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Molecular identification of python species: Development and validation of a novel assay for forensic investigations

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ABSTRACT

Python snake species are often encountered in illegal activities and the question of species identity can be pertinent to such criminal investigations. Morphological identification of species of pythons can be confounded by many issues and molecular examination by DNA analysis can provide an alternative and objective means of identification. Our paper reports on the development and validation of a PCR primer pair that amplifies a segment of the mitochondrial cytochrome *b* gene that has been suggested previously as a good candidate locus for differentiating python species. We used this DNA region to perform species identification of pythons, even when the template DNA was of poor quality, as might be the case with forensic evidentiary items. Validation tests are presented to demonstrate the characteristics of the assay. Tests involved the cross-species amplification of this marker in non-target species, minimum amount of DNA template required, effects of degradation on product amplification and a blind trial to simulate a casework scenario that provided 100% correct identity. Our results demonstrate that this assay performs reliably and robustly on pythons and can be applied directly to forensic investigations where the presence of a species of python is in question.

1. Introduction

Pythons have aesthetic appeal, due to the wide range of colour and pattern variants available, with rarity driving their monetary value [1,2]; novel variants can fetch prices for a single individual of around AUD $5000 in Australia and up to $10,000 in international trade [3]. All python species are listed in Appendix II of the Convention on International Trade in Endangered Species of wild flora and fauna (CITES,www.CITES.org) [4]; their trade between international borders is controlled through licensing systems by countries that are signatories to CITES. Pythons are native to many countries including Australia. No Australian export permits can be issued for live native specimens destined for commercial use (as set out in Part 13A, Division 2, Subdivision B of the Environmental Protection and Biodiversity Conservation Act, 1999). It has been reported that live reptiles, including pythons, are taken from the wild to be smuggled into overseas markets, with particular popularity in Europe and Japan [2,5]. Often animals do not survive the smuggling process leading to remains that might be morphologically unrecognisable. Individuals belonging to different python species can look very similar, and snakes of the same species can be mistaken as different species due to their varied appearance, complicating visual identification by wildlife enforcement officers (S. James, OEH NSW, *pers. comm.*). A molecular species identification test is therefore required to conclusively identify the species of seized snake, whether live or dead, to enforce international and national legislation.

Mitochondrial DNA markers are used commonly for forensic wildlife species identification due to characteristics that make them more suitable than nuclear markers [6]. Amplification and sequencing using universal primers is the most common method of species identification (e.g. [7–9]). This method has been referred to by different names, such as Forensically Informative Nucleotide Sequencing (FINS) [10] and barcoding [11]. Snake species identification work has previously utilised the cytochrome *b* [12,13], 16S rRNA [14] and cytochrome oxidase I loci [15], although none of these studies extensively examined python species. In

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some examples, sequences are simply compared to a reference sequence or an online database, such as GenBank (e.g. [16,17]). More commonly, the technique involves placing a sequenced haplotype into a reference set via sequence alignment and subsequent phylogenetic reconstruction (e.g. [18]). Phylogenetic reconstruction is a preferable technique for court going investigations, as the species can be identified from its relationship to the other reference species and it is accompanied by an assessment of statistical support.

All techniques developed for forensic investigation must undergo validation to demonstrate their suitability for application to the legal system; that is, reliability of results and demonstrated conditions and limitations of use. A commonly used example of the steps involved in validation of a new forensic method has been published by the Scientific Working Group on DNA Analysis Methods [19]. Subsequent recommendations have focused on the validation of techniques for non-human species [20–22].

Previous investigation of a continuous segment of mitochondrial DNA sequence spanning the ND6 and the cytochrome *b* (Cyt *b*) open reading frames [23] identified a putative 278 bp region suitable for differentiating species of *Morelia* pythons, providing the theoretical foundation to create a species identification test for all pythons. We report on the development and validation of a PCR primer pair that will amplify a larger region of the mitochondrial cyt *b* gene, yielding 300 bp of sequence encompassing the recommended region, for identification of python species. Phylogenetic analysis of the new larger mitochondrial section produces higher statistical support than the smaller DNA segment.

Table 1
Details of the 43 samples utilised in this study. Superscript letters indicate the section in which each sample was used. The three right hand columns show the results of the species specificity testing for the H1478/L1091 universal primer set and the MSFCB primer set using two different annealing temperatures. The H1478/L1091 primer set was used to demonstrate that the quality of the DNA extract was suitable to amplify a product.

ABTC #	Species	Common name	Family	Voucher#	H1478/L109 Product^{+ve} control	MSFCB Product 64 °C anneal	MSFCB product 67 °C anneal
70157[b]	*Aspidites ramsayi*	Woma python	Pythonidae	SAMAR54050	√	√	√
72828[b]	*A. melanocephalus*	Black-headed python	Pythonidae	SAMAR54373	√	√	√
43885[b]	*Morelia amethistina*	Amethistine python	Pythonidae	AMSR115347	√	√	√
68275[d]	*M. amethistina*	Amethistine python	Pythonidae	–			
49652[b]	*M. boeleni*	Boelen's python	Pythonidae	BPBM11611	√	√	√
112609[b]	*M. bredli*	Centralian carpet python	Pythonidae	–	√	√	√
12154[d]	*M. bredli*	Centralian carpet python	Pythonidae	–			
12155[d]	*M. bredli*	Centralian carpet python	Pythonidae	–			
51987[a]	*M. carinata*	Rough-scaled python	Pythonidae	–			
67641[b]	*M. kinghorni*	Scrub python	Pythonidae	QMJ66806	√	√	√
68272[e]	*M. kinghorni*	Scrub python	Pythonidae	–			
67163[b]	*M. nauta*	Tanimbar python	Pythonidae	–	√	√	√
29590[b]	*M. oenpelliensis*	Oenpelli python	Pythonidae	–	√	√	√
55499[a]	*M. spilota*	Carpet python	Pythonidae	SAMAR26877			
62456[c]	*M. spilota*	Carpet python	Pythonidae	WAMR96970			
68310[b]	*M. spilota imbricata*	Southern carpet python	Pythonidae	–	√	√	√
66327[e]	*M. spilota imbricata*	Southern carpet python	Pythonidae	–			
67162[b]	*M. tracyae*	Halmahera python	Pythonidae	–	√	√	√
45444[a]	*M. viridis-S*[f]	Green tree python	Pythonidae	AMSR122363			
49784[b]	*M. viridis-N*[f]	Green tree python	Pythonidae	BPBM11617	√	√	√
46281[e]	*M. viridis-S*[f]	Green tree python	Pythonidae	AMSR122364			
128046[b]	*Python reticulatus*	Reticulated python	Pythonidae	WAMR107781	√	√	–
128029[b]	*P. timoriensis*	Timor python	Pythonidae	WAMR105205	√	√	√
125915[b]	*P. curtus*	Sumatran short-tailed python	Pythonidae	–	√	√	–
125918[b]	*P. brongersmai*	Blood python	Pythonidae	–	√	–	–
125921[b]	*P. breitensteini*	Borneo short-tailed python	Pythonidae	–	√	–	–
48454[b]	*Candoia aspera*	Ground boa	Boidae	AMSR124363	√	√	√
48456[b]	*Boiga irregularis*	Brown tree snake	Colubridae	AMSR124365	√	–	–
127945[b]	*Simoselaps bertholdi*	Banded snake	Elapidae	SAMAR67356	–	√	√
127944[b]	*Ramphotyphlops bituberculatus*	Peter's Blindsnake	Typhlopidae	SAMAR67340	√	–	–
55463[b]	*Acrochrodus arafurae*	Arafura File snake	Acrochordidae	NTMR10687	√	–	–
32313[b]	*Bipes biporus*	Mexican Mole lizard	Amphisbaenidae	–	√	–	–
32261[b]	*Cnemidophorus uniparens*	Whiptail lizard	Teiidae	UMMZ182960	√	–	–
127940[b]	*Ctenotus schomburgkii*	Skink	Scincidae	SAMAR67341	√	–	–
127928[b]	*Nephrurus levis*	Gecko	Gekkonidae	SAMAR67333	√	–	–
127934[b]	*Varanus gilleni*	Goanna	Varanidae	SAMAR67350	√	–	–
127917[b]	*Ctenophorus cristatus*	Dragon	Agamidae	SAMAR67332	√	–	–
16390[b]	*Crocodylus porosus*	Crocodile	Crocodylidae	SAMAR34532	√	–	–
14309[b]	*Chelodina longicollis*	Turtle	Chelidae	SAMAR33946	√	–	–
48478[b]	*Litoria infrafrenata*	Frog	Hylidae	AMSR124387	√	–	–
–[b]	*Homo sapiens*	Human	Hominidae	–	√	–	–
–[b]	*Ovis aries*	Sheep	Bovidae	–	√	–	–
–[b]	*Gallus gallus*	Chicken	Phasianidae	–	√	–	–

[a] Initial gradient amplification (Section 2.2).
[b] Species specificity testing (Section 2.4).
[c] Limit of detection (Section 2.5).
[d] Poor quality DNA template (Section 2.6).
[e] Blind trial (Section 2.7).
[f] Two highly divergent populations of *Morelia viridis* identified by Rawlings and Donnellan [31] that likely represent separate species, labelled – S = southern populations and – N = northern populations.