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TECHNICAL NOTE

Microsatellite primers for Australian and New Guinean pythons isolated with an efficient marker development method for related species

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Abstract

Microsatellites are powerful molecular genetic markers for many evolutionary and biotechnological investigations, however, development of sufficient microsatellite markers is time-consuming and expensive especially considering the vast numbers of species for which they could be used. In light of the conservative nature of microsatellite loci between related species we describe an alternative approach to microsatellite development. A single round of microsatellite isolation enabled the characterization of sufficient loci for a large number of related python species. From 21 loci isolated in the focal species, an average of 86.2% were conserved within the other species while an average of 60.5% were polymorphic in all 13 python species analysed. Our approach will decrease significantly the expense and time required for microsatellite development for large numbers of related species.

Keywords: DNA fingerprinting, microsatellite, python, snake, wildlife management

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Microsatellites, an established form of forensic evidence, are the optimal DNA fingerprinting technology for identification of individuals (Goldstein & Schlötterer 1999). Such technology is needed to assist with the regulation of the legislative and conservation problems caused by the illegal trafficking of native wildlife. Worldwide, the illegal trade in wildlife species is second only to drug trafficking in organized crime activity (McDowell 1997). However, the development of microsatellites will require considerable time and expense for the very large number of wildlife species affected by illegal activities if loci are isolated and developed on a species-by-species basis.

There are some properties of the evolution of microsatellite loci that makes the development of loci for a wide range of species possible without a species-by-species approach. Notably, several studies (e.g. FitzSimmons *et al*. 1995; Rico *et al*. 1996) have shown that microsatellites can be conserved between distantly related species, indeed in one case between species that last shared a common ancestor 450 Ma. Typically, in the 'standard approach', microsatellites are isolated and characterized from a focal species then screened for both their presence and level of polymorphism on a range of related species, often as an afterthought. If isolating and characterizing microsatellites from a large number of related species is the aim, then the standard approach is limited because of inherent selection biases and the pattern of rapid decay of conservation and polymorphism with increasing evolutionary distance from the focal species (Brown *et al*. 1996; Primmer *et al*. 1996). Furthermore, as the microsatellite loci have been specifically selected for analysis in the focal species they tend to be suboptimal for related species often resulting in low levels of conservation, monomorphism, and null alleles. The approach that we trial here differs in that loci were screened simultaneously for presence and polymorphism on the focal species and related species, the aim being to obtain sufficient polymorphic loci for each species through a single round of isolation.

We trial our approach on the pythons, a snake family of 26 species in eight genera (Kluge 1993), that last shared a common ancestor at least 40 Ma (Szyndlar & Böhme 1993). Pythons are an Australian reptile group that is subject to high levels of illegal trade. Microsatellite loci were

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Table 1 Microsatellite locus details, motifs, primer sequences and GenBank accession numbers. Unless specified, the annealing temperature (T_a) of both primers at a locus is identical

Locus	Repeat motif	Primer Sequence (5′ → 3′) (F/R)	T_a (°C)	Expected product size (bp)	GenBank Accession no.
MS1	$(AAAG)_{18}$	M13-CACCATCCCCATCCTGAG	58	302	AF403193
		GAGAAGGATAATGCTGGATG			
MS2	$(AG)_9(AAAG)_{18}$	TGTCTATCAGGGCTCACC	56	438	AF403194
		M13-GGGAAAAGTTACCTATCG	52		
MS3	$(AAAG)_{15}$	M13-CCACAACCTAACCCAATC	54	165	AF403195
		ATCTGGAGCCTGAAGTCC			
MS4	$(AAAG)_{18}$	TATTTCATTTTCCCTATCTTCG	58	312	AF403196
		M13-CAACTCAGTAGGGTGTCAG			
MS5	$(AAAG)_{19}$	M13-TAGGGTGTCAGTCATTGCTC	60	339	AF403197
		TGGCATCCAGCAGTCATAG	58		
MS6	$(AAAG)_{12}AAAAGAAAG(AG)_9(AAAG)_{19}$	M13-CCAGTCACTCTTTCTCAGC	58	378	AF403198
		ATGATACTCTGCACAGCACC	60		
MS7	$(AAAG)_9G(AAGG)_{16}$	M13-GCTCAAGAGAATACAAGACC	58	171	AF403199
		CTTCCCACATCTCTCCTTG			
MS8	$(AGAAAG)_6(AAAGAG)_4$	M13-AGATTAGAGGTGAAGAGACG	58	430	AF403200
		CCGAAAGAAAGAATGAGTGC			
MS9	$(AAAG)_{18}$	CAGTGGGCTTGAGATTGAC	58	204	AF403201
		M13-CATTCCTTAAAACACTCTCAC			
MS10	$(AAAG)_{20}$	M13-AACATCAGAAGACCCAACAC	58	202	AF403202
		AAATCTCAGCCAGCCAACC			
MS11	$(AAGAAAG)_6(AAGACAG)_7$	M13-TGTCTCCAGAAACATCCAG	56	335	AF403203
		ATGGTGAGACTTGAACTTAG			
MS12	$(AAAG)_8$	TTAATTTCTGTCCCCACTGC	58	133	AF403204
		M13-AATTCAGTTCTTTTTAGGGTAG			
MS13	$(AAAG)_{10}$	M13-AACAGAGAAGCACAATCACC	58	185	AF403205
		TGGCTCTCACTTGATATATTAG	60		
MS14	$(AAAG)_{22}$	M13-ACTCCCATTTCATAGCACAG	58	333	AF403206
		CGACAACCCCTTTATTTATGG	60		
MS15	$(AAAG)_{21}(ACAG)_{11}$	M13-ACCTGAAGTAATGCCTCCTG	60	471	AF403207
		AGAAGATACGGTGCCTACTG			
MS16	$(AAAG)_{11}$	GAGTTCTGGTCTTGCTTTCG	60	356	AF403208
		M13-CAGGTACAACTTTCTCCAAC	58		
MS17	$(AAAG)_{19}AG(AAAG)_3$	M13-CAGAAGGTCAGTAAATAGTCC	60	175	AF403209
		ATCAGTTACACGCTCCATTAG			
MS18	$(AAGG)_8AAAGAG(AAAG)_{20}$	M13-CTTTAAAAGCACAGTATTCAGG	60	477	AF403210
		AAATGCTTACTCAGATGCTGG			
MS19	$(AAAG)_{13}$	M13-CATCCATCCATCCAATACAG	58	511	AF403211
		GCAGCAGCCAGTCACAAAC	60		
MS20	$(AAAG)_{13}$	AGAAGCTGGTCCATCCGTC	60	433	AF403212
		M13-AGTGAGGAGAACCAGGATG	58		
MS21	$(GT)_{24}AT(AG)_9$	M13*CTACATAAACCAACATAAGCAG	60	425	AF403213
		GAATAGCATTGTTCAGACACC			
MS22	$(AG)_{10}(AAGG)_{19}AAGAG(AAAG)_{19}$	M13*CAGCAAGAAGGAAGTGAAGG	60	226	AF403214
		TTGGTGGGGTTTATTTTCAGC			
MS23	$(AG)_9(AAAG)_{17}$	M13-GGAGGGGGGGTGTCATAC	60	240	AF403215
		GTGATTCCTCTGACCTATTAC			
MS24	$(AG)_8(AAAG)_{15}$	M13-TAGACAAACCCAGGAAAACC	58	366	AF403216
		TTTGACTTGAGTGAGGACAC			
MS25	$(AAAG)_{24}$	GTGCTGCCCATTTGCTGAC	60	168	AF403217
		M13-GAAACTAAGAAAAGGGGAACC			
MS26	$(AAAG)_{24}$	M13-ACAGTTTCAAGAGGGAGTTAC	60	454	AF403218
		GATTACGCCAAGCTATTTAGG			
MS27	$(AG)_{11}(AAAG)_3(GTTG)_4(AAAG)_4AG(AAAG)_7$	TTACACAACAACCGCCATAG	58	358	AF403219
		M13-CTTCTTATCCTGTTTACTCTG			

screened for microsatellite conservation and polymorphism in the focal species (*Morelia spilota*) and simultaneously in another 12 species representative of the eight python genera.

All tissue samples are from the Australian Biological Tissue Collection (ABTC), South Australian Museum. Species, ABTC voucher numbers and collection locations are available from the corresponding author. We chose the tetranucleotide motif $(AAAG)_n$ because of its abundance in python genomes (Jordan, unpublished data) and because tetranucleotide loci are less prone to PCR stutter (Goldstein & Schlötterer 1999). $(AAAG)_n$ enrichment of *Sau*3A partially digested fragments of *M. spilota* was performed with a magnetic bead-based enrichment protocol and the enriched fragments were amplified using long-range polymerase chain reaction (LR–PCR) (Gardner *et al*. 1999). LR–PCR products were ligated into a pGEM®-T Vector (Promega) and transformed into competent *Eschericia coli* JM109 cells (Promega). Transformants with vectors that contained an insert (ampicillin resistant and white in the presence of X-GAL) were screened using a general colony hybridization process (Sambrook *et al*. 1989) with a biotinylated $(AAAG)_6$ probe and an alkaline phosphatase/streptavidin colourimetric reaction (Boehringer Mannheim).

Positive colonies were sequenced on an ABI 377 DNA Sequencer (PE Biosystems) using the Big Dye™ cycle sequencing kit (PE Biosystems). Primers were designed for 27 microsatellite loci (Table 1), one primer for each locus was synthesized with the M13 (5′-TGT AAA ACG ACG GCC AGT-3′) sequence at the 5′ end, to enable the fluorescent labelling of PCR products (Schuelke 2000).

To analyse conservation and polymorphism of *M. spilota* microsatellite loci in other pythons, 12 python species were selected to represent all eight python genera. Six specimens of *M. spilota* and two specimens of the other 12 python species were screened using a nested touchdown PCR protocol with fluorescent labelling. PCR conditions were: one cycle of 92 °C for 9 min, eight cycles of 92 °C for 30 s, 65–51 °C for 45 s (annealing temperature decreased by 2 °C per cycle), 72 °C for 1 min, 27 cycles of 92 °C for 30 s, 50 °C for 45 s, 72 °C for 1 min, one cycle of 72 °C for 8 min, 26 °C for 10 s.

Conservation of a microsatellite locus in a nonfocal species was deemed positive when PCR amplification resulted in one or two products of a similar size to the product(s) from the focal species. Polymorphism of conserved microsatellite loci was assessed by fluorescence analysis on an ABI 377 DNA Sequencer.

Twenty-one of the 27 microsatellite locus primer pairs were found to amplify one or two products (putative alleles) in the focal specimen that were of a size compatible with that of the cloned sequence. Following the successful amplification of 21 loci from the focal specimen, the conservation of these loci was assessed in 12 other python species. All species, except *Bothrochilus boa*, displayed a high level of microsatellite locus conservation, with 17–20 (81–95%) of the 21 loci assessed amplifying PCR product(s) of a similar size to the product(s) observed for *M. spilota* (Fig. 1). Nine (43%) of the 21 *M. spilota* loci were conserved in *B. boa*.

Polymorphism of the 21 microsatellite loci was also assessed in the 13 species of pythons. Most species analysed possessed between 13 and 16 (62–76%) polymorphic loci, although only 5–9 (24–43%) loci were polymorphic in *B. boa*, *Python reticulatus*, and *P. timoriensis* (Fig. 1). The average number of loci that were conserved in the 12 nonfocal python species was 18.1 and the average number of loci that were polymorphic was 12.7. The average percentage of conserved microsatellite loci that were also polymorphic in each of the nonfocal species was 70%, ranging between 46% (*P. reticulatus* and *P. timoriensis*) and 94% (*Aspidites ramsayi*).

The ‘standard approach’ to isolating microsatellites usually involves screening initially for polymorphism in the focal species. Microsatellite loci that display the highest level of polymorphism are characterized further and the other loci discarded. It is only the few microsatellite loci, typically between six and eight, selected for further characterization that are used to find polymorphic microsatellites in species that are closely related to the focal species. To compare our method of microsatellite characterization to the ‘standard approach’, we first assessed the level of polymorphism of all 21 loci by genotyping six specimens of *M. spilota*. In order to mimic the ‘standard approach’ we selected eight loci (MS1, MS3, MS4, MS5, MS9, MS13, MS16, MS27) that satisfied the ‘standard approach’ criteria of a high level of polymorphism, small allele size range, allele length below 500 bp, and the ability to be used together in a multiplex genotyping system. When we assessed the performance of these selected eight loci in the nonfocal species, we found 33% of the nonfocal species displayed polymorphism in seven of the eight selected loci and only 17% displayed polymorphism in all eight loci (Fig. 1). In contrast, the method of microsatellite development used in the present study showed that 92% of the nonfocal species displayed at least eight polymorphic loci (Fig. 1). To obtain more loci for species with a low level of microsatellite conservation, e.g. *B. boa*, it would be a simple matter to sequence a small number of the remaining transformed colonies that contain putative *M. spilota* microsatellite sequences, rather than carry out a another cycle of microsatellite isolation in a new focal species. This approach is feasible since during the process of microsatellite isolation, only 31 of the 174 (19%) colonies containing putative microsatellite inserts were sequenced to obtain the 21 microsatellite loci that were useful here.

The high level of microsatellite conservation observed here may appear to be surprising as the majority of microsatellite sequences, including the flanking regions, are likely to be noncoding DNA and subject to the neutral rate

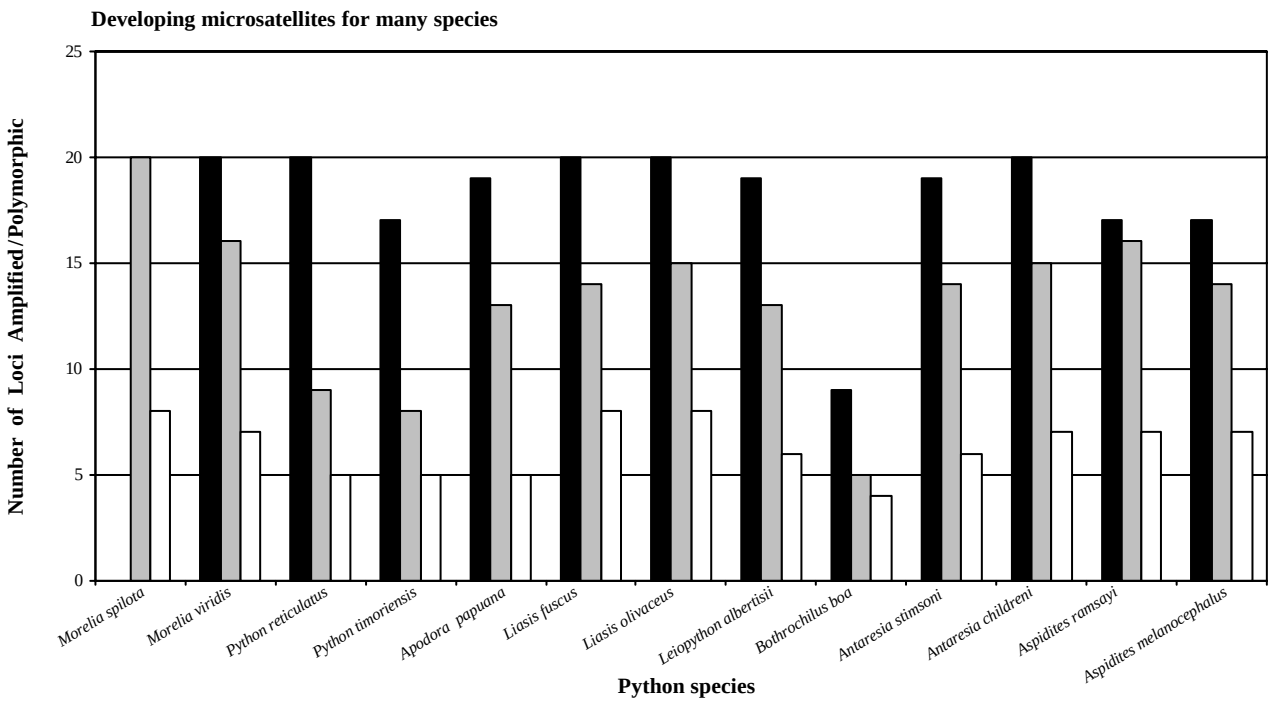

Fig. 1 A histogram showing the conservation and polymorphism of the 21 microsatellite loci isolated from *Morelia spilota* among 13 species of pythons, and a direct comparison between our alternative method of microsatellite development and the standard approach to microsatellite development. The 21 microsatellite loci isolated from *M. spilota* were assessed for conservation in 12 related python species and the polymorphism was assessed among all 13 species of pythons. Black columns = number of conserved loci; grey columns = number of polymorphic loci; white columns = number of loci selected according to the standard approach criteria that are also polymorphic in the nonfocal species.

of nucleotide substitution. As neutral sequences in animals typically have a divergence rate of about 1.0% Myr^{-1} (Wilson *et al*. 1987), it is expected that such sequence divergence would rapidly diminish the likelihood of PCR amplification of homologueous sequences in distantly related taxa. For instance in comparisons of the focal species *M. spilota* and its most distant relatives among the pythons (40 Ma), we would expect to observe on average 16 substitutions for a pair of primers of 40 bp sequence length, an average length for the 21 primer pairs utilized here. Some empirical comparisons of microsatellite conservation support the inverse relationship between evolutionary distance and successful cross-species amplification (Primmer *et al*. 1996), but other studies demonstrate microsatellite conservation over large evolutionary distances (FitzSimmons *et al*. 1995; Rico *et al*. 1996). These latter studies and our observations suggest that there are selective constraints on the nucleotide substitution process in microsatellite flanking regions.

To date the few observations of conservation of individual microsatellite loci over substantial evolutionary periods (e.g. FitzSimmons *et al*. 1995; Rico *et al*. 1996) provide a limited perspective of how large a proportion of loci overall show such conservation. Thus, it would be desirable to assess our approach as a general method for the development of microsatellite markers on other groups comprising large numbers of related species with a longer evolutionary history and to test the upper limits of our approach in terms of the overall evolutionary diversity of the group.

The high proportion of polymorphic loci obtained for each species will allow the development of DNA fingerprinting markers for all 13 python species surveyed. The findings here set a path for future microsatellite isolation/characterization work and development of DNA fingerprinting identification processes required for the large number of wildlife species under threat from illegal activities. Our approach will also be useful to molecular ecologists as they often use a comparative approach to investigate evolutionary questions. The ability to develop microsatellites quickly for a number of related species will substantially increase the power of the comparative approach.

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