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PERMANENT GENETIC RESOURCES

Isolation of microsatellite loci from the endangered plant *Sibara filifolia* (Brassicaceae)

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Abstract

***Sibara filifolia* (Brassicaceae) is a federally endangered annual herb found on two of the California Channel Islands. Previous studies based on allozymes revealed little genetic variability on San Clemente Island. Nine polymorphic microsatellite loci were isolated from individuals on San Clemente Island. We found low levels of allelic variation (mean N_A = 2.3), with seven loci exhibiting significant deviations from Hardy–Weinberg equilibrium ($P < 0.01$) and 10 pairs of loci exhibiting significant linkage disequilibrium ($P < 0.01$). Most of the observed variability (mean H_O = 0.003) occurred among populations or in rare homozygous individuals.**

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

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Sibara filifolia (E. Greene) E. Greene (Brassicaceae), the Santa Cruz Island Rockcress, is a federally endangered, annual herb endemic to the California Channel Islands (USFWS 1997). This species was presumed extinct until it was rediscovered on San Clemente Island in 1986 and Santa Catalina Island in 2001. Currently, there are seven known occurrences on San Clemente Island that are separated by less than 1 km and a single population on Santa Catalina Island. A previous study of three populations on San Clemente Island based on 29 allozyme loci revealed only two polymorphic loci (Helenurm 2003). Greenhouse-grown samples of *S. filifolia* set copious amounts of viable seed, suggesting that this species is self-compatible and autogamous (Helenurm personal observation). Consequentially, the small populations (10–200 individuals) that currently constitute this species may represent highly related selfing lineages. Moreover, the rare nature of this species and recent demographic changes has likely impacted levels of genetic variability. Here, we report the characterization of nine microsatellite loci isolated from *S. filifolia* to reexamine genetic variation and structure among populations.

Genomic DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (QIAGEN). Isolation of microsatellite loci was preformed 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 (Glenn & Schable 2002) and the Phototope chemiluminescent detection system (New England Biolabs). A total of 340 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 either using a PEG precipitation procedure (Johnson & Soltis 1995) or using 16 U of Exonuclease I and 3 U of Antarctic Phosphatase (New England Biolabs) followed by an 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 one-eighth volume reactions. Sequences were electrophoresed on an Avant 3100 Genetic Analyser (Applied Biosystems). For inserts containing a microsatellite motif, the T7 primer

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Table 1 Primer sequences, reaction conditions, and diversity statistics for nine microsatellite loci isolated from *Sibara filifolia*. 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 201 individuals on San Clemente Island

Locus	Primer sequence (5′–3′)	5′-tag	Repeat motif	T_a (°C)	Magnesium	Dye	Allele size range	N_A	H_O	H_E	GenBank Accession no.
Sifi162	F-TCCAGCGAGTGTCGTGATAG R-GGCTCAAACCATCTTTGTCC	M13R	$(TC)_8$	58	2 mM $MgCl_2$	VIC	254–256	2	0.000*	0.216	EF600687
Sifi187	F-AGCATTGTTCGAGAGAAAGTGG R-ACGGTTAAGACCGACCTAGGAG	M13R	$(AAG)_9$	58	2 mM $MgCl_2$	6-FAM	207–210	2	0.000*	0.236	EF600688
Sifi195	F-ACCAAGGCTGAGTGTTGGAC R-TCTGAAGAGACAAGACCTTAAGAC	M13R	$(AG)_3TGA$	52.5	3 mM $MgSO_4$	VIC	205–207	2	0.000*	0.010	EF600689
Sifi207	F-CATGTTTTTGGAGTCAAAGAG R-CTAGCTCTTGTCCGAACTGG	M13R	$(CTT)_2CTC(CTT)_5$	52.5	3 mM $MgSO_4$	6-FAM	216–219	2	0.000*	0.234	EF600690
Sifi210	F-CACAGCAACCTATGCACCAC R-TGCAGAGACAAGACATTAAGAC	M13R	$(AG)_3TG(AG)_7$	64	3 mM $MgSO_4$	PET	259–269	4	0.025	0.035	EF600691
Sifi214	F-GAGGAGGAAGAAGAGGAAACG R-CCAAAATTGGGTCAGTGTACG	M13R	$(GAA)_{10}$	61.5	3 mM $MgSO_4$	PET	210–213	2	0.000*	0.228	EF600692
Sifi224	F-CCAAATTCCCATGTATAATGTCG R-CATCTTCTGCTGCTTCACAGG	CAGT	$(GA)_2(AG)_{16}$	52.5	3 mM $MgSO_4$	VIC	204–216	3	0.000*	0.020	EF600693
Sifi264	F-CTATTTTGCCGCCACTGACT R-ACTCCGCTCGACTAAAGAGC	M13R	$CT(TC)_{13}$	59.8	3 mM $MgSO_4$	PET	233–235	2	0.005	0.035	EF600694
Sifi336	F-TCGAGCGACCAGAATACCAA R-AGGGGCTGAAATACAATAACC	M13R	$(GA)_{10}$	61.5	4 mM MgCl2	6-FAM	299–303	2	0.000*	0.234	EF600695
Mean								2.3	0.003	0.139	

*Significant departure from Hardy–Weinberg equilibrium as a result of a lack of heterozygous individuals.

was used to generate a complementary reverse sequence. All sequences were aligned using SEQUENCHER 4.1 (GeneCodes).

Of the 88 inserts that were sequenced, 44 contained a region of at least six repeat units, but only 24 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). This technique allows for the use of common tags labelled with fluorescent dyes, which can be incorporated into the PCR products, precluding the need for labelling locus-specific primers. Two common tags were used, M13R (AGGAAACAGCTATGACCAT) and CAGT (ACAGTCGGGCGTCATCA). Primers were optimized for a range of temperatures and $MgCl_2$ or $MgSO_4$ concentrations. We chose nine 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 201 individuals from five populations on San Clemente Island was used to evaluate variability in *S. filifolia*. Microsatellite loci were amplified individually in 20 μL reactions containing 1–3 μL template DNA (*c*. 20–40 ng), 1× Go*Taq* 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, varying concentrations of $MgCl_2$ or $MgSO_4$, and 0.2 U Go*Taq* Flexi DNA polymerase (Promega). The reactions were carried out under the following thermal cycling 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 (H_O) and expected (H_E) heterozygosities for all loci using MSA version 4.05 (Dieringer & Schlotterer 2003), and tested for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using GENEPOP version 3.4 (Raymond & Rousset 1995). MICROCHECKER version 2.2.3 was used to infer the presence of null alleles with 1000 bootstrap replicates (Van Oosterhout *et al*. 2004).

Although all loci were found to be polymorphic, levels of observed genetic diversity were very low across the sampled individuals. No heterozygotes were observed at seven of the nine loci, resulting in highly significant deviations from HWE ($P < 0.01$); the other two loci (Sifi 210 and Sifi 264) had few heterozygotes, but deviations were not significant. Expected heterozygosity ranged from 0.010 to 0.236 (Table 1). Observed deviations from HWE are likely

driven by self-fertilization and small population size, leading to the fixation of alleles. The occurrence of null alleles could also result in a deficiency of observed heterozygotes, but this seems an unlikely general explanation because it would require the existence of seven null alleles for loci at which only 15 total alleles were observed. Tests for null alleles ($P < 0.01$) identified two loci (Sifi 162 and Sifi 264) that exhibited potential null alleles for one population each. Of the 36 interlocus comparisons, 10 pairs of loci exhibited significant ($P < 0.01$) genotypic linkage disequilibrium. The observed linkage disequilibrium is likely driven by fixed differences between one population and the other four populations at four of the loci (Sifi 187, Sifi 207, Sifi, 214, and Sifi 336). However, without additional information, physical linkage of loci cannot be distinguished from disequilibrium due to population processes such as nonrandom mating (Hedrick 2005).

The markers described in this study will be used to investigate the population structure, levels of genetic variability, and past demographic events for *S. filifolia*. Furthermore, these markers are intended for use throughout the genus *Sibara* (Brassicaceae), which is composed of 10 North American species (Al-Shehbaz 1988) including five narrowly endemic species of conservation concern.

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