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Source: Applications in Plant Sciences, 1(5)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1200393

Applications in Plant Sciences 2013 1(5): 1200393

PRIMER NOTE

ISOLATION OF MICROSATELLITE MARKERS IN A CHAPARRAL SPECIES ENDEMIC TO SOUTHERN CALIFORNIA, *CEANOTHUS MEGACARPUS* (RHAMNACEAE)[1]

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- *Premise of the study:* Microsatellite (simple sequence repeat [SSR]) markers were developed for *Ceanothus megacarpus*, a chaparral species endemic to coastal southern California, to investigate potential processes (e.g., fragmentation, genetic drift, and interspecific hybridization) responsible for the genetic structure within and among populations distributed throughout mainland and island populations.
- *Methods and Results:* Four SSR-enriched libraries were used to develop and optimize 10 primer sets of microsatellite loci containing either di-, tri-, or tetranucleotide repeats. Levels of variation at these loci were assessed for two populations of *C. megacarpus*. Observed heterozygosity ranged from 0.250 to 0.885, and number of alleles ranged between four and 21 per locus. Eight to nine loci also successfully amplified in three other species of *Ceanothus*.
- *Conclusions:* These markers should prove useful for evaluating the influence of recent and historical processes on genetic variation in *C. megacarpus* and related species.

Key words: *Ceanothus*; chaparral; microsatellites; Rhamnaceae.

Ceanothus megacarpus Nutt. (Rhamnaceae) is a diploid perennial shrub endemic to both coastal regions of the southern California Floristic Province and multiple Channel Islands off the coast of California (Fross and Wilken, 2006). This species has experienced considerable range fragmentation as a result of several factors including urbanization, increased fire frequency, and episodic droughts (Schlesinger and Gill, 1978; Witter et al., 2007). As a nonsprouting species of chaparral shrub, *C. megacarpus* relies on the seedbank for recovery from fire, which initiates seed germination. Nevertheless, this species tends to disappear in areas of high fire frequency (Witter et al., 2007), such as seen in the Santa Monica Mountains, thus creating a mosaic of fragmented stands of *C. megacarpus* that differ in both age and density. Such fragmentation can have potential genetic consequences, including loss of genetic variation within fragmented stands and a decrease in gene flow between isolated fragments (Young et al., 1996). Interspecific hybridization is another potential factor that can influence patterns of genetic variation within species of *Ceanothus* L. (California lilac). Although introgressive hybridization between species within each subgenus (*Cerastes* and *Ceanothus*) has been documented, it is presumably rare between members of different subgenera (McMinn, 1942; Nobs, 1963).

Most genetic studies of *Ceanothus* have focused on phylogenetic relationships among species (Hardig et al., 2002; Burge et al., 2011), whereas little is known about genetic variation within and among species. Implementation of detailed genetic studies related to the effects of fragmentation and interspecific hybridization requires genetic markers variable enough to examine patterns of variation at the level of populations. Therefore, we developed a panel of microsatellite markers for *C. megacarpus* that is useful for detailed population studies and comparisons between species. This panel should prove useful for studies of some of the more dominant members of the chaparral shrub communities in California.

METHODS AND RESULTS

Tissue was collected from a single individual of *C. megacarpus* (34°02.395′N, 118°42.072′W) and sent to Genetic Identification Services (GIS; Chatsworth, California, USA). Genomic DNA was extracted, and four CA-, AAC-, ATG-, and TAGA-enriched libraries were developed using *E. coli* cells (strain DH5α) with the recombinant plasmid pUC19. Positive clones (n = 144) with inserts between 350 and 700 bp were sequenced, and 86 contained microsatellite loci. The criteria used to select clones for primer design included: (1) Microsatellite motifs were required to have enough flanking sequence for primer design. Clones containing microsatellite motifs near the ends of the sequence were excluded. (2) Flanking sequences had to meet standard design criteria. These included primer length (min = 18 bp, max = 22 bp), melting temperature

[1]Manuscript received 26 July 2012; revision accepted 26 September 2012.

This research was supported by grants from the National Science Foundation (DBI-1062721), the U.S. National Parks Service, and a Dean's Research grant from Pepperdine University. We thank Mark Todd (GIS) for microsatellite library development, as well as Daphne Green and many undergraduates at Pepperdine University for assistance in the field and laboratory.

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doi:10.3732/apps.1200393

TABLE 1. Characterization of 10 nuclear microsatellite primers developed in *Ceanothus megacarpus*.[a]

Locus	GenBank accession no.	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[b]	Label[c]
CmegA4	JX415516	F: TGGGTCAAGGTCCTCATAA R: ACAAACATCTGGGTTCATAGTG	$(GT)_{17}$	285–355	6-FAM
CmegA9	JX415517	F: GAGAGGGAAGGGGCATAC R: TCCAAAGCCTGAATGTGTC	$(GT)_{9}$	261–269	HEX
CmegA110	JX415518	F: TCCAAACACCAATATCTCC R: GTGCCAAGTGTAAGGTTG	$(CA)_{12}$	326–338	6-FAM
CmegA125	JX524558	F: AGAACCCCTCTGGTGGAGAT R: TGATAGTGAACCCCTTTTGTCA	$(CT)_4(CA)_5(CG)(CA)_{16}$	166, 175–237[d]	HEX
CmegB8	JX415519	F: AACGAGACCTTGAGGGATTG R: ACGACCCGCTAACAGTGAGT	$(CTT)_2(GTT)_9$	134–218	HEX
CmegB126	JX415520	F: CCTCAGCCACTCCAAGAAC R: TCCACCAAAATAAAATCTCCAC	$(GTT)_8$	231–249	HEX
CmegC11	JX415521	F: GTCGCTGTGATTCCTCTG R: TTACGAACCACGCAAACT	$(GTT)_{11}$	186–201	HEX
CmegC107	JX415522	F: TTTCCACAACCACAATGTCC R: CCCTGAACTAGCCACTGACC	$(CAT)_8$	141–198	HEX
CmegC121	JX415523	F: AATGGCTCTGTTTCCCAA R: CATCACTTCCTCTTCTTCC	$(GAT)_{14}$	197–242	6-FAM
CmegD3	JX415524	F: CACCTTGAGGTTTGAACTTGAT R: CGATTCTTGGGATCATAGTTT	$(CTAT)_{15}$	180–232	6-FAM

[a]All primers were run at an annealing temperature of 57.0°C with the exception of CmegA110, which was run at 55.5°C.
[b]Based on a pooled sample of 52 individuals from two populations located in the Santa Monica Mountains of southern California.
[c]Fluorescent tags used to label the 5′ ends of the forward primers.
[d]Allele 166 represents a polymorphism resulting from an indel involving one rather than two bases.

(min = 55°C, max = 60°C), % GC (min = 35%, max = 65%), and PCR product length (min = 100 bp, max = 300 bp). Both DesignerPCR version 1.03 (Research Genetics, Huntsville, Alabama, USA) and Primer3Plus (Untergasser et al., 2007) were used to design primers for 20 unique loci, and these loci were initially screened across 20 individuals. A final panel of 10 microsatellite loci was optimized for detailed genetic analysis (Table 1). These loci were selected based on the following criteria: (1) demonstration of polymorphism; (2) production of fragment patterns allowing for accurate allele calling; and (3) consistent PCR amplification. Genetic variation was assessed for two populations (34°04.888′N, 118°45.513′W; and 34°04.856′N, 118°45.959′W; see Appendix 1) located in the Santa Monica Mountains (Malibu, California, USA) (n = 27, 25).

Microsatellite loci were amplified using a BIOLASE PCR Kit (Bioline, Boston, Massachusetts, USA). Reactions were performed in 10-μL volumes containing the following: 0.3 μL labeled forward primer (FAM or HEX) (6 pM), 0.3 μL reverse primer (6 pM), 1 μL PCR buffer (10×), 0.8 μL dNTPs (2.5 mM each), 0.4 μL $MgCl_2$ (50 mM), 3 μL Polymate Additive (3×), 3.15 μL H_2O, 0.05 μL BIOLASE polymerase (0.25 U), and 1 μL template DNA (20–50 ng). PCR was performed in a Bio-Rad MyCycler (Bio-Rad Laboratories, Hercules, California, USA) with the following conditions: (1) 94°C for 1 min; (2) 35 cycles of 94°C for 40 s, 55.5°C or 57°C for 40 s, 72°C for 30 s; and (3) 72°C for 4 min. Genotyping reactions contained 8.8 μL of Hi-Di Formamide (Applied Biosystems, Carlsbad, California, USA) and 0.2 μL of GeneScan 400HD Rox standard (Applied Biosystems). An ABI 3130 genetic analyzer (Applied Biosystems) was used for fragment analysis, and allele sizes were determined using the software GeneMapper version 3.7 (Applied Biosystems). Potential scoring errors resulting from null alleles and allele dropout were evaluated with MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004).

GenAlEx version 6.4 (Peakall and Smouse, 2006) was used to calculate overall number of alleles per locus and heterozygosity and to test for Hardy–Weinberg equilibrium (HWE; Table 2). For both populations combined, the number of alleles per locus ranged between four and 21 with a mean across all loci of 11.8. Observed heterozygosity ranged from 0.250 to 0.885 (mean: 0.6273), while expected heterozygosity ranged from 0.274 to 0.889 (mean: 0.7224). Tests for HWE were performed separately for both populations of *C. megacarpus* (Table 2). CmegA125 and CmegB126 revealed an excess of homozygotes, and according to MICRO-CHECKER, a potential explanation was the presence of null alleles. Therefore, the Oosterhout correction algorithm (Van Oosterhout et al., 2004) was used to adjust genotypes for the presence of null alleles, and corrected values of heterozygosity were obtained (Table 2). Although significant deviation from HWE was not observed for CmegC121, MICRO-CHECKER did suggest the presence of null alleles in population 1. Deviations from HWE for CmegA125, CmegB126, and Cmeg121 were also observed when both populations were combined, but this is likely the result of these two populations differing in the alleles present at individual loci.

Although no detailed analysis was conducted, we did test to see if these microsatellite markers could prove useful for genetics studies of other species in the genus *Ceanothus* (Table 2). These species were selected based on their contrasting distribution patterns (Appendix 1): (1) one similar to *C. megacarpus* (*C. crassifolius* Torr.); (2) a more cosmopolitan species, *C. cuneatus* (Hook.) Nutt.; and (3) *C. arboreus* Greene, a species restricted to California islands. *Ceanothus arboreus* occurs in the subgenus *Ceanothus*, whereas all other species are in the subgenus *Cerastes*. Eight of the 10 loci consistently amplified across these species, and CmegA110 also amplified in *C. arboreus*. Although no data are shown, a preliminary study suggests that most of these loci, with the exception of CmegB8 and CmegB107, can be amplified for *C. ferrisiae* McMinn, a species occurring in serpentine soils. Given the low level of phylogenetic divergence within each subgenus (Burge et al., 2011), these markers should prove useful for detailed genetic studies of many species in both subgenera.

CONCLUSIONS

We developed 10 microsatellite markers for the native California chaparral shrub, *C. megacarpus*, and have demonstrated that these loci are polymorphic across two populations. These markers are currently being used to investigate population structure, patterns of gene flow, and fragmentation of *C. megacarpus* in the Santa Monica Mountains of southern California. In addition, we confirmed cross-amplification of these microsatellite loci in additional species from both subgenera. Therefore, these markers will be highly advantageous for performing comparative studies on genetic diversity in *Ceanothus* with the potential application to more than 50 species from Baja California, California, and Oregon.

TABLE 2. Patterns of variation observed across the 10 loci (CmegA4 to CmegD3) for *Ceanothus megacarpus*, *C. cuneatus*, *C. crassifolius*, and *C. arboreus*.[a]

	C. megacarpus (Pop. 1, $n = 27$)			*C. megacarpus* (Pop. 2, $n = 25$)			*C. megacarpus* (Pop. 1 and 2)			*C. cuneatus* ($n = 10$)		*C. crassifolius* ($n = 10$)		*C. arboreus* ($n = 10$)	
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	Size range (bp)	*A*	Size range (bp)	*A*	Size range (bp)	*A*
CmegA4	16	0.852	0.877	11	0.800	0.820	21	0.827	0.871	285–299	6	276–300	7	277–295	5
CmegA9	5	0.407	0.423	3	0.080	0.078	5	0.250	0.274	259–267	4	255–265	4	263–265	2
CmegA110	4	0.519	0.457	3	0.480	0.519	4	0.500	0.491	—	—	—	—	328–340	4
CmegA125	14	0.654 (0.769)	0.876*** (0.897)	16	0.920	0.901**	21	0.784	0.923***	—	—	—	—	—	—
CmegB8	12	0.815	0.883	14	0.880	0.853	16	0.846	0.889	140–176	6	140–179	10	134–158	5
CmegB126	7	0.481 (0.852)	0.807* (0.867)	5	0.292 (0.833)	0.767*** (0.806)	7	0.392	0.796***	174–325	11	231–339	9	231–276	3
CmegC11	5	0.481	0.457	4	0.480	0.554	6	0.481	0.512*	192–201	4	189–201	4	195	1
CmegC107	9	0.630	0.748	10	0.680	0.673	13	0.654	0.731	141–153	3	150–156	3	144–171	6
CmegC121	7	0.556	0.816	5	0.760	0.862	12	0.654	0.863***	203–224	6	196–221	6	197–239	10
CmegD3	13	0.926	0.870	10	0.840	0.853	13	0.885	0.874	181–197	3	177–229	8	188	1

Note: A = number of alleles; H_e = mean expected heterozygosity; H_o = mean observed heterozygosity; n = sample size.

[a]Values in parentheses represent observed and expected heterozygosities obtained after genotypes were adjusted for potential null alleles based on the Oosterhout correction algorithm (Van Oosterhout et al., 2004). Asterisks (*) denote loci that were not in Hardy–Weinberg equilibrium: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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APPENDIX 1. Voucher data for examined specimens of *Ceanothus*. The individual chaparral shrubs were marked with a metal tag, and stem, bud, and leaf samples were removed and are being maintained in a −70°C freezer. Each voucher specimen was given a separate number, and a GPS coordinate was recorded. All vouchers are deposited at Pepperdine University.

C. megacarpus: Cm6-152–178, Santa Monica Mountains, Los Angeles Co., Malibu, CA, USA (*n* = 27; 34°04.888′N, 118°45.513′W). *C. megacarpus*: Cm7-101–125, Santa Monica Mountains, Los Angeles Co., Malibu, CA, USA (*n* = 25; 34°04.856′N, 118°45.959′W). *C. cuneatus*: Ccu10-75–78, Ccu10-81–86, Santa Monica Mountains, Los Angeles Co., Malibu, CA, USA (*n* = 10; 34°05.949′N, 118°44.564′W). *C. crassifolius*: Ccr7-126–135, Santa Monica Mountains, Los Angeles Co., Malibu, CA, USA (*n* = 10, 34°04.856′N, 118°45.959′W). *C. arboreus*: Ca21–30, Santa Catalina Island, Los Angeles Co., CA, USA (*n* = 10; 33°23.295′N, 118°24.246′W).