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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *CANAVALIA CATHARTICA* AND *C. LINEATA* (FABACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for the sea-dispersed legume *Canavalia cathartica* to investigate its genetic diversity and population structure. We also applied these microsatellite markers to the closely related species *C. lineata*.
- *Methods and Results:* Ten primer sets were developed for *C. cathartica*, and all of the primers were amplified in *C. lineata*. The number of alleles per locus ranged from two to 13 and one to 10 for *C. cathartica* and *C. lineata*, respectively. The expected heterozygosity ranged from 0.375 to 0.870 and from 0.071 to 0.877 for *C. cathartica* and *C. lineata*, respectively.
- *Conclusions:* The 10 microsatellite markers developed in this study can be used to analyze the population genetic structure of *C. cathartica* and other related taxa.

Key words: *Canavalia cathartica*; *Canavalia lineata*; Fabaceae; microsatellite; polymorphism.

Coastal plants often exhibit continuous distribution and are widely distributed. Coastal plant populations have been affected not only by Quaternary climatic oscillations but also by modern sea currents (Westberg and Kadereit, 2009), and they provide an opportunity to study various aspects of the phylogeography of flowering plants. However, except for mangrove species (e.g., Dodd et al., 2002), relatively little information is available regarding the patterns, levels of gene flow, and population genetic structure among coastal plants (e.g., Kadereit et al., 2005; Takayama et al., 2008; Westberg and Kadereit, 2009). *Canavalia cathartica* Thouars occurs in coastal regions from South to Southeast Asia and in East Africa (Sauer, 1964), and its seeds are dispersed by sea currents. The development of highly polymorphic microsatellite markers will provide valuable insight into the population genetic structure and dispersal mechanisms of sea-dispersed plants. In this study, we report the isolation and characterization of 10 microsatellite markers for *C. cathartica* and their applicability to the closely related sea-dispersed species *C. lineata* (Thunb.) DC.

METHODS AND RESULTS

Genomic DNA was extracted from leaf samples collected at Takana, Iriomote Island, Japan (see Appendix 1 for voucher information) using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommended protocol. An enriched genomic DNA library was constructed as described by Bloor et al. (2001), with slight modifications. The genomic DNA was digested with three restriction enzymes, *Eco*RVI, *Alu*I, and *Ssp*I (Toyobo Co., Osaka, Japan), and the resulting fragments, ranging from 0.4 to 1 kb, were excised from 1% low-melting-point agarose gels and purified. The fragments were then ligated into a blunt-end adapter as described by Bloor et al. (2001). Di- or trinucleotide sequences found within the library were enriched by hybridization to $(AG)_{10}$, $(GT)_{15}$, and $(CAG)_8$ biotinylated oligonucleotides bound to streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). Additionally, to obtain a compound microsatellite primer (Lian et al., 2006), fragments flanked by a microsatellite at one end were amplified from the above libraries using the compound SSR primer $(AC)_6(AG)_5$ and oligo A (Bloor et al., 2001). The enriched fragments and amplified fragments were then ligated into pGEM-T Easy Vector (Promega Corporation) and transformed into DH5α-competent cells (Toyobo Co.). The recombinant DNA was sequenced in both directions on an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, California, USA) using M13 primers (F: 5′-GTTGAAAACGACGGCCAGT-3′; R: 5′-GGAAACAGCTATGACCATGA-3′) and a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). A total of 22 cloned sequences were used to design locus-specific primers with Primer3 Plus (Untergasser et al., 2007). Ten of the 22 primer pairs successfully amplified the target region. To evaluate the loci for polymorphisms, 34 and 43 leaf samples were used for *C. cathartica* (Ishigaki Island [*N* = 22] and Iriomote Island [*N* = 12]) and *C. lineata* (Nichinan, Miyazaki [*N* = 27] and Minami, Tokushima [*N* = 16]), respectively. Voucher specimens representing the sampled populations have been deposited in the herbarium at the College of Education, University of the Ryukyus (URO) (Appendix 1). PCR was performed using 3 ng of template DNA, 0.5 μM each primer, 0.25 mM each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, and 0.25 U of *Ex Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan) in a final volume of 10 μL. The forward primers were labeled with one of three Beckman Coulter WellRED dyes (D2–4; Sigma-Aldrich, St. Louis, Missouri, USA). The cycling conditions were 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at the annealing temperature (T_a; Table 1), and 45 s at 72°C, with a final extension of 10 min at 72°C. The reactions were run using a PC-818S program temperature control system (Astec, Fukuoka, Japan). Genotypes were determined using CEQ 8000 Fragment Analysis software (CEQ 8000; Beckman Coulter, Pasadena, California, USA) after electrophoresis with a CEQ 8000 Genetic Analysis System (Beckman

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TABLE 1. Characteristics of the 10 microsatellites developed in *Canavalia cathartica*.

Locus	Primer sequences (5′–3′)	Repeat motif	WellRED dye[a]	T_a (°C)	Size range (bp)[b]	GenBank accession no.
CANA1	F: TTTCATTTGCTGGTGAACGC R: CTTCATTGACCCATACACAC	$(GT)_{30}$	D2	55	83–111	AB618267
CANA2	F: ATGGAGGGTTCGGTGGGGAGA R: CACCACCATCCTTTCCCTTTT	$(AG)_{16}$	D2	58	214–236	AB618268
CANA3	F: TTAGATGTTGCAAGTGCATGGG R: GCAGCATTTCAAACTTGGGA	$(GT)_{30}$	D4	55	208–260	AB618269
CANA4	F: ATTTATGACCAAATAGTCTC R: ACCCCTGAAAAAGTGGTGTGT	$(AG)_{35}$	D3	55	252–296	AB618270
CANA5	F: CAACAACAACGAAGAGGAAGA R: GAGGTGGACACACACTCCTT	$(AC)_{19}$	D2	55	267–293	AB618271
CANA6	F: TCAAATTGGTCACAACTCGC R: ATGAACAGCCAGAATGCATC	$(TGC)_{11}$	D2	55	148–196	AB618272
CANA7	F: TGTTTTTCCAGTGCCGGGAGGA R: AATTTTTCTCCTCCTAACCCAGCC	$(CAG)_{11}$	D4	60	255–294	AB618273
CANA-C1	F: ACACACACACACAGAGAGAGAG R: TTGGATTCTTCTTCTTCACCTTCCG	$(AC)_6(AG)_{18}$	D3	60	212–240	AB618274
CANA-C2	F: ACACACACACACAGAGAGAGAG R: CTGATAGATCCTCAGGGGAA	$(AC)_6(AG)_{14}$	D3	55	126–154	AB618275
CANA-C3	F: ACACACACACACAGAGAGAGAG R: TCTGCTATCTGTTTTGCCC	$(AC)_6(AG)_7$	D4	55	134–140	AB618276

Note: T_a = annealing temperature.
[a] Forward primer label.
[b] Observed size range from 34 samples of *C. cathartica.*

TABLE 2. Results of initial primer screening in *Canavalia cathartica* and *C. lineata*.

	C. cathartica								*C. lineata*							
	Ishigaki Island (N = 22)				Iriomote Island (N = 12)				Nichinan (N = 27)				Minami (N = 16)			
Locus	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	HWE
CANA1	12	0.818	0.865	0.087	11	0.750	0.858	0.140	4	0.704	0.736	0.188	7	0.688	0.779	0.168
CANA2	8	0.727	0.806	0.276	6	0.833	0.813	0.488	2	0.370	0.346	0.791	3	0.563	0.510	0.603
CANA3	13	0.864	0.870	0.256	7	0.667	0.840	0.046	9	0.852	0.834	0.093	4	0.563	0.455	1.000
CANA4	8	0.682	0.669	0.298	3	0.333	0.392	0.411	7	0.852	0.694	0.078	1	—	—	—
CANA5	8	0.864	0.635	1.000	5	0.500	0.691	0.052*	3	0.444	0.501	0.245	2	0.313	0.264	1.000
CANA6	12	0.682	0.751	0.246	11	0.917	0.840	0.863	6	0.778	0.653	0.173	8	0.688	0.807	0.082
CANA7	7	0.591	0.760	0.104	8	0.750	0.806	0.466	5	0.444	0.453	0.485	1	—	—	—
CANA-C1	10	0.864	0.819	0.734	10	0.818	0.847	0.132	7	0.815	0.747	0.313	10	0.813	0.877	0.082
CANA-C2	12	0.864	0.843	0.127	9	0.750	0.847	0.101	2	0.074	0.071	1.000	2	0.313	0.342	0.566
CANA-C3	4	0.818	0.681	0.922	2	0.500	0.375	1.000	2	0.259	0.226	1.000	2	0.875	0.492	1.000

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = number of individuals.
* Deviations from HWE ($P < 0.05$).

Coulter). A GenomeLab DNA Size Standard Kit (400; Beckman Coulter) was used to determine allele size. PCR using the 10 primer sets resulted in polymorphic, single-locus amplification products (Table 1).

The results of our initial screening of *C. cathartica* and *C. lineata* are summarized in Table 2. Ten loci were polymorphic, with two to 13 and two to 10 alleles for *C. cathartica* and *C. lineata*, respectively, except for CANA4 and CANA7 in the Minami population. The observed heterozygosity values ranged from 0.333 to 0.919 and from 0.074 to 0.875 for *C. cathartica* and *C. lineata*, respectively. The expected heterozygosity values ranged from 0.375 to 0.870 and 0.071 to 0.877 for *C. cathartica* and *C. lineata*, respectively. Analyses of Hardy–Weinberg equilibrium and linkage disequilibrium were performed using GENEPOP version 3.3 (Raymond and Rousset, 1995). No significant heterozygote deficiency ($P < 0.05$) was detected for any of the loci except for CANA5 in the Iriomote Island population. For each population, no significant linkage disequilibrium was found among the 45 possible pairwise comparisons ($P = 0.05$ after sequential Bonferroni correction [Rice, 1989]).

CONCLUSIONS

The 10 microsatellite markers for *C. cathartica* revealed high levels of polymorphism, suggesting that they can be used to analyze the population genetic structure and genetic diversity of the species. Moreover, the ability to cross-amplify *C. lineata* sequences indicates that these markers may be used in the cross-amplification of other *Canavalia* species.

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Appendix 1. Voucher specimens of *Canavalia cathartica* and *C. lineata* used in this study. Voucher specimens are deposited at the Herbarium of the College of Education, University of the Ryukyus (URO).

Species	Locality	Latitude	Longitude	Voucher no.
C. cathartica	Takana, Iriomote Island, Okinawa	24°22′57″N	123°53′18″E	*T. Yamashiro 11109*
	Banna, Ishigaki Island, Okinawa	24°22′19″N	124°09′39″E	*T. Yamashiro 11113*
C. lineata	Nichinan, Miyazaki	31°40′35″N	131°27′15″E	*T. Yamashiro 11203*
	Minami, Tokushima	33°70′61″N	134°51′68″E	*T. Yamashiro 11120*