Molecular Ecology Resources (2008) **8**, 1386–1389 doi: 10.1111/j.1755-0998.2008.02291.x

PERMANENT GENETIC RESOURCES

Development and characterization of nine polymorphic microsatellite markers for Mexican spadefoot toads (*Spea multiplicata*) with cross-amplification in Plains spadefoot toads (*S. bombifrons*)

A. M. RICE,*# D. E. PEARSE,† T. BECKER,‡ R. A. NEWMAN,‡ C. LEBONVILLE,* G. R. HARPER*§ and K. S. PFENNIG*

**Department of Biology, University of North Carolina, Chapel Hill, NC 27599, USA, †Southwest Fisheries Science Center, 110 Shaffer Road, Santa Cruz, CA 95060, USA, ‡Department of Biology, University of North Dakota, Grand Forks, ND 58202, USA*

Abstract

We developed nine polymorphic microsatellite markers for the Mexican spadefoot toad, *Spea multiplicata*. Allele numbers range from five to 12, with observed heterozygosities from 0.48 to 0.87. Because two loci are in linkage disequilibrium, these nine loci provide eight independent markers. Three loci exhibit departure from Hardy–Weinberg equilibrium, possibly resulting from null alleles or population admixture. These markers will be useful for assessing population structure and relatedness in *S. multiplicata*. Based on our success at cross-amplification in the Plains spadefoot toad (*Spea bombifrons*), these loci also may be useful in this species with additional optimization.

Keywords: amphibian, cross-amplification, population genetics, SSR markers

Received 23 February 2008; revision accepted 7 April 2008

Mexican spadefoot toads (*Spea multiplicata*) range from southern Mexico into the southwestern USA (Stebbins 2003). This species has been studied for decades, on topics as diverse as phenotypic plasticity, character displacement, hybridization, speciation and sexual selection (e.g. Bragg 1965; Forester 1975; Pomeroy 1981; Simovich & Sassaman 1986; Pfennig 2000; Pfennig *et al*. 2007). Despite the attention to this species, few population genetic markers have been developed for spadefoot toads (but see Simovich & Sassaman 1986; Chan 2007). Here we report the development and characterization of nine polymorphic microsatellite markers for studies of population structure, gene flow, kinship and inbreeding in *S. multiplicata*.

To isolate microsatellite loci for *S. multiplicata*, we extracted DNA from the blood of one adult using phenol–chloroform (blood samples were stored in lysis buffer before extraction). Following standard protocols (Prodöhl *et al*. 1996; Pearse *et al*. 2001), whole genomic DNA was digested using the *Mbo*I restriction enzyme, and the products were size selected using agarose gel electrophoresis. Selected fragments were ligated into linearized pBluescript II SK + vector (Stratagene) and transformed into competent cells to create a bacterial clone library. Successfully transformed bacterial colonies were screened using ^{32}P-labelled probes for di-, tri- and tetranucleotide microsatellite repeat motifs. After hybridization, positive colonies were grown overnight in liquid media and plasmid DNA was purified using QIAprep minipreps (QIAGEN). The vector primers T3 and T7 were used to sequence 25 positive clones using the *fmol* DNA sequencing system (Promega) and ^{32}P autoradiography. From these sequences, primer pairs were designed for eight loci, seven of which proved useful and are presented here (Sm1, Sm4, Sm14, Sm17, Sm20, Sm23 and Sm25; see Table 1).

We developed two additional loci (Sb15 and Sb28; see Table 1) from a *Spea bombifrons* microsatellite library, and later optimized these loci for *S. multiplicata*. We used

Correspondence: Amber M. Rice, Fax: +46 18 471 6484; E-mail: amber.rice@ebc.uu.se

§Present address: Department of Biology, Hendrix College, Conway, AR 72032, USA

#Present address: Department of Animal Ecology, Evolutionary Biology Centre, Uppsala University, SE-75236 Uppsala, Sweden

Table 1 Primer sequences, annealing temperatures and locus variation summaries for *Spea multiplicata* microsatellite loci, with cross-amplification results for *Spea bombifrons*

				S. multiplicata			*S. bombifrons*		
Locus/GenBank Accession no.	Primer sequences (5′–3′)†	Repeat motif	T_a	N_A (size range)	H_O/H_E	N_G/N_S	N_A (size range)	H_O/H_E	N_G/N_S
Sm1	F: NED-TAACATCCATAGAGTAAAT	GT	50 °C	5 (103–125)	0.55/0.47	40/40	8 (106–132)	0.75/0.76	8/21
EU285450	R: GTCTATAATACAAAGTAATATC								
Sm4	F: 6-FAM-TATGCCTGGTTAAGACTTTATTATC	$(GT)_8(TA)_{15}TTAA(TA)_4$	55 °C	12(163–201)	0.48/0.90**	40/40	4 (158–185)	0.33/0.87	3/22
EU285451	R: CCTGTTCCTAATATAAACACTTTG								
Sm14	F: VIC-GGAAAACTGCCCATGAAAAA	$(GATA)_{14}GACA(GATA)_4$	50 °C	11(170–242)	0.78/0.80	40/40	8 (164–200)	0.34/0.59	35/42
EU285452	R: CCATGAGCTGTCACTAGTTTGC	$(GACA)_6GATA$							
Sm17‡	F: NED-CAATAACCATCTATTTCTTCACTGT	$(GATA)_{13}(GACA)_2$	55 °C	9 (141–181)	0.80/0.83	40/40	6 (93–172)	0.40/0.89	5/44
EU285453	R: GAACCCAGCGAGAAACTTACT								
Sm20‡	F: 6-FAM-TTACTGTACTCCTTTGTAATATACT	GATA	55 °C	10 (163–199)	0.87/0.87	39/40	2 (93–171)	0.25/0.25	4/44
EU285454	R: TATACTGCCTGTTTACCTACC								
Sm23	F: 6-FAM-TGCTAGTGTACGATGCATATATT	CT	50 °C	12 (133–167)	0.85/0.89	40/40	5 (97–155)	0.20/0.51	10/44
EU285455	R: CAGGGGTGTGAGTTATATGTTT								
Sm25	F: 6-FAM-TGCATCAAAACCCATAAGTGAA	$(GATA)_{14}GACAGATA$	50 °C	11 (143–191)	0.63/0.75*	40/40	13 (138–198)	0.79/0.89	42/43
EU285456	R: CAGCCCATGTCGTTTTTAAATAG								
Sb15	F: PET-ATAAATCCTGGATCTTTCTC	AAT	50 °C	7 (47–65)	0.53/0.77**	40/40	12 (59–92)	0.86/0.89	42/42
EU285444	R: GGGAAGTAGATTAAATTATTG								
Sb28	F:PET-GGGCAACTTTAGCGTCTT	AAT	50 °C	6 (314–344)	0.53/0.54	40/40	4 (312–332)	0.40/0.55	40/44
EU285445	R:AACTGTTGGCGCTATATAAAT								

F, forward primer; R, reverse primer; T_a, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; N_G, number of individuals successfully genotyped; N_S, number of individuals screened. *Significant deviation from Hardy–Weinberg equilibrium: *$P = 0.005$; **$P < 0.0001$. †Type of fluorescent label used (DS-33, manufactured by Applied Biosystems) is indicated at the beginning of forward primer sequences. ‡Locus pair in linkage disequilibrium ($P < 0.001$).

phenol–chloroform to extract genomic DNA from muscle tissue of one adult *S. bombifrons*, restriction enzyme (*Dpn*II) digested the DNA, and selected fragments of 200–600 bp. We then ligated these fragments into phage genomes, mixed the phage with XLI Blue *Escherichia coli*, and plated the bacterial suspension. After a 12-h incubation at 37 °C, we screened this library for clones containing the trinucleotide repeat AAT using nylon membranes (Immobilon) treated with ^{32}P-labelled probes. We identified positive clones by exposing X-ray film (Kodak XAR) to the labelled membranes, and then excised these clones from the phage genome. We sequenced 33 positive clones using vector primers (PBKCMV-1 and PBKCMV-2). Sequencing reactions included ^{32}P, and 30 cycles were carried out in an Omnigene thermocycler (Hybaid) under the following conditions: 95 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s. We ran the sequencing reactions on denaturing acrylamide gels, and designed primer pairs for the seven clones that had at least eight repeats and appropriate flanking sequences. Two of these primer pairs were optimized for *S. multiplicata*, and are presented here (Sb15 and Sb28, Table 1).

We genotyped 40 *S. multiplicata* tadpoles collected from breeding sites in the San Simon Valley of Cochise County, Arizona. We extracted DNA from tail tissue using either saturated NaCl or chloroform/isoamyl alcohol, and then polymerase chain reaction (PCR)-amplified each locus in all 40 individuals. Each 10-μL PCR was carried out in the PTC-200 DNA Engine thermocycler (MJ Research) with 1-μL template DNA, 1× buffer (including 1.5 mM $MgCl_2$), 0.1 mM dNTPs, 0.3 μM fluorescently labelled forward primer (Applied Biosystems DS-33; Table 1), 0.3 μM reverse primer, and 1 U *Taq* DNA polymerase (Promega). After an initial 3-min denaturation at 94 °C, we carried out 25 cycles of the following profile, ending with a final extension of 60 min at 72 °C: 94 °C for 60 s, annealing temperature for 60 s (see Table 1), 72 °C for 60 s, and 55 °C for 45 s. PCR products were submitted to the UNC-Chapel Hill Genome Analysis Facility for genotyping on an ABI PRISM 3730 Genetic Analyser. Peaks were scored based on an internal size standard (GeneScan500 LIZ; Applied Biosystems) using GeneMapper version 3.7 software (Applied Biosystems).

Using the successfully genotyped individuals, we characterized variation at each locus for *S. multiplicata* (Table 1). We used GenePop 4.0.6 (Rousset 2008) to test loci for departure from Hardy–Weinberg equilibrium, and loci pairs for linkage disequilibrium. Statistical significance was estimated using the Markov chain method, with 10 000 dememorization steps, 100 batches and 5000 iterations per batch. We adjusted α-values for multiple comparisons using the Bonferroni correction. Two loci, Sm17 and Sm20, were in linkage disequilibrium ($P < 0.001$); therefore, these nine loci provide eight independent markers for *S. multiplicata*. Three loci (Sm4, Sm25, Sb15) exhibited significant departure from Hardy–Weinberg equilibrium ($P < 0.006$), resulting from heterozygote deficiencies. Heterozygote deficiencies may have numerous causes, including null alleles and population admixture. We used Micro-Checker (van Oosterhout *et al*. 2004) to test for the presence of null alleles, and found evidence of null alleles at Sm4 and Sb15. However, because population structure has been detected in *S. multiplicata* (A. Rice, unpublished data), population admixture may also be a cause of the observed heterozygote deficiencies; the samples we used to characterize variation may have been drawn from multiple populations.

Using the optimal PCR conditions for *S. multiplicata* (Table 1), we tested the nine loci for cross-amplification in up to 44 individual *S. bombifrons* collected from the San Simon and Sulphur Springs Valleys in Cochise County, Arizona. All of the loci amplified in at least three individuals, while four of the loci (Sm14, Sm25, Sb15, Sb28) had genotyping success rates of 83% or more (Table 1). Moreover, all nine loci were polymorphic in *S. bombifrons*.

These microsatellite loci will be useful for studies of population structure, gene flow, kinship and inbreeding in *S. multiplicata*. Moreover, our success at cross-amplification in *S. bombifrons* suggests that with additional optimization, these loci may be useful for population genetic studies in this species as well.

Acknowledgements

We thank Dr Colin Hughes for invaluable assistance with primer development. This research was supported by NSF Grants DEB-0542566 and IOB-0455380 to KSP, and funding from the USGS Northern Prairie Wildlife Research Center to RAN. DEP developed the *Sm* loci in John Avise's laboratory at the University of Georgia Department of Genetics.

References

Bragg AN (1965) *Gnomes of the Night: The Spadefoot Toads*. University of Pennsylvania Press, Philadelphia.

Chan LM (2007) Characterization of microsatellite markers for Couch's spadefoot toad (*Scaphiopus couchii*) and cross-amplification in other species of the family Scaphiopodidae. *Molecular Ecology Notes*, **7**, 318–320.

Forester DC (1975) Laboratory evidence for potential gene flow between two species of spadefoot toads, *Scaphiopus bombifrons and Scaphiopus hammondii*. *Herpetologica*, **31**, 282–286.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Pearse DE, Janzen FJ, Avise JC (2001) Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles. *Heredity*, **86**, 378–384.

Pfennig KS (2000) Female spadefoot toads compromise on mate quality to ensure conspecific matings. *Behavioral Ecology*, **11**, 220–227.

Pfennig DW, Rice AM, Martin RA (2007) Field and experimental evidence for competition's role in phenotypic divergence. *Evolution*, **61**, 257–271.

Pomeroy LV (1981) *Developmental Polymorphism in the Tadpoles of the Spadefoot Toad Scaphiopus Multiplicatus.* PhD dissertation, University of California, Riverside, California.

Prodöhl PA, Loughry WJ, McDonough CM, Nelson WS, Avise JC (1996) Molecular documentation of polyembryony and the micro-spatial dispersion of clonal sibships in the nine-banded armadillo, *Dasypus novemcinctus. Proceedings of the Royal Society B: Biological Sciences*, **263**, 1643–1649.

Rousset F (2008) GenePop '007: a complete reimplementation of the GenePop software for Windows and Linux. *Molecular Ecology Resources*, **8**, 103–106.

Simovich MA, Sassaman CA (1986) Four independent electrophoretic markers in spadefoot toads. *Journal of Heredity*, **77**, 410–414.

Stebbins RC (2003) *A Field Guide to Western Reptiles and Amphibians*, 3rd edn. Houghton Mifflin Company, New York.