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TECHNICAL REPORT

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

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Abstract Species identification techniques commonly utilized in Australian Forensic Science laboratories are gel immunodifussion 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.

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

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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 immounodifussion 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 ($\sim$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), is the focus of a worldwide 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.

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

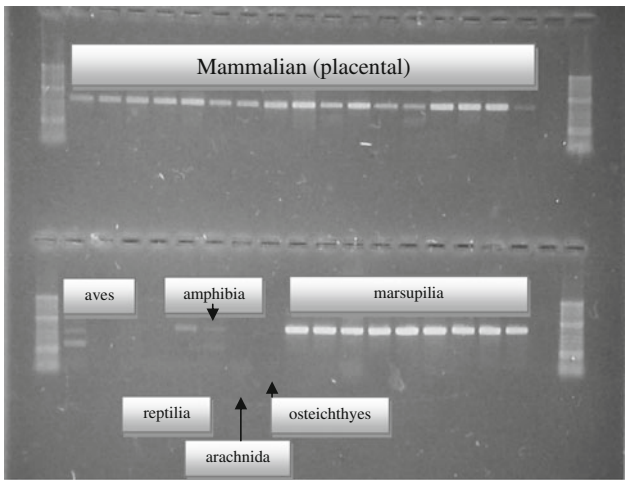

Fig. 1 Amplification success using the new COI primer pair. All mammalian samples amplified with strong results using the new COI primer pair. However, amplification in other taxa was not successful

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 *Ornithorhychus 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 *Trichosuarus 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 Lepertoteria) 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

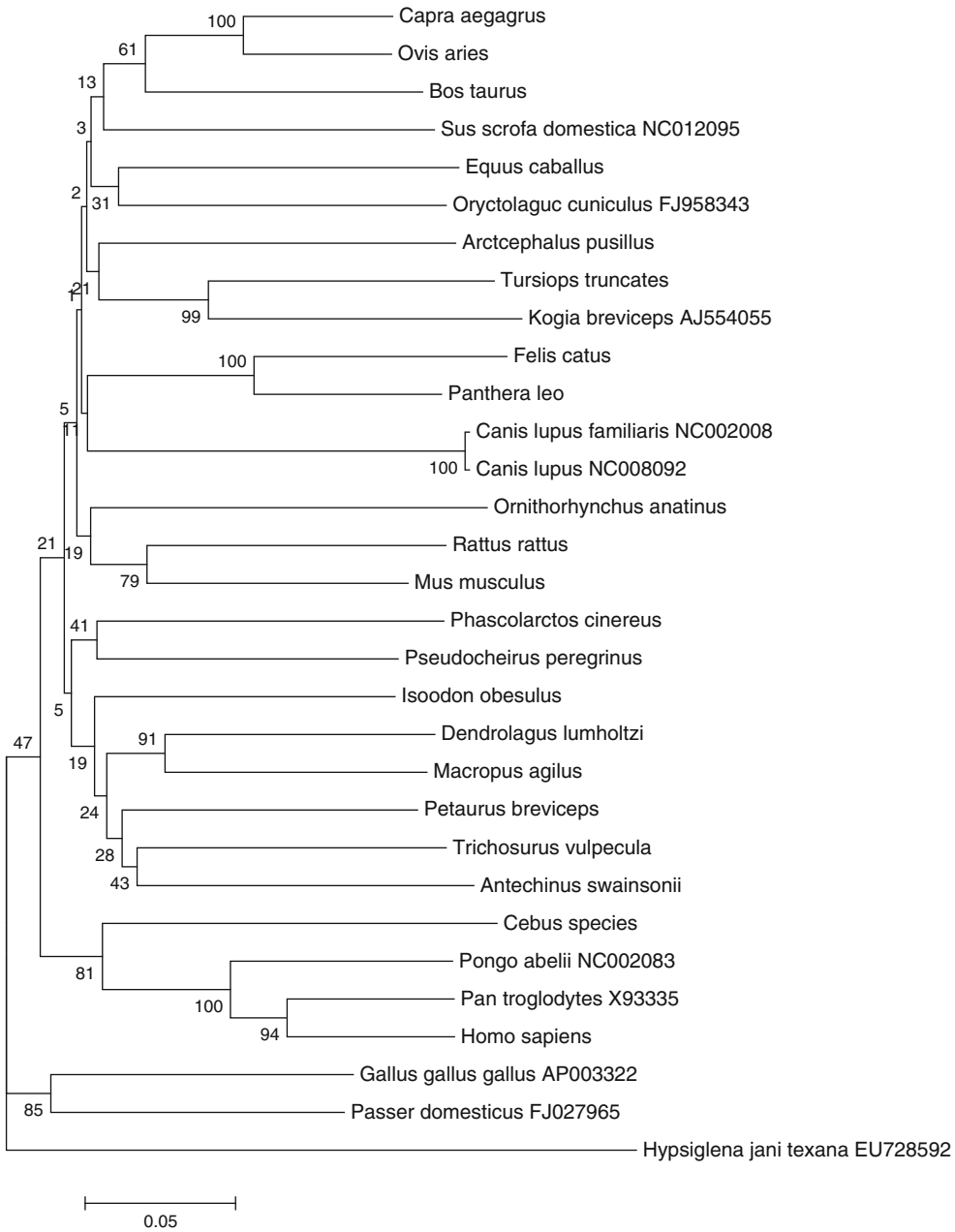

Fig. 2 Neighbourhood joining tree of sequences obtained coupled with those sourced from Genbank. Bootstrap values given 100 replicates are provided

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

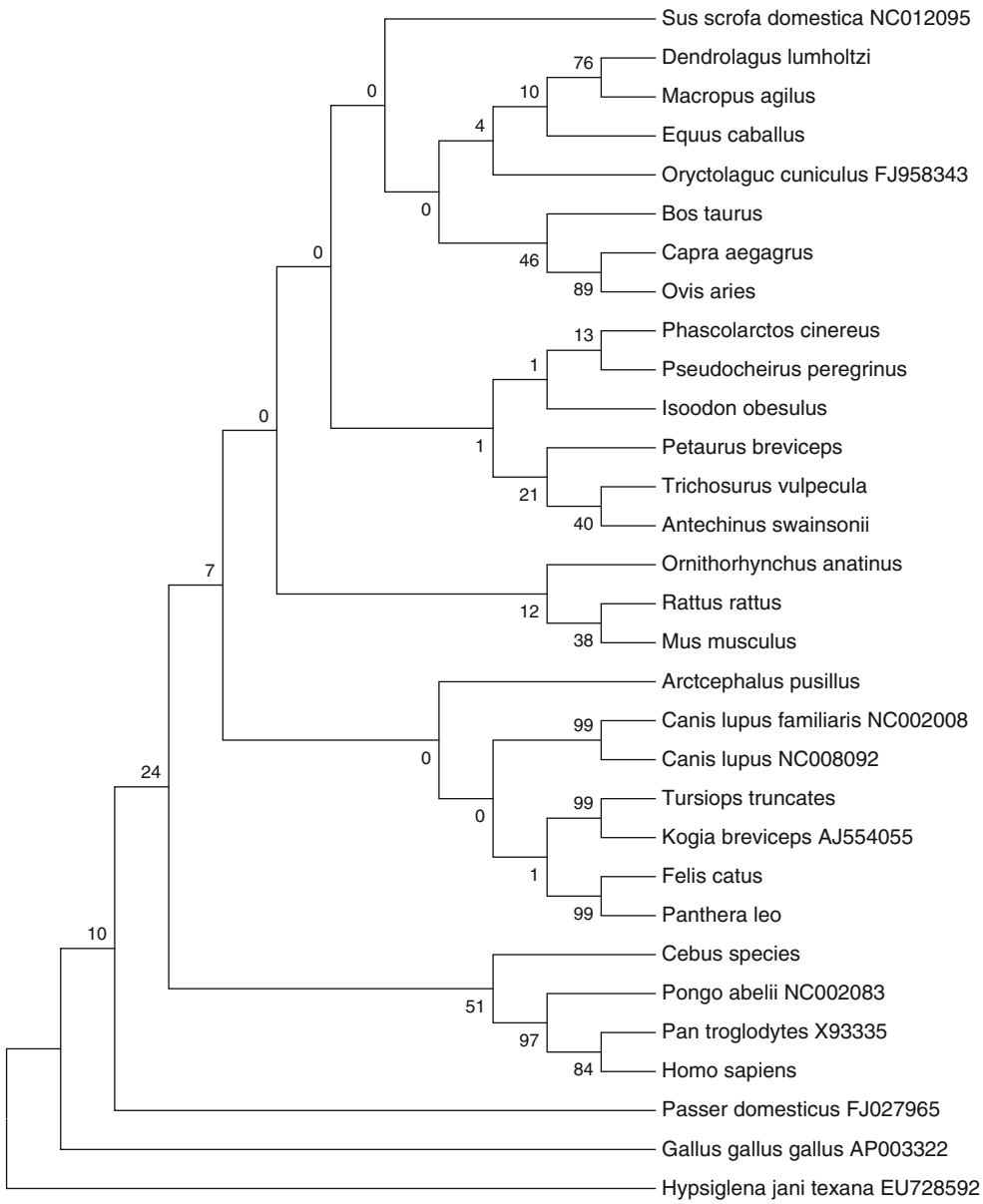

Fig. 3 Maximum parsimony tree of sequences obtained coupled with those sourced from Genbank. Bootstrap values given 100 replicates are provided

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.

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