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TECHNICAL NOTE

Twelve novel microsatellite markers for the marbled salamander, *Ambystoma opacum*

Schyler O. Nunziata · David E. Scott · Kenneth L. Jones · Cris Hagen · Stacey L. Lance

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Abstract We isolated and characterized a total of 12 microsatellite loci from the marbled salamander, *Ambystoma opacum.* Loci were screened in 24 individuals from a single location on the Savannah River Site in South Carolina. The number of alleles per locus ranged from 3 to 15, observed heterozygosity ranged from 0.167 to 0.917, and the probability of identity values ranged from 8.5×10^{-2} to 3.2×10^{-1}. These new loci will provide tools for examining the genetic diversity, structure, and mating system of *A. opacum* populations.

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S. O. Nunziata · D. E. Scott · C. Hagen · S. L. Lance (✉)
Savannah River Ecology Laboratory, University of Georgia, Drawer E, Aiken, SC 29802, USA
e-mail: lance@srel.edu

S. O. Nunziata
Department of Biological Sciences, Eastern Kentucky University, Richmond, KY 40475, USA

K. L. Jones
Department of Biochemistry and Molecular Genetics, School of Medicine, University of Colorado, Aurora, CO 80045, USA

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Ambystoma opacum, the marbled salamander, is pond-breeding species distributed throughout much of the eastern United States from eastern Texas to southern New England (Petranka 1998). Adults are most frequently encountered during migrations to breeding sites in late summer or fall; population sizes of >10,000 individuals have been reported (Scott 2005). In the Southeast *A. opacum* occur most frequently in association with short hydroperiod isolated seasonal wetlands (Snodgrass et al. 2000), although many populations have likely been extirpated due to anthropogenic destruction of seasonal wetlands (Scott 2005). Currently, there are eight microsatellite loci developed for *A. opacum* (Croshaw et al. 2005), but not all of these have been successful when applied in genetic studies (Bartoszek and Greenwald 2009, Greenwald et al. 2009). To increase the number of available loci we developed an additional 12 loci for this species.

Total DNA was extracted from one individual of *Ambystoma opacum*, using the DNeasy tissue kit protocol (Qiagen, Valencia, CA) for the construction of the microsatellite library. DNA was then serially enriched twice for microsatellites following Glenn and Schable (2005) with the changes described in Lance et al. (2010). There were two primary changes to the Glenn and Schable (2005) protocol. First, a different linker was used (SimpleX-6 Forward 5′-AAAA GCACGAGCGGAACT and SimpleX-6 Reverse 5′-pAGTTCCGCTCGTGC). Second, the enriched libraries were sequenced on a 454 using titanium chemistry following standard Roche 454 library protocols (454 Life Sciences, a Roche company, Branford CT). All methods for sequencing, microsatellite identification,

Table 1 Details for 12 polymorphic microsatellite loci developed for *Ambystoma opacum*

Locus	Primer Sequence 5′–>3′	Repeat motif	Size (bp)	N	*K*	H_o	H_e	PI	TD
Amop14	F: CCTAGCAGCAAAGAGGAGG[a]	ATCT(14)	390–454	24	14	0.792	0.884	0.024	65
	R: GTTGACCCACTTACATCGCC								
Amop15[b]	F: CCTTCTTCCCAACACCTC[a]	CTTT(15)	127–147	24	5	0.458	0.790	0.078	65
	R: ATGACATCAGTGCCACCTAC								
Amop18	F: CTCGAGCACAAAGTGGC[a]	AAAG(10)	431–467	24	8	0.583	0.771	0.085	65
	R: CGCCCAGTATCTGGCTTTCT								
Amop27	F: GGCGAGGGAGTGATATACGA[a]	AGAT(21)	247–287	24	9	0.875	0.807	0.063	65
	R: CCGGTTAGCTCAATGTCTGC								
Amop29	F: GGTCTTCTCCAGGGCCAATA[a]	CTTT(12)	445–509	24	15	0.792	0.891	0.021	65
	R: TTCTGGTGTCATGAGCAAGG								
Amop31[b]	F: GTTCACTCTCCTGTGCATCAA[a]	ATCT(18)	244–312	23	12	0.348	0.788	0.06	65
	R: CTGTGGTTAGTGGCGCA								
Amop33	F: TCCAAGCAAACAGGTCCACT[a]	AGAT(14)	320–364	24	10	0.917	0.866	0.032	65
	R: TCGTGGAGGTCCAACTTACC								
Amop34[b]	F: TCCGTCCCTACCATTTGTTCA[a]	CTTT(14)	354–386	24	8	0.542	0.806	0.065	65
	R: TCCTTGTTGGCTGTTTGACG								
Amop39	F: CTCTGAGGGAGACATCCTGC	GAGT(11)	320–328	24	3	0.542	0.508	0.32	65
	R: GAAGAAAGCTGCTGGCAAGA[a]								
Amop40	F: GACTCTGAGACCTATGGGCG	AGAT(17)	298–370	24	11	0.875	0.807	0.03	65
	R: TTCACTTGGTCCGAGACAGG[a]								
Amop42	F: GCGATAGGTCATGCAGTGTG	ATCT(13)	329–369	24	9	0.750	0.845	0.042	65
	R: GGCATGCAGTAAGAAATCCTGT[a]								
Amop47[b]	F: TGCAAACTTGTGAAGGCCAG	ATCT(23)	159–275	24	8	0.167	0.782	0.077	65
	R: TAGTGGCTGAATAGACAGGT[a]								

The size indicates the range of observed alleles in base pairs and includes the length of the CAG tag; number of individuals genotyped is *N*; *k* is number of alleles observed; H_o and H_e are observed and expected heterozygosity, respectively; *PI* is the probability of identity for each locus, and *TD* refers to the touchdown protocol used for pcr (see text)

[a] CAG tag (5′-CAGTCGGGCGTCATCA-3′) label; [b] significant deviations from Hardy–Weinberg expectations after Bonferroni corrections

primer design, and primer screening are as described in Lance et al. (2010) with the exception that the sequence GTTT was added to primers without the universal CAG tag addition.

Forty-eight primer pairs were tested for amplification and polymorphism using DNA obtained from eight individuals. PCR amplifications were performed in a 12.5 µl volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabeled primer, 0.04 µM tag labeled primer, 0.36 µM universal dye-labeled primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 units AmpliTaq Gold® Polymerase (Applied Biosystems), and 20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Touchdown thermal cycling programs (Don et al. 1991) encompassing a 10°C span of annealing temperatures ranging between 65 and 55°C (TD65) was used for all loci. Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95°C followed by 20 cycles of 95°C for 30 s, highest annealing temperature (decreased 0.5°C per cycle) for 30 s, and 72°C for 30 s; and 20 cycles of 95°C for 30 s, lowest annealing temperature for 30 s, and 72°C for 30 s. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 3.7 (Applied Biosystems). Twelve of the tested primer pairs amplified high quality PCR product that exhibited polymorphism.

We assessed the variability of the 12 polymorphic loci in 24 specimens collected from Rainbow Bay, a Carolina Bay on the Savannah River Site (Aiken, SC, USA). Conditions and characteristics of the loci are provided in Table 1. We estimated the number of alleles per locus (*k*), observed and expected heterozygosity (H_o and H_e), and probability of identity (PI) using GenAlEx v6.4 (Peakall and Smouse 2006). Tests for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0 (Rousset 2008). After Bonferroni correction for multiple comparisons four loci showed significant deviations from

expectations under HWE and no linkage disequilibrium was detected for any of 45 paired loci comparisons. We used Microchecker (Van Oosterhout et al. 2004) to look for evidence of null (non-amplifying) alleles, and found evidence for null alleles at the four loci not in HWE (Amop15, Amop31, Amop34, Amop47).

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