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PERMANENT GENETIC RESOURCES

Characterization of tri- and tetranucleotide microsatellite loci for the slatey-grey snake (*Stegonotus cucullatus*, Colubridae)

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Abstract

We characterized nine polymorphic microsatellite (six trinucleotides and three tetranucleotides) loci for the slatey-grey snake (*Stegonotus cucullatus*) from Australia, in order to study the mating system of this species. Based on a total of 100 samples, the number of alleles per locus ranged from three to 10, and the observed and expected heterozygosities ranged from 0.62 to 0.86 and from 0.53 to 0.83, respectively.

Keywords: Colubridae, microsatellite, paternity, sexual dimorphism, snake

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Most species of snakes show sexual dimorphism in mean adult body size, with females growing larger than conspecific males (Shine 1994). Larger body size in females may be favoured because bigger females typically produce more offspring per clutch (Rivas & Burghardt 2005). But why, in a minority of snake species, do males attain larger body sizes than females? The likely explanation is that larger body size enhances male success in physical combat over females, and hence increases male mating opportunities (Shine 1994). Larger males also may be better at mate-finding or at overcoming female resistance during courtship attempts (Madsen & Shine 1993; Shine *et al*. 2005). Female reproductive success also may be enhanced by body size in ways other than simple increases in fecundity; for example, larger females tend to attract more courtship and hence have a higher frequency of multiple mating (Prosser *et al*. 2002), which may increase offspring viability (Madsen *et al*. 1992). To clarify the reasons why males sometimes attain much larger sizes than females, we need field studies on reproductive success.

The slatey-grey snake (*Stegonotus cucullatus* Duméril, Bibron & Duméril 1854), a terrestrial colubrid distributed in tropical Australia and New Guinea (Cogger 2000), provides an extreme example of male-biased sexual size dimorphism among snakes (Shine 1994). In animals studied by Shine (1994), adult males were 13% larger than females. Hence, the evolutionary ecology of the slatey-grey snake warrants detailed study. Here, we describe nine polymorphic microsatellite loci for paternity analyses in this species.

Total cellular DNA was isolated from scales. Tissue of each individual was placed in 200 μL of 5% Chelex containing 0.2 mg/mL of proteinase K, incubated overnight at 56 °C, and boiled at 100 °C. Enriched genomic libraries from *S. cucullatus* were constructed by Genetic Identification Services (GIS, http://www.genetic-id-services.com). Methods for DNA library construction, enrichment and screening were as described by Jones *et al*. (2002). Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV; New England Biolabs). Fragments in the size range of 300–750 bp were ligated to adaptors and enriched for microsatellites via a biotin-capture method using streptavidin-magnetic beads (CPG, Inc.). Libraries were prepared in parallel using Biotin-AAC_{12}, Biotin-CAG_{10}, Biotin-$CATC_8$ and Biotin-$TAGA_8$ as capture molecules in a protocol provided by the manufacturer. Captured molecules were amplified with the forward strand of the adapter sequence (5′-ACGACGTTGTAAAACGACGGAAGCTT-3′), and restricted with *Hin*dIII to remove the adapters. The resulting fragments were ligated into the *Hin*dIII site of pUC19. Recombinant molecules were electroporated into *Escherichia coli* (strain DH5α; ElectroMax-, Invitrogen). Recombinant clones were selected at random by standard blue/white colony selection on Luria-Bertani–ampicillin plates, and sequenced using an M13-based sequence 5′- to the insert (5′-AGGAAACAGC-TATGACCATG-3′). Sequences were obtained on an ABI

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Table 1 Characteristics of microsatellite loci for the snake *Stegonotus cucullatus*, with GenBank Accession nos. Values reported are the range with clone size (bp), the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, and amplification conditions (final mM of $MgCl_2$ and annealing temperature)

Loci	Repeat motif	Primer sequence (5′–3′) with fluorescent label	Accession no.	Range (bp)	N_A	H_O	H_E	PCR
Steg_A4	$(CAA)_9$	F: VIC-CGAGCTGCTGAGCTATTAACC	EU022681	143–152 (152)	3	0.62	0.53	1.25
		R: CAATGCAATTCCAGTTCCTCT						57
Steg_A5	$(CAA)_{16}$	F: VIC-ATGCTTCATGTCCCATCAATC	EU022682	280–301 (298)	8	0.86	0.83	1.25
		R: ACAACTCTGGGTGCCTTACAG						58.5
Steg_A105	$(CAA)_{10}$	F: VIC-GCTTCCGTAATCCTTTGC	EU022683	138–159 (156)	8	0.79	0.73	1.25
		R: CTGAACATCTGGCTGACTG						57
Steg_B104	$(CAT)_8$	F: PET-CCCCACTACAAAAGTCAAAGG	EU022684	164–200 (176)	9	0.81	0.73	1.25
		R: GTGCTGTTCTCTTCCTCCATC						57
Steg_B105	$(CAT)_{12}$	F: 6-FAM-CCTGAGTCATCTGGAGAGAGG	EU022685	160–184 (163)	9	0.72	0.66	1.25
		R: TAAACCTTGTAGGAGCGAGGAC						57
Steg_C109	$(CATC)_9$	F: NED-CCTTATCCCCTGAAGAGGAG	EU022686	239–275 (259)	8	0.81	0.80	1.25
		R: GGCTTATTATGGCTTGCTTG						57
Steg_D1	$(CTAT)_{15}$	F: 6-FAM-GCAGAATCCATTGATCTGAAG	EU022679	157–185 (177)	8	0.81	0.77	1.25
		R: GACGGACAGACAGACAGCTAC						57
Steg_D2	$(CTAT)_{12}$	F: NED-TTATGGGATGACAGAGATGATG	EU022680	172–208 (188)	10	0.78	0.79	2.0
		R: ATGCCTACTACAGAAGACCCTG						57
Steg_D114	$(CTAT)_{11}$	F: PET-AAGTCAAGATGGCAATCACAAC	EU022687	157–181 (161)	6	0.82	0.75	1.25
		R: GGTCTCAACGGTGTCATAAGTC						57

PRISM 377 genetic analyser (Applied Biosystems, Inc.), using ABI PRISM *Taq* dye terminator cycle sequencing methodology. Twenty-five clones were sequenced from each library. Microsatellite yields were AAC, 18; ATG, 18; CATC, eight; and TAGA, 22, and oligonucleotide primers were designed for 53 loci using the software DESIGNERPCR version 1.03, 1994 (Research Genetics, Inc.), and nine were tested for polymorphism (Table 1).

Polymerase chain reaction (PCR) amplifications were performed in a 9800 Fast thermal cycler (Applied Biosystems) with 5-μL reactions containing 0.075 U *Taq Ti* DNA polymerase (Biotech), 0.1 mM dNTPs, 0.4 μM of each primer, 20 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.25 or 2.0 mM $MgCl_2$ (Table 1), and 15 ng of DNA. Cycling conditions included a hot-start denaturation of 95 °C for 3 min; followed by 35 cycles of 95 °C for 30 s, 57–58.5 °C (Table 1) annealing temperature for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 30 min.

PCR products were amplified with one primer of each primer pair end–labelled with a fluorescent dye, either 6-FAM, NED, PET, VIC (Table 1). Four and five PCR products of different loci were multiplexed together and added to a loading buffer (containing formamide) and Liz-500 (Gene Scan) as the internal size standard and separated by electrophoresis on an ABI PRISM 3130xl genetic analyser (Applied Biosystems). Microsatellite allele sizes were determined with GENEMAPPER software version 3.7 (Applied Biosystems).

Variability of these microsatellite loci was tested on 100 individuals from two adjacent populations located in Northern Territory, Australia (Fogg Dam, 73 samples: 12°34′13″S, 131°17′53″E and Harrison Dam, 27 samples: 12°34′39″S, 131°20′16″E). The number of alleles, observed and expected heterozygosities, linkage disequilibrium and tests for Hardy–Weinberg equilibrium per locus were calculated with the program FSTAT (Goudet 1995; Table 1). Significance values were corrected for multiple tests using the sequential Bonferroni correction (Rice 1989). Detection of null alleles was tested according to Chakraborty & Jin (1992).

The nine loci were polymorphic within the two populations, with a total number of alleles ranging from three to 10 (mean 7.7); observed heterozygosity ranged from 0.62 to 0.86 and expected heterozygosity ranged from 0.53 to 0.83. No linkage disequilibrium and null alleles were detected, and all loci were in Hardy–Weinberg equilibrium. Consequently, the loci described here should be useful for assessing genetic structure and parentage analysis in this snake species, and hence may shed light on the unusual pattern of sexual size dimorphism within this taxon.

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