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TECHNICAL NOTE

Development of microsatellite loci for the western slimy salamander (*Plethodon albagula*) using 454 sequencing

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Abstract Terrestrial salamanders of the genus *Plethodon* are closely tied to abiotic environmental habitat features and are particularly sensitive to land use and habitat change. To better understand the effects of land use and habitat fragmentation on population genetic characteristics, we screened 137 primers developed from 454 sequence libraries, and developed 27 microsatellite primers for the western slimy salamander, *Plethodon albagula*. Twenty-two of these primers cross-amplified in *P. shermani*. These new loci will be invaluable tools to study habitat variables affecting gene flow, population genetic structure, and genetic diversity across heterogeneous landscapes.

Keywords *Plethodon shermani* · Population genetics · Landscape genetics

Habitat loss and fragmentation are key factors affecting the stability of amphibian populations and can negatively affect dispersal and population connectivity (Cushman 2006; Stuart et al. 2004). Salamanders of the genus *Plethodon* are closely tied to cool, moist abiotic conditions, making them particularly sensitive to habitat alteration and fragmentation (Marsh et al. 2008; Petranka 1998). Western slimy salamanders (*Plethodon albagula*) inhabit forested areas throughout the Ozark Highlands (Conant and Collins 1998). The ability to disperse through and persist in fragmented landscapes can have a great impact on genetic diversity among populations (Noël et al. 2007), and an understanding of gene flow across the landscape is critical to forming sound conservation and management decisions (Storfer et al. 2010).

Samples from *P. albagula* were collected by taking 0.5 cm of tail tissue from salamanders at Danville (n = 51) and Reform (n = 50) Conservation areas, Missouri, USA, which are separated by ~25 km. Samples were stored in 95 % EtOH at −20 °C prior to DNA extraction. DNA was extracted using the Wizard SV 96 Genomic DNA Purification System (Promega, Madison, WI, USA). Sequences were generated using 454 technology (Roche, Branford, CT, USA) in two ways; a microsatellite-enriched library was developed using methods outlined by Lance et al. (2010) for *P. albagula*, while traditional whole genome sequencing (WGS) was used to generate sequences for *P. glutinosus*, a sister species to *P. albagula*. The *P. albagula* tissue was collected in Garland County, Arkansas , USA, approximately 500 km from the sources of the samples were screened. For the WGS sequence data for *P. glutinosus*, we used RepeatMasker with Tandem Repeats Finder (Smit et al. 2012; Benson 1999) to identify reads containing microsatellites and then used a custom PerlScript to identify reads with ≧ 40 bp of sequence flanking each side of the microsatellite region. We used Primer3 implemented within MSATCOMMANDER to identify and design primers for screening (Faircloth 2008; Rozen and Skaletsky 2000). Primers for 137 di-, tetra-, and penta-nucleotide microsatellites were developed from *P. albagula*

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Table 1 Primer sequences, repeat motif, multiplex pool, and concentration in reaction (μM) for 27 microsatellite loci for *P. albagula*

Locus	Primer sequence 5′ to 3′	Motif	Label	Multiplex	Concentration	GenBank #
PG_184b	F: GTGCCCAGTGTGCTTTACAA	AACAT(6)	NED	1	0.1	JX946151
	R: TTTGGGTCAAACCCTCTTTG					
PG_3XI	F: AGCGGTGGATAGTCGTACAC	AACAT(12)	NED	2	0.05	JX946152
	R: ATAGCACATAGGCAGATCAGTC					
PG_43M	F: AGTCATTGTCAGCTTGCGC	AATG(13)	FAM	2	0.05	JX946153
	R: GGGAGCTTGCATCAGGAAAG					
PG_P0G	F: ACCTGTATTTCACGCTGCAC	AATG(11)	VIC	1	0.05	JX946154
	R: CTGCACCTCTCACCCTACTG					
PG_OLQ	F: AACTGCGGATAGTGGTCACC	AACAT(10)	–	–	–	JX946155
	R: AAGCAAACCAATGTCCCTGC					
PG_QWZ	F: TCGTCTGATTATTGCGCTGC	AATG(8)	PET	2	0.05	JX946156
	R: ACCTATCTCATCCACCACTGC					
PG_RIH	F: AGCAGAGGGTTAGGGTATCG	AAAG(16)	FAM	2	0.05	JX946157
	R: GCGCTCCGACACCTAAGG					
PG_V58	F: CTGTGCCACCTTGTTTCCTG	AATG(10)	PET	1	0.075	JX946158
	R: TTGTGAGTCTCCTGCCCTTG					
PLAL_084	F: ACTCCACAAACTCACTACCTG	ACAG(11)	VIC	1	0.1	JX946132
	R: TGTGGACCCTATTCTTGGCC					
PLAL_124	F: TCTCTCTAGCCTGACGTTCTG	AG(12)	NED	2	0.1	JX946133
	R: TGCCGGGAATAAGACAAAGC					
PLAL_127	F: ATGTCCGAGCTATGAAACCC	AATG(6)	VIC	2	0.05	JX946134
	R: GCACTCGCCTTGACCATTAC					
PLAL_190b	F: AGAAGCTGTTGGCATGAGGT	AGAT(26)	FAM	1	0.1	JX946135
	R: AGGTGCTATGGTGTGGCTTC					
PLAL_241	F: CAAAGGTAGGCAATGGTCTCG	AATG(15)	NED	2	0.1	JX946136
	R: ACGAGCTAGACCTCTATTTGGG					
PLAL_315	F: CGGTGAATAACTCTTAAACCGC	AGAT(20)	NED	1	0.1	JX946137
	R: TTAGCGTCATGTTGCCTCTG					
PLAL_331b	F: GCTATAATGGCGGACAATGG	AGAT(14)	FAM	1	0.1	JX946138
	R: TTTTCTAGTCGCCAGGATGC					
PLAL_402	F: AGTGGTGAGGGAGATGGATG	ATCC(14)	FAM	1	0.1	JX946139
	R: TGGACTGTTGCTTTCTTGTGC					
PLAL_542	F: ATGCCTTAGGACCGCAGTAG	AGAT(32)	PET	1	0.1	JX946140
	R: TGGGTTTCCTGGCATACTCC					
PLAL_545b	F: TGGGCCTGGAGCATTACATA	AGAT(27)	VIC	2	0.1	JX946141
	R: GCTTAGTGCAAGGTGTCTTCC					
PLAL_615	F: CCTAAGAGCACGGGACAGAG	AGAT(14)	VIC	2	0.1	JX946142
	R: TATGAGGTCGATCGGTGAGC					
PLAL_701	F: CATGCGTACAGGATTAGGTCAG	ACAG(16)…AGAT(16)	FAM	2	0.1	JX946143
	R: CAGTCTGCCTCTTTGTAAGGC					
PLAL_791	F: GCTTGTTTACTTGATGGAGC	AATG(11)	VIC	1	0.075	JX946144
	R: ACGTAATCCCAGGTTACACTCC					
PLAL_815b	F: GCTTCGTGCATTAAGTCGTG	AATG(8)	–	–	–	JX946150
	R: GATGCCACCGTTATTACCA					
PLAL_909	F: ATCGGAAGTGGGACCATAATG	AACT(21)	VIC	1	0.05	JX946145
	R: TCGGAAGTGGGACCATAATG					
PLAL_AOYX3	F: AAATGGTTTCAGGCTGGCTC	AATG(11)	–	–	–	JX946149
	R: GGGACTGCAAAGTGACACATG					

Table 1 continued

Locus	Primer sequence 5′ to 3′	Motif	Label	Multiplex	Concentration	GenBank #
PLAL_B8DRY	F: TGTGGACAGTGGGATCAAGG	AGAT(10)	VIC	2	0.1	JX946146
	R: TGATGGAGCCAGAGAGCATG					
PLAL_C8W7K	F: AGGACCTTGGCACTCTTAGG	AGAT(9)	PET	2	0.05	JX946147
	R: CAGAGCGTCATTTCACCCTG					
PLAL_EIXNY	F: TTTGTTTGTGGCTAGTCGTG	ACAG(9)…AGAT(11)	PET	2	0.15	JX946148
	R: GCCCAGTCCTTGCTTCTTTC					

Table 2 Size ranges, number of samples successfully amplified (N), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, for microsatellite loci in *P. albagula* and *P. shermani*

Locus	*P. albagula*					*P. shermani*				
	Size (bp)	N	N_A	H_O	H_E	Size (bp)	N	N_A	H_O	H_E
PG_184b	182–192	99	2	0.475	0.436	166–206	8	6	0.750	0.817
PG_3XI	130–156	101	2	0.039	0.038	120–130	8	3	0.500	0.575
PG_43M	105–125	101	4	0.378	0.347	100–138	8	7	0.750	0.742
PG_OLQ	+	+	+	+	+	164–204	8	2	0.375	0.508
PG_POG	229–245	101	5	0.308	0.378	220–264	7	8	0.625	0.900
PG_QWZ	152–156	101	2	0.256	0.294	152–160	8	2	0.250	0.233
PG_RIH	204	101	1	0.000	0.000	190–204	8	2	0.125	0.125
PG_V58	140–156	101	3	0.307	0.399	132–168	8	6	0.375	0.792
Plal_084	345–413	101	3	0.29	0.324	245–421	8	10	0.625	0.950
Plal_124	302–358	101	8	0.593	0.704	240–294	8	10	1	0.95
Plal_127	87–103	101	3	0.436	0.512	84–116	8	4	0.625	0.692
Plal_190b	272–380	95	18	0.673	0.832	307–413	8	9	0.875	0.917
Plal_241	211–247	101	7	0.455	0.424	190–230	8	8	0.875	0.908
Plal_315	281–399	98	19	0.745	0.827	243–297	8	10	0.875	0.933
Plal_331b	166–186	101	6	0.653	0.638	129–177	8	7	0.875	0.867
Plal_402	107–147	101	7	0.394	0.417	59–147	8	7	0.750	0.850
Plal_542	200–248	101	7	0.652	0.65	138–231	8	10	0.750	0.917
Plal_545b	326	101	1	0.000	0.000	–	–	–	–	–
Plal_615	165–181	101	4	0.2	0.208	–	–	–	–	–
Plal_701	302–356	100	11	0.8	0.81	–	–	–	–	–
Plal_815b	+	+	+	+	+	213–253	8	7	0.875	0.850
Plal_791	106–122	101	4	0.229	0.232	–	–	–	–	–
Plal_909	156–192	101	7	0.632	0.676	122–182	8	7	1.000	0.867
Plal_AOYX3	+	+	+	+	+	405–453	8	9	0.625	0.917
Plal_B8DRY	245–281	100	7	0.344	0.444	238–358	8	9	0.500	0.908
Plal_C8WYK	193–237	101	7	0.339	0.383	164–292	8	12	0.875	0.958
Plal_EIXNY	265–381	95	21	0.643	0.92	–	–	–	–	–
Mean[a]		100.14	7.14	0.447	0.495		7.95	7.05	0.676	0.781

[a] Excluding monomorphic loci. "+" signifies loci that worked in *P. albagula*, but could not be fit into multiplex reactions; "−"signifies loci with no or poor amplification in *P. shermani*

(n = 87) and *P. glutinosis* (n = 50) sequences and screened using a single sample of total genomic DNA from *P. albagula*. 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, USA), 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, 60 °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) and 100 bp ladder. Forty primers yielding clear PCR products were further screened for polymorphism on seven samples. A total of 27 polymorphic loci were identified and forward primers were fluorescently labeled (Table 1). Primers were divided into two multiplexes (Table 1) using Multiplex Manager (Holleley and Geerts 2009) and screened in 101 *P. albagula* samples from the two conservation areas. Multiplex PCR reactions were done with the Qiagen Multiplex PCR kit (Valencia, CA, USA) in an 8 μL volume using the following cycling conditions: initial denaturing at 95 °C for 15 min, followed by 35 cycles of 94 °C denaturing for 30 s, annealing at 60 °C for 135 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, USA). We calculated expected and observed heterozygosities using the Excel Microsatellite Toolkit (Park 2001), Hardy–Weinberg equilibrium and linkage disequilibrium using GENEPOP version 4.0.10 (Rousset 2008), and pairwise differentiation between population pairs in GENODIVE version 2.0b22 (Merriman and VanTienderen 2004). Lastly, we cross-amplified all of our primers in eight specimens of the IUCN vulnerable species *P. shermani* using PCR conditions described for initial primer screening.

Alleles per locus for *P. albagula* ranged from 1 to 21 (mean = 7.14, Table 2) and the observed heterozygosity ranged from 0.039 to 0.800 (mean = 0.447). The populations were significantly differentiated ($F_{ST} = 0.209$; $p = 0.001$); the average number of alleles was 5.04 for Danville and 5.21 for Reform. Loci *Plal_190b* and *Plal_EIXNY* deviated significantly from expected values of Hardy–Weinberg equilibrium (Table 2). Loci *PG_RIH* and *Plal_545b* were monomorphic in the screened samples, although both are polymorphic across a broader geographic range (WEP, unpublished results). Cross-amplification was successful in 22 of the 27 screened primers with 2–12 alleles (mean = 7.05) and observed heterozygosity from 0.125 to 1.000 (mean = 0.676). This is only the third species of *Plethodon*, the most species genus of amphibians in North America (Collins and Taggart 2009), in which microsatellite loci have been characterized. Based on our cross-species amplification with *P. shermani*, these primers appear to have generality within the genus *Plethodon*, making them invaluable tools for gaining a greater understanding of ecology, population biology, and conservation of these species.

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