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PRIMER NOTE

Isolation of microsatellite loci in spotted salamanders (*Ambystoma maculatum*)

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Abstract

To assess the level and spatial pattern of genetic diversity in the spotted salamander, *Ambystoma maculatum*, we characterized hypervariable molecular markers by screening genomic libraries enriched for microsatellite motifs. We designed primers that reliably amplify twelve polymorphic loci and checked for variability in individuals from populations in the vicinity of Ithaca, New York. Loci show high variability in the number of alleles and heterozygosities, suggesting they will be useful for determining local population differentiation and mating systems in this pond-breeding amphibian.

Keywords: *Ambystoma*, microsatellites, salamander

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The spotted salamander (*Ambystoma maculatum*) is widespread in eastern North America. These salamanders are terrestrial as adults and aggregate in ephemeral ponds during the early spring for breeding (Petranka 1998); their dependence on semi-isolated patches of temporary habitat likely influences genetic structuring among populations. Here we report on the development of microsatellites for *A. maculatum* that will be useful for studies of connectivity among breeding aggregations, estimates of gene flow, and characterization of the mating system in this amphibian.

Loci were cloned and characterized from three independent libraries. For locus AmaA, we prepared an enriched library according to the protocol of Kijas *et al*. (1994). Genomic DNA was extracted from one individual (CU# 12678) by standard phenol-chloroform extraction (Sambrook & Russell 2001), digested with Sau3AI, size-selected for fragments 300–1000 bp in length, and ligated into M13mp18RF vectors (Gibco BRL). M13 primers were used in asymmetrical polymerase chain reaction (PCR) and products were enriched for microsatellites with a biotynilated $(CA)_n$ probe bound to streptavidin-coated magnetic beads (Dynal, Inc.); these were then ligated in to the pCRII® vector (Invitrogen Corp.). For loci Ama 5–1, 9–4, 4–10, 2C2, 12–7, 11–2B, and 3–3 we followed Kandpal *et al*. (1994) and Amador *et al*. (2001). Fragments ranging from 400 to 1500 bp were ligated to Sau3A1 linkers, then amplified with linker-specific primers. Products were hybridized to a biotynilated probe, and targets captured with VECTREX® Avidin D matrix. The enriched pool of fragments was amplified and cloned into a PCR2.1 TOPO® TA vector (Invitrogen Corp.). Loci Ama61, 07, 34, and 13 were cloned following the methods of Castleberry *et al*. (2000). A series of di-, tri-, and tetranucleotide repeat probes were used to screen Magnagraph nylon membranes (Micron Separation, Inc.) containing lifted colonies with inserts and positive clones were detected by chemiluminescence (Lifecodes Corp.). For all libraries, positive clones were amplified with vector-specific primers and sequenced directly with dGTP BigDye terminator cycle sequencing components on an ABI Prism® 377 or 310 (Applied Biosystems).

We designed PCR primers in the flanking regions of 41 microsatellites using PRIMER 3 (Rozen & Skaletsky 1996). Following optimization, 20 loci reliably yielded specific PCR product of good concentration. In some cases, we amplified with Ampli*Taq* Gold® Polymerase (Applied Biosystems) for increased specificity. Each PCR reaction (10 μL total volume) contained 100–200 ng of genomic DNA, 0.5–1.0 U *Taq* polymerase (Roche Taq, Ampli*Taq*® or Ampli*Taq* Gold®), 1X Roche Taq PCR buffer (10 mM

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Table 1 Primers, amplification conditions, and measures of polymorphism for *Ambystoma maculatum* microsatellite loci

Locus	Primer sequences (5′–3′)	Core Motif	Size	T_a (°C)	[$MgCl_2$]	Taq	Alleles	H_O	H_E	GenBank
Ama61	(F)-CCAATCTAGTGCTCTCTCCC (R)-ACATACTCCCCTCTGCTCAC	$(GA)_n(GT)_n$	229–255	57	1.5	P	5	0.352	0.600	AF108919
Ama5–1	(F)-GCATGGCTGAAAAACGATTG (R)-GTGCTGAGGGAGGGGTAGTG	$(CA)_n$	386–400	59	1.5	P	8	0.672	0.783	AF452178
Ama9–4	(F)-TATGCATCCACCATTTGTGC (R)-TGTCACCTATTGGGCCTTTC	$(GT)_n$	205–245	54	2.0	G	16	0.504	0.780	AF452179
Ama11–2B	(F)-AGCCATCACCATAATCAGG (R)-CAGAGTGGGAGGCTTGTC	$(CA)_n$ AA$(CA)_n$	237–275	54	2.0	G	12	0.440	0.696	AF452180
Ama4–10	(F)-AAGTGTGGGTATCGTGTCC (R)-ACCTTGTGGGACACCATAA	$(GA)_n$ $(GT)_n$	235–269	54	1.5	G	9	0.480	0.783	AF452181
AmaA	(F)-CGCCACATAAGAGTTACAGTGC (R)-TGGTTTTCCCTGAAGTGAGC	$(CA)_n$	151–197	55	2.5	G	14	0.464	0.609	AF452184
Ama3–3	(F)-ACACTCAGCTCACATTCAG (R)-AACTTCTTCCTTCCAGACTTA	$(CA)_n$	169–263	54	1.5	G	17	0.488	0.779	AF452182
Ama2C2	(F)-GCTTTCAAGTCCAACATAAAC (R)-CCTTAACCCCTCCCTTTC	$(GT)_n$	203–221	48	2.0	G	11	0.480	0.820	AF452183
Ama12–7	(F)-ATTAAAGTTGGTGTGGTTG (R)-TGGATGCTTCAGAGTTTGT	$(AT)_n$	290–310	49	2.0	G	10	0.409	0.571	AY12205
Ama07	(F)-TGTAAACAACCACTTCGGG (R)-AGCTCAGGTGTTGGGTGG	$(GT)_n$	182–226	59	1.5	P	10	0.496	0.616	AF108913
Ama34	(F)-GAACCGCTTGTTCAGTATAG (R)-TCAGGTAAGCACGATTTAAC	$(CA)_n$	90–118	53	1.5	P	12	0.288	0.329	AF108918
Ama13	(F)-GGTCTTATTTTGTTTACAGGAGG (R)-TTATTTATTTATTGTGCTTGTTGG	$(AT)_n$	180–240	56	1.5	P	8	0.537	0.607	AF108914

Taq, polymerase used for amplification: G = Ampli*Taq* Gold®, P = other brands of polymerase. T_a, annealing temperature.

Tris-HCl, pH 8.3; 50 mM KCl), or Ampli*Taq* Gold® PCR buffer (15 mM Tris-HCl, pH 8.0; 50 mM KCl), 1.5–2.5 mM $MgCl_2$, 0.2–0.25 mM dNTPs, and 5 pmol of each primer. Forward primers were 5′-labelled with a fluorescent dye (HEX, TET or 6-FAM). Loci were amplified in a PTC-100 or PTC-200 (MJ Research) thermal cycler under the following conditions: 2–5 min denaturation at 95 °C (10 min for AmpliTaq Gold); 35 cycles of 30 s at 95 °C, 30 s at specific annealing temperatures, and 45 s to 1 min at 72 °C; and a final extension of 72 °C for 10–30 min (Table 1).

Amplified products with different fluorescent labels or nonoverlapping size ranges were pooled, and either electrophoresed on a 5% polyacrylamide gel on an ABI 377 DNA sequencer or subjected to capillary electrophoresis on an ABI 310 Genetic Analyser. Fragment sizes were determined with the TAMRA-500 standard using GENESCAN version 3.1 and GENOTYPER version 2.1. We genotyped 100 individuals from populations in Tompkins Co., New York at all loci except Ama 13, for which we used 44 individuals. Twelve loci were polymorphic (Table 1); the number of alleles ranged from four to 17 (average = 11.6); observed heterozygosity (H_O) ranged from 0.288 to 0.672 (average = 0.467), and expected heterozygosity (H_E) ranged from 0.329 to 0.820 (average = 0.664). All loci conform to Hardy–Weinberg expectations using an exact test in GENEPOP version 3.3 (Raymond & Rousset 1995). Considerable polymorphism will make these markers valuable for studies of local population differentiation and mating systems in this species.

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