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PRIMER NOTE

Polymorphic microsatellite loci in *Lithophragma maximum* (Saxifragaceae), an endemic plant of San Clemente Island

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Abstract

In this paper, we report the isolation of 11 polymorphic microsatellite loci from *Lithophragma maximum* (Saxifragaceae), a rare species restricted to San Clemente Island, California. We found moderate levels of allelic variation (mean N_A = 5.5) and high levels of expected heterozygosity (mean H_E = 0.531) across sampled individuals. However, eight loci showed a significant absence of heterozygous individuals, which we attribute to small population size and demographic history of the species.

Keywords: California Channel Islands, *Lithophragma*, microsatellite

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Lithophragma maximum Bacigal. (Saxifragaceae) is a rare perennial herb endemic to San Clemente Island, California. Considered extinct until 1979, approximately 500 individuals are now known from 15 populations located in deep canyons along 2–3 km of the southeast coast of San Clemente Island (Junak & Wilken 1998; Helenurm, personal observation). A previous study of five *L. maximum* populations based on 24 allozyme loci did not reveal any genetic variation (K. Helenurm, unpublished data), but rare species with a restricted distribution often do not exhibit allozyme variation. In this study, we report the characterization of 11 microsatellite loci that were isolated from *L. maximum* and used to re-examine population genetic structure and assess the historical impact of human disturbance on populations on San Clemente Island.

Genomic DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (QIAGEN). Tandem repeat regions were isolated using the subtractive hybridization method of Hamilton *et al*. (1999). Digested DNA was enriched for eight oligonucleotide repeats $(AC)_{15}$, $(AG)_{15}$, $(AT)_{15}$, $(CG)_{15}$, $(CCG)_{10}$, $(AAC)_{10}$, $(AGG)_{10}$ and $(CAC)_{10}$. Enriched polymerase chain reaction (PCR) products were cloned using pBluescript II SK (+) and XL-1 MRF' Supercompetent cells (Stratagene). Colonies were screened using X-gal (5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and IPTG (isopropyl-beta-D-thiogalactopyranoside) (Sigma-Aldrich). Approximately 400 colour-positive (i.e. white) colonies were suspended in 100 μL TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0), and 2 μL of the colony solution was used to screen for a microsatellite region by hybridization with oligonucleotides (Glenn & Schable, unpublished) and the Phototope detection system (New England Biolabs). Subsequently, 214 positive clones were further screened for insert size by PCR. Reactions were 25 μL in volume and contained 1 μL template DNA, 0.8 μM each of primers T3 and T7 (Integrated DNA Technologies), 1× ThermoPol Reaction Buffer (10 mM KCl; 10 mM $(NH_4)_2SO_4$; 20 mM Tris-HCl, pH 8.8; 2 mM $MgSO_4$; 0.1% Triton X-100; New England Biolabs), 200 μM of each dNTP and 0.2 U of Vent (exo$^-$) DNA polymerase (New England Biolabs). The thermal cycler program consisted of 5 min at 96 °C, followed by 30 cycles at 96 °C for 45 s, 51 °C for 1 min and 72 °C for 2 min. PCR products were run electrophoretically in a 1.5% agarose TBE gel and visualized by ethidium bromide and UV light. Clones that exhibited a single amplified band of 500–1000 bp were cleaned with 16 U of Exonuclease I and 3 U of Antarctic Phosphatase (New England Biolabs) followed by ethanol precipitation. Cleaned PCR products were sequenced using the T3 primer, BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and Better Buffer (The Gel Company) in 1/8th volume reactions. Sequences were electrophoresed on an Avant 3100 Genetic Analyser (Applied Biosystems).

Of the 58 inserts that were sequenced, 51 contained a region of at least four repeat units, but only 21 clones proved suitable for primer design. For inserts containing a

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Table 1 Primer sequences, reaction conditions, and diversity statistics for 11 microsatellite loci isolated from *Lithophragma maximum*. Repeat motif refers to the allele of the sequenced clone. The labelled primer is indicated by an asterisk. The number of observed alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) were determined from 122 individuals on San Clemente Island. * a touchdown program was used with a final annealing temperature of 52 °C for 35 cycles; + significant departure from Hardy–Weinberg equilibrium as a result of a lack of heterozygous individuals; – significant departure from Hardy–Weinberg equilibrium as a result of an excess of heterozygous individuals

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Allele size range	N_A	H_O	H_E	GenBank Accession No.
Lima5	F-FAM CATCATCGACAACCATAGATCC R-CCTTCATGAATGCCTATTTTCC	$(CT)_{11}$ $(CACT)_2(C)_8$	62	272–288	3	0.058	0.088	DQ270410
Lima6	F-PET TCGCCAACTGAAAGAGTGAG R-GTTTCAATATGTACCCCAGTTGAG	$(AG)_{13}$	50	214–228	6	0.041+	0.735	DQ270411
Lima13	F-VIC AACAGTGAGGGTACCAAAATGC R-GAGAGAAAGAGGGACAATTTGC	$(CT)_{13}(C)_{14}$	63	260–280	5	0.115+	0.344	DQ270412
Lima31	F-FAM GTCTGATCTCTTTCCCTTTTCATC R-CACGAAAATGACCAAAATACCC	$(AG)_{32}$	62	204–237	13	0.246+	0.875	DQ270413
Lima47	F-VIC ATGTAGAGATGAGAATGCCAACC R-GCCCCCTCATAACAAAACC	$(GA)_2G_2$ $(GA)_4CA(GA)_{13}$	56–52*	184–186	2	0+	0.152	DQ270414
Lima49	F-CACAAAAATGGTACTCGTACTTCC R-PET TCGACGATTTACCAGAGAGG	$(C)_4A_2(C)_7(TA)_8$	60	160–180	4	0.036+	0.599	DQ270415
Lima53	F-NED CAATTCTCCGGTGGCTTACC R-CGACATTGACGGTGATGC	$(AG)_{15}$	65	162–182	4	0.402	0.372	DQ270416
Lima77	F-FAM GGAAGCCTGCATCCCTAAATATAG R-GGGTTGACACAAGAAGAACCA	$(AG)_{18}$	65	155–167	6	0.213+	0.671	DQ270417
Lima132	F-FAM CAGAACTTCCACTTTACTTCC R-CTTTCACAAGAAACAACACG	$(AG)_{15}$	61	377–399	5	0.140+	0.655	DQ270418
Lima134	F-TTGGTTTTTCCATACAGTCG R-NED ATTCACCTGCTTTTCAATCC	$(AG)_2AT(AG)_8$	50	168–176	5	0.479+	0.586	DQ270419
Lima142	F-CGAATCCACTGTTATGAGC R-VIC TTCTCTGGCTAAGAGACC	$(AG)_8ACTC$ $(AC)_8(AG)_{21}$	61	220–234	8	0.934–	0.768	DQ270420
Mean					5.5	0.242+	0.531	

microsatellite motif, forward and reverse sequences were generated and aligned in SEQUENCHER 4.1 (GeneCodes). Primers were designed using the program PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers that amplified a single band from original genomic DNA were further screened for polymorphism (i.e. indicated by visual identification of bands of different sizes on an agarose gel) in a sample of five individuals from different populations. We chose 11 primers that yielded consistent amplification products to survey genetic variation in *L. maximum*. One of the primers for each locus was labelled at the 5′ end with one of four fluorescent dyes, 6-FAM, VIC, NED or PET (Applied Biosystems; Table 1). The LIZ 500 marker (Applied Biosystems) was run with each locus as an internal size standard.

A sample of 122 individuals from multiple populations was used to evaluate variability for the 11 loci in *L. maximum*. Microsatellite loci were amplified individually in 10 μL reactions containing 0.5–1 μL template DNA (*c*. 50–60 ng), 1× ThermoPol Buffer, 0.2 mM of each dNTP, 0.3 μM of forward and reverse primers and 0.2 U Vent (exo⁻) DNA polymerase. The reactions were carried out under the following thermal cycler conditions: 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 50–65 °C (Table 1) for 1 min, 72 °C for 1 min and an elongation step at 72 °C for 30 min. A sample of the PCR product was run in a 1.2% agarose TBE gel to check the quality and quantity of each product. PCR products were diluted and mixed with Hi-Di formamide and the LIZ 500 size standard before electrophoresis on an Avant 3100 genetic analyser. Fragments were sized using the GENEMAPPER software (Applied Biosystems). We calculated observed (H_O) and expected (H_E) heterozygosity for all loci using POPGENE version 1.32 (Yeh & Boyle 1997), and we tested each of the loci for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using FSTAT version 2.9.3.2 (Goudet 1995) with the application of a Bonferroni correction in assessing significance for both measures.

Although all loci were found to be polymorphic, only moderate levels of genetic diversity were found across the sampled individuals. For example, the number of alleles per locus ranged from two to 13, but there was a general lack of heterozygous individuals in the sample of individuals, resulting in substantial deviations from HWE ($P < 0.01$) for eight loci (Table 1). Locus Lima 142, though, exhibited a significant excess of heterozygous individuals ($P < 0.01$),

and loci Lima5 and Lima53 did not depart significantly from expectations. Expected heterozygosity values ranged from 0.088 to 0.875 (Table 1). Of the 55 interlocus comparisons, 27 pairs of loci exhibited significant genotypic linkage disequilibrium at $P < 0.01$. Locus Lima31 was in disequilibrium with all loci except Lima5 and Lima134. Lima5 was the only locus that appeared to be in equilibrium with all other loci. However, without information on gametic phase, physical linkage of loci cannot be distinguished from disequilibrium due to population processes such as nonrandom mating (Hedrick 2005). Furthermore, the rare nature of this species due to population size and demographic history has likely affected gene and genotypic combinations resulting in the observed deviations from HWE and genotypic disequilibrium. Populations of *L. maximum* occupy small moist sites within canyons and range from 10 to 100 individuals. Moreover, populations have likely experienced reductions in size as a result of intense grazing pressure by feral goats during the last century. Finally, many of the populations appear to be fixed, or nearly fixed, for different alleles. Consequently, genetic drift may be expected to have caused loss of genetic variation at many loci within these small populations, resulting in an overall deficiency of heterozygotes. Although nothing is known of the mating and pollination system of this species, all other species of *Lithophragma* are self-incompatible, and pollination is effected primarily by moths and solitary bees (Taylor 1965). Additional analyses with larger sample sizes are currently underway to more fully characterize population genetic structure in *L. maximum* and to examine further the potential effects that historical factors have had on these populations.

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