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Isolation of microsatellite loci from the endangered plant *Galium catalinense* subspecies *acrispum* (Rubiaceae)

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Abstract

***Galium catalinense* subspecies *acrispum* (Rubiaceae) is a state-endangered perennial shrub endemic to San Clemente Island. Eight polymorphic microsatellite loci were isolated from *G. catalinense* ssp. *acrispum*. These loci show high levels of variability, averaging 6.5 alleles per locus and an expected heterozygosity of 0.550. One locus exhibited significant deviations from Hardy–Weinberg equilibrium ($P < 0.01$) and one pair of loci exhibited significant linkage disequilibrium.**

Keywords: California Channel Islands, conservation genetics, *Galium*, microsatellite

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Galium catalinense A. Gray ssp. *acrispum* Dempster (Rubiaceae), the San Clemente Island bedstraw, is a California endangered, perennial shrub endemic to San Clemente Island, California. This species occurs primarily on rocky outcrops of steep canyon walls but is also found on canyon bottoms and open coastal slopes; populations are often small, consisting of fewer than 30 individuals, although several of these may be found in the same canyon (Junak & Wilken 1998). It is likely that *G. catalinense* ssp. *acrispum* was decimated by extensive overgrazing by goats during the past century and by the spread of non-native plants after grazers were eliminated. Due to this recent history of overgrazing and introduced competitors on San Clemente Island, understanding the population structure, effective population size, and rates of gene flow among populations is important to managing this rare plant. Here, we report the characterization of eight polymorphic microsatellite loci isolated from *G. catalinense* ssp. *acrispum*.

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Genomic DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (QIAGEN). 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 $(CAC)_{10}$. Fragments were cloned using pBluescript II SK-Phagemid vector and the XL1-Blue MRF′ bacterial host strain (Stratagene). Colour-positive clones were screened for microsatellite regions using a membrane-based 'dot blot' method (T. Glenn and M. Schable, unpublished) and the Phototope chemiluminescent detection system (New England Biolabs). A total of 126 positive clones were screened for insert size by polymerase chain reaction (PCR) using an MJ Research PTC-200. The 25-μL reactions contained 1 μL template DNA, 0.8 μM each of primers T3 and T7 (Integrated DNA Technologies), 1× Themopol Reaction Buffer (New England Biolabs), 200 μM of each dNTP, and 0.2 U of Vent (exo-) DNA polymerase (New England Biolabs). Clones that exhibited a single amplified band of 400–1000 bp were cleaned using a PEG precipitation procedure and sequenced

Table 1 Primer sequences, reaction conditions, and diversity statistics for eight microsatellite loci isolated from *Galium catalinense* ssp. *acrispum*. Repeat motif refers to the allele of the sequenced clone. The 5′ tag used for incorporation of fluorescent tag is indicated; M13R (AGGAAACAGCTATGACCAT) and CAGT (ACAGTCGGGCGTCATCA). The number of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) were determined from 83 *G. catalinense* individuals on San Clemente Island

Locus	Primer sequence (5′–3′)	5′ tag	Repeat motif	T_a (°C)	Dye	N amplified	Allele size range (bp)	N_A	H_O	H_E	GenBank Accession no.
Gaca_3	F-AAATTCACCATGTCGGTGGT	CAGT	$(GT)_{13}$	57.8	VIC	75/83	270–288	11	0.813	0.778	FJ373886
	R-GATTTGCTGGCTTCACACAG										
Gaca_32	F-CTCCTCCAAGTAGCGTCT	CAGT	$(CT)_{10}$	57.8	VIC	81/83	188–210	10	0.728	0.798	FJ373887
	R-TGCGAAACAAAGAAACACCA										
Gaca_94	F-CCACTGAAACAGAGCAGTCG		$(GT)_{16}$	57.8	VIC	79/83	272–292	10	0.494	0.612	FJ373888
	GCCATCACCAAAAGTCCAACT	CAGT									
Gaca_102	F-CTCTCAGCACCCTCCTCTC		$(AC)_{10}$	54.8	PET	81/83	171–183	5	0.642	0.572	FJ373889
	R-GCGTGCGTGTTTTCTGTTTGA	CAGT									
Gaca_148	F-TCTAACCAATTCCCAACCTG		$(CCT)_8$	54.8	VIC	81/83	114–126	4	0.284	0.308	FJ373890
	R-TGATGGAACCCAGAAAACAA	M13R									
Gaca_178	F-CAGACATATTGGGCACACT		$(GTT)_5(CTT)_6$	54.8	6-FAM	83/83	181–187	3	0.169	0.158	FJ373891
	R-TCATACTCCTCCTCATCTGG	CAGT									
Gaca_198	F-GCTATTTTGATGATTTGTTATG	M13R	$(CT)_{10}$	52.5	VIC	79/83	168–180	5	0.481	0.647	FJ373892
	R-ACTAGTAAGGTTTGCTTT										
Gaca_208	F-TCCATTACCGTCACTTTCGTT		$(AG)_{10}$	57.8	6-FAM	78/83	262–270	4	0.462	0.523	FJ373893
	R-AACAACAGCAGCCCAGAAAC	M13R									
Mean									6.5	0.509	0.550

N amplified, number of successful amplifications out of the 83 sampled individuals.

using the T3 primer, BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) in one-eighth volume reactions. Sequences were electrophoresed on an Avant 3100 Genetic Analyser (Applied Biosystems). For inserts containing a microsatellite motif, the T7 primer was used to generate a complementary reverse sequence. All sequences were aligned using Sequencher 4.1 (GeneCodes).

Of the 120 inserts that were sequenced, 40 contained a region of at least six repeat units but only 27 proved suitable for primer design. Primers were designed using the program Primer 3 (Rozen & Skaletsky 2000). One primer of each pair was designed with a common tag at the 5′ end following the procedure of Boutin-Ganache *et al*. (2001; Table 1). Two common tags were used, M13R (AGGAAACAGCTATGACCAT) and CAGT (ACAGTCGGGCGTCATCA). Primers were optimized for a range of temperatures (Table 1). We chose eight primers that yielded consistent amplification products. Loci were amplified with a common tag containing one of three fluorescent dyes, 6-FAM, PET, or VIC (Applied Biosystems)

A sample of 83 individuals from four populations on San Clemente Island was used to evaluate variability in *Galium catalinense* ssp. *acrispum*. Microsatellite loci were amplified individually in 12-μL reactions containing 1 μL template DNA (*c*. 10–20 ng), 1× GoTaq Flexi buffer (Promega), 0.2 mM of each dNTP, 0.25 μM of untagged primer, 0.25 μM of common fluorescent tag, 0.03 μM of the tagged primer, 2.5 mM $MgSO_4$, and 0.3 U Go*Taq* Flexi DNA polymerase (Promega). The reactions were carried out under the following thermal cycler conditions: 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 52.5–64 °C (Table 1) for 1 min, 72 °C for 1 min, and an elongation step at 72 °C for 30 min, using an MJ Research PTC-200. PCR products were diluted with water and mixed with Hi-Di formamide and LIZ 500 size standard (Applied Biosystems) before electrophoresis on an Avant 3100 Genetic Analyser. Fragments were sized using the GeneMapper software (Applied Biosystems). We calculated observed and expected heterozygosities, and tested for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium using GenePop version 3.4 (Raymond & Rousset 1995). Micro-Checker version 2.2.3 was used to infer the presence of null alleles with 1000 bootstrap replicates (Van Oosterhout *et al*. 2004).

All loci were found to be polymorphic and levels of observed genetic diversity were high across the sampled individuals. The number of alleles per locus ranged from 3 to 11, with an average of 6.5 (Table 1). Expected heterozygosity ranged from 0.158 to 0.798 (mean 0.550, Table 1). One locus, Gaca_198, showed significant ($P < 0.01$) deviations from HWE in two of the four populations. When populations were pooled, Gaca_94 and Gaca_198 showed significant deviations from HWE. In both cases, the presence of private alleles in at least two populations lead to overestimation of expected heterozygosity. No null alleles were observed when populations were analysed individually. Of the 28 interlocus comparisons, only one pair (Gaca_148 and

Gaca_208) exhibited significant ($P < 0.01$) linkage disequilibrium. The observed linkage disequilibrium is likely driven by population differentiation. Each population has only two or three of the four observed alleles for each locus and apparently elevated co-occurrence of those alleles.

The markers described in this study will be used to investigate the population structure, levels of genetic variability, and past demographic events for *G. catalinense* ssp. *acrispum*. Furthermore, these markers are intended for use throughout the genus *Galium* (Rubiaceae), which is composed of *c*. 400 species worldwide, including 18 North American species of conservation concern.

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Isolation and characterization of 16 polymorphic microsatellite loci for *Frangula alnus* (Rhamnaceae)

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Abstract

We report the first 16 polymorphic nuclear microsatellite markers developed for *Frangula alnus* (Rhamnaceae). Markers were tested on all three subspecies as well as on three local populations, including analyses of both leaf and seed endocarps. A total of 87 alleles were found (mean number of alleles per locus was 5.44) for 72 individuals genotyped. Observed and expected heterozygosities ranged from 0.097 to 0.792 and from 0.093 to 0.794, respectively. The levels of polymorphism and exclusionary power of the developed markers render them applicable for parentage analyses and measurements of seed dispersal through direct comparison of endocarps and adult tree genotypes.

Keywords: endocarp, *Frangula*, Rhamnaceae, seed dispersal, SSR

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Recent advances in seed dispersal studies allow the direct estimation of dispersal distances based on assignment procedures that use the genotype of maternally derived seed endocarps and the genotype of candidate maternal trees (Godoy & Jordano 2001). These techniques require reliable sets of microsatellite markers allowing robust exclusion of candidate source trees and applicable to tissues of both seeds and established seedlings.

Frangula alnus Mill. (Rhamnaceae) is a shrub or small tree widely distributed over Europe and West Asia; most of the native range is occupied by *Frangula alnus alnus*, while *F. a.*

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