American Journal of Botany

American Journal of Botany: e000–e000. 2012.

AJB Primer Notes & Protocols in the Plant Sciences

Microsatellite markers from *Ceanothus roderickii* (Rhamnaceae) using next-generation sequencing technology[1]

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- *Premise of the study: Ceanothus roderickii* is an endangered shrub endemic to California. To investigate the population genetics of this species, including the genetic consequences of population fragmentation and hybridization, 10 microsatellite markers were developed.
- *Methods and Results:* Using next-generation sequencing (454) data from a single *C. roderickii* individual, 10 microsatellite markers were developed. A group of 12 individuals representing all of the major *C. roderickii* populations were analyzed. All loci were found to be polymorphic, with a range from two to 12 alleles per locus. Observed heterozygosity ranged from 0.08 to 0.83 across loci. All 10 loci were also amplifiable in at least one other *Ceanothus* species.
- *Conclusions:* Results presented here indicate the utility of our new microsatellite primers in ongoing and future studies concerning population genetics and gene flow in *C. roderickii*, as well as the potential applicability of these primers in similar studies on other *Ceanothus* species.

Key words: *Ceanothus*; endangered; gabbro; GS FLX (454); microsatellite; Rhamnaceae.

Ceanothus roderickii W. Knight (Rhamnaceae) is a diploid shrub species endemic to the Sierra Nevada foothills of western El Dorado County, California, where it occurs exclusively on nutrient-poor soils derived from the mafic rock gabbro (Burge and Manos, 2011; Fig. 1). *Ceanothus roderickii* is a pioneer species of the chaparral that depends upon fire for effective recruitment from seeds, but reproduces clonally via root-layering during fire-free intervals (Boyd, 2007). Specialized ecology, limited geographic range, and threats from urban development led to the listing of this species as Endangered at both the U.S. Federal and California State level (USFWS, 2002). To provide for the perpetuation of this species, the United States Fish and Wildlife Service Recovery Plan for Gabbro Soil Plants of the Central Sierra Nevada Foothills (USFWS, 2002) calls for a program to maintain the genetic diversity of *C. roderickii*. Therefore, basic knowledge is needed about the genetic diversity of this species, gene flow among populations, and gene flow between *C. roderickii* and other species of *Ceanothus*. To this end, we developed and present here microsatellite markers for population genetic research on *C. roderickii*.

[1]Manuscript received 3 September 2011; revision accepted 6 December 2011.

We would like to thank Lisa Bukovnic and the staff of the Duke Institute for Genome Sciences and Policy (IGSP) Sequencing Core Facility for their assistance. This research was supported by grants from the Bureau of Land Management Pine Hill Preserve and the California Department of Fish and Game to D.O.B., and a grant from the Lawrence R. Heckard Endowment Fund of the Jepson Herbarium to S.W.K.

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doi:10.3732/ajb.1100431

METHODS AND RESULTS

Total genomic DNA of one *C. roderickii* individual (*D. O. Burge 1288-S2*, Appendix 1) was extracted from leaf tissue as in Burge and Manos (2011). DNA was shotgun sequenced on the Roche (454) GS FLX at the Duke Institute for Genome Sciences and Policy Sequencing Core Facility using GS FLX Titanium reagents. One-eighth of a plate generated 79 382 reads; average read length was 334 bases, with a median at 385; a total of 26.4 Mb of sequence data were generated. Raw reads were screened for di-, tri-, tetra-, penta-, and hexanucleotide repeats using the program MSATCOMMANDER (Faircloth, 2008), with minimum repeats set to 10, 7, 6, 5, and 4, respectively. All other parameters were kept at their default values. Primers were designed using the MSATCOMMANDER workflow, which includes Primer3 (Rozen and Skaletsky, 2000). A total of 173 primer pairs were identified. We culled those primers that amplified overly long, overly short, or compound-interrupted simple sequence repeat (SSR) stretches. We also eliminated primers that were located very close to the vector, or that were based on a sequence represented by another, superior pair of primers. From the remaining primer pairs, a total of 48 were selected and ordered from Eurofins MWG Operon (Huntsville, Alabama). Fluorescent labeling was done according to Schuelke (2000). Four individuals representing two separate populations of *C. roderickii* (Fig. 1) were prescreened for the quality of amplified SSR loci (*D. O. Burge 1288-S2*, *1288-S3*, *1090-S1*, and *1090-S3*; Table 1). Total DNA for these and all other amplified plants was extracted as described above. PCR was performed as in Csencsics et al. (2010) using a Perkin Elmer GeneAmp thermocycler (Waltham, Massachusetts, USA). Amplification products were separated by capillary electrophoresis at the Georgia Genomics Facility (GGF), University of Georgia (Athens, Georgia), using an Applied Biosystems AB 3170 automatic sequencer (Applied Biosystems, Carlsbad, California, USA). Alleles were sized using a ROX-labeled size standard manufactured by GGF according to DeWoody et al. (2004) and the program GeneMarker 1.95 (SoftGenetics LLC, State College, Pennsylvania, USA). Based on the prescreening, 10 SSR loci were found to be heterozygous in at least one individual and have easily scorable bands that were polymorphic across individuals. The other 38 loci were eliminated because they amplified poorly, had pronounced stutter, or yielded monomorphic or multibanding patterns.

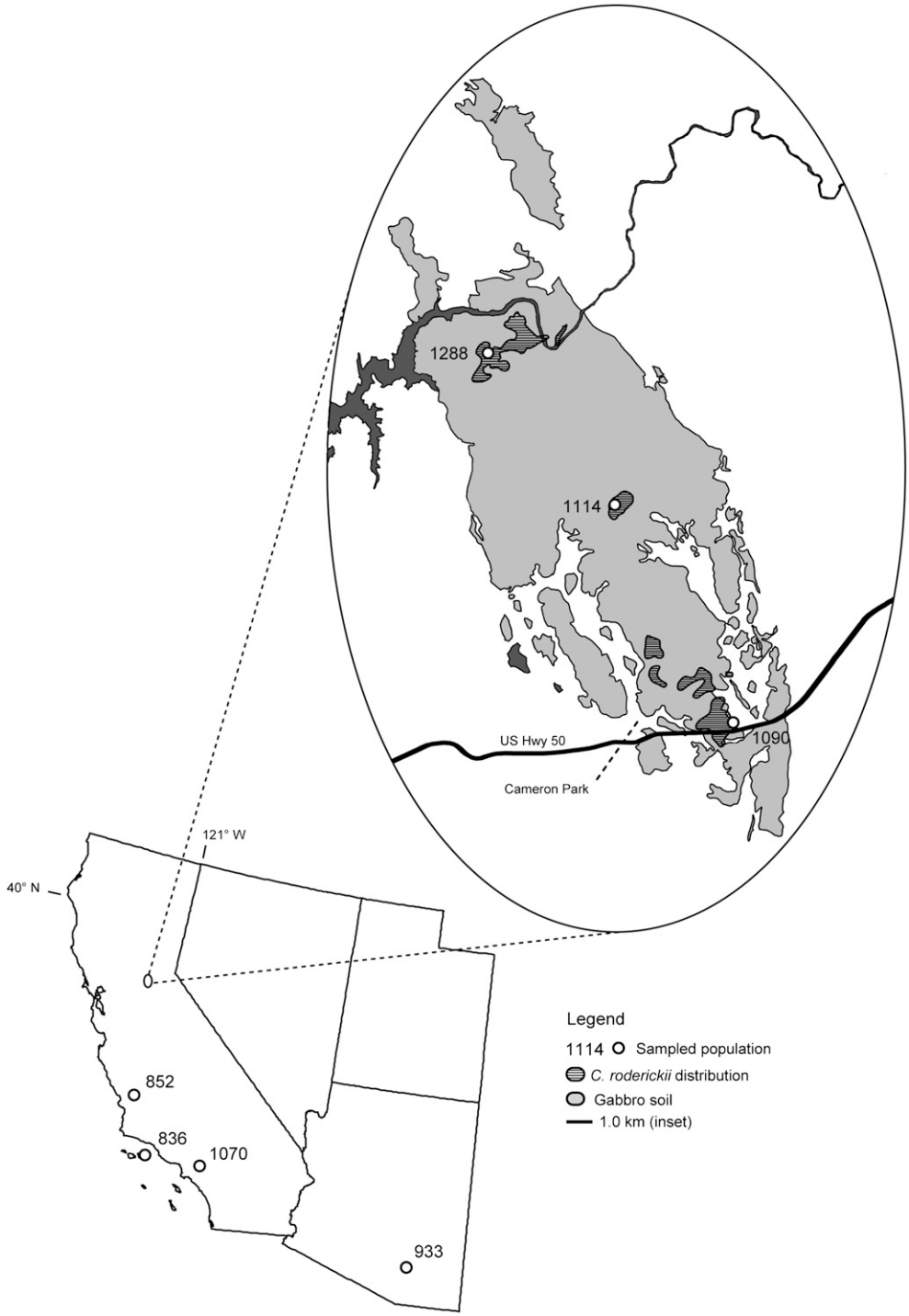

Fig. 1. Sampling of *Ceanothus* in western North America. Background map of western North America shows sampling of species other than *C. roderickii* (*C. cuneatus*, *D. O. Burge 1070*; *C. foliosus*, *D. O. Burge 852*; *C. fendleri*, *D. O. Burge 933*; *C. megacarpus*, *D. O. Burge 836*; see also Appendix 2). Inset map shows localities where individuals of *C. roderickii* were collected from the Pine Hill Region, El Dorado County, California (*D. O. Burge 1090*, *D. O. Burge 1114*, and *D. O. Burge 1288*, n = 4 in all cases). Inset map also shows distribution of gabbro-derived soil type, an important indicator of *C. roderickii* distribution in western El Dorado County (Burge and Manos, 2011).

To characterize the 10 confirmed loci, 12 individuals of *C. roderickii* were sampled from the same two populations used in the prescreening, supplemented with one additional population (Table 1). Together, these samples represent all three of the known population centers for *C. roderickii* (Fig. 1). Loci were amplified and scored as described above. Using all loci providing clear electropherograms, we calculated the overall number of alleles and observed heterozygosity by hand. We found between two and 12 alleles per locus (mean: 6.6); observed heterozygosity ranged from 0.08 to 0.83 (mean: 0.50; Table 1). The high number of alleles detected at most loci should provide sufficient power for analyses of population genetic diversity and structure.

TABLE 1. Characterization of new microsatellite primers in *Ceanothus roderickii*.

Locus	GenBank accession no.	Primer sequences (5′–3′)	T_m (°C)	Repeat motif	Individuals[a]	Allele size range (bp)	No. of alleles	H_o
CR3	JN621796	F: AGTTTGCAGAGCTTGATCTTAAC	58.1	AT	8	208–226	5	0.50
		R: TCTCCAGGATGAAGGTAAGTG	57.2					
CR5	JN621797	F: CCTGCCCAGGAAGTGAGG	60.1	AG	11	233–249	6	0.82
		R: ATGTTAGACCACGTGTAGGG	57.4					
CR8	JN621798	F: CAATTTGTTTGAAGGACCGTTG	57.7	AT	10	297–317	8	0.30
		R: ACCAAACGGACATGCCTTC	59.1					
CR14	JN621800	F: TTTCAAGGCGGCGACAAAG	60.1	GATGGT	12	195–201	2	0.08
		R: TCTTGGCTGGCCACTCTAC	59.8					
CR25	JN621801	F: AGGGAGTTTGAGGGCATGG	60.1	ATTT	10	183–199	6	0.70
		R: ATGAAGTACGAGCATTTCCAC	57.1					
CR27	JN621802	F: CAATGGCGCCGATGACAG	60.0	CTT	12	322–356	8	0.75
		R: AGTTACCTGGTGAAGCGGG	60.1					
CR33	JN621803	F: AAGGTGACCGAACCATTGC	59.1	GAT	12	163–245	12	0.83
		R: CATGGCAAGCAGTATGGGC	60.0					
CR41	JN621804	F: TGCGGGTCCAATACAAAGTG	59.2	CTT	12	251–258	3	0.17
		R: TGACCTTGCACCACCCAAG	60.9					
CR46	JN621805	F: AACCGAGGACCCAACTGAC	60.0	AGG	11	166–199	7	0.45
		R: CCGTCTGCTTACCAACAGC	59.6					
CR48	JN621806	F: GACTCCTAAAGGATCGCAACC	59.2	ATC	12	163–203	9	0.42
		R: AAGCCGCAAACTCATGGTG	59.8					

Note: H_o = observed heterozygosity across all genotyped individuals; T_m = melting temperature.

[a] "Individuals" indicates the number of individuals that were successfully genotyped (out of 12, including four plants from each of three populations: *D. O. Burge 1090*, *D. O. Burge 1114*, and *D. O. Burge 1288*; Appendix 1; Fig. 1; all voucher specimens deposited at DUKE).

In addition to the characterization of SSR loci in *C. roderickii*, we also attempted cross-amplification in four other species of *Ceanothus* representing both *Ceanothus* subgenera (*Ceanothus* subgen. *Cerastes*: *C. cuneatus* Nutt. and *C. megacarpus* Nutt.; *Ceanothus* subgen. *Ceanothus*: *C. foliosus* Parry and *C. fendleri* A. Gray; Fig. 1, Appendix 2). The two subgenera of *Ceanothus* represent recent and rapid (~5 Ma) diversifications that have taken place independently, but simultaneously, in North America, particularly within the California Floristic Province (Burge et al., 2011). Because of the low level of phylogenetic divergence within each subgenus (Burge et al., 2011), we thought it probable that many of the same SSR loci identified in *C. roderickii* would be shared with other species of *Ceanothus*. Individuals from the four additional *Ceanothus* species were genotyped as described above; results show that all 10 of the primer pairs developed here are effective in at least one of these species (Appendix 2).

CONCLUSIONS

Using data from next-generation sequencing technology, we developed 10 microsatellite markers for the shrub *C. roderickii*. We demonstrated that these loci are highly polymorphic in a sample of 12 individuals representing all three of the major *C. roderickii* population centers (Fig. 1). In addition to verifying their utility in *C. roderickii*, we confirmed that each of these loci cross-amplifies in at least one other species of *Ceanothus*. Primers for SSR loci developed here will be used for ongoing studies on *C. roderickii* to determine how population fragmentation affects genetic diversity, and how substrate conditions influence gene flow between *C. roderickii* and its close relative *C. cuneatus*. This latter question is of particular interest given recent results from a combination of population genetic (amplified fragment length polymorphism) and experimental growth trials, which indicate that gene flow between these two species is influenced by substrate conditions (unpublished data). This finding suggests that disturbance of soil by human activities has the potential to increase gene flow between these species, possibly leading to genetic swamping of the endangered *C. roderickii*. Our results also demonstrate that the microsatellite loci developed for *C. roderickii* work in other *Ceanothus* species. Thus, the markers presented here will probably have great value in ongoing and future studies on genetic diversity and gene flow in *Ceanothus*, a diverse and ecologically dominant North American plant genus (Fross and Wilken, 2006).

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APPENDIX 1. *Ceanothus* voucher data. Information is alphabetical by species, with collection number and locality description followed by number of individuals genotyped and geographic coordinates (NAD84). All vouchers are deposited at DUKE.

C. cuneatus—D. O. Burge 1070a, Rialto Municipal Airport, San Bernardino Co., CA [n = 1; 34°08.022′N, 117°24.639′W]. *C. foliosus—D. O. Burge 852a*, Cuesta Ridge, San Luis Obispo Co., CA [n = 1; 35°22.552′N, 120°41.360′W]. *C. fendleri—D. O. Burge 933a*, Santa Catalina Mountains, Pima Co., AZ [n = 1; 32°24.357′N, 110°42.202′W]. *C. megacarpus—D. O. Burge 836b*, Santa Cruz Island, Santa Barbara Co., CA [n = 1; 33°59.680′N, 119°44.967′W]. *C. roderickii—D. O. Burge 1090*, Cameron Park, El Dorado Co., CA [n = 4; 38°39.621′N, 120°57.108′W]; *D. O. Burge 1114*, Pine Hill, El Dorado Co., CA [n = 4; 38°43.148′N, 120°59.488′W]; *D. O. Burge 1288*, South Fork American River Watershed, El Dorado Co., CA [n = 4; 38°45.560′N, 121°01.879′W].

APPENDIX 2. Performance of microsatellite primers in other *Ceanothus* species.[a,b]

Locus	*C. megacarpus* (*D. O. Burge 836*)	*C. foliosus* (*D. O. Burge 852*)	*C. fendleri* (*D. O. Burge 933*)	*C. cuneatus* (*D. O. Burge 1070*)
CR3	—	206, 212	—	216
CR5	241	—	247	241
CR8	303	—	—	295
CR14	—	365	365	195
CR25	187	191, 206	179	185, 197
CR27	325, 337	352, 358	328	325, 331
CR33	—	215	215	—
CR41	255	—	—	—
CR46	193, 196	184	187	187, 190
CR48	163, 169	175	172, 175	200, 206

[a] Number(s) indicate the size of the fragment(s) (in base pairs) amplified by each of the 10 primer pairs; — indicates that the primers did not amplify a fragment, or the fragment was not scorable (pronounced stutter or no amplification).

[b] All specimen vouchers deposited at DUKE (see Appendix 1).