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TECHNICAL NOTE

Development and characterization of 22 microsatellite loci for the ringed salamander (*Ambystoma annulatum*) using paired-end Illumina shotgun sequencing

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Abstract We isolated and screened 150 microsatellite loci from the ringed salamander, *Ambystoma annulatum*, an ambystomatid salamander endemic to the Interior Highlands. Twenty-two polymorphic loci were identified and pooled into two multiplex reactions. These loci will be valuable tools for assessing population genetic structure and connectivity across the landscape, and informing management of this species.

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

Pond breeding amphibians such as ambystomatid salamanders are generally philopatric to breeding ponds, and have limited dispersal ability (Gamble et al. 2007). Further, ambystomatid salamanders are often sensitive to land use (Semlitsch et al. 2009), which can increase population isolation and genetic differentiation (Greenwald et al. 2009). The ringed salamander, *Ambystoma annulatum*, is a species endemic to the Interior Highlands of Missouri, Arkansas, and Oklahoma, USA, and is a poorly studied species (Petranka 1998). The ability to disperse through and persist in fragmented landscapes can have a great impact on genetic diversity among populations (Greenwald et al. 2009), and an understanding of gene flow across the landscape is critical to forming sound conservation and management decisions for this species.

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Tissue samples for *A. annulatum* were collected from a breeding pond at Fort Leonard Wood, Missouri, USA (37.92°N, 92.17°W). A single developing embryo was carefully removed from a clutch of eggs (n = 43) 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. One hundred fifty tetra- and pentanucleotide loci from 749 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 35 polymorphic loci were identified. Twenty-two of these primers were divided into two multiplexes using Multiplex Manager (Holleley and Geerts 2009), and forward primers were fluorescently labeled (Table 1). Primers were screened in 43 *A. annulatum* samples from a single breeding pond.

Table 1 Primer sequences, repeat motif, fluorescent label, multiplex pool, amplicon size, and summary statistics for 22 microsatellite loci for *Ambystoma annulatum*

Locus	Primer sequence 5′–3′		Motif	Label	Multiplex	Size (bp)	N	N_A	H_O	H_E
Aa_31	F:	GGTTGGACCCAGAAATGAAGG	ATCT	FAM	1	173–201	43	7	0.744	0.804
	R:	TTTCAATGTGCTGTTATGGGC								
Aa_311	F:	TTGTTTGATGGAATGCCTGG	AAAG	FAM	1	261–319	43	11	0.907	0.813
	R:	CGGTGGATGTCTCTGCTCC								
Aa_36[a]	F:	CTGAACGGTCACTTTGCAGG	AAAG	VIC	1	262–310	43	10	0.512	0.803
	R:	CCTCCTCTTTGCGTACCTCC								
Aa_19	F:	TAACTTGTGGGCCAGATTTCC	ATCT	VIC	1	387–455	43	17	0.953	0.908
	R:	CCCTTTGCACGGAGTAGG								
Aa_21	F:	TTCCACCTCCATAGAAATGAATTGG	TTCC	NED	1	112–153	43	9	0.767	0.793
	R:	AATAGGTGCTTACGTATGGAAGGAAGG								
Aa_27	F:	GATGCCTTCGGGTCTTTGC	ATAC	NED	1	198–218	43	3	0.535	0.561
	R:	CCTAATCACCATAGCATTCGGC								
Aa_28	F:	TGCTGAATGTATTGTGCAGGG	ATCT	NED	1	267–279	43	5	0.465	0.522
	R:	GCTGTTGTCTCTTCAACATGCG								
Aa_86	F:	TTCTTGCAACTGGTAGATGCG	AGTG	NED	1	320–332	43	3	0.465	0.421
	R:	ATGAGTCTCGGTGTCCCTGG								
Aa_153	F:	TTTGGCATATGTCACACCCG	TCTG	NED	1	375–399	43	4	0.512	0.574
	R:	CGTGCCTCCTTAACGTATTGG								
Aa_84	F:	GGAGTTGGTTGGTTGCTTGC	ATGG	PET	1	116–140	43	3	0.674	0.659
	R:	TCTCTCTGACCTCATGCATTCC								
Aa_314	F:	CCTCAAGCTCATTAATTGTTCTCC	AAAG	PET	1	256–272	43	4	0.628	0.537
	R:	GAACAGCACTGCATCAAGGG								
Aa_37	F:	CCGCAGTAAACAAGTGACACC	ATCT	FAM	2	232–242	40	6	0.675	0.648
	R:	CTTCTCAGGAGGCCTGTGG								
Aa_20	F:	AAATGAAGCAACACGGAGG	AAAG	FAM	2	144–160	43	5	0.442	0.572
	R:	CTTCGTCCAAACCAAACAGC								
Aa_50	F:	TGAACACATACAAGTTTGATGCC	ATCT	FAM	2	258–286	43	8	0.791	0.825
	R:	TTGGTATTGATGCAAAGCTTCC								
Aa_25	F:	TCCTTACTGGCTGCTATTGCC	ATAC	VIC	2	209–247	43	8	0.907	0.843
	R:	AAGCTCTGCGACTGCATGG								
Aa_258	F:	CGCACACACTATCTCATTCCC	AATG	VIC	2	275–299	43	5	0.558	0.595
	R:	TGTGCACAGGCTCATTTAGG								
Aa_85	F:	GAGAACAAGACAATTAGTGGGATGG	ATCT	NED	2	131–151	43	6	0.744	0.794
	R:	TCTGTCTGCTTATCCATTGATCC								
Aa_46[a]	F:	GAAACAGGGAAACATCATGCC	AAAG	NED	2	162–190	42	6	0.571	0.833
	R:	TTGTTTGTTGGTGGAGAGGC								
Aa_44	F:	TTTGTCCGATATGCGTGTGC	AAAT	NED	2	98–120	35	6	0.629	0.608
	R:	TCTTCATATTTCGCCTTTGCC								
Aa_39	F:	TTCACCACCACAAGAGCAGG	ATGG	NED	2	475–511	43	4	0.744	0.664
	R:	AATTCCAGGATCCAAGATAGGG								
Aa_40	F:	AAGATAAGAGTGAACTCCATGAGGG	AAGAG	PET	2	154–188	43	8	0.860	0.881
	R:	GGTTGTTGCTCTAGCTTTCACC								
Aa_312	F:	TGTGACAACCTGGAACTCGG	AGTG	PET	2	248–308	43	7	0.628	0.708
	R:	ACTGTCAGGAGGGCATTTCC								

All primers were screened on 43 samples. All annealing temperatures were 58 °C

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

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 eleven loci. Alleles per locus ranged from 3 to 17 (mean = 6.59, Table 1) and the observed heterozygosity ranged from 0.442 to 0.953 (mean = 0.669). Loci *Aa_36* and *Aa_46* deviated significantly from Hardy–Weinberg equilibrium expectations. Locus *Aa_44* failed to amplify in 20 % of the samples, suggesting the presence of null alleles. Linkage disequilibrium between loci was not detected. A previous study attempted to cross amplify existing *Ambystoma* spp. microsatellite loci in *A. annulatum* with minimal success (Peterman et al. 2012). The loci described in this study will be valuable and powerful tools to assess population structure, dispersal, and connectivity in the poorly studied *A. annulatum*, facilitating management of this endemic species at biologically relevant scales.

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