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TECHNICAL NOTE

Microsatellite markers for *Pseudoeurycea leprosa,* a plethodontid salamander endemic to the Transmexican Neovolcanic Belt

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Abstract We isolated and characterized 11 polymorphic microsatellite markers for the plethodontid salamander *Pseudoeurycea leprosa* to obtain population genetic data necessary for the proper management of this threatened species endemic to Central México. We tested polymorphism of these loci among 50 individuals from two populations (Texcalyacac and Calpan) in the states of México and Puebla, across the Transmexican Neovolcanic Belt. The number of alleles per locus ranged from three to 33 (mean; Na = 14.5). Observed and expected heterozygosities ranged from 0.20 to 0.88 and 0.22 to 0.93, respectively. We found deviations from Hardy–Weinberg equilibrium expectations for both populations at two loci (Plt028 and Plt066) and for Calpan population at locus Plt009. Evidence of significant linkage disequilibrium between pairs of loci was found only in one population. We found no evidence of large allele dropouts or stuttering, although null alleles could be present at loci Plt028 and Plt066, which showed significant excess of homozygotes. These markers will be useful for resolving fine-scale population genetic structure, gene flow, and population genetic diversity in *Pseudoeurycea leprosa*.

Keywords Amphibian · Genetic diversity · Microsatellite · *Pseudoeurycea leprosa* · Plethodontid · Salamander · Transmexican Neovolcanic Belt

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Amphibian species are declining worldwide (Stuart et al. 2004) and one of the primary causes is the continued anthropogenic habitat modification (Dodd and Smith 2003). Especially affected are species that are habitat specialists and thus more vulnerable to habitat loss and discontinuity. Recently, surveys of historical populations showed that densities of plethodontid salamanders in Central America and México have also declined (Rovito et al. 2009). *Pseudoeurycea leprosa* is endemic to pine and pine-oak forests at high elevations (2,500–3,200 m asl.) along the Transmexican Neovolcanic Belt (TNB). The species is listed as 'vulnerable' by the World Conservation Union (IUCN 2008) due to decreasing population sizes. During the last two decades, an increase in unplanned forest logging along the TNB has caused local population extinctions and a fragmented distribution in the remaining areas of the species' range, potentially threatening the viability of the species (Ochoa-Ochoa and Flores-Villela 2006). Understanding the spatial distribution of genetic diversity across the range will be crucial for development of effective conservation measures. Here we report on the development of polymorphic microsatellite loci which provide sufficient resolution for detecting fine-scale population genetic structure, gene flow, and population genetic diversity.

We cloned microsatellite loci from an enriched partial genomic library prepared with tissue from three individuals of *P. leprosa* from Calpan (state of Puebla; IBH 18226), Texcalyacac (state of México; IBH 18205) and Llano Grande populations (state of México). Genomic DNA was extracted using a Qiagen DNeasy kit, digested with Alu I/Hae III (New England Bio Labs), size selected for 500–700 bp in length, and ligated to SNX linkers using T4 DNA Ligase (New England Bio Labs). Linked fragments were enriched for microsatellites with biotinylated dimer, trimer,

Table 1 Description of 11 microsatellite polymorphic loci isolated for the plethodontid salamander *Pseudoeurycea leprosa*

Locus ID	Primer sequences (5′–3′) Fluorescence labelling	Repeat motif	T^a (°C)	Allele size range (bp)	N	N_A	H_O	H_E	GenBank accession no.
Pld 005	F: ^{NED} GCCTGGAGGGTTTTTGGAGCAC	$(CA)_{24}$	56	249–257	50	3	0.200	0.219	FJ873180
	R: GTGGAGACTGGGCAACATCAAGAAG								
Plt 009	F: ^{VIC} GAAGCCTCTCAATGAAGCAACA	$(GAA)_{79}$	50.5	239–397	49	33	0.878	0.932	FJ873181
	R: ATAGTAGAAGGACAGAAAGATGATTA								
Plt 109	F: ^{VIC} ATATACGATGTGGCAGATGCTGGATGGTC	$(TCAA)_6(TCAAA)_4$	53	132–149	45	5	0.583	0.642	FJ873182
	R: TACTGTGGCTTGAAGGCTTAAGGTAATTTTGACTG								
Plt 087	F: ^{6FAM} TGTAGCCTTGAGTGACAATGCACAAAGAGA	$(AG)_{39}$	49	241–307	46	25	0.935	0.886	FJ873183
	R: GCTTACTATAACTCCGGACTGCTCACCACTTGAC								
Plt 039	F: ^{VIC} CTAGCCTGTCCGCTGGTATTAGAATTAGCACATCT	$(TATC)_{24}$	53	229–289	49	16	0.862	0.894	FJ873184
	R: CTCCTGGCCTCCATCCCTTGATTGAAC								
Plt 064	F: ^{6FAM} CTAAGGATGGTTCTCAATGGCAAGATAG	$(TAGA)_{17}$	56	163–215	48	14	0.891	0.869	FJ873185
	R: CATTTTATGTGCGATTTCTGGTTATTTTTAT								
Plt 107	F: ^{VIC} ATATAAGGAATGAATGAACGAACGAATCAATG	$(TAAG)_{40}$	56.5	387–427	50	9	0.710	0.780	FJ873186
	R: GTGTGGATCGCTGACTTTCTGACC								
Plt 042	F: ^{6FAM} GCCACTTCCTGCATGCCTTGTTC	$(TAGA)_{11}$	56	144–165	50	6	0.563	0.622	FJ873187
	R: GGCTGGGCTGGTTGGAGGTGGGATGG								
Plt 045	F: ^{VIC} TCCCATGAAGATTGCCCAGAAACA	$(GA)_{14}$	56	267–285	48	8	0.524	0.455	FJ873188
	R: GGCATACTAAGTCACCAAAATCCCTCAAT								
Plt 28	F: ^{6FAM} CCTTAGCCCAGTACAAGCCCATGCTCTCAT	$(CT)_{20}$	62	458–521	41	23	0.601	0.881	FJ873189
	R: CTCTCGAGGAGGGTGAACAATAAGGT								
Plt 66	F: ^{VIC} ACGGGGTGTTTGTTATTTGCTATG	$(TCTA)_{58}$	54	407–560	30	18	0.344	0.879	FJ873190
	R: CTCCTGGCGATTTGTGCTGTA								
Total mean						15	0.645	0.733	

For each locus, we list the primer pair, the repeat motif from the original clone, the annealing temp (°C) and size range of alleles present in both populations, the number of individuals genotypes (N), the number of alleles (N_A), and the observed (H_O) and expected (H_E) heterozygosities across both populations

and tetramer probes bound to streptavidin-coated magnetic beads (Dynabeads, Dynal Biotech). DNA fragments containing microsatellites were captured magnetically and amplified via PCR with linker-specific primers. Amplification products were digested with Nhe I, cloned into pUC19 vector, and transformed using DH5α competent cells (Invitrogen). Colonies were grown on X-Gal/IPTG-coated agar plates and transferred to Magna Lift nylon membranes (Osmonics Inc.) that were later probed with the same series of di-, tri-, and tetranucleotide radio-labeled repeats. Positive clones were cultured and plasmid DNA was extracted with QIAGEN miniprep columns. Template DNA was sequenced directly with vector-specific primers (M13 F and R) using dGTP BigDye terminator cycle sequencing components on an ABI 3730 Genetic Analyzer (Applied Biosystems).

We designed primers for 34 microsatellite loci using Primer Select (DNA Star software version 5.05) and tested them for amplification using one sample of *P. leprosa*. Of these loci, 22 reliably amplified a product of the correct size; these were tested for polymorphism across samples from eight different populations. Of those, 11 were polymorphic and used for genotyping 50 tissue samples of *P. leprosa* collected from two populations (Texcalyacac and Calpan) in the states of Mexico and Puebla. We extracted genomic DNA from tail clips or liver using QIAGEN extraction kits (Qiagen Inc., Valencia, California, USA). The concentration of genomic DNA was quantified with the use of the NanoDrop-1000 (NanoDrop Tecnologies) and adjusted to a concentration of ~100 ng/ul for DNA amplification. Polymerase chain reaction (PCR) amplifications were performed in a total volume of 12 μl volume including ~100 ng of DNA template, 0.5 U Taq polymerase (Applied Biosystems), 1× PCR buffer with $MgCl_2$, 0.4 mM dNTPs, and 0.5 μM forward and reverse primers. For each locus, the forward primer was 5′-labelled with a fluorescent dye (VIC, PET, 6-FAM, or NED). PCR cycling conditions consisted of an initial denaturation at 94° for 5 min, followed by 30 cycles of 94° for 1 min, annealing at 49–62°C (Table 1) for 1 min, and 72° for 1 min, followed by a final elongation step at 72° for 10 min. Loci Plt009, Plt28, Plt107 and Plt109 amplified better with shorter denaturation, annealing and extension times (40 s). Loci were multiplexed according to amplicon size and fluorescent tags, and electrophoresed on an ABI 3730 genetic analyzer. Fragment sizes were determined using LIZ-500 size standard; amplicons for Loci Plt028 and Plt066 were greater than 500 base pairs in length and were sized with LIZ-600. Fragments were scored and binned using GeneMapper version 3.7 (Applied Biosystems).

The number of alleles per locus ranged from three to 33 (mean; Na = 14.5). Observed and expected heterozygosities ranged from 0.20 to 0.88 and 0.22 to 0.93, respectively (Table 1). We tested for evidence of linkage disequilibrium and departures from Hardy–Weinberg equilibrium using the software Genepop on the web version 3.4 (Raymond and Rousset 1995). A Markov chain method with 10,000 dememorization steps and 1,000 batches of 10,000 iterations per batch was used to determine significance. Significant deviation from HWE (heterozygote deficit) was detected for loci Plt28 and Plt66 in both populations, and Plt009 in the Calpan population, after Bonferroni correction for multiple comparisons (Rice 1989). We further tested for the presence of null alleles using MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). No large allele dropouts or stuttering was detected at the 99% confidence level across all loci. However, loci Plt028 and Plt066 showed significant excess of homozygotes, possibly due to null alleles. Linkage disequilibrium was identified among six loci, but only in Calpan population (Plt039-Plt028, Plt009-Plt028 and Plt039-Plt064).

These polymorphic microsatellites will be useful for population genetic studies of *P. leprosa* throughout its range, and for specific tests of the effects of habitat loss and fragmentation on the genetic variability and persistence of this species.

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