are on the database identification of unknown samples may be possible. Whether the COI gene is capable of this enormous task is still to be determined and it has already been postulated that additional markers may need to be used [43–47]. In addition, applying any new technique from the general scientific arena to a forensic one presents many problems, both practically and theoretically. Whilst a preliminary validation study has been conducted against forensic standards [48] the system is still to be tested and implemented in a forensic environment in Australia.

This study aims to look at the current method of species identification and how the COI methodology could be applied in a forensic environment. It examines some of the potential issues that could arise for laboratories, and by examining some forensically relevant species recommendations can be derived for the implementation of this type of species identification test.

## Materials and methods

### DNA extraction

Samples obtained for the study are detailed in Table 1 and reflect forensically relevant domesticated and Australian

native species. Species were identified based on those that would be applicable from a forensic perspective along with those that might be used to test the COI methodology by incorporating distantly and closely related species. Those from the Victoria Police Forensic Science Centre were control blood samples on cloth, whilst those obtained from Museums were tissue samples (usually heart, liver or muscle tissue). The human sample was liquid blood in EDTA and the spider sample was collected by the author. All tissue samples were either extracted using standard phenol chloroform procedures [49] or Qiagen DNeasy® Blood and Tissue Kit (Qiagen part # 69506) using the animal tissue bench protocol. Blood samples were extracted using the Qiagen DNAeasy kit blood bench protocol. Negative controls were used in all extractions.

### Polymerase chain reaction and sequencing

Polymerase chain reactions were performed in a 25 uL reaction using 12.5 uL of master mix made up of 12.5 uL Go Taq Green® Master Mix 2× (Promega cat # M712C), 0.75 uL primer (see below) and 0.3 uL 25nM magnesium chloride (Qiagen) per sample. 12.5 uL of master mix was added to a 96 well plate (cat # T323-96 N) after which 12.5 uL of each sample was added to the appropriate wells. Negative controls were used in all PCRs. Samples were amplified under the following reaction conditions: initial incubation of 94°C 3 min; followed by 40 cycles of 94°C 30 s, 50–57°C 30 s, 72°C 60 s; and a final extension step of 72°C 7 min.

PCR amplification success rates for the Folmer [35] primers were found to be variable (data not shown) requiring a new forward primer to be designed using aligned mammalian sequences obtained from Genbank. The primer was designed and quality checked using Primer 3 [50]. The reverse primer was that of Folmer. The primer sequences used were COILWW26F caa tgc tta cct cag cca ttt tac and LC1490 taa act tca ggg tga cca aaa aat ca and targeted a region of ~700 base pairs at the 5' end of the COI gene. Sequences provided above are excluding M13 tails; forward: gta aaa cga cgg cca gt and reverse: cag gaa aca gct atg ac, which were attached according to Australian Genome Research Facility (AGRF) requirements for high throughput barcoding analysis [www.agrf.org.au](http://www.agrf.org.au).

PCR products were visualized on a 1.2% agarose gel with Hyperladder II (Bioline) and ethidium bromide staining. PCR products were forwarded to AGRF for PCR clean-up, sequencing reaction targeting the M13 tails and sequencing clean-up. All samples were then sequenced in both directions by AGRF with raw data files returned for analysis. Run data raw files were then edited using the SEQUENCHER version 4.1.4 (GeneCodes Corporation

**Table 1** Origin for samples used in this study

Class	Species	Common name	Source	Reference number
Mammalia	<i>Homo sapiens</i>	Human	Authors	NA
	<i>Felis catus</i>	Cat	VPFSC	NA
	<i>Canis lupus familiaris</i>	Dog	VPFSC	NA
	<i>Canis lupus</i>	Wolf	Genbank	NC008092
	<i>Capra aegagrus</i>	Goat	VPFSC	NA
	<i>Ovis aries</i>	Sheep	VPFSC	NA
	<i>Equus caballus</i>	Horse	VPFSC	NA
	<i>Bos taurus</i>	Cow	VPFSC	NA
	<i>Sus scrofa domestica</i>	Pig	Genbank	NC012095
	<i>Panthera leo</i>	Lion	VM	W5235
	<i>Arctocephalus pusillus</i>	Australian fur seal	VM	W5371
	<i>Tursiops truncatus</i>	Bottlenose dolphin	VM	W5076
	<i>Kogia breviceps</i>	Pygmy whale	Genbank	AJ554055
	<i>Oryctolagus cuniculus</i>	Rabbit	Genbank	FJ958343
	<i>Rattus rattus</i>	Black rat	ANWC	M29955
	<i>Mus musculus</i>	Mouse	VPFSC	NA
	<i>Cebus species</i>	Capuchin monkey	VM	W5282
	<i>Pongo abelii</i>	Orangutan	Genbank	NC002083
	<i>Pan troglodytes</i>	Chimpanzee	Genbank	X93335
	<i>Phascolarctos cinereus</i>	Koala	ANWC	M24360
	<i>Trichosurus vulpecula</i>	CBT possum	ANWC	M28611
	<i>Petaurus breviceps</i>	Sugar glider	ANWC	M16819
	<i>Pseudocheirus peregrinus</i>	CRT possum	ANWC	M16323
	<i>Dendrolagus lumholtzi</i>	Tree kangaroo	ANWC	M16996
	<i>Macropus agilis</i>	Agile wallaby	ANWC	M16443
	<i>Isodon obesulus</i>	Southern brown bandicoot	ANWC	M29952
	<i>Antechinus swainsonii</i>	Dusky antechinus	ANWC	M29456
	<i>Ornithorhynchus anatinus</i>	Platypus	ANWC	M29302
Aves	<i>Gallus gallus gallus</i>	Chicken	Genbank	AP003322
	<i>Gymnorhina tobica</i>	Australian magpie	ANWC	51356
	<i>Passer domesticus</i>	House sparrow	Genbank	FJ027965
	<i>Chenonetta jubata</i>	Australian wood duck	ANWC	51280
Reptilia	<i>Tiliqua scincoides</i>	Eastern blue tongue lizard	ANWC	R06901
	<i>Pseudechis porphyriacus</i>	Red-bellied black snake	ANWC	R06672
	<i>Hypsiglena jani texana</i>	Texas night snake	Genbank	EU728592
Amphibia	<i>Crinia signifera</i>	Common Eastern Froglet	ANWC	A02048
Arachnida	<i>Pholcidae</i>	Spider	Authors	NA
Osteichthyes	<i>Amphiprion latezonatus</i>	Wide banded anemone fish	VM	JR1

Key: VPFSC Victoria Police Forensic Science Centre, VM Victoria Museum, ANWC Australian National Wildlife Collection

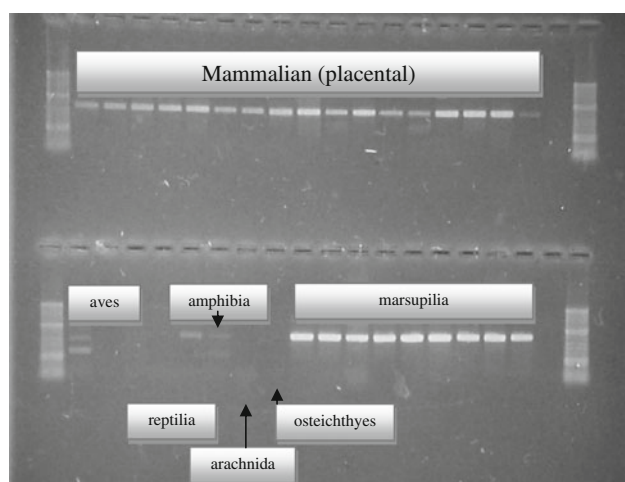
1991–2002) software package and consensus sequences produced for each sample.

Consensus sequences were aligned using the CLUSTAL module in MEGA version 4 [51] with manual adjustments. Sequence divergence was calculated using the Kimura 2-parameter (K2P) model of pairwise base substitution [52]. A neighborhood-joining tree [53] and a maximum parsimony tree [54] were developed using K2P distances

showing intraspecific variation in MEGA, including bootstrap analysis using 1,000 replications.

## Results

The COI paired primers amplified well in mammals (Fig. 1) but as expected did not amplify well in aves,



**Fig. 1** Amplification success using the new COI primer pair. All mammalian samples amplified with strong results using the new COI primer pair. However, amplification in other taxa was not successful

reptilia, amphibia, arachnida or osteichthyes. To include those groups in the phylogenetic analysis, we supplemented our data where possible with Genbank sequences (refer Table 1 for details). The amplification consistency of the new primer pair was found to be much better than the original Folmer primer pair, particularly in the marsupial group and good quality sequences were generated from the new primer set.

The neighbourhood joining and the maximum parsimony trees were manually rooted using *Hypsiglena jani texana* and generally illustrate similar topography (Figs. 2 and 3). The overall groupings for both trees are as expected; for instance aves group together, primates also group together and *Felis catus* group with *Panthera leo*. There were however, some significant anomalies, particularly *Ornithorhynchus anatinus* grouping among the eutherians. Most exceptions are at the lower branch level where species are more closely related, in particular the marsupialia did not always group as expected; for instance *Trichosurus vulpecula* groups with *Antechinus swainsonii* in both trees.

Additionally, whilst *Dendrolagus lumholtzi* and *Macropus agilis* group together in both trees and with marsupilia in the neighbourhood joining tree, they group with *Equus caballus* in the maximum parsimony tree. However, there is no support for the positioning of *Dendrolagus lumholtzi* and *Macropus agilis* with *Equus caballus* in the maximum parsimony tree; with a bootstrap value of 10.

Whilst there is strong support at the lower branch levels for paired species with bootstrap values between 70 and 100, at the deeper nodes there is little or no support. This would indicate that if the unknown sample had an equivalent or closely related reference sample on the database a strong indication as to the identification of the species

could be obtained. However, if no relevant comparison sample was held on the database little information may be gained as to the identification of the unknown sample.

## Discussion

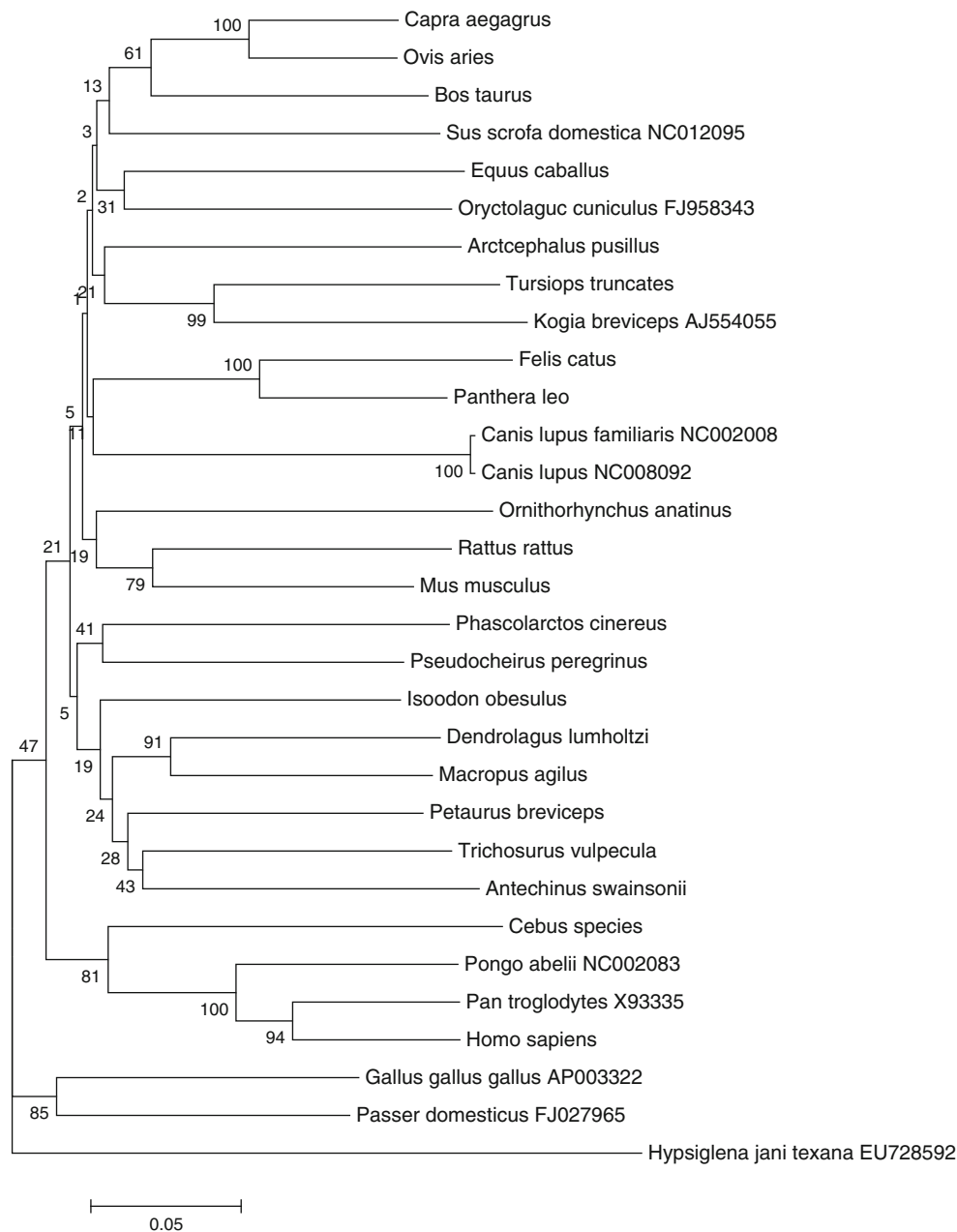
We found that the COI methodology was easy to use and fairly robust on the samples tested, however, these consisted mainly of blood and tissue samples which have sufficient levels of genomic DNA. Standard PCR equipment is used to conduct the analysis and samples could be run on the type of sequencer currently used by forensic laboratories. Once suitable universal primers have been developed the methodology is similar to current human DNA analysis methodology. However, review of run data sequences to obtain consensus sequences would require some training for scientists, including in the use of appropriate software to analyze the consensus sequences produced and, if required, the calculation of a weighting of any match. Additionally, dedicated laboratory space should be identified to separate the mitochondrial DNA analysis processes from the nuclear DNA based processes to reduce the chance of a contamination event. To support this clear policy and procedures need to be introduced along with appropriate validated methods.

With the set of data tested here we found that high confidence matches could be obtained for all domesticated species. However, when analyzing Australian native species further work to develop appropriate reference sequences is required.

One of the most significant issues for COI analysis and indeed all species identification using DNA analysis is the lack of robust primers. The most recognized ‘universal primers’ are the Folmer primers. The Folmer primers were originally designed for use in moths and butterflies (genus *Lepertotertia*) but they also tamplify in other phyla. However, as with all universal primers they do not amplify consistently in all species in a genus/family/order (but in the Folmer primer case they were not designed to). Here an additional new forward primer assisted in the amplification of mammalian samples but further primers would need to be designed to achieve full amplification of all species. It was evident that there were possible point mutations at the primer binding sites for some ad hoc species causing ineffective primer binding resulting in incomplete amplification. These sites could be investigated further for use as a possible Single Nucleotide Polymorphism based identification test.

Another major issue is the current lack of an extensive database, particularly one relevant in an Australian context. Whilst some Barcode of Life related groups have targeted certain classes or orders (e.g. fish) there has not been a law enforcement push to include relevant species, such as



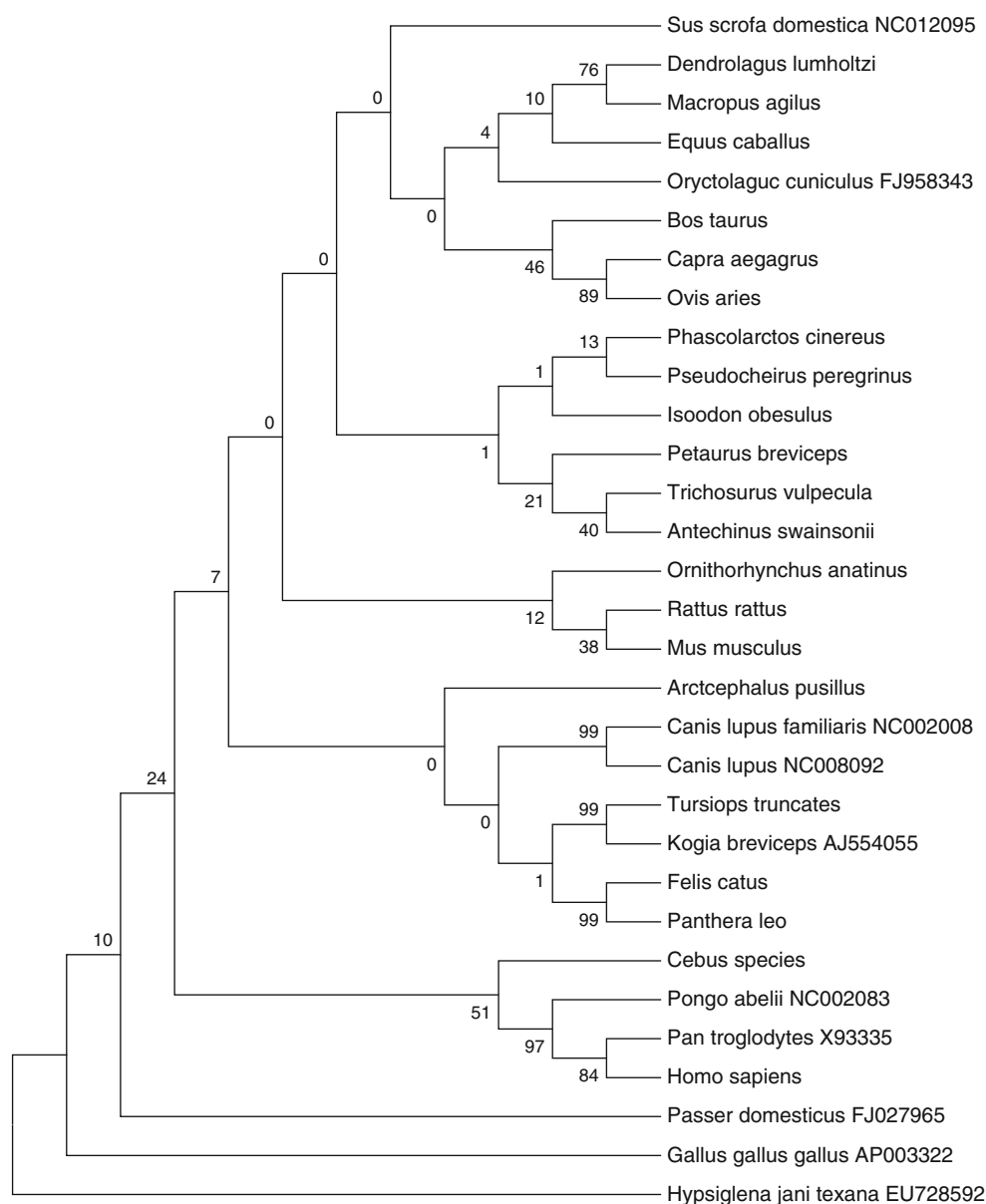


**Fig. 2** Neighbourhood joining tree of sequences obtained coupled with those sourced from Genbank. Bootstrap values given 100 replicates are provided

CITES listed species that would be more useful in a wildlife crime context. Without a good quality database of relevant species the COI marker cannot be in a position to provide a solution for species identification in a forensic context. However, a targeted drive from law enforcement in collaboration with research institutions could achieve this goal.

Training of personnel would also be required in the underpinning information needed to conduct and interpret species identification analysis (taxonomy, biogeography

and phylogenetic evolution). The significance of positioning on a phylogenetic tree along with the interpretation of bootstrap values would need to be understood, as would the concept of a species and what constitutes a species, such as the percent sequence variation between species. Whilst some species might be more confidently identified than others, such as with the domesticated animals studied here, it should never be forgotten that this is opinion evidence; regardless of the technique used in the identification process. Currently no forensic laboratory in Australia has staff



**Fig. 3** Maximum parsimony tree of sequences obtained coupled with those sourced from Genbank. Bootstrap values given 100 replicates are provided

with the expertise to present species identification evidence based on either mitochondrial or nuclear DNA analysis.

With the appropriate investment in the establishment of relevant databases, validated methods and procedures, and training of scientists, the COI marker system could be useful in species identification. However, the results presented here support the use of DNA markers in addition to COI such as cytochrome b for law enforcement purposes in the investigations of wildlife crime. It is recommended that a suite of markers are available, including multiple mitochondrial and nuclear markers and appropriate markers that might resolve more distantly related populations (and

therefore the deeper nodes on the tree) that could provide more information if the database does not hold a relevant reference species sample.

Due to the investment required in research, training and infrastructure to implement species identification it is unlikely all forensic laboratories will adopt species testing for a broad range of fauna. A more effective approach would be for one or two laboratories to develop a centre of excellence approach in collaboration with relevant academic partners. Before DNA based species testing could be introduced significant foundation research is still required, followed by more specific forensic validation. This would

be no trivial matter. The scale of wildlife crime is such that the development of a centre of excellence in Australia would be well justified.

### Key points

1. Species identification is opinion evidence based on underpinning information.
2. Prior to implementation of any species identification method into a law enforcement forensic environment relevant dedicated laboratory space, method validation and staff training would need to be conducted.
3. The COI gene offers a viable option for species identification, however, would need to be backed up by relevant databases and further research.

**Acknowledgments** The authors would like to thank the Australian Federal Police for funding this research. Specimens were donated by the Australian National Wildlife Collection Victoria Police Forensic Services Department and the Victoria Museum. The authors would also like to thank the two anonymous reviewers for their comments and suggestions in the improvement of this manuscript.

### References

1. Keller RP, Lodge DM. Species invasions from commerce in live aquatic organisms problems and possible solutions. *Bioscience*. 2007;57:428–36.
2. Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L, Pessier AP, Collins JP. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc Nat Acad Sci USA*. 2006;103:3165–70.
3. McDowell D. Wildlife crime policy and the law. Canberra: Australian Government Publishing Service; 1997.
4. Cook D, Roberts M, Lowther J. The international wildlife trade and organised crime: a review of the evidence and the role of the UK. United Kingdom: World Wildlife Fund; 2002.
5. Wyler LS, Sheikh PA. CRS report for congress—international illegal trade in wildlife: threats and U.S. policy; 2008.
6. Brack D. The growth and control of international environmental crime. *Environ Health Perspect*. 2004;112:80–1.
7. Holden J. By hook or by crook a reference manual on illegal wildlife trade and prosecutions in the United Kingdom. Bedfordshire: The Royal Society for the Protection of Birds; 1998.
8. Symondson WOC. Molecular identification of prey in predator diets. *Mol Ecol*. 2002;11:627–41.
9. Berry O, Sarre SD, Farrington L, Aitken N. Faecal DNA detection of invasive species: the case of feral foxes in Tasmania. *Wildl Res*. 2007;34(1):1–7.
10. Berry O, Sarre SD. Gel-free species identification using melt-curve analysis. *Mol Ecol Notes*. 2007;7(1):1–4.
11. Macedo-Silva A, Macedo-Silva SC, Barbosa MGA, Alkmin AJ, Vaz M, Shimokomaki M, Tenuta-Filho A. Hamburger meat identification by dot-ELISA. *Meat Sci*. 2000;56:189–92.
12. Martinex I, Danielsdottir AK. Identification of marine mammal species in food products. *J Sci Food Agric*. 2000;80(4):527–33.
13. Wong KL, Wang J, But PPH SPC. Application of cytochrome b DNA sequences for the authentication of endangered snake species. *For Sci Inter*. 2004;139:49–55.
14. Peppin L, McEwing R, Carvalho GR, Ogden R. A DNA based approach for the forensic identification of Asiatic black bear (*Ursus thibetanus*) in traditional Asian medicine. *J Forensic Sci*. 2008;53:1358–62.
15. Fumière O, Veys P, Boix A, von Holst C, Baeten V, Berben G. Methods of detection, species identification and quantification of processed animal proteins in feedingstuffs. *Base*. 2009;13:59–70.
16. Brunner H, Coman BJ. The identification of mammalian hairs. Melbourne: Inkata Press; 1974.
17. Robertson J, editor. Forensic examination of hair. 2nd ed. London: Taylor & Francis; 1999.
18. Ouchterlony O. Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor: Ann Arbor Science Publishers Inc; 1968.
19. Ouchterlony O, Nilsson LA. Immunodiffusion and immunoelectrophoresis. In: Weir DM, Herzerberg LA, Blackwell C, Herzerberg LA, editors. Handbook of experimental immunology, vol 1. 4th ed. Oxford: Blackwell; 1986. p. 32.1–32.50.
20. Saferstein R. Identification and grouping of Bloodstains. In: Saferstein R, editor. Forensic science handbook. New Jersey: Prentice Hall Inc; 1982. p. 267–96.
21. Fugate HG, Penn SR. Immunodiffusion technique for the identification of animal species. *J Assoc Off Anal Chem*. 1971;54:1152–6.
22. Bird GWG. Paradoxical findings in Ouchterlony tests. *Cell Mole Life Sci*. 2005;17:408.
23. Wilson MW, Pringle BH. Cross-reactions in the Ouchterlony plate: analysis of native and halogenated bovine serum albumins. *J Immun*. 1956;77:324–31.
24. Alacs E, Georges A, Fitzsimmons NN, Robertson J. DNA detective: a review of molecular approaches to wildlife forensics. *Forensic Sci Med Path*. 2010. doi:10.1007/s12024-009-9131-7.
25. Ogden R, Dawnay N, McEwing R. Wildlife DNA forensics—bridging the gap between conservation genetics and law enforcement. *End Sp Res*. 2009. doi:10.3354/esr00144.
26. DeYoung RW, Honeycutt RL. The molecular toolbox: genetic techniques in wildlife ecology and management. *J Wildl Manage*. 2005;69:1362–84.
27. Mallet J. A species definition for the modern synthesis. *Tree*. 1995;10:294–9.
28. Harrison RG. Molecular changes at speciation. *Annu Rev Ecol Syst*. 1991;22:281–308.
29. Olsen SJ. Origins of the domestic dog: the fossil record. Tucson: The University of Arizona Press; 1985.
30. Avise JC. Phylogeography: the history and formation of species. Massachusetts: Harvard University Press; 2000.
31. Parson W, Pegoraro K, Niederstätter H, Föger M, Steinlechner M. Species identification by means of the cytochrome b gene. *Int J Legal Med*. 2000;114:23–8.
32. Verma SK, Singh L. Noverl universal primers establish identify of an enormous number of animal species for forensic application. *Mol Ecol Notes*. 2002;3:28–31.
33. Bellis C, Ashton KJ, Freney L, Blair B, Griffiths LR. A molecular genetic approach for forensic animal species identification. *For Sci Inter*. 2203;134:99–108.
34. Hsieh HM, Haung LH, Tsai LC, Kuo YC, Meng HH, Linacre A, Lee JC. Species identification of rhinoceros horns using the cytochrome b gene. *For Sci Inter*. 2003;136:1–11.
35. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for the amplification of mitochondrial cytochrome c oxidase 1 from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol*. 1994;3:294–9.

36. Lynch M, Jarrell PE. A method for calibrating molecular clocks and its application to animal mitochondrial DNA. *Genetics*. 1993; 135:1197–208.
37. Hebert PDN, Cywinska A, Ball SL. Biological identifications through DNA barcodes. *Proc R Soc Lond B*. 2003;270:313–21.
38. Hebert PDN, Ratnasingham S, deWaard JR. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc R Soc Lond B*. 2003;270(Suppl):S96–S9.
39. Blaxter ML. The promise of molecular taxonomy. *Phil Trans R Soc B*. 2004;359:669–79.
40. Hebert PD, Stoeckle MY, Zemlak TS, Francis CM. Identification of birds through DNA barcodes. *PLoS Biol*. 2004;2(10):e312. doi:[10.1371/journal.pbio.0020312](https://doi.org/10.1371/journal.pbio.0020312).
41. Hanner RH, Schindel DE, Ward RD, Hebert PDN. FISH-BOL workshop report, August 26, 2005. For the workshop held at the University of Guelph, June 5–8, 2005 Ontario Canada 2005 <http://www.fishbol.org/news.php>.
42. Budowle B, Garofano P, Hellman A, Ketchum M, Kanthaswamy S, Parson W, van Haeringen W, Fain S, Broad T. Recommendations for animal DNA forensic and identity testing. *Int J Legal Med*. 2009;119:295–302.
43. Mallet J, Willmott K. Taxonomy: renaissance or tower of babel? *Trends Ecol Evol*. 2003;18:57–9.
44. Moritz C, Cicero C. DNA barcoding: promise and pitfalls. *PLoS Biol*. 2004;2(10):1529–31. doi:[10.1371/journal.pbio.0020354](https://doi.org/10.1371/journal.pbio.0020354).
45. Johnson NK, Cicero C. New mitochondrial DNA data affirm the importance of pleistocene speciation in North American birds. *Evolution*. 2004;58(5):1122–30.
46. Will KW, Rubinoff D. Myth of the molecule: DNA barcodes for species cannot replace morphology or identification and classification. *Cladistics*. 2004;20:47–55.
47. Erpenbeck D, Hooper JNA, Worheide G. CO1 phylogenies in diploblasts and the ‘Barcoding of Life’—are we sequencing a suboptimal partition? *Mol Ecol Notes*. 2006;6:550–3.
48. Dawnay N, Ogden R, McEwing R, Carvalho RS. Validation of the barcoding gene COI for use in forensic genetic species identification. *Forensic Sci Int*. 2007;173(1):1–6.
49. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
50. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, editors. *Bioinformatics methods and protocols: methods in molecular biology*. Totowa: Humana Press; 2000. p. 365–86.
51. Tamura K, Dudley J, Nei M. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol*. 2007;24:1596–9 (Publication PDF at <http://www.kumarlab.net/publications>).
52. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparison studies of nucleotide sequences. *J Mol Evol*. 1980;16:111–20.
53. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4:406–25.
54. Felsenstein J. Parsimony in systematics: biological and statistical issues. *Annu Rev Ecol Syst*. 1983;14:313–33.

Wilson-Wilde L, Norman J, Robertson J, Sarre S, Georges A. Current issues in species identification for forensic science and the validity of using the cytochrome oxidase I (COI) gene. *Forensic Sci Med Pathol*. 2010 Sep;6(3):233–41.

## Lethal consequences of ingested foreign material in seabirds

Ella Carapetis · Aaron J. Machado ·  
Roger W. Byard

Accepted: 20 April 2010 / Published online: 19 May 2010  
© Springer Science+Business Media, LLC 2010

A pied cormorant (*Phalacrocorax varius*) was found sleeping on rocks in a coastal region near Adelaide. Examination revealed entanglement by fishing line with superficial wounds to the left leg. The cormorant was also reported to have ingested a number of fishing hooks, however, the exact location of the hooks was not known. In addition, the bird was found to be underweight and shocked. X-ray examination revealed two ingested fishing hooks embedded in the esophagus in the midneck, and in the stomach (Fig. 1). These had caused the cormorant to be unable to extend its neck for diving, feeding and/or flying. Surgical intervention was undertaken.



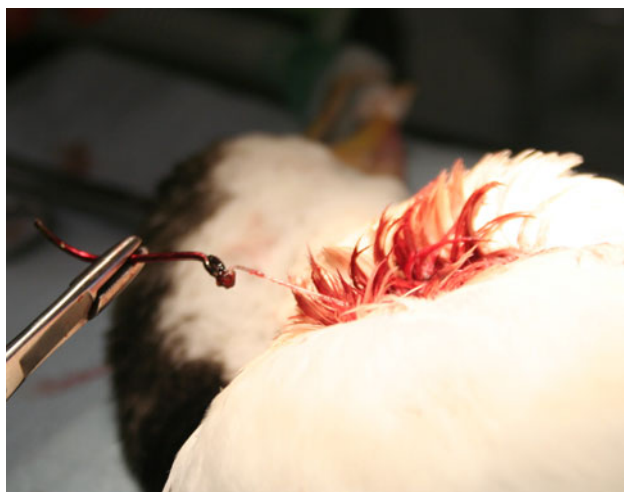
**Fig. 1** An antemortem X-ray of a pied cormorant (*Phalacrocorax varius*) showing two ingested fishing hooks embedded in the esophagus in the midneck and in the stomach

---

E. Carapetis · A. J. Machado · R. W. Byard (✉)  
Discipline of Pathology, The University of Adelaide,  
Adelaide, SA, Australia  
e-mail: roger.byard@sa.gov.au

E. Carapetis · A. J. Machado · R. W. Byard  
Australian Marine Wildlife Research and Rescue Organization,  
Torrens Island, SA, Australia





**Fig. 2** Removal of a portion of the upper hook during surgery revealing nylon fishing line running down the esophagus attaching to the hook embedded in the stomach

The upper hook was attached by nylon fishing line to the lower hook (Fig. 2) resulting in significant injuries to the stomach wall when the animal had attempted to extend its neck whilst trying to fly and/or dive. The bird died before the second hook could be retrieved.

## Discussion

Ill and/or injured marine animals may be rescued and taken to wildlife centers for examination and treatment. Evaluation of injuries is undertaken to establish causes of death, to identify situations that may be preventable, and to also exclude or identify inflicted injury by humans. This may involve assessment by both veterinarians and forensic pathologists [1].

Seabirds may be particularly vulnerable to injury from fishing material given their scavenging and fishing activities in areas that are also used by humans. They are often attracted to baited hooks because of the resemblance to natural prey. However, once encountered hooks may attach to external parts of seabirds, including the legs and wings, be ingested and attach to the mouth, or be swallowed and cause internal problems. The location of the attached hook usually determines the severity of injury. For example, a fully ingested hook may cause death within a short period of time as a result of perforation of the stomach, heart or great vessels [2–4].

**Table 1** Range of problems that may be identified at necropsy in seabirds resulting from fishing line/hook ingestion

Aerodigestive tract
Injuries to the mouth
Closure of the mouth
Injuries to the oropharynx
Sepsis
Gastrointestinal tract
Perforation
Localized inflammation/sepsis
Intussusception/intestinal obstruction
Intestinal transection
Generalized sepsis

Ingestion of the attached fishing line may also be a problem for birds and animals, as a long line may extend into the intestine causing intussusception or intestinal transection [3]. In addition, two hooks attached to a single piece of fishing line may both embed in the gastrointestinal tract at different levels causing specific problems related to the length of the line, as occurred in the reported case. On occasion this may involve two birds that have become attached to each other and thus are not able to effectively fly, forage, or feed.

The reported case also demonstrates the usefulness of imaging in seabirds that may have ingested fishing gear. The range of lesions and conditions that may be associated with ingested hooks and line is summarized in Table 1 [2–4].

## References

1. Byard RW, Gilbert JD, Kemper CM. Dolphin deaths: forensic investigations. *Med J Aust.* 2001;175:623–4.
2. Dau BK, Gilardi KVK, Gulland FM, Higgins A, Holcomb JB, Leger JS, et al. Fishing gear-related injury in California marine wildlife. *J Wild Dis.* 2009;45:355–62.
3. Casale P, Freggi D, Rocco M. Mortality induced by drifting longline hooks and branchlines in loggerhead sea turtles, estimated through observation in captivity. *Aquat Conserv Marine Freshwater Ecosyst.* 2008;18:945–54.
4. Cooper JM. Fishing hooks associated with albatrosses at Bird Island, South Georgia. *Mar Ornithol.* 1995;23:17–21.

## **Adrian Linacre (ed): Forensic science in wildlife investigations**

**CRC Press, Taylor and Francis Group, Florida USA, 2009, 162 pp,  
ISBN: 978-0-8493-0410-1**

**Linzi Wilson-Wilde**

Accepted: 14 January 2010 / Published online: 23 February 2010  
© Springer Science+Business Media, LLC 2010

Forensic Science in Wildlife Investigations is the latest book in a series on forensic science topics from the Taylor and Francis Publishers. This book is aimed at a basic level and as such is targeted at police, lawyers, students and teachers as well as those scientists looking for a basic overview on wildlife forensics. The book is not overly long at 162 pages.

The book features 6 chapters written by various experts and covers a general overview of the discipline, an explanation of the Convention of International Trade in Endangered Species of Wild Fauna and Flora, better known as CITES, microscopic examination of hair, species identification using DNA analysis, DNA individualisation of animals and isotopic ratio analysis to determine geographic origins. The book is somewhat let down by the differing writing styles of the various authors which leads to a slightly disjointed reading experience; even the referencing styles vary between chapters. It is also of significant note that the world's largest forensic wildlife laboratory is not included as a contributing author nor are they mentioned.

The book does, however, provide a good general overview of the issues. The chapter on microscopic examination of hair would have benefited from a case example. The two chapters covering DNA analysis provide good overviews and highlight many important concepts, issues and need for further work in the area. However, the explanations of the various types of statistical analysis that can be applied to the DNA analysis results could be confusing to the novice reader, as it is not always clear how they are interrelated.

The last chapter looking at geographical origins of samples using isotope ratios is a good introduction to what is a difficult subject. However, the chapter does stray with examples of human and drug analysis, used presumably in lieu of wildlife forensic related examples. This is probably reflective of the infancy of this discipline and the lack of casework experience of the chapter author in this area. The book should not be viewed as a 'how to' but rather provides a good summary and raises the issues to be considered if investigating or studying in this area.

---

L. Wilson-Wilde (✉)  
ANZPAA National Institute of Forensic Science, Melbourne,  
VIC, Australia  
e-mail: Linzi.wilson-wilde@anzpaa.org.au

## **John E. Cooper and Margaret E. Cooper: Introduction to veterinary and comparative forensic medicine**

**Blackwell Publishing, ISBN: 978-14051-1101-0, Cost: US\$125.99**

**Runa Daniel**

Published online: 23 March 2010  
© Springer Science+Business Media, LLC 2010

Introduction to Veterinary and Comparative Forensic Medicine is a comprehensive, highly practical and well organised text. The book features 13 chapters and covers a range of topics including veterinary medicine, animal welfare, wildlife crime and conservation.

The introduction outlines the major aspects of the new discipline of ‘comparative forensic medicine’ and includes case studies. It provides an overview of the links between veterinary, medical and forensic science specialisations not previously described using a structured approach. The introduction discusses the many emerging disciplines in the veterinary and medical fields, many of which are biology based, that constitute contemporary forensic science.

Of particular importance to the forensic community, the chapter on conservation and wildlife crime highlights the increasing need for a more coordinated effort from scientific, law enforcement and legislative bodies in dealing with this issue. The extensive methodology section also

provides excellent guidelines and templates for evidence collection, storage, chain of custody, providing expert testimony in court and quality assurance and management of forensic evidence in criminal investigations.

The methodology section also covers clinical, laboratory and field work, pathology and post-mortem examinations. The relevant and necessary information has been collated and provided in a summarised format making this section highly practical for practitioners in all related fields. However, it should be noted that the guidelines and suggestions provided are not standard operating procedures and should be developed and adapted, as appropriate, to adhere to quality management and laboratory accreditation requirements prior to use in forensic and clinical investigations.

This book greatly benefits from the vast and diverse experience of the authors. With over 150 illustrations and an extensive bibliography, this is a valuable resource for the veterinary, medical and wider forensic communities.

---

R. Daniel (✉)  
Victoria Police Forensic Services Department, Macleod, Victoria  
3085, Australia  
e-mail: runa.daniel@police.vic.gov.au