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ORIGINAL ARTICLE

Species identification of protected carpet pythons suitable for degraded forensic samples

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Abstract In this paper we report on the identification of a section of mitochondrial DNA that can be used to identify the species of protected and illegally traded pythons of the genus *Morelia*. Successful enforcement of wildlife laws requires forensic tests that can identify the species nominated in the relevant legislation. The potentially degraded state of evidentiary samples requires that forensic investigation using molecular genetic species identification is optimized to interrogate small fragments of DNA. DNA was isolated from 35 samples of *Morelia spilota* from which the complete cytochrome b was sequenced. The ND6 gene was also sequenced in 32 of these samples. Additional DNA sequences were generated from 9 additional species of *Morelia*. The sequences were aligned by Geneious and imported into MEGA to create phylogenetic trees based on the entire complex of approximately 1,706 base pairs (bp). To mimic degraded DNA, which is usually found in forensic cases, short sub-sections of the full alignment were used to generate phylogenetic trees. The sub-sections that had the greatest DNA sequence information were in parts of the cytochrome b gene. Our results highlight that legislation is presently informed by inadequate taxonomy. We demonstrated that a 278 bp region of the cytochrome *b* gene recovered the topology of the phylogenetic tree found with the entire gene sequence and correctly identified species of *Morelia* with a high degree of confidence. The locus described in this report will assist in the successful prosecution of alleged illegal trade in python species.

Keywords Carpet python · Cytochrome b · Illegal wildlife trade · Mitochondrial DNA · *Morelia* · ND6

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Introduction

All python species are listed in Appendix 2 of the Convention on International Trade in Endangered Species of Flora and Fauna (CITES) and are therefore subject to trade sanctions and protection by signatory countries [1]. Despite this protection, reptiles including pythons are regularly acquired and traded unlawfully [2]. Native species provide an easy resource for captive breeding ventures [3, 4] and additional measures Australia wide (i.e., State and Federal legislation) still fall short of preventing the illegal take of endemic python species (H. Dridan, DEWNR, personal communication). Native and exotic snake trade is frequently associated with other organized criminal activities [4–7].

Variability in morphology and color pattern makes Australasian pythons attractive to the national and international pet trade, while also complicating visual species identification for enforcement officers tasked to regulate the trade (S. James, OEH NSW, personal communication).

Investigators locating animals during searches require reliable and definitive tools to identify whether the animals in question are smuggled exotic species or local species. Identification of the species is essential for an evidentiary basis and has previously been inadequate to progress these cases to court (H. Dridan, DEWNR, personal communication). In our experience, the condition of evidentiary samples can be very poor; samples might be stored in unfavorable conditions prior to submission for laboratory testing. The samples provided to our own forensic laboratory might be scale clippings or poorly stored tissue samples. Swabs from an evidentiary item suspected to have come into contact with a snake (e.g., [8]) might also be submitted, with the instruction to identify the species present. From such samples there is an expectation, based on previous experience, that the quality of the DNA template could be highly compromised.

There has been sustained debate over the adequacy of the popular species identification genes: cytochrome *b* (cyt *b*) is a standard gene used for species testing (e.g., [9]) and cytochrome oxidase *c 1* (*COI*) [10] is the gene selected by the Barcode of Life consortium (www.barcodinglife.org) [11]. A small number of studies have considered other candidate gene sequences encoded by the mitochondrial genome for use in species identification [12–16]. While any of these genes might be equally as informative, their use for species identification hinges on a demonstration that the relationships between the mitochondrial DNA sequences for a particular locus reflect the evolutionary relationships among the individuals carrying the genes, i.e., the species tree relationships.

In most snakes, the cyt *b* gene is 1,114 base pairs (bp) in length and encodes a protein of 371 amino acids and the ND6 gene encodes a protein of 171 amino acids and is 513 bp in length. The ND6 gene is the only protein-encoding gene coded by the light strand in snakes [17] and is adjacent to the cyt *b* gene, separated by the tRNA-glutamine gene.

Forensic species identification tests should be designed to obtain results from remnant DNA fragments in case the forensic sample is in poor condition, which is not uncommon for biological exhibits; an example of such a test is nested PCR of the cyt *b* gene used to identify species of turtle from processed turtle shell [18]. "Mini barcoding" of the COI gene has been used to interrogate very small fragments of the COI barcoding gene in eleven species of protected Indian snakes [19]. In this study [19], a figure of 98 % similarity was claimed to be a species match, however it was based upon one sample per species with no apparent consideration of intraspecific variation. It should be acknowledged that these authors assessed their technique according to recommendations of the Scientific Working Group on DNA Analysis Methods (SWGDAM).

Alignment of existing GenBank data indicated that alongside cyt *b*, chosen both for its high variability and reputation in previous species identification research (e.g., [20, 21]), ND6 exhibited a high degree of interspecies variation (unpublished data). The ND6 gene was the second most variable of all genes in the mitochondrial genome (determined by methods described by Tobe et al. [22]); the most variable mitochondrial gene locus, ATPase 8, was suspected by the authors to be too variable for species identification purposes as it is a very small gene (171 bp in *M. spilota*) and identification of PCR primer sites conserved across the snake species would likely be problematic.

We report on the identification of a mitochondrial DNA section that would identify CITES Appendix 2 listed and legislatively protected members of the python genus *Morelia*. This genus, which comprises 11 species [23], is found in Australia, New Guinea and the eastern part of the Indonesian archipelago. Through phylogenetic reconstruction, we demonstrate the ability of the ND6 and cyt *b* genes to distinguish between the widespread and morphologically variable carpet python species and all other Australasian *Morelia* species. Within these genes, we show that short segments can provide accurate species identification comparable to the entire respective gene region. Further, we report on the identification of a small fragment of mitochondrial DNA with the ability to identify, to species level, scale clippings suspected to have been taken from a species of *Morelia*. Our work was conducted in line with the recommendations of Linacre et al. [24].

Materials and methodology

Primer design

Primers were designed using the software Primer3 [25] as part of the Geneious software package (Geneious v5.4.3 created by Biomatters). Primer sequences and locations are shown in Fig. 1. An alignment of the 39 snake mitochondrial genomes available on Genbank and one mitochondrial genome obtained during this study was used to design primers universal for snake species. Primers with a redundant sequence were designed for cyt *b* to maintain universality. Primer sequences, located within the ND5 and tRNA-gGlutamine genes, were designed to amplify the entire ND6 gene. Two overlapping primer pairs were designed to amplify the entire cyt *b* gene. The first primer pair is situated at the (5′) end of ND6 gene and amplifies the *tRNA-Glutamine* gene and 471 bp of the first half of the cyt *b* gene. The second primer set amplifies bases 377-1,114 of the cyt *b* gene and 35 bp (5′) of the *tRNA-Threonine* gene, creating a sequence overlap of 94 bp between the two amplification products.

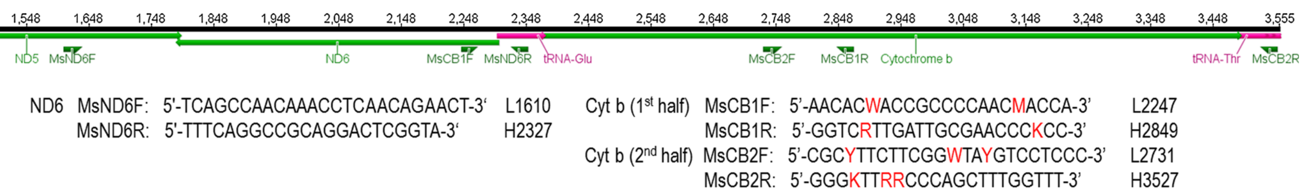

Fig. 1 Primer sequences and locations with respect to mitochondrial genome features. Numbers refer to base pairs from base one of the ND5 start codon, as no complete mitochondrial genome reference sequence is available for *Morelia*

Samples

The specimens used (Table 1) were representative of ten of the eleven species that comprise the genus *Morelia*. Sequence data for the eleventh species, *M. clastolepis*, were obtained from GenBank (see Electronic supplementary material 2). Geographic origins of the samples are shown in Fig. 2. Sampling aimed to include examples from the broadest geographic spread possible from each species, given the biological samples and literature available, following the expectation that those from close geographical locations should have a higher similarity at the DNA level when compared to more geographically distant specimens. All specimens listed in Table 1, excluding the four GenBank samples, were obtained from the Australian Biological Tissue Collection (ABTC) of the South Australian Museum. These samples were stored in ethanol or frozen at -80 °C. Scale clippings from four snakes, seized under warrant during an investigation in New South Wales in 2011 which were suspected to be, and are confirmed as, *Morelia spilota* carpet pythons, were also included (samples M001, M002, M003, and M004).

DNA extraction and sequencing

Samples were thawed or removed from solution and placed on a heating-block at 56 °C for 10 min to remove any traces of ethanol. DNA extraction was performed using the QIAamp DNeasy Blood and Tissue Kit (QIAGEN, Australia) following the protocol for the Purification of Total DNA from Animal Tissues with the exception that the volume for the ATL buffer, Proteinase K, and DTT was doubled for large skin samples, as well as the Isolation of Total DNA from Tissues (QIAGEN, Australia), following the manufacturer's protocol. Elution volumes ranged from 40 to 600 μL according to the quality of the sample.

Amplifications were performed using 2 μL extracted DNA, 200 nM dNTPs, 1 μM of each primer, and 2 Units of DNA Polymerase (BIOLINE, Australia), in a total volume of 20 μL. PCR conditions for amplifying the ND6 gene were initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Amplification of cyt *b* was performed as above but for 30 cycles and with an annealing temperature of 56 °C. Product concentration was estimated by 2 % agarose gel electrophoresis and visualization by ethidium bromide using a Bio-Rad Gel Doc and Image Lab software (Bio-Rad, Australia). PCR products were prepared for sequencing using ExoSap (GE Healthcare, Australia), following the manufacturer's protocol. DNA sequencing was performed by the Australian Genome Research Facility.

Sequence alignment and data analysis

DNA sequence data were edited and aligned using Geneious 5.4.3. Alignments of consensus sequences were imported into MEGA v5.0 for phylogenetic analysis. Sequences have been deposited on GenBank (accession numbers KJ666583-KJ666640). Phylogenetic trees were constructed using Neighbor-Joining and Maximum Likelihood algorithms using *Python regius* as an out-group. Neighbor-Joining trees were constructed using MEGA v5.0 with a p-distance model. Maximum Likelihood trees were constructed using RAxML Blackbox v7.6.3 (Cipres Science Gateway [26]) with partitioning strategy and nucleotide substitution models chosen using Partition Finder v1.1.1 [27] (see Electronic supplementary material 3). Bootstrapping was used to provide statistical confidence for the tree topology.

Gene fragment analysis

A continuous sequence containing the ND6 and cyt *b* genes from 48 reference samples including 32 *Morelia spilota*, one each of *M. amethistina* and *M. kinghorni* and two each of *M. boeleni*, *M. bredli*, *M. carinata*, *M. nauta*, *M. oenpelliensis*, *M. viridis* (northern population), and *M. viridis* (southern population) were divided into half and quarter fragments. Eighth fragments were also created for the larger cyt *b* gene. Phylogenetic trees were constructed to test if any of the 6 smaller fragments from ND6 gene and 14 smaller fragments from cyt *b* gene could reproduce the ability of the larger gene fragments to separate individuals to species.

Five extra samples, for which only the complete cyt *b* sequence could be obtained (due to poor sample quality),

Table 1 Samples genotyped

Location #	ABTC #	Species	Locality	Voucher #	Sample type	Use
1	117064	*M. spilota*	Merauke, PNG	–	Scale	Reference
2	123504	*M. spilota*	Port Moresby, PNG	–	Scale	Reference
3	62456	*M. spilota*	Mount Connor, WA	WAMR96970	Liver	Reference
4	30087	*M. spilota*	Melville Isl., NT	NTMR23019	Tissue	Reference
5	55482	*M. spilota*	Darwin, NT	SAMAR21456	Liver	Reference
6	81659	*M. spilota*	Cannington Mine, Qld.	–	Scale	Reference
7	81028	*M. spilota*	East McIlwraith Range, Qld.	ANWCR05271	Tissue	Reference
8	67578	*M. spilota*	Cape Tribulation, Qld.	–	Scale	Blind trial "test"
9	82975	*M. spilota*	Tully, Qld.	–	Tail	Reference
10	82974	*M. spilota*	Townsville, Qld.	–	Tail	Reference
11	86768	*M. spilota*	near Mackay, Qld.	–	Tail	Reference
12	17464	*M. spilota*	Brisbane, Qld.	–	Blood	Reference
13	81307	*M. spilota*	Nevertire, NSW	AMSR155203	Muscle	Reference
14	68285	*M. spilota*	Caparra, NSW	–	Skin	Reference
15	13446	*M. spilota*	Gosford, NSW	–	Scale	Reference
16	68293	*M. spilota*	Tilba, NSW	–	Skin	Reference
17	71384	*M. spilota*	Warby Ranges, Vic.	–	Skin	Reference
17	71389	*M. spilota*	Warby Ranges, Vic.	–	Skin	Reference
18	71390	*M. spilota*	Gunbower Isl. Vic.	–	Skin	Reference
19	83875	*M. spilota*	Kinchega NP, NSW	–	Skin	Blind trial "test"
20	76268	*M. spilota*	Sturt NP, NSW	–	Scale	Blind trial "test"
21	50868	*M. spilota*	Bobbiemoonga WH, SA	–	Skin	Reference
21	76530	*M. spilota*	Bobbiemoonga WH, SA	–	Scale	Blind trial "test"
22	55499	*M. spilota*	Goyder Lagoon, SA	SAMAR26877	Tissue	Reference
23	34289	*M. spilota*	Blanchetown, SA	SAMAR39773	Liver	Reference
24	106072	*M. spilota*	Waikerie, SA	–	Scale	Reference
24	106074	*M. spilota*	Waikerie, SA	–	Scale	Blind trial "test"
25	68297	*M. spilota*	Depot Springs, SA	–	Skin	Reference
26	68341	*M. s. imbricata*	Buckleboo, SA	–	Scale	Reference
27	76225	*M. s. imbricata*	Dog Fence Beach, SA	–	Scale	Reference
27	68298	*M. s. imbricata*	Fowler's Bay, SA	–	Blood	Reference
28	68304	*M. s. imbricata*	St Francis Isl. SA	–	Skin	Reference
29	81224	*M. s. imbricata*	Yalata, SA	–	Tail	Reference
30	51576	*M. s. imbricata*	Norseman, WA	–	Blood	Reference
31	66327	*M. s. imbricata*	Dryandra, WA	–	Scale	Reference
32	68310	*M. s. imbricata*	Northern Twin Peak Isl., WA	–	Skin	Reference
33	68308	*M. s. imbricata*	Mondrain Isl., WA	–	Skin	Reference
34	68330	*M. bredli*	Alice Springs, NT	–	Skin	Reference
35	112609	*M. bredli*	Trephina Gorge, NT	–	Scale	Reference
36	45444	*M. viridis*-S[a]	Namosado, SHP	AMSR122363	DNA extract	Reference
37	50176	*M. viridis*-S[a]	Guleguleu, MBP	AMSR129716	DNA extract	Reference
38	29590	*M. oenpelliensis*	Ex Territory Wildlife Park, NT	–	DNA extract	Reference
39	68277	*M. oenpelliensis*	Kakadu, NT	–	DNA extract	Reference
40	49652	*M. boeleni*	Morobe, PNG	BPBM11611	DNA extract	Reference
41	67161	*M. boeleni*	Wamena, Indo.	–	DNA extract	Reference
42	51987	*M. carinata*	Mitchell Plateau, WA	–	Skin	Reference
42	53435	*M. carinata*	Mitchell Plateau, WA	–	Skin	Reference
43	67641	*M. kinghorni*	Heathlands, Qld.	QMJ66806	Liver	Reference (cyt b only)

Table 1 continued

Location #	ABTC #	Species	Locality	Voucher #	Sample type	Use
44	83041	*M. kinghorni*	Tully, Qld	QMJ82111	Muscle/Scale	Reference
45	43885	*M. amethistina*	Doido, CHP	AMSR115347	Liver	Reference
46	46076	*M. amethistina*	Namosado, SHP	AMSR122690	Liver	Reference (cyt b only)
47	49784	*M. viridis*-N[a]	Wau, MP	BPBM11617	Liver	Reference
47	49785	*M. viridis*-N[a]	Wau, MP	BPBM13798	Liver	Reference
48	67160	*M. tracyae*	Halmahera, Indo.	–	Scale	Reference (cyt b only)
48	67162	*M. tracyae*	Halmahera, Indo.	–	Scale	Reference (cyt b only)
49	67163	*M. nauta*	Tanimbar, Indo.	–	Scale	Reference (cyt b only)
49	128066	*M. nauta*	Yamdena, Indo.	WAMR112269	Liver	Reference
49	128067	*M. nauta*	Yamdena, Indo.	WAMR112277	Liver	Reference
50	AF241401	*M. clastolepis*	Ambon/Seram, Indo.	–	Genbank	Reference (partial cyt b)
50	AF241397	*M. clastolepis*	Ambon/Seram, Indo.	–	Genbank	Reference (partial cyt b)
50	AF241390	*M. clastolepis*	Ambon/Seram, Indo.	–	Genbank	Reference (partial cyt b)
50	AF241389	*M. clastolepis*	Ambon/Seram, Indo.	–	Genbank	Reference (partial cyt b)

No sample was available for *Morelia clastolepis*

[a] Two highly divergent populations of *Morelia viridis* identified by Rawlings and Donnellan [30] that likely represent separate species, labeled –S = southern populations and –N = northern populations. Sequences available on GenBank under accession numbers KJ666583–KJ666640

were included in the comparisons within cyt *b* fragments. These samples increased the cyt *b* dataset to 53 individuals (samples marked cyt *b* only in Table 1). Five blind trial or "test" samples were used to test the ability of the best smaller gene fragment to identify species as per the larger gene fragment (see Table 1 for sample details). The species identity of these samples was checked in the ABTC records after testing.

In order to examine every member species of *Morelia*, a separate dataset comprising four 715 bp cyt *b* sequences of *M. clastolepis* retrieved from GenBank and the corresponding sequence data for our 53 cyt *b* reference samples was used to demonstrate the phylogenetic placement of *M. clastolepis* in relation to other *Morelia* species.

Results and discussion

Mitochondrial DNA analysis

Phylogenetic analysis of the complete mitochondrial sequence spanning the ND6 and the cyt *b* genes (1,706 bp) for the 48 reference samples, resulted in a tree that grouped all *M. spilota* and *M. bredli* individuals in a separate clade to all other species (see 1b in Electronic supplementary material 1). For those species where we sampled two or more individuals, the conspecific (individuals of the same species) samples grouped in a single clade with bootstrap proportions of 100 %. These data demonstrate the ability of this entire genetic locus to identify the python samples to species level.

The position of *M. bredli* within *M. spilota* and the clustering of individuals of the *M. spilota imbricata* subspecies as a separate group to the remainder of *M. spilota* requires some comment. *Morelia bredli* was recently reclassified as a separate species from *M. spilota* without a formal taxonomic treatment [28]. Our data suggest two possibilities for the relationship of *M. bredli* with *M. spilota*: either that *M. bredli* is not sufficiently divergent to be a separate species, or that *M. spilota* actually comprises several species, among which two would be *M. bredli* and *M. s. imbricata*. Resolution of these issues will require extensive morphological analysis and likely nuclear gene data, neither of which is trivial for this widespread group of pythons. As an interim we hereafter use "*M. spilota* complex" to refer to *M. spilota*, *M. bredli,* and *M. s. imbricata in lieu* of future taxonomic resolution of the complex. Further, these data show that our species identification test is suitable for correctly grouping individuals within the "*M. spilota* complex."

Phylogenetic analysis using the ND6 gene

We correctly identified all samples to species level with the phylogenetic tree constructed from the 513 bp ND6 gene (see 1a in Electronic supplementary material 1). Trees constructed using two halves of ND6 (bases 1–256 and 257–513) were able to reproduce all species groupings. Only the last of the four ~128 bp ND6 segments (bases 385–513) was able to group *Morelia spilota* complex to species, all other fragments producing trees that split the

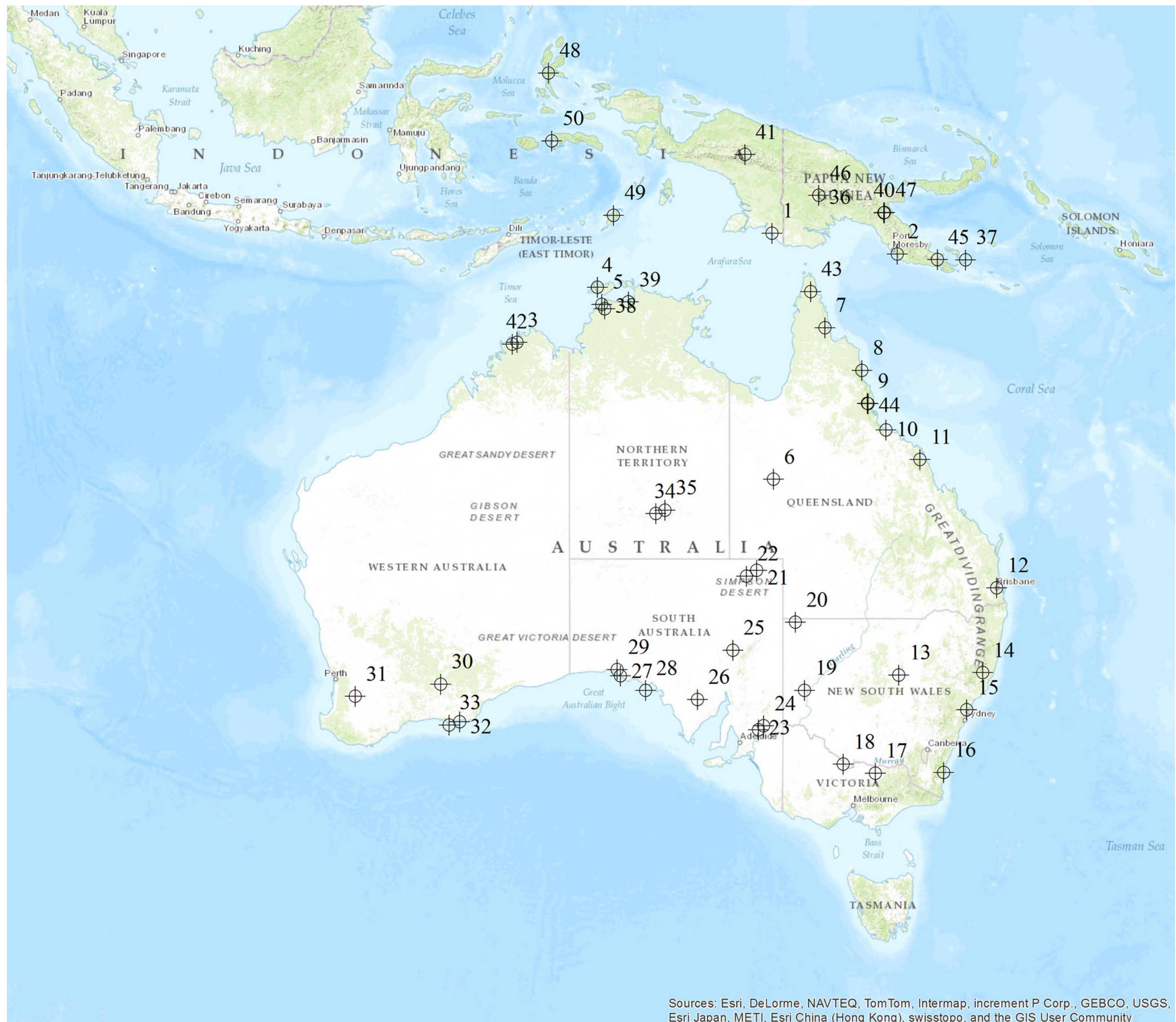

Fig. 2 Geographic locations of the samples used in study. Numbers correspond to the location # column in Table 1

carpet pythons into several groups that each clustered with other species. Our data show that the first and second half and the last quarter of the ND6 gene are suitable for the identification of many python species.

Phylogenetic analysis using the complete cytochrome b gene

Construction of a phylogenetic tree using the entire cyt *b* gene (1,114 bp) resulted in the 34 individuals of the *M. spilota* complex grouping together and all other species groupings replicating the tree constructed from the contiguous sequence (see 1c in Electronic supplementary material 1). The two *M. bredli* grouped together, again within the *M. spilota* complex. The phylogenetic tree constructed from 715 bp of cyt *b* including *M. clastolepis* and an additional individual of each of *M. amethistina* and *M. kinghorni* (see Electronic supplementary material 2) shows that the conspecific *M. clastolepis* sequences cluster with strong support to the exclusion of all other *Morelia* species. *Morelia clastolepis* grouped most closely with other scrub python species, consistent with previous findings [29]. The *M. kinghorni* sequences do not cluster together however; instead the sequence from the northern tip of Cape York clustered with *M. amethistina* sequences from southern New Guinea. Our finding is consistent with either *M. amethistina* being present in northern Cape York Peninsula (and currently unrecognized) or the species boundary between these two taxa being in need of revision. Further sampling of mitochondrial and nuclear gene diversity in northern Cape York Peninsula will be required to resolve this issue.

Within the *M. spilota* complex, samples from northern Western Australia and the Northern Territory clustered

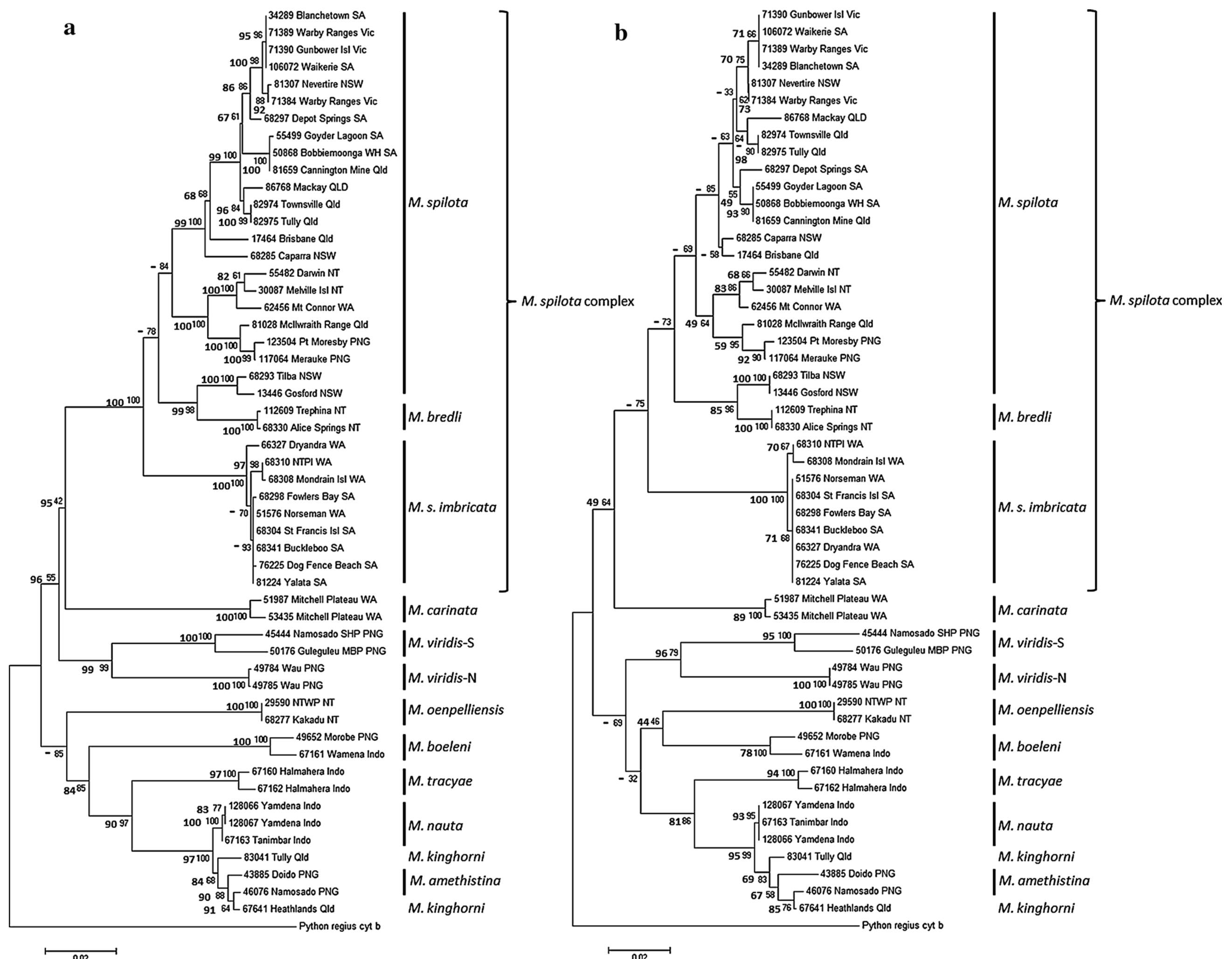

Fig. 3 Phylogenetic trees constructed using the entire *cyt b* gene region (**a**) and the 278 bp fragment of bases 558–835 inclusive (**b**). Phylogenetic trees were constructed using Neighbor-Joining with p-distance model and 500 bootstrap replicates including 53 *Morelia* samples and rooted using *Python regius* as outgroup. Maximum Likelihood support values are shown in bold next to Neighbor-Joining bootstrap values, denotes a NJ node that was not present in the ML tree

together as did the samples collected from the neighboring regions of western Victoria and New South Wales and eastern South Australia. The samples from southern Western Australia and western coastal South Australia grouped most distantly from the other carpet pythons. The topology of the cyt *b* gene tree more closely resembles that of the contiguous sequence tree than does the ND6 gene tree, indicating this to be the more accurate gene for recovering relationships between individuals.

Phylogenetic analysis using decreasing sections of the cyt *b* gene

Reducing the overall sequence length used in the comparison was undertaken to mimic forensic samples where the DNA has been degraded. Five extra individuals for which ND6 sequence data could not be obtained were added to the cyt *b* dataset for intra-gene tree comparison. These represented two *M. tracyae*, one *M. nauta*, one *M. amethistina,* and one *M. kinghorni*. Phylogenetic trees were constructed by dividing the gene into two equal 557 bp fragments, four fragments of 278 or 279 bp and eight fragments of 139 or 140 bp. The smaller sections of the gene were unable to recover a tree of identical topology to the whole gene tree (Fig. 3a); in many cases however variations were very minor and did not affect the ability of the tree to group conspecific sequences and closely related species. While the two halves of cyt *b* gave more accurate tree reconstructions than the smaller fragments with only minor differences to the entire gene tree, a sequence of 557 bp still might not be achievable for forensic samples involving degraded DNA.

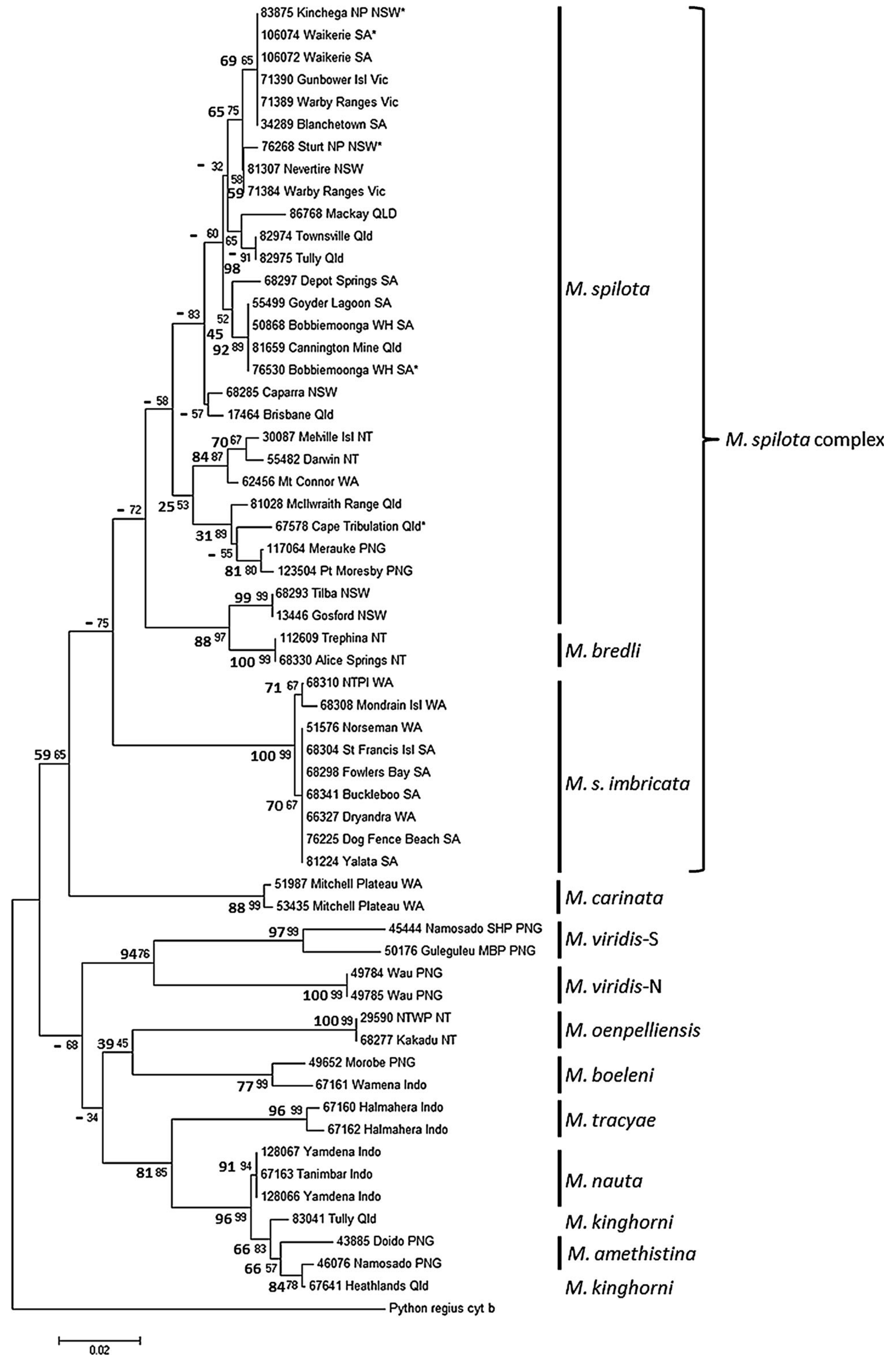

Fig. 4 Phylogenetic tree of the 278 bp segment of *cyt b* including 53 reference samples of *Morelia* and the five "test" samples. "Test" samples are denoted with an (*). Phylogenetic trees were constructed using Neighbor-Joining with p-distance model and 500 bootstrap replicates. The tree was rooted using *Python regius* as outgroup. The Maximum Likelihood support values are shown in bold next to the Neighbor-Joining bootstrap values, *line* denotes a NJ node that was not present in the ML tree

One quarter section of the gene (a 278 bp section from base 558 to 835 inclusive) reconstructed the entire cyt *b* tree more accurately than any other fragment 279 bp or smaller (Fig. 3b), based on comparison to the tree created with the entire gene. The only change in topology using the section from 558 to 835 relative to the entire cyt *b* tree was the placement of the *M. spilota* samples collected from the eastern interior of Australia (e.g., Goyder Lagoon, Depot Springs, Townsville) which shifted positions in the tree relative to each other. Bootstrap values were generally

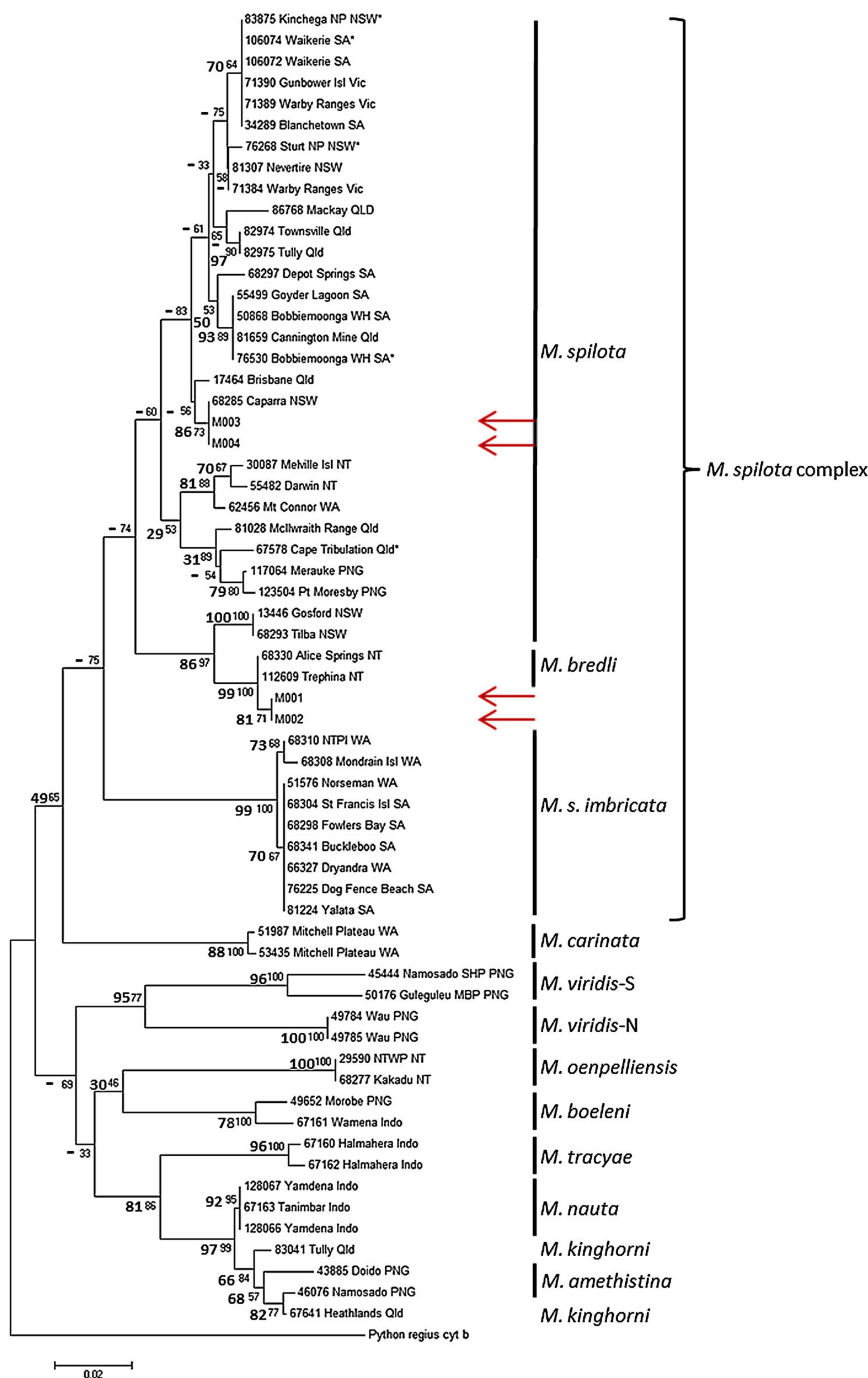

Fig. 5 Phylogenetic tree of 53 reference samples, five test samples and four forensic samples of questioned origin (M001–M004). Test samples are identified by (*). Forensic samples are denoted by red arrows. Phylogenetic trees were constructed using Neighbor-Joining with p-distance model and 500 bootstrap replicates. The tree was rooted using *Python regius* as outgroup. Maximum Likelihood support values are shown in bold next to the Neighbor-Joining bootstrap values, *line* denotes a NJ node that was not present in the ML tree

lower using the 558–835 fragment, consistent with less data being available for analysis (Fig. 3). Further indication that the remaining smaller fragments are, in general, unsuitable or less suitable for species identification is that bootstrap support was generally lower across each tree and that the tree topology varied substantially in comparison with the entire cyt *b* tree.

Our results support the use of the 278 bp (bases 558–835) segment of the cyt *b* sequence for species identification of forensic evidence samples believed to contain

Morelia pythons. Amplification of this short region is aimed at generating amplification products from highly degraded DNA templates, but a PCR test is necessary to confirm practical application to compromised DNA samples.

Evaluating the use of cyt *b* with test samples

We further evaluated the efficacy of the 278 bp segment of cyt *b* sequence as a species identification test on five samples chosen as blind trials. These samples came from the ABTC and their origin could be verified through the ABTC database after testing. We amplified and sequenced the full length cyt *b* gene from these samples but used just the 278 bp target region from these and the 53 reference samples to construct a phylogenetic tree. The topology of the tree produced (Fig. 4) closely resembled that constructed previously using the 53 reference samples (Fig. 3). The five unknown samples grouped within the *M. spilota* complex. Based on reference to ABTC records, these samples were *M. spilota* and grouped not only with the correct species, but alongside specimens collected from neighboring geographical locations.

Test case with forensic samples

The 278 bp cyt *b* segment was analyzed for each of the four samples of scale clippings (M001, M002, M003, and M004) believed to be taken from carpet pythons, using the MsCB2 primer pair (Fig. 1). These sequences were aligned against those already generated and a phylogenetic tree was constructed (Fig. 5). Two samples (M001, M002) grouped with *M. bredli* supported by a bootstrap value of 100 %, while the other two samples (M003, M004) grouped with other *M. spilota* well within the *M. spilota* complex. These test case samples can therefore be allocated to this species complex with a high degree of confidence.

These samples were provided as part of a police investigation and we have recently received information that the seized snakes were a breeding pair of Centralian carpet pythons (*M. bredli*) and two *M. spilota* (S. James, DPC, personal communication). Samples M003 and M004 grouped most closely with individuals originating from midway down eastern coastal Australia, suggesting that they also originate from that region. An indicative geographic location can provide useful intelligence where no other investigative information might be available. While our preliminary results suggest that the cyt *b* gene shows potential for establishing the geographic region from which an unknown sample originated, a comprehensive geographic reference set is required to establish confidence in the findings. Mitochondrial loci alone can provide limited geographical assignment data and the results should be complemented using a more highly resolving marker type such as a robust set of microsatellites, if they are available.

Conclusion

Evidentiary tools such as DNA testing are crucial to the effective enforcement of legislation to protect highly targeted species from criminal activity including illegal trade. Legislation names the protected species, yet this is complicated by frequent revision of the taxonomy of many of these species. It also critically relies on the taxonomy accurately portraying the real underlying systematic relationships of the species, which in the case of the genus *Morelia*, and likely many others, it clearly does not. While further studies on the systematics of Australian python species are on-going, our data indicate that the cyt *b* gene can provide an accurate means of species identification for all *Morelia* species. For forensic testing involving compromised samples, a 278 bp region of the cyt *b* gene has been found to demonstrate high congruence with the entire gene tree and allow species testing where only small fragments of DNA remain. Within the *M. spilota* complex, the fidelity of groupings of individuals from similar geographic localities (as seen in the larger tree) suggests that this information might be of further value for investigative purposes. The data provided in our report are a model for the taxonomic information required prior to using DNA as a species test. This is the first study to use phylogenetic reconstruction to examine a marker's ability to identify, to species, every member of *Morelia*, providing increased confidence about an unknown sample's identity when the phylogenetic relationships of all species are known. We report a cyt *b* test which is compatible with degraded forensic samples and can identify multiple carpet python species with a high degree of certainty.

Key Points

1. Carpet pythons are traded illegally and molecular analysis offers the best method for identification of these species when visual identification is not possible and the submitted sample is of poor quality.
2. Phylogenetic analysis of the complete cyt *b* and ND6 loci identified ambiguities in current taxonomic designations.
3. Greater interspecific variation was apparent from phylogenetic analyses based on the cyt *b* locus compared to those using the ND6 locus.
4. Sequence comparisons led to the identification of a 278 bp section of cyt *b* gene that could identify the species in line with the entire gene locus.

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