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PRIMER NOTE

Polymorphic microsatellite loci for tiger salamanders, *Ambystoma tigrinum*

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Abstract

Microsatellites have been developed for few amphibian species. However, developing genetic markers for population genetic studies in amphibians is critical because amphibians are declining globally. The tiger salamander, *Ambystoma tigrinum*, is widespread throughout the United States and includes the endangered subspecies, *A. t. stebbinsi*. We present primers and amplification conditions for 10 polymorphic microsatellite loci that have produced successful results in three subspecies of *A. tigrinum*. Number of alleles per locus ranged from one to 11 and heterozygosity ranged from 0 to 0.815 depending on the subspecies and locus analysed. These markers should prove useful for future studies of genetic diversity and population subdivision.

Keywords: *Ambystoma tigrinum*, amphibian, microsatellite, tiger salamander

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Microsatellites have been developed for few amphibian species, including the Pacific chorus frog (*Hyla regilla*) and Northern spotted frog (*Rana lutiventris*; Call & Hallett 1998), the common toad (*Bufo bufo*; Brede *et al*. 2001), the natterjack toad (*Bufo calamita*; Rowe *et al*. 1997), newts (*Triturus cristatus*, and *T. marmoratus*; Jehle *et al*. 2001), and Pacific giant salamanders (*Dicamptodon tenebrosus*; Curtis & Taylor 2000). Developing useful genetic markers for population genetic studies in amphibians is critical because amphibians are indicator species of habitat degradation and are now declining globally (Wake 1998).

The tiger salamander, *Ambystoma tigrinum*, is widespread throughout the United States, southern Canada, and Mexico (Gehlbach 1967). The species complex contains eight subspecies, including the endangered *A. t. stebbinsi*. Mitochondrial DNA markers, such as the control region and an insert between proline and threonine tRNAs have been developed for *A. tigrinum*, but have not been useful for detecting fine-scale population structure (Shaffer & McKnight 1996). Thus, genetic markers with high resolution, such as microsatellites, are needed to conduct population genetic studies within subspecies (Goldstein & Schlötterer 1999). We developed a microsatellite library for *A. tigrinum* that yielded 10 polymorphic loci that successfully amplified in three subspecies.

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Genomic DNA was isolated using Puregene® DNA (Gentra, Inc.) isolation kits following a modified version of the standard animal tissue isolation protocol. Our modifications followed three basic steps: (i) tissues were homogenized in Puregene® cell lysis buffer and incubated at 55 °C for 1 h with 10 μL of 20 mg/mL Proteinase-K solution; (ii) tissues were re-homogenized and incubated at 55 °C overnight; (iii) after incubation, samples were homogenized a third time. Genomic DNA was then digested with *Sau3*AI restriction enzyme and 400–1500 bp fragments were selected via agarose gel electrophoresis and size fractionation using Chromo Spin® columns (Clonetech Laboratories). We then ligated fractionated DNA to *Sau*3AI linkers and amplified using standard PCR conditions and followed a standard enrichment protocol using Vectrex Avidin D matrix (Vector Laboratories, Inc.) to create a library enriched for $(CA)_n$ and $(GA)_n$ repeats. PCR products from the enriched library were then directly ligated to a TOPO vector (Invitrogen, Inc.). We then transformed plasmid vectors into One Shot™ *E. coli* (Invitrogen, Inc.).

Colonies were screened using standard nitrocellulose membranes and hybridized with a $(CA)_n$ chemiluminescent probe (Lifecodes, Inc.) and sprayed with Lumi-Phos 480 (Lifecodes, Inc.). Positive clones were then detected

Table 1 Microsatellite primer information for *Ambystoma tigrinum*

Locus (GenBank ID)	Repeat Unit	Primer Sequence*	$MgCl_2$ (mM)	Temp (°C)/ Time (s)	Annealing Extension Time (s)	No. of cycles	Allele size range (bp)	Subspecies	Sample Size	Heterozygosity†		
										No. of alleles	H_E	H_O
ATS4-11 (AY157747)	$(GA)_3TT(GA)_3AT(GA)_5$	F: GTGTTACCCACCTTTTTCTATCCC	1.0	56.5 (30)	50	32	175, 187–193	*mavortium*	18	4	0.640	0.722
		R: GGCCGCACAACCTAAGTG						*nebulosum*	17	3	0.469	0.353
								stebbinsi	15	2	0.129	0.000
ATS4-20 (AY157748)	$(CA)_9$	F: TGTTTTGCCCTTATGTCG	1.5	59.5 (30)	50	31	321,	*mavortium*	17	11	0.889	0.765
		R: GCCCAAATCCTAAAGAGTAAGT					325–347, 372	*nebulosum*	26	1‡	0.000	0.000
								stebbinsi	19	2	0.512	0.000
ATS4-25 (AY157749)	$(CA)_7$	F: ATAGGGGCCTCAAGTTAAG	1.5	56 (30)	50	30	229–237	*mavortium*	20	4	0.706	0.500
		R: GGCTACTAGATGGCGTTGT						*nebulosum*	27	3	0.671	0.519
								stebbinsi	19	3	0.284	0.000
ATS5-6 (AY157750)	$(CA)_3TA(CA)_4TC(CA)_7$	F: TATGGGTGAGAATCTAGGAG	1.5	50.9 (30)	75	30	193,	*mavortium*	20	2	0.481	0.350
		R: GCACCATCCAGTCAAAC					199–207	*nebulosum*	27	6	0.715	0.815
								stebbinsi	19	2	0.053	0.053
ATS5-7 (AY157751)	$(CA)_{10}$	F: GGGCTTGAATCATGTAGTGG	1.5	54.7 (50)	70	32	240–244,	*mavortium*	19	6	0.647	0.105
		R: GGGAAGACTAGATGGCAATAAC					252–268, 282	*nebulosum*	27	8	0.751	0.704
								stebbinsi	19	1	0.000	0.000
ATS5-8 (AY157752)	$(CA)_{11}$	F: AGTCCCTCTCTATCTAATCTCG	1.5	56.5 (50)	50	34	354–366	*mavortium*	20	3	0.445	0.400
		R: ATTCTCCTGCCTGTATGTTT						*nebulosum*	27	4	0.240	0.185
								stebbinsi	19	1	0.000	0.000
ATS10-7 (AY157753)	$(GA)_6$	F: GAGGCAGGATGATTTAGA	2.0	58 (50)	70	31	296–302	*mavortium*	20	3	0.678	0.500
		R: CTTGGCATTACTGATTAGG						*nebulosum*	26	1	0.000	0.000
								stebbinsi	19	3	0.432	0.000
ATS12-3 (AY157754)	$(CA)_8CG(CA)_3CG(CA)_{14}$	F: TGTAGCAGAAGACGGGTAT	1.5	52.8 (30)	50	34	193–200,	*mavortium*	20	5	0.501	0.400
		R: AGTAAAGCGAAGATATGGG					211–225	*nebulosum*	23	4	0.661	0.087
								stebbinsi	19	2	0.102	0.000
ATS13-1 (AY157755)	$(CA)_{12}TTCGCGCG(CA)_{17}$	F: AGGTCTTCTTACAGCACAA	1.5	56.3 (50)	70	33	311–341, 369	*mavortium*	17	3	0.314	0.235
		R: TCACCAGGGTAGGGATA						*nebulosum*	18	9	0.813	0.111
								stebbinsi	19	2	0.491	0.474
ATS14-3 (AY157756)	$(CA)_{18}$	F: GGGCACTGAAACGGAACACT	1.5	56 (30)	50	31	103–109,	*mavortium*	19	2	0.149	0.053
		R: CCCCAAATGGCGTCCCT					125–129	*nebulosum*	27	4	0.312	0.296
								stebbinsi	18	2	0.203	0.000

*F and R are forward and reverse primers, respectively.

†H_O and H_E are observed and expected heterozygosities, respectively, estimated using BIOSYS (Swofford & Selander 1981).

‡*A. t. nebulosum* sample is fixed for allele 372. Neither other subspecies had this allele.

using standard autoradiography film and sequenced using M13 primers on an ABI 377 automated sequencer. Visual inspection was used to confirm presence of repeats and to determine whether there was sufficient flanking sequence to construct PCR primers.

Primer pairs for selected loci were developed using OLIGO 6.0 software (Molecular Biology Insights, Inc.). One primer from each set was fluorescently labelled. Optimization for each primer pair was carried out using individuals from two common subspecies from Arizona (*A. t. nebulosum* and *A. t. mavortium*), and one endangered subspecies (*A. t. stebbensi*). DNA was extracted from adult and larval tail tissue stored in 70% ethanol using Qiagen's DNAEasy kit or standard Phenol/Chloroform extractions. Reaction volumes were optimized using 13 μL TV consisting of 50 mM KCl, 100 mM Tris-HCl, 0.96 mM each dNTP, 25 μM each oligonucleotide, 1 unit of *Taq* polymerase (Fisher), 75–150 ng template DNA and primer specific $MgCl_2$ concentrations (Table 1).

All PCR profiles began with 2 min at 94 °C (denaturation), followed by a primer-specific number of cycles of 30 s 94 °C denaturation, primer-specific annealing time and temperature, and primer-specific extension time at 72 °C (see Table 1 for primer-specific data). Each profile ended with 5 min at 72 °C to complete the products. Optimization and amplification reactions were performed on a Bio-Rad iCycler® gradient thermocycler. All products were run on an ABI 377 Automated Sequencer using ROX 500 size standard and analysed using GENESCAN and GENOTYPER software (ABI).

Of the 15 primer-pairs tested, three were not variable and two others could not be optimized. The remaining 10 loci showed variation in at least one subspecies (Table 1). Observed and expected heterozygosities were calculated and tested using the GENEPOP 3.3 software package (Raymond & Rousset 1995) and BIOSYS (Swofford & Selander 1981). Four loci (ATS4-25, ATS5-7, ATS10-7 and ATS12-3) had significant heterozygote deficiencies in *A. t. mavortium*, and one locus (ATS13-1) had a heterozygote deficiency in *A. t. stebbinsi*; note also that this locus works well for *A. t. stebbinsi*, but has shown limited success with other tiger salamander subspecies. Appropriate repeat sizes for alleles with substantially different lengths were confirmed by sequencing individuals homozygous for the allele.

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References

Brede EG, Rowe G, Trojanowski J, Beebee TJC (2001) Polymerase chain reaction primers for microsatellite loci in the Common Toad *Bufo bufo*. *Molecular Ecology Notes*, **1**, 308–310.

Call DR, Hallett JG (1998) PCR primers for microsatellite loci in the anurans *Rana luteiventris* and *Hyla regilla*. *Molecular Ecology*, **7**, 1085–1087.

Curtis JM, Taylor EB (2000) Isolation and characterization of microsatellite loci in the Pacific giant salamander, *Dicamptodon tenebrosus*. *Molecular Ecology*, **9**, 116–118.

Gehlbach FR (1967) Ambystoma Figrinum (Green). Catalogue of American Amphibians and Reptiles. pp. 52.1–52.4. *American Society of Ichthyologists and Herpotologists*. Kansas City, MO.

Goldstein DB, Schlötterer C (1999) *Microsatellites: Evolution and Applications*. Oxford University Press, Oxford.

Jehle R, Arntzen JW, Burke T, Krupa AP, Hoedl W (2001) The annual number of breeding adults and the effective population size of syntopic newts (*Triturus cristatus*, *T. marmoratus*). *Molecular Ecology*, **10**, 839–850.

Raymond M, Rousset F (1995) GENEPOP Version 1.2.: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rowe G, Beebee TJC, Burke T (1997) PCR primers for polymorphic microsatellite loci in the anuran amphibian *Bufo calamita*. *Molecular Ecology*, **6**, 401–402.

Shaffer HB, McKnight ML (1996) The polytypic species revisited: Genetic differentiation and molecular phylogenetics of the tiger salamander, *Ambystoma tigrinum* (Amphibia: Caudata) Complex. *Evolution*, **50**, 417–433.

Swofford DL, Selander RB (1981) BIOSYS-1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *Journal of Heredity*, **72**, 281–283.

Wake DB (1998) Action on amphibians. *Trends in Ecology and Evolution*, **13**, 379–380.