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Primer Note

Development of microsatellite markers for *Vitex rotundifolia* (Verbenaceae), an endangered coastal plant in Lake Biwa, Japan[1]

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- *Premise of the study:* Microsatellite loci were developed for *Vitex rotundifolia*, an endangered species isolated to Lake Biwa, to investigate its genetic diversity and population structure.
- *Methods and Results:* Ten primer sets were identified in Japanese populations of *V. rotundifolia.* The number of alleles per locus ranged from one to six and gene diversity per locus ranged from 0.040 to 0.697 between two populations. In addition, all loci could be successfully amplified in *V. trifolia*.
- *Conclusions:* These markers will be useful for studies of genetic diversity and population structure of endangered species isolated to Lake Biwa of *V. rotundifolia*, to aid in the development of conservation strategies.

Key words: conservation genetics; gene diversity; microsatellite; population structure; *Vitex rotundifolia*; *Vitex trifolia*.

Vitex rotundifolia L. f. is a deciduous, sprawling shrub of the family Verbenaceae that commonly occurs in sandy, temperate, coastal areas of Japan, Southeast Asia, the Pacific Islands, and Australia (Murata, 1989). This species proliferates by sexual reproduction as well as clonal propagation through the elongation of rhizomes and root systems. In Japan, *V. rotundifolia* is a common species of sandy coastal vegetation and also occurs on the sandy shores of Lake Biwa, an ancient lake that was established ca. 400 mya and harbors coastal plant taxa. Populations of *V. rotundifolia* occurring at Lake Biwa are assumed to have migrated from coastal populations and have been isolated from them for a very long period of time (Kitamura, 1968). The lakeshore environment at Lake Biwa has been heavily disturbed by development, and *V. rotundifolia* has been protected as a threatened species in Shiga Prefecture. In the current study, we developed simple sequence repeat (SSR) markers to clarify the genetic structure of *V. rotundifolia* between Lake Biwa and coastal populations and also described successful amplification of the related species *V. trifolia* L.

METHODS AND RESULTS

Genomic DNA of *V. rotundifolia* was extracted from fresh leaves of a single individual collected from a population from Matsunase, Japan, for development of the DNA library. Genomic DNA of *V. rotundifolia* from a coastal population (Matsunase, Japan) and a Lake Biwa population (Sabae, Japan) and two populations of *V. trifolia* from Amami-Oshima Island was extracted to determine population genetic diversity parameters. All population vouchers were deposited in Kyoto University Museum (KYO) (T. Ohtsuki JP13001–JP13003 and Y. Kaneko JP1101). Microsatellite markers were developed using an improved technique for isolating codominant compound microsatellite markers (Lian and Hogetsu, 2002; Lian et al., 2006). An adapter-ligated, restricted DNA library for *V. rotundifolia* was constructed according to the following procedure: genomic DNA was extracted from fresh leaves using liquid nitrogen to assist in the grinding of plant material and the 2-4-(2-hydroxyethyl-1-piperanzinyl) ethanesulfonic acid/cetyltrimethylammonium bromide (HEPES/CTAB) method with a HEPES wash step adapted from Setoguchi and Ohba (1995). Total DNA was extracted from each pellet using CTAB (Doyle and Doyle, 1990), and a total volume of 100 µL containing ~2.5 µg genomic DNA was digested with the blunt-end restriction enzymes *Ssp*I, *Eco*RV, and *Alu*I. The restriction fragments were then ligated with a specific blunt adapter (consisting of the 48-mer: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGC-TGGT-3′ and an 8-mer with the 3′-end capped with an amino residue: 5′-ACCAGCCC-NH_2-3′) using a DNA ligation kit (TaKaRa Bio Inc., Ohtsu, Shiga, Japan). Fragments were amplified by PCR from the DNA library using a compound SSR primer [$(AC)_6(AG)_5$, $(TC)_6(AC)_5$] and an adapter primer (5′-CTATAGGGCACGCGTGGT-3′). PCR was performed in a total reaction volume of 50 µL containing 38.25 µL of sterilized water, 0.2 mM dNTP mixture, 0.125 unit of AmpliTaq Gold (Applied Biosystems, Foster City, California, USA), 1.5 mM reaction buffer with $MgCl_2$ (Applied Biosystems), 0.5 µM of each primer, and 5 ng of template DNA. The amplification profiles included initial denaturation at 94°C for 9 min; followed by 20 cycles of 30 s at 94°C, 30 s at 66–60°C (reduced 0.3°C/cycle), and 1 min at 72°C; 20 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C; and a final extension at 72°C for 6 min. The amplified fragments, ranging from 400 to 800 bp, were then separated on a 1.5% LO3 agarose gel (TaKaRa Bio Inc.) and purified using Gene Clean II Kit (Qbiogene, Solon, Ohio, USA). The purified DNA fragments were cloned using the QIAGEN PCR Cloning^{plus} Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The cloned fragments were identified using blue/white screening on Lennox-L-Agar plates containing ampicillin, X-gal, and isopropyl-β-D-l-thiogalactopyranoside (IPTG). Two hundred forty insert-positive clones were amplified using the M13 forward and reverse primers from the plasmid DNA. PCR amplifications included initial denaturation at 95°C for 9 min; followed by 40 cycles of 30 s at 94°C, 1.5 min at 54°C, and 1 min at

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TABLE 1. Characteristics of 10 microsatellite primers developed in *Vitex rotundifolia*.[a]

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
r06	F: TCATGAACATCAATCTCG	$(TG)_{10}$	108 (100–108)	57	AB725769
	R: <HEX>TCTCTCTCTCTCACACACACAC				
r15	F: CTTCAAGTGTGAAGTAACAATCTCC	$(TG)_8$	156 (154–158)	57	AB725770
	R: <HEX>TCTCTCTCTCTCACACACACAC				
r12	F: ACCGACTGCTCACATACCTG	$(TG)_8$	200 (186–202)	57	AB725771
	R: <HEX>TCTCTCTCTCTCACACACACAC				
r14	F: CATGCCCAAGTCCTCAACAC	$(TG)_8$	133 (131–143)	57	AB725772
	R: <HEX>TCTCTCTCTCTCACACACACAC				
r20	F: CCTGATAAACTCAGGGCTAACACCC	$(TG)_7$	249 (249–255)	57	AB725773
	R: <HEX>TCTCTCTCTCTCACACACACAC				
r23	F: TTGCCCCCTAAAGGGACAGTG	$(TG)_7$	193 (191–193)	57	AB725774
	R: <HEX>TCTCTCTCTCTCACACACACAC				
r01	F: ATGTGGGATGTCACAATTACT	$(CT)_{14}$	127 (123–135)	57	AB841368
	R: <6-FAM>ACACACACACACAGAGAGAGAG				
r27	F: GGACAGATCCGAATTTGCAGG	$(CT)_6$	137 (137–139)	57	AB841369
	R: <6-FAM>ACACACACACACAGAGAGAGAG				
r28	F: AAGTTCAACTCACGACCGCCGGAG	$(CT)_7$	90 (84–92)	57	AB859620
	R: <6-FAM>ACACACACACACAGAGAGAGAG				
r33	F: GTATAAACTCTTCTATTGTTGC	$(CT)_{12}$	193 (193–205)	57	AB859621
	R: <6-FAM>ACACACACACACAGAGAGAGAG				

Note: T_a = annealing temperature.

[a] All values are based on 50 samples representing Japanese populations located in Sabae and Matsunase (n = 25 for each). See Table 2 for geographic coordinates.

72°C; and a final extension at 72°C for 4 min. PCR was performed in a total reaction volume of 10 µL containing 6.75 µL of sterilized water, 0.08 mM dNTP mixture, 0.25 unit TaKaRa *Ex Taq* (TaKaRa Bio Inc.), 1.0 mM reaction buffer (TaKaRa *Ex Taq*), 0.2 µM of each primer, and 10 ng of template DNA. Forty-eight ambiguous and/or multiple bands for amplified PCR products were discarded. Amplified fragments were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). After sequencing, 37 identical sequences were found. Given that cloned DNA may result from a substantial number of PCR errors and that it is not appropriate to develop primer pairs using these sequences, we removed them. In addition, 122 clones were discarded because amplified fragments containing compound SSR sequences were removed. For each fragment containing a compound SSR sequence at one end, a specific primer was designed from the sequence flanking the compound SSR using Primer3 (version 0.4.0; Rozen and Skaletsky, 2000). Each primer was designed to have a total size of approximately 18–25 bp and an annealing temperature of 55–60°C with <60% of GC content. PCR amplifications were performed following the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN) in a final volume of 6 µL, which contained 3 ng of extracted DNA, 3 µL of 2× Multiplex PCR Master Mix, and 0.2 µM of each multiplexed primer. Compound SSR primers, $(AC)_6(AG)_5$ and $(TC)_6(AC)_5$, were labeled with the fluorochromes 6-FAM or HEX (Applied Biosystems). The amplification profiles included initial denaturation at 95°C for 15 min; followed by 35 cycles of 30 s at 94°C, 1.5 min at 57°C (Table 1), and 1 min at 72°C; and a final extension at 60°C for 30 min. The size of the PCR products was measured using the ABI PRISM 3100 Genetic Analyzer with GeneScan 350 ROX Size Standard (Applied Biosystems) and GeneMapper analysis software (Applied Biosystems).

Thirty-three primer sets were initially screened using four individuals for each population of *V. rotundifolia*, and 10 polymorphic loci were identified that showed a clear, strong, single band for each allele (Table 1). The levels of polymorphism of the microsatellites were evaluated by screening 25 individuals for each population of *V. rotundifolia*. The genetic diversity parameters were calculated using FSTAT version 2.9.3.2 (Goudet, 1995). The number of alleles per locus ranged from one to six in *V. rotundifolia* populations. The observed heterozygosity and genetic diversity per locus (Nei, 1987) ranged from 0.000 to 1.000 and 0.040 to 0.697, respectively (Table 2). Deviation

TABLE 2. Characteristics of 10 microsatellite primers in populations of *Vitex rotundifolia* and *V. trifolia*.[a]

	Vitex rotundifolia								*Vitex trifolia*	
	Sabae (n = 25)			Matsunase (n = 25)			Total		Ayamaru (n = 8)	Maehida (n = 8)
Locus	A	H_o	H_s	A	H_o	H_s	A_T	H_T	A (size range)[b]	A (size range)[b]
r06	4	0.441	0.697*	4	0.400	0.588*	5	0.728	3 (101–107)	3 (101–107)
r15	3	0.080	0.223*	3	0.080	0.155	3	0.187	3 (158–162)	2 (158, 162)
r12	2	0.320	0.373	4	0.360	0.319	4	0.537	3 (198–202)	2 (198, 202)
r14	2	0.040	0.040	5	0.840	0.743	5	0.564	1 (143)	1 (143)
r20	3	0.040	0.255*	4	0.400	0.686*	4	0.523	1 (262)	1 (262)
r23	2	0.080	0.078	2	0.000	0.080	2	0.078	2 (188, 192)	2 (188, 192)
r01	6	0.200	0.618*	4	0.280	0.554	7	0.594	4 (134–140)	3 (132–136)
r27	1	NA	NA	2	0.120	0.115	2	0.059	3 (135–139)	3 (135–139)
r28	2	0.512	0.390	3	0.440	0.555*	3	0.478	3 (84–94)	2 (84, 94)
r33	2	1.000	0.500*	3	0.961	0.628*	3	0.572	1 (206)	1 (206)

Note: A = number of alleles per locus; A_T = total number of alleles per locus; H_o = average observed heterozygosity; H_S = gene diversity; H_T = corrected total heterozygosity; n = sample size; NA = not applicable.

[a] Geographic coordinates for populations: Sabae = 35°08′53″N, 136°01′23″E; Matsunase = 34°36′19″N, 136°34′47″E; Ayamaru = 28°28′25″N, 129°43′06″E; Maehida = 28°27′14″N, 129°39′33″E.

[b] Size range of alleles (bp) observed for each locus.

* Deviations from Hardy–Weinberg equilibrium: $P < 0.01$.

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from Hardy–Weinberg equilibrium (HWE) at each locus was calculated using FSTAT version 2.9.3.2 (Goudet, 1995). Three significant deviations ($P < 0.01$) from HWE were detected in both the Sabae and Matsunase populations (r06, r20, and r33). No significant linkage disequilibrium was found in the pairwise comparisons of the polymorphic loci. All microsatellite markers developed for *V. rotundifolia* were successfully amplified in *V. trifolia* with the annealing temperatures at 57°C, and seven pairs showed polymorphism (Table 2).

CONCLUSIONS

The microsatellite markers presented here will be useful for investigating the genetic diversity and population structure of the isolated populations of *V. rotundifolia* at ancient Lake Biwa. All of the primers can amplify microsatellites in *V. trifolia*, suggesting the potential utility of these primers for a variety of population genetic studies across *Vitex* species.

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