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PRIMER NOTE

Novel tetranucleotide microsatellite markers from the Del Norte salamander (*Plethodon elongatus*) with application to its sister species the Siskiyou Mountain salamander (*P. stormi*)

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Abstract

Eleven tetranucleotide microsatellite loci were developed for the Del Norte salamander (*Plethodon elongatus*). The loci were variably polymorphic, ranging from two to 20 alleles per locus, with expected heterozygosities ranging from 0.07 to 0.86. The loci also amplified in a congener, the Siskiyou Mountain salamander (*P. stormi*). The microsatellite loci will be used to assess the utility of highly polymorphic markers to assay within- and between-species differentiation between these two closely related species.

Keywords: Del Norte salamander, microsatellites, Plethodon, Siskiyou Mountain salamander

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Plethodontid salamanders are model organisms for addressing a variety of questions in evolutionary biology (Highton & Larson 1979; Wake & Larson 1987; Arnold *et al*. 1993; Houck & Verrell 1993). Of particular interest are contact zones that provide windows on evolutionary processes important in speciation (Harrison 1993; Highton 1995). Most studies of plethodontid contact zones have used either allozymes or mitochondrial genes. However, it would be advantageous to use more variable nuclear markers to analyse complex patterns of gene flow across species' boundaries (Wake & Jockusch 2000; Mead *et al*. 2001). Microsatellites can fill this role but have never been used to analyse plethodontid contact zones. Here, we developed 11 microsatellite markers in order to clarify patterns of gene flow at the contact zone between two closely related western North American species of plethodontid salamanders (*Plethodon elongatus* and *P. stormi*).

Microsatellites were developed from two unrelated *P. elongatus* individuals (MEP0133 and LSM0267). Genomic DNA was extracted using a proteinase K digestion and phenol–chloroform technique (Hillis *et al*. 1996) from tail tissue removed nonlethally from each salamander in the field. We used a degenerate oligonucleotide primer–polymerase chain reaction (DOP–PCR) to develop the microsatellite markers following the general procedure of Cabe & Marshall (2001). This method employs a degenerated primer (K6-MW) and a gradient of annealing temperatures to amplify many genomic fragments from 200 to 2000 bp (Macas *et al*. 1996). The DOP–PCR conditions were identical to those used by Hoffman *et al*. (2003). The PCR fragments were enriched for microsatellite repeats by hybridization to biotinylated $GATA_8$ oligonucleotides, using the conditions of Hoffman *et al*. (2003). These repeat-rich fragments were captured using streptavidin-coated magnasphere beads (Promega Corporation). A second enrichment step was carried out following the procedure of Cabe & Marshall (2001). The eluate from this second enrichment was run in a final DOP–PCR to create double-stranded product which was cloned and plated using a Topo cloning kit (Invitrogen Corporation) and subsequently incubated overnight at 37 °C. Colonies were screened using the PCR protocol of Cabe & Marshall (2001). Of the 480 colonies screened, approximately 145 positive colonies were isolated (30% efficiency) and purified using a PCR Purification Kit (QIAGEN Inc.). Purified samples were quantified and sent to the Nevada Genomics Center (University of Nevada, Reno, NV, USA) and sequenced in one direction with the sequencing primer T7.

Thirty-seven unique sequences had microsatellite motifs and adequate flanking regions for primer design. Eleven of the designed primer pairs amplified successfully and so the forward primer was fluorescently labelled to test for

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Table 1 Microsatellite primers for the Del Norte and Siskiyou Mountain salamanders [*Plethodon elongatus* (n = 15) and *P. stormi* (n = 10)]

Locus	Sequence (5′–3′)	Repeat motif	Annealing temp. (°C)	GenBank Accession no.	*P. elongatus*			*P. stormi*		
					H_O	H_E	Size range	H_O	H_E	Size range
*Plel*100	*F-ATGAAATGGCACGACTTGTTTA CACATGGGGTAGGAAATGACTT	$(TCTA)_{23}$	53	AY532595	0.73	0.80	296–344	0.80	0.86	276–302
*Plel*101	*H-TATGCGAGTTATCTCACTATGC TTGTACTGTCCATTGCTTTCT	$(AGAT)_{12}$	56	AY532596	0.80	0.76	160–180	0.60	0.66	164–200
*Plel*103	*F-AGCACAGTCCCAACCTCAAATC CCATGCGAATGTGGCACTATAC	$(TCTA)_{30}$	56	AY532597	0.67	0.76	181–215	0.30	0.74	173–201
*Plel*104	*F-GATGCTGCTTTGGAAATGTGT CGCCTCGAAATTACTTATAGTTGT	$(GATA)_{15}$	62.5	AY532598	0.47	0.52	261–281	0.70	0.79	261–315
*Plel*105	*H-CCTGCTGTCAAGTACCAT CTACGCTCTATTCAAAATCA	$(ATCT)_{19}$	61	AY532599	0.87	0.75	284–334	0.60	0.78	292–314
*Plel*107	*F-CTGGGGATTTATCGCTAGTC CAGGCAGGGGTAAAAACTAT	$(AGAT)_{16}$	61	AY532600	0.60	0.57	427–459	0.40	0.40	403–407
*Plel*108	*F-GGGGTGGCAGCTGTAGACT ATACGTCATGCCGCCCAGTAGTTA	$(GATA)_8$	62	AY532601	0.20	0.19	210–214	0.40	0.47	210–214
*Plel*109	*H-GAGCCGATCCAAGCGAGGT AGGGGGAGCACGGACTTGT	$(GATA)_5$	61.5	AY532602	0.07	0.07	265–269	0.70	0.73	261–301
*Plel*110	*H-CTATTTGACTGAACCTGTA CAAGTCCCTCTATGTAGTA	$(GATA)_{18}$	55	AY532603	0.67	0.69	326–368	0.80	0.84	318–368
*Plel*111	*F-GTCTCACCCCACTCACTTTGCTA GTATGTCCACTGCTCGTCTTTCTT	$(TAGA)_{22}$	55	AY532604	0.80	0.85	342–368	0.50	0.41	334–342
*Plel*112	*F-GGCTGTGGAATATGTACTTT GTGGAGTGGCGAGAGAGA	$(TCTA)_{14}$	59	AY532605	0.47	0.53	365–385	0.70	0.78	369–417

*Primer labelled with 6-FAM or 5-HEX (QIAGEN).
H_O, Observed heterozygosity; H_E, expected heterozygosity.

locus variability. Samples of both *P. elongatus* and the congeneric species *P. stormi* successfully amplified using the following PCR conditions: 94 °C for 5 min, 35 cycles of 94 °C for 1.5 min, locus-specific annealing temperature (Table 1) for 45 s, and 72 °C for 45 s followed by 72 °C for 10 min. Amplified DNA was analysed for fragment size in the ABI 3100 capillary system at Oregon State University.

All loci were analysed in GENEPOP version 3.3 (Raymond & Rousset 1995). One population of *P. elongatus* (n = 15) and one population of *P. stormi* (n = 10) were used to estimate allelic frequencies and to test for Hardy–Weinburg and genotypic equilibria (exact probabilities). Sequential Bonferroni corrections for multiple comparisons were applied to both equilibrium tests (Sokal & Rohlf 1995). The 11 scored loci were polymorphic with two to 20 alleles per locus (mean alleles/locus = 9.7) in the combined sample of *P. elongatus* and *P. stormi* (Table 1). Expected heterozygosities (H_E) ranged from 0.067 to 0.853 (mean H_E = 0.590) for *P. elongatus* and from 0.400 to 0.859 (mean H_E = 0.679) for *P. stormi* (Table 1). Neither population was out of Hardy–Weinburg equilibrium ($P > 0.05$) and there was no evidence of linkage disequilibrium ($P > 0.05$). These loci represent the first microsatellite markers to be diagnosed for any of the western North American plethodontids.

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