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PRIMER NOTE

Characterization of microsatellite loci in polyploid *Lavatera assurgentiflora* ssp. *assurgentiflora* and ssp. *glabra* (Malvaceae)

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Abstract

***Lavatera assurgentiflora* (Malvaceae) is one of four species of the genus *Lavatera* native to California and Baja California. Two geographically defined subspecies are recognized: *L. a. assurgentiflora* on the northern islands and *L. a. glabra* on the southern islands. We isolated nine polymorphic microsatellite loci that amplify in both subspecies of *L. assurgentiflora*. Substantial levels of polymorphism were observed at many of the loci. Four loci exhibited more than 10 alleles, polymorphism information content ranged from 0.4 to 0.8, and up to six alleles were found in some individuals, supporting reports that these taxa are hexaploid. All loci also amplified in *Lavatera lindsayi* from Guadalupe Island, and we anticipate that they will cross-amplify in other California *Lavatera* species as well.**

Keywords: California Channel Islands, conservation genetics, *Lavatera*, microsatellite, polyploidy

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Lavatera (Malvaceae) is a genus of approximately 25 species occurring in the Mediterranean, Baja California, the California Channel Islands and Australia. All species are perennial shrubs or small trees with large pink, purple or white flowers. Several species of *Lavatera* are polyploid, with haploid chromosome counts ranging from 14 to 56 chromosomes in the genus (Ray 1995; Fryxell 1997). The four species of *Lavatera* native to Baja California and the California Channel Islands form a closely related group (Ray 1995). The most widely distributed species of the California *Lavatera* is *Lavatera assurgentiflora* Kellogg, within which two subspecies are recognized. *Lavatera a. assurgentiflora* is native to the northern Channel Islands of Anacapa and San Miguel, whereas *L. a. glabra* is native to the southern islands of Santa Catalina and San Clemente; the two subspecies differ in leaf and floral characteristics (Junak *et al*. 1995). Like many other *Lavatera* species, *L. assurgentiflora* is thought to be hexaploid with a chromosome number of $2n = 6x = c.40$ (Ray 1995; Fryxell 1997). Here, we report results of recent efforts to develop microsatellite loci in *L. assurgentiflora* to re-examine genetic variation and structure among populations located on the California Channel Islands and to test the taxonomic validity of the two subspecies.

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Genomic DNA was isolated from leaf tissue using the cetyltrimethyl ammonium bromide (CTAB) method of Doyle & Doyle (1987). Isolation of microsatellite loci was performed following the subtractive hybridization method of Hamilton *et al*. (1999) with some modifications. 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(+) as a vector and XL-1 MRF' Supercompetent bacterial 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); colour-positive (i.e. white) colonies were subsequently screened for microsatellite regions using a 'dot blot' method (Glenn & Schable, unpublished) and the Phototope chemiluminescent detection system (New England Biolabs). Individual 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. A total of 358 positive clones were screened for insert size by PCR. The 25 μL reaction 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). After a 5-min 96 °C denaturation step, 30 cycles were performed as follows: 45 s at 96 °C, 1 min at 51 °C and 2 min at 72 °C. The PCR product was run electrophoretically in a 1.5% agarose TBE gel. Bands were visualized by ethidium bromide and UV light. Amplification products from clones that produced a single band of 500–1000 bp were cleaned with QIAquick columns (QIAGEN) or Exonuclease I and Antarctic Phosphatase (New England Biolabs) followed by ethanol precipitation. Cleaned PCR products were sequenced directly using one of the amplification primers, BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and Better Buffer (The Gel Company) in one-eighth volume reactions. Sequences were electrophoresed on an Avant 3100 genetic analyser.

Of the 85 inserts that were sequenced, 45 contained a repeat region. However, only 23 clones proved suitable for primer design. For inserts containing a microsatellite motif, forward and reverse sequences were generated and aligned in SEQUENCHER 4.1 (Gene Codes). Primers were designed using the program PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Unlabelled primers (Integrated DNA Technologies) were screened in a two-step process using the plasmid followed by the original DNA sample as template. Primers that amplified a single band in the control sample 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. Primers were labelled at the 5′ end of the sequence with one of four fluorescent dyes, 6-FAM, VIC, NED or PET (Applied Biosystems). The LIZ 500 marker (Applied Biosystems) was run with each locus as an internal size standard. Although 16 of the loci appeared polymorphic on agarose gels, only nine loci were consistently genotyped using fluorescently labelled primers. The combination of stutter bands and the potential for multiple alleles of consecutive size precluded allelic determination for the remaining seven loci.

A sample of 165 individuals, including 10 individuals of *L. a. assurgentiflora* and 155 individuals of *L. a. glabra*, were used to evaluate variability for the nine loci in *L. a. assurgentiflora*. Microsatellite loci were amplified individually in 25 μL reactions containing 2 μL template DNA (*c.* 50–60 ng), 1× ThermoPol Buffer, 200 μM of each dNTP, 1.6 μM of forward and reverse primers, 0.4 U Vent (exo-) DNA polymerase. Samples were amplified in an MJ Research PTC 200 thermal cycler with a denaturing step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 50 °C–62 °C (Table 1) for 1 min and 72 °C for 1 min, and a final 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

Table 1 Primer sequences, reaction conditions and diversity statistics for nine microsatellite loci in *Lavatera assurgentiflora*. The total number of alleles (N_a), number of alleles per individual (N_i) and polymorphism information content (PIC) are based on a survey of 165 individuals from San Clemente, Santa Catalina and Santa Cruz Islands. The fluorescent label is indicated after the primer sequence for each locus; T_a, annealing temperature

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size range	N_a (N_i)	PIC	GenBank Accession no.
Laas10	F: CTGAATGAAGGTAACTCAACC R: AAAGAACAGAAGACGAAAGC (VIC)	$(AC)_9(AT)_5$	55	187–209	7 (1–3)	0.617	DQ222229
Laas21.122	F: CTAGTTTTGGTTCGATCAGC R: TATGGTACTGGATTCGTTGG (6-FAM)	$(GT)_{18}$	62	229–249	11 (1–6)	0.859	DQ222230
Laas21.139	F: ATACCGATCAACCCCTTTCC (PET) R: TGTCGAATCAGGATCTACCG	$(GT)_{16}$	54	194–221	14 (2–6)	0.883	DQ222231
Laas24.10	F: CGTTTTGGGAAGTGTCCTACG R: ATTGAAAATCATGTCCCGTTGG (6-FAM)	$(GT)_{18}$	50	152–178	14 (2–6)	0.862	DQ222232
Laas27.32	F: GGTTCAGTAGATGTTGAGGTTTCC R: TTACGACCACCCCATCTACC (6-FAM)	$(AAG)_7$	54	151–167	4 (1–2)	0.411	DQ222233
Laas27.114	F: TATAGGGTTAAACCGTGAGG (6-FAM) R: AAAGACGACGATGATGAGG	$(CTT)_3(CAT)_4$ $(TA)_2T_2(CTT)_8$	55	197–209	4 (1–3)	0.515	DQ222234
Laas27.166	F: TGAACCAGAACTCCATCG (VIC) R: TAATGGATGAGACCATGTCC	$(CTT)_{13}$	54	212–221	3 (1–3)	0.604	DQ222235
Laas27.169	F: TAGTTCTGCAAACAGTAGCC (VIC) R: TGGGATCTTTGACTCTGC	$(AG)_{14}$	57	232–242	6 (1–3)	0.736	DQ222236
Laas30.101	F: AAGGGAAAACTCAGAAGC (6-FAM) R: CTTCAACTTGGATAAGTTCC	$(AC)_8$	60	143–167	11 (1–5)	0.844	DQ222237

the diluted products were 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). The number and range of amplified alleles were determined across all individuals, and polymorphism information content (PIC) values were calculated for each of the loci following the method of Baruah *et al*. (2003) using a web-based calculator (http://www.agri.huji.ac.il/~weller/Hayim/parent/PIC.htm).

All loci amplified equally well in the two taxa and collectively exhibited high levels of polymorphism. The number of observed alleles per locus ranged from three at Laas27.166 to 14 at Laas21.139 and Laas24.10 (Table 1). PIC values ranged from 0.411 to 0.883, with eight of the nine loci exhibiting PIC values greater than 0.5%. Consistent with a hexaploid genome, both subspecies of *L. assurgentiflora* exhibited one to six alleles per locus. Fewer alleles were found in *L. a. assurgentiflora* than in *L. a. glabra*, an expected result given the much fewer individuals of the former taxon that were examined. Only one allele at locus Laas30.101 was unique to *L. a. assurgentiflora*. All nine primers also amplified readily in *Lavatera lindsayi* Moran on Guadalupe Island and may be cross-amplifiable in other species of *Lavatera* as well. Because we were unable to assign allelic copies to individual duplicated loci for each primer pair, traditional measures of genetic variability, including deviations from Hardy–Weinberg equilibrium, could not be determined. However, other means of characterizing population genetic structure and genetic differentiation of the two subspecies of *L. assurgentiflora* on the California Channel Islands are under investigation using the developed microsatellite markers.

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