Molecular Ecology Notes (2005) **5**, 246–248 doi: 10.1111/j.1471-8286.2005.00893.x

PRIMER NOTE

Isolation of polymorphic microsatellite loci in *Crossosoma californicum* (Crossosomataceae)

LISA E. WALLACE and KAIUS HELENURM
Department of Biology, University of South Dakota, Vermillion, SD 57069

Abstract

Crossosoma californicum **(Crossosomataceae) is a rare shrub species endemic to the California Channel Islands. Previous studies based on allozymes revealed little genetic variability in this plant species. We have isolated 13 polymorphic microsatellite loci from *C. californicum*. These loci show intermediate levels of variability, averaging 4.2 alleles per locus and expected heterozygosity of 0.376. Two loci did not fit Hardy–Weinberg expectations with significant deficits of heterozygous genotypes consistent with the presence of null alleles or population subdivision.**

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

Received 28 October 2004; revision accepted 2 December 2004

Crossosoma californicum Nutt. (Crossosomataceae) is a perennial shrub endemic to the southernmost California Channel Islands. On San Clemente Island, populations are numerous but each contains only one to seven individuals. By contrast, the six extant populations on Santa Catalina Island each contains 31–100 individuals. Because this is a rare, endemic species with limited distribution, it is expected to harbour little genetic variation and substantial structure (Hamrick & Godt 1989). Previous studies of allozyme diversity revealed little variation in *C. californicum* (K. Helenurm, unpublished). Nevertheless, the lack of detectable allozyme variation can result from factors other than the erosive effects of fragmentation and genetic drift, including a recent origin from an invariable ancestor. Additionally, greater levels of genetic variability are often observed with polymerase chain reaction-based (PCR-based) markers, which survey variation at the level of DNA rather than the expression of DNA sequences. Here, we report results of recent efforts to develop microsatellite loci in *C. californicum* to re-examine genetic variation and structure among populations and islands.

Genomic DNA was isolated from leaf tissue using a modification of the cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987), which included the addition of 4 L of RNase A (100 mg/mL; QIAGEN). The 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 $(CAC)_{10}$. Color-positive clones were screened for microsatellite regions using a 'dot blot' method (Glenn & Schable 2002) and the Phototope chemiluminescent detection system (New England Biolabs). Individual colonies were suspended in 100 L T.E. buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0), and 2 L of the colony solution was used to screen for a microsatellite region. A total of 793 positive clones was screened for insert size by PCR. The 25- L reaction contained 1 L template DNA, 0.8 M 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 M of each dNTP, and 0.2 units of Vent Exo$^-$ DNA polymerase (New England Biolabs). Denaturation was carried out at 96 C for 5 min followed by 30 cycles of 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. A single band of 500–1000 base pairs was amplified for 153 clones; these clones were subsequently 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

Correspondence: Lisa E. Wallace, Fax: 605-677-6557; E-mail: lwallace@usd.edu

Buffer (Gel Company) in 1/8th volume reactions. Sequences were electrophoresed on an Avant 3100 genetic analyser.

Of the 153 inserts that were sequenced, 76 contained a repeat region. However, only 30 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 PRIMER3 (http://frodo.wi.mit.edu/cgibin/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. Subsequently, 13 loci were chosen to assess genetic variability in *C. californicum*. Primers were labelled 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.

A sample of 36 individuals from Santa Catalina and San Clemente islands and the Palos Verdes Peninsula, California was used to evaluate variability for the 13 loci. Microsatellite loci were amplified in 25- L reactions containing 2 L template DNA (*c.* 50–60 ng), 1× ThermoPol Buffer, 200 M of each dNTPs, 1.6 M of each primer, 0.4 units Vent Exo⁻ DNA polymerase, and 2–6 mM $MgSO_4$ (Crca25.45, Crca28.46, Crca31.25, and Crca31.156 required 2 mM; Crca22.122, Crca25.21, Crca25.47, and Crca28.38 required 2.4 mM; Crca7.23 required 3 mM; Crca28.42 required 3.2 mM; Crca7.26, Crca28.5, and Crca28.26 required 6 mM). Locus Crca28.46 required the addition of 1× BSA for successful amplification. Most loci were amplified using a step-down protocol wherein the initial annealing temperature is at or above the T_a of the primers and is stepped down 1 C each cycle to an appropriate annealing temperature (Table 1). After a 5 min 95 C denaturation step, each step consisted of a single

Table 1 Primer sequences, reaction conditions, and diversity statistics for 13 microsatellite loci in *Crossosoma californicum*. Repeat motif refers to the allele of sequenced clones. 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 36 individuals from Santa Catalina Island, San Clemente Island, and the Palos Verdes Peninsula

Locus	Primer sequences (5′ to 3′)	Repeat motif	T_a (C)	Allele size range	N_A	H_O	H_E	GenBank Accession no.
Crca7.23	F-TCTTTGGTAGGGGTGAGTGG* R-CCTGCTACCCTGAAGTTCTCC	$(AG)_{10}$	59	203–221	6	0.472	0.481	AY825270
Crca7.26	F-GCTCTGGTTCCATCACAAGC R-CCCCCACTCTCTCACTAGTCACC*	$(GT)_{11}$	60	204–210	4	0.583	0.624	AY825271
Crca22.122	F-GAGAGATTCCGATTGAGATTAGC R-TCACACATCTATCAACAACATGC*	$(GT)_{13}$	56–53†	175–201	7	0.694	0.664	AY825272
Crca25.21	F-ACTCGCCATAGTGTTTGACC* R-GCATATGCTTGGAGTGGTTGC	$(AC)_2(ACC)_6(AAC)_2$	56–53†	234–252	2	0	0.054	AY825273
Crca25.45	F-GGTGGTCACCCTAACAAC R-TCCCTGGAAAACTTGAATTG*	$(CCA)_7$	57	152–164	3	0.222	0.222	AY825274
Crca25.47	F-CGTCGGTAATGTTAGGGCTG* R-GGAAGCTCTAATCGCCTATGG	$(GGT)_8(GCC)_3(GGT)_3$	56–53†	146–168	3	0.083	0.081	AY825275
Crca28.5	F-AGAGAACAAGTAGAGCAGATGACG* R-CCATGATTATGAATCCTCTTTGC	$(AT)_{18}(AG)_{12}$	58	198–202	2	0.333	0.318	AY825276
Crca28.26	F-TCCATACTGATCCCAAGTTGC* R-GATCTTCAGCTCCATACATCAGG	$(AG)_{17}$	55–51†	239–297	8	0.333	0.684#	AY825277
Crca28.38	F-TCAATACCACTCGCGTTCAC* R-CCAACCTGCTCATTCCAAAG	$(CTT)_8(GTT)_5$	56–52†	194–209	4	0.250	0.315	AY825278
Crca28.42	F-TTTGTGCTTCTCTCTTTTGTGTC* R-TTGTGATAATCAGGCATGCAGC	$(AAG)_8$	57–50†	233–248	3	0.139	0.155	AY825279
Crca28.46	F-AATTTTTGCAGCCTTTTCTCC* R-GTACACAATTTTAGCAAACAAGC	$(CCCAAG)_3(AT)_8$	54–50†	241–245	3	0.056	0.326#	AY825280
Crca31.25	F-GGAACCAAGAATGGCGTAGC R-TGATGAACTCCTACGCTCTCG*	$(ATG)_{10}(AGG)_7$	57–54†	210–225	5	0.694	0.681	AY825281
Crca31.156	F-AATGTATCTCGTTGGTGTTCG* R-CGAGACAAATGGCACATCC	$(GGT)_{12}$	55–51†	139–169	5	0.250	0.294	AY825282
Mean					4.2	0.316	0.376.6.	

†Indicates the use of a step-down thermal cycler protocol; the upper and lower temperatures are indicated. See text for additional details.
#Indicates a significant departure from Hardy–Weinberg equilibrium.

cycle of 1 min at 95 C, 1 min at the appropriate annealing temperature, and 1 min at 72 C; at the end of the step cycles, 30 cycles of 1 min at 95 C, 1 min at the appropriate annealing temperature, and 1 min at 72 C were performed, followed by a final elongation at 72 C for 30 min. All reactions were run in an MJ Research PTC 200 thermal cycler.

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 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).

Allele frequencies and heterozygosities for each locus were calculated in POPGENE 1.32 (Yeh & Boyle 1997). All loci were tested for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using FSTAT version 2.9.3.2 (Goudet 1995) and applying a Bonferroni correction to assess significance levels. Moderate levels of genetic variability were observed at these microsatellite loci. Among the 36 individuals examined, the number of alleles per locus ranged from two to eight with an average of 4.2 alleles (Table 1). Expected heterozygosity ranged from a low of 0.054 for locus Crca25.21 to a high of 0.684 for locus Crca28.26. Two loci, Crca28.26 and Crca28.46, exhibited significant deviations from HWE ($P < 0.05$), and specifically a deficit of heterozygotes, which is consistent with the presence of null alleles or population subdivision. There was no evidence of linkage among any of the loci. Collectively, these microsatellite loci showed variation where little had been found at allozyme loci. Thus, they will be useful for assessing genetic structure and breeding system in *C. californicum*.

Acknowledgements

This research was funded by the Natural Resources Office, Staff Civil Engineer, Naval Air Station, North Island, San Diego, California. LEW was supported by the National Science Foundation/EPSCoR Grant no. 0091948 and by the State of South Dakota. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. We thank Matthew Hamilton and Travis Glenn for technical assistance.

References

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small amounts of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11–15.

Glenn TC, Schable M (2002) *Microsatellite Isolation with Dynabeads 2002*. Unpublished manual. Savannah River Ecology Laboratory, Aiken, South Carolina.

Goudet J (1995) FSTAT (version. 1.2): a computer program to calculate *F*-statistics. *Journal of Heredity*, **86**, 485–486.

Hamilton MB, Pincus EL, DiFiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.

Hamrick JL, Godt MJW (1989) Allozyme diversity in plant species. In: *Plant Population Genetics, Breeding, and Genetic Resources* (eds Brown AHD, Clegg MT, Kahler AL, Weir BS), pp. 43–63. Sinauer, Sunderland, Massachusetts.

Yeh FC, Boyle TJB (1997) Population genetic analyses of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany*, **129**, 157.