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TECHNICAL NOTE

Development and characterization of 18 microsatellite loci for the spotted salamander (*Ambystoma maculatum*) using paired-end Illumina shotgun sequencing

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Abstract We isolated and screened 68 microsatellite loci from the spotted salamander, *Ambystoma maculatum*, in Missouri, USA. Eighteen polymorphic loci were identified and pooled into two multiplex reactions. These loci are a needed addition to the known loci for *A. maculatum*, which were developed in a different geographic region and are unsuitable for population or landscape genetic analyses in Missouri. To effectively protect and manage this species, regionally-relevant studies of population genetic structure and connectivity are necessary.

Keywords *Ambystoma* · Population genetics · Landscape genetics · Multiplex PCR · PAL_Finder

Ambystoma maculatum is a widely distributed species occurring in forested habitats throughout eastern North America, from Texas, USA to Ontario and Nova Scotia, Canada (Petranka 1998) and is comprised of two distinct lineages (Zamudio and Savage 2003). *Ambystoma maculatum* have been the focus of numerous population and landscape genetic studies (Zamudio and Wieczorek 2007; Purrenhage et al. 2009; Richardson 2012) using the 31 identified di- and tetranucleotide microsatellite primers (Wieczorek et al. 2002; Julian et al. 2003). These microsatellites were isolated from populations in New York and Maryland, respectively, and have been found to be minimally polymorphic, monomorphic, or unsuitable in populations in Missouri (WEP unpublished). To conduct population and landscape genetic studies throughout the range of *A. maculatum* suitable microsatellite loci are needed.

Tissue samples for *A. maculatum* were collected from two ponds at Fort Leonard Wood, Missouri, USA (37.92°N, 92.17°W) that are separated by ~10 km. A single developing embryo was carefully removed from a clutch of eggs (n = 51 and 56) and stored in 95 % EtOH at −20°C prior to DNA extraction. DNA was extracted using DNeasy tissue kits (QIAGEN) according to the manufacturer's protocols. An Illumina paired-end shotgun library was prepared as described by Nunziata et al. (2013). Resulting reads were analyzed with the program PAL_FINDER_v0.02.03 (Castoe et al. 2012) to extract reads containing di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Positive reads were then analyzed with Primer3 (Rozen and Skaletsky 2000) for primer design. Only primer sequences occurring a single time among the reads were considered. Sixty-eight tetranucleotide loci from 591 loci meeting our selection criteria were screened for amplification and polymorphism. Primers were initially screened using seven samples of total genomic DNA. PCR conditions were standardized to 15 μL containing 1× PCR Gold buffer, 2.0 mM $MgCl_2$, 2.0 mM dNTP, 0.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.8× bovine serum albumin (BSA), 0.4 μM of forward and reverse unlabeled primer, and 1.0 μL (50 ng) genomic DNA. The PCR profile included an initial denaturing step at 95°C for 10 min, followed by 35 cycles of 95°C denaturing for 45 s, 58°C annealing for 45 s, extension at 72°C for 45 s; and a final 5-min extension at 72°C. PCR products were separated in a 4 % agarose gel with Gel Star (Lonza, Allendale, NJ) and 100 bp ladder. A total of 34 polymorphic loci were identified. Eighteen loci were divided into two multiplexes using

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Table 1 Primer sequences, repeat motif, fluorescent label, multiplex pool, amplicon size, and summary statistics for 18 microsatellite loci for *Ambystoma maculatum*

Locus	Primer sequence 5′–3′	Motif	Label	Multiplex	Size (bp)	N	N_A	H_O	H_E
Am_56	F: CCTTCGGGTGATATGGCG R: TTTGTGGCTCGTGCACCC	ATCT	FAM	1	89–109	107	5	0.391	0.420
Am_34[a]	F: GGAGGAAGGCTAGAGGCAGG R: AGCAGCCAATTCCTGTGTGC	AAAG	FAM	1	239–267	102	8	0.688	0.817
Am_62	F: CTGTGTCTCTGGAGTTGGGC R: CCAATACAACATTCAATTCACGC	AATC	VIC	1	147–159	107	3	0.446	0.469
Am_4	F: GCTTGTGATTTGGATGGATGC R: TGAAGAAGACATCTAATTGCTACATGG	AAAG	VIC	1	201–258	106	10	0.819	0.793
Am_9	F: GGTCCCTCAGTGGAAGAAAGG R: GCTGAGTTGAACTTGCCAGG	TTCC	NED	1	124–184	106	10	0.777	0.720
Am_39	F: ATTGGCCTTGTCCTGACTGG R: GGACATAACCATCACTGTCTCGC	ATCT	NED	1	226–252	107	6	0.728	0.730
Am_21	F: TCCCTCCTTCTTCTGTCTTTGC R: CAAAGGATGCAAGCAAATGG	AAAG	PET	1	109–133	107	7	0.747	0.685
Am_10	F: CCCTCCTAAAGAAGCAAAGAAGG R: TGTCACCCAGGAAGGTTTGC	AAAG	PET	1	191–217	106	12	0.736	0.782
Am_29	F: CCAGTAAGAACCTGCTTGCCC R: CTTGCTCCCTCAATACAATCCC	TTCC	PET	1	285–305	106	4	0.404	0.442
Am_43	F: ATCGAAAGGAGGAACACATGG R: GTGTTTGTAATTGGCTTGAGCG	ATGG	FAM	2	141–149	107	3	0.418	0.389
Am_33	F: GAGCAGGACGATGAAGAGGG R: GGCGTTACTTTAGTACCATTATATCCC	AAAG	FAM	2	253–315	107	4	0.139	0.132
Am_13	F: TGTAATTCACTGTGCTAAGGCG R: TGAACTCTATTTCCATCCTTGGC	AAAC	VIC	2	118–134	107	4	0.237	0.217
Am_30	F: TTTGTCACTCAATTGCAACCC R: TGTCTTCGAGAACATTAGAATCCC	ATCT	VIC	2	162–182	106	6	0.708	0.678
Am_38	F: CTCCCAGATGTTCCCTACGC R: CGATTAAATGGAGGCCAGACC	ATAC	VIC	2	331–343	107	4	0.550	0.545
Am_3	F: CCATCTGTATATTTCCATTCTCCC R: CTCCGTGGGATGTCTGAGG	AAAG	NED	2	223–283	107	14	0.859	0.872
Am_55	F: CCTTTGAGCTGTATTTGATAGTCC R: GGACTGCACACCAAGATGC	AATG	PET	2	116–132	107	5	0.673	0.623
Am_7	F: AAACCCTCGGGAAGCAGG R: GGACTCGATGTGAGAAAGGAGG	AAAG	PET	2	205–237	106	8	0.791	0.796
Am_37	F: CACATATTCAAACATCTGTGCCC R: CTCCCGAGTGGGAACCTACC	ATCT	PET	2	301–317	107	5	0.541	0.538

All primers were screened on 107 samples. All annealing temperatures were 58°C

[a] Locus deviated significantly from heterozygosity values expected under Hardy-Weinberg equilibrium after Bonferroni correction

Multiplex Manager (Holleley and Geerts 2009), and forward primers were fluorescently labeled (Table 1). Primers were screened in 107 *A. maculatum* samples from the two breeding ponds. Multiplex PCR reactions were performed using Platinum Multiplex PCR Master Mix (Applied Biosystems, Foster City, CA) following the manufacturer protocols, but in 8 µL volume reactions with GC enhancer solution added to a final concentration of 10 %. PCR cycling conditions consisted of: initial denaturing at 95°C for 2 min, followed by 30 cycles of 95°C denaturing for 30 s, annealing at 58°C for 90 s, and 72°C extension for 60 s; and a 30-min extension at 60°C. Amplification products were sized on an ABI 3730 DNA Analyzer (Applied Biosystems) using Liz 600 size standard at the University of Missouri DNA Core Facility, and results were scored using GENEMARKER version 1.97 (Softgenetics, State College, PA). We calculated expected and observed heterozygosities using GENODIVE version

2.0b23 (Meirmans and Van Tienderen 2004) and tested for linkage disequilibrium using GENEPOP version 4.0.10 (Rousset 2008) .

Loci were arranged into two multiplex reactions, each containing nine loci. Alleles per locus ranged from 3 to 14 (mean = 6.26, Table 1) and the observed heterozygosity ranged from 0.139 to 0.859 (mean = 0.592). Locus *Am_34* deviated significantly from Hardy–Weinberg equilibrium expectations. Linkage disequilibrium between loci was not detected. These loci have been isolated and characterized from *A. maculatum* in the western part of their distribution where previously developed microsatellite loci have proven uninformative. Their use in population and landscape genetic studies will provide insight into population dynamics and responses to land use in a new region, allowing for a broader understanding of the species throughout its range.

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