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PRIMER NOTE

Characterization of microsatellite markers for Couch's spadefoot toad (*Scaphiopus couchii*) and cross-amplification in other species of the family Scaphiopodidae

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Abstract

I developed 12 di- and tetranucleotide microsatellite loci for Couch's spadefoot toad (*Scaphiopus couchii*). These loci have 3–37 alleles per locus and observed heterozygosities ranging from 0.157 to 0.941 among 85 individuals from four populations. Global and within-population exact tests do not reveal departure from Hardy–Weinberg expectations and all loci pairs are in linkage equilibrium. These independent markers will be useful for studies of population structure and kinship in this commonly studied amphibian. Additionally, several of these loci may be applicable for studies of other North American toads of the family Scaphiopodidae.

Keywords: amphibian, anuran, desert, frog, population genetics, relatedness

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Couch's spadefoot toad (*Scaphiopus couchii*) is a frog in the family Scaphiopodidae that is common throughout arid central and northern Mexico and the southwestern United States (Morey 2005). The ecology and larval development of *S. couchii* has been well studied (e.g. Tinsley & Tocque 1995; Newman 1998; Dayton & Fitzgerald 2001) and they are notable among North American amphibians because they can metamorphosis in as little as 8 days (Newman 1987). However, relatively little is known about population genetics and mating system biology of this species. I developed microsatellite markers to quantify reproductive success, larval survivorship, and population connectivity in this species.

Whole genomic DNA was extracted from a single adult individual using the QIAquick DNeasy Extraction Kit (QIAGEN). I digested 300 ng of DNA with the restriction enzymes *Alu*I and *Hae*III in two reactions and ligated SNX linkers to the fragments using T4 DNA ligase (Hamilton *et al*. 1999). The pool of ligated DNA fragments was enriched by hybridization with biotin-labelled di-, tri-, and tetrameric oligonucleotides in reactions consisting of a 10 min incubation at 97 °C followed by a 60 min incubation at 52°, 60°, or 65 °C. I recovered single-stranded fragments containing microsatellite repeats using streptavidin-coated magnetic beads (New England Biolabs) and made fragments double-stranded with a polymerase chain reaction (PCR) using the SNX primer. PCR products were cut with *Nhe*I and the digested product was run on a 1% TAE agarose gel for size selection. I excised fragments from 300 to 900 base pairs in length and purified these using a QIAquick Gel Extraction Kit (QIAGEN). Fragments were ligated to pUC19 plasmids and transformed into DH5α T1 phage-resistant competent cells (Invitrogen). I plated cells on LB agar/ampicillin plates and transferred colonies to nylon membranes that were hybridized to ^{32}P radiolabelled probes with the same repeat motifs used in the enrichment and visualized on autoradiographs (Sambrook & Russell 2001). Clones were cultured overnight and DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN).

I sequenced 344 clones using the primer M13F (5′-GTTGTAAAACGACGGCCAGTG-3′) and the Big Dye termination sequencing chemistry (PerkinElmer) on an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems). I trimmed the pUC19 vector and SNX linker from sequences and aligned all sequences containing microsatellites to identify duplicate clones. I found 136 unique sequences containing microsatellites and designed

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primers for the flanking regions of these sequences using the programs PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and PRIMERSELECT (DNASTAR). Sequences of the clones used for primer design are accessioned in GenBank Accession nos DQ922795–DQ922806.

I incubated tissue for 120 min at 55 °C and 10 min at 99 °C in 150 μL of a 5% Chelex solution (Chelex-100; Bio-Rad) with 1 μL proteinase K to purify template DNA from spadefoot toe clips. All PCRs were done at 10 μL with 1 μL template DNA, 1 × buffer, 0.4 μM dNTPs, 1.5 mM $MgCl_2$, and 0.25 U *Taq* polymerase. Primer concentrations differed across loci (see Table 1). PCR for most loci used two primers with the forward primer fluorescently labelled for visualization on a capillary sequencer (1–9, Table 1). Four two-primer loci amplified well in multilocus PCRs; I combined scoD097 and scoT135 in a single PCR and scoD106 and scoT019 in another single PCR (1–4, Table 1). Five loci amplified optimally in single-locus PCRs (5–9, Table 1). For three loci I used a three primer system described by Waldbieser *et al*. (2003) with a fluorescently labelled 'universal' primer (5′-TTTATTCTACAGCCTTCACTTTCA-3′), a forward primer with 5′-tail of 5′-CGAGTTTTCCCAGTCACGAC-3′, and a reverse primer with a 5′-tail of 5′-GTTT-3′. All thermocycler profiles consisted of a 94 °C initial denaturing for 5 min, 35 cycles of 1 min at 94 °C, 1 min at a locus specific annealing temperature (Table 1), and 1 min at 72 °C, and 5 min final extension at 72 °C. I optimized 12 microsatellite markers that were genotyped in two multiplex groups (1–6, 7–12, Table 1).

I chose 85 adult individuals from four breeding populations in the San Simon Valley in Arizona for the test of genetic variation, departure from Hardy–Weinberg equilibrium (HWE), and independence at these 12 loci. Statistical significance was assessed using the software GENEPOP on the web version 3.4 (Raymond & Rousset 1995). A Markov chain method (Guo & Thompson 1992) with 2500 dememorization steps and 100 batches of 5000 iterations per batch was used to determine significance. I found no

Table 1 Primer sequences, amplification conditions, and diversity in 12 microsatellite loci for *Scaphiopus couchii*

	Locus/GenBank Accession no.	Primer (5′–3′)	Primer concentration (μM)	Repeat	T_a	Size	N_a	H_O/H_E
1	scoD097	F: PET – CTGGCTTATTTGTCGGATTGG	0.1	$(TG)_{11}$	60	283–303	8	0.706/0.756
	DQ922797	R: CTCGCTGCTCATGTAGAAAAAGTT	0.1					
2	scoT135	F: VIC – GGCTCCAACCACAACAAACA	0.2	$(TATC)_{15}(TACC)_3$	60	229–265	10	0.729/0.844
	DQ922801	R: GAAGCCTATAAAAGCAGAGTGA	0.2	$TATA(TATC)_3$				
3	scoD106	F: NED – AGGGACATGTGGCTGGAT	0.05	$(TG)_9$	62	210–215	3	0.560/0.642
	DQ922798	R: CATACACTCGCACTGACTCTAAC	0.05					
4	scoT019	F: 6-FAM – GTACAGGGGCAACAACT	0.2	$(GATA)_{16}$	62	258–310	13	0.906/0.875
	DQ922799	R: GAACCAGAAAGCCAAACCATA	0.2					
5	sco126	F: VIC – GCACCAATAAGCATAAGT	0.2	$(TG)_8$	58	83–92	6	0.157/0.286
	DQ922805	R: GGCAGGCCCTCATCACC	0.2					
6	scoT043	F: 6-FAM – TGCCCCTCCAGATTACAGA	0.2	$(ACT)_{18}$	46	131–169	11	0.762/0.701
	DQ922800	R: TGCCCAGCGCCCTTTTC	0.2					
7	scoD002	F: 6-FAM – GGCCCATTATCTTCCAGGTTAG	0.2	$(GT)_{22}$	50	122–150	15	0.734/0.860
	DQ922795	R: CCTAGGGGGTTAATTTTT	0.2					
8	scoD089	F: VIC – AGCCGGGGCCCTCTTCTC	0.2	$(AATGT)_3 56 (GT)_7$	60	282–288	3	0.457/0.480
	DQ922796	R: ATAACCTGCTCACTTTGCCTTTCA	0.2					
9	scoT142	F: PET – AGGATATTTAAAGGGATTGTTGC	0.2	$(TCTA)_{14}(TCTG)_5$	60	262–375	37	0.941/0.950
	DQ922802	R: TCCCATGTGGTTTAGTTTAGAT	0.2	$(TCTA)_{15}$				
10	sco130	U: PET – CGAGTTTTCCCAGTCACGAC	0.2	$(AC)_5A(AC)_6$	62	162–170	6	0.624/0.575
	DQ922806	F: UNIV – CTCTGAGCAGCCTACCTA	0.1					
		R: GTTTACCCCCTTTTAATACCGAGACA	0.4					
11	scoT145	U: NED – CGAGTTTTCCCAGTCACGAC	0.2	$(ATCT)_{19}$	60	296–362	16	0.807/0.890
	DQ922803	F: UNIV – AATTATTTTCCCTACAACAACAT	0.1					
		R: GTTTTAGATACCAGCCTGACATTACATA	0.4					
12	scoT151	U: 6-FAM – CGAGTTTTCCCAGTCACGAC	0.2	$(TAGA)_2TACA$	58	269–325	16	0.824/0.904
	DQ922804	F: UNIV – TTACTGTGCCCGTGTTGTTA	0.1	$(TAGA)_{13}TA(GT)_2$				
		R: GTTTACTTCCCCTCATGCTACTTTC	0.4					

F, forward primer; R, reverse primer; U, universal primer; 'UNIV' corresponds to the sequence 5′-CGAGTTTTCCCAGTCACGAC-3′ T_a, annealing temperature; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

evidence of departure from HWE for global and population based tests and did not detect any linkage disequilibrium for global tests. This indicates that these loci are independently evolving genetic markers and are useful for studies of population genetic structure, kinship, and parentage in *S. couchii*.

In southwestern deserts, *S. couchii* is syntopic with two other species in the family Scaphiopodidae, *Spea bombifrons* and *Spea multiplicata.* Adults and older tadpoles of these species are easily distinguishable; however, young larvae (< 1 week old) can be difficult to identify. To be certain that my sample did not include misidentified tadpoles, I tested cross-specific amplification of these markers in *Spea*. Using the same PCR conditions optimized for *Scaphiopus couchii*, I genotyped eight adult individuals of each species of *Spea*. None of the loci showed strong and consistent amplification in *Spea*, corroborating previous reports of low cross-amplification success even among closely related anuran species (e.g. Rowe *et al*. 2000; Primmer & Merilä 2002). However, six loci (sco126, scoD002, scoD106 scoT019, scoT043, scoT135) amplified weak products, but with readable signal on an ABI 3100 and at different fragment sizes than alleles for *S. couchii*. Therefore, with additional optimization, these loci may yield markers that are polymorphic and useful for studies in *Spea* as well.

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