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Primer Note

Development and characterization of microsatellite markers for *Veratrum maackii* (Melanthiaceae)[1]

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- *Premise of the study:* Microsatellite markers were developed in *Veratrum maackii* (Melanthiaceae) to assess the pattern of population genetic structures across the species' distribution.
- *Methods and Results:* Ten polymorphic loci were isolated from *V. maackii* using 454 shotgun pyrosequencing and the biotin capture method. The number of alleles per locus ranged from three to 19. The observed heterozygosity and expected heterozygosity ranged from 0.143 to 0.929 and 0.269 to 0.904, respectively.
- *Conclusions:* These newly developed microsatellite markers are useful for assessing the genetic diversity, population structure, and demographic history of *V. maackii* across its distribution range.

Key words: 454 shotgun sequencing; genetic structure; Melanthiaceae; microsatellite; multiplex PCR; *Veratrum maackii.*

Veratrum maackii Regel (Melanthiaceae) is distributed widely in Japan, Korea, northern China, and eastern Siberia. The species is a polymorphic perennial, and many infraspecific taxa have been described. In Japan, three varieties, namely, var. *maackii*, var. *japonicum* (Baker) T. Shimizu, and var. *parviflorum* (Miq.) Hara & Mizushima, and two forms, var. *japonicum* f. *atropurpureum* (Honda) Kitam. and var. *parviflora* f. *alpinum* (Nakai) Kitam., are recognized (Satake, 1982). These infraspecific taxa are distinguished based on perianth color, leaf shape, and capsule size, although these characteristics are continuous, and delimitation among taxa is difficult. Takada and Kawanobe (1996) examined variations in perianth colors and leaf shapes in populations of *V. maackii* in the Japanese Archipelago, reporting clinal variations in leaf shape. Individuals with green perianths were found in the northern part of Honshu Island, those with dark purple perianths were observed in other areas, and those with intermediate color perianths were observed in the zones where green and dark purple perianth individuals were sympatric. They inferred that these character variations reflect the distribution contraction and expansion of the species in the past. To obtain phylogeographic inferences regarding the species, molecular genetic data are indispensable but have not been available to date. In this study, we developed microsatellite markers for *V. maackii* to investigate its genetic diversity and population structure in Japan and surrounding areas. Furthermore, we explored cross-amplification of the developed markers in closely related taxa.

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METHODS AND RESULTS

The genomic DNA of one individual of *V. maackii* from a population located in Sendai, Japan (Appendix 1), was used for the development of microsatellites. Genomic DNA was extracted from frozen leaf tissues using a modified cetyltrimethylammonium bromide (CTAB) method (Maki and Horie, 1999). The GS Junior Titanium Series Kit (Roche 454 Life Sciences, Branford, Connecticut, USA) was used to generate a shotgun library of genomic DNA. A 500-ng aliquot of genomic DNA was nebulized at 0.21 MPa for 1 min. The DNA fragments were purified using the MinElute PCR Purification Kit (QIAGEN, Hilden, Germany). The purified fragments were end-repaired, A-tailed, ligated to the Rapid Library Adapter, and suitably sized by removing small fragments (<350 bp) using the GS FLX Titanium Rapid Library Preparation Kit (Roche 454 Life Sciences). The fragments were then mixed with capture beads and amplified through emulsion PCR (emPCR) using the GS Junior Titanium emPCR Kit (Roche 454 Life Sciences). After emPCR, beads capturing the DNA library were enriched with sufficient amounts of template DNA for sequencing. The enriched beads were annealed with sequencing primers, and the amplified fragments were pyrosequenced on the GS Junior 454 System (Roche 454 Life Sciences).

A total of 33,542 sequences with an average read length of 450 bp were obtained and further screened for putative microsatellites using the MISA Perl script (Thiel et al., 2003). A total of 1158 microsatellite repeats were identified, including 1064 di-, 40 tri-, 27 tetra-, seven penta-, and 15 hexanucleotide repeat motifs, with minimum contiguous repeat units of eight, eight, five, five, and five, respectively. The most common motifs were AG/CT, AT, AC/GT, and ACC/GGT. We additionally developed and sequenced a microsatellite-enriched library using a modified biotin-capture method (Fischer and Bachmann, 1998). The detailed library construction procedure was presented in Li and Maki (2013). We obtained 30 sequences containing microsatellite repeats using the biotin-capture method.

Fifty sequences containing microsatellite repeats with sufficient flanking regions were chosen for primer design. Of the 50 sequences, 44 came from the 454 sequencing and six came from the modified biotin-capture method. Primer pairs were designed with an expected product size ranging from 120 to 350 bp using Primer3 version 2.0 (Rozen and Skaletsky, 2000). For the loci, the forward primer was ligated to a 454A adapter primer sequence (5′-GCCTCCCTCGCGCCATCAG-3′; Margulies et al., 2005), and the reverse was tagged with a PIG-tail (5′-GTTTCTT-3′; Brownstein et al., 1996). To evaluate amplifications of the primer pairs and polymorphisms at each locus, a panel of six individuals (two individuals from each population) collected from three natural populations located across the *V. maackii* range

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TABLE 1. Sequence and multiplex characteristics of the 10 microsatellites developed for *Veratrum maackii*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent dye	Multiplex set	GenBank accession no.
maackii-003	F: CTTCAGCTCAGCTCTCCCATCAC	$(GA)_{18}$	VIC	1	LC020526
	R: CCTCCTATTGCCTCTCACACTTCTC				
maackii-011	F: TGCGAGGAGGACAAGCTGTGTTCAT	$(CT)_{12}$	NED	3	LC020527
	R: GGGTAGGGTTTGGCTGAGTGAGAGG				
maackii-015	F: CGAAGAAACCTCAACGAATCACAACTG	$(GA)_{16}$	NED	1	LC020534
	R: TCCAGCGGATTCCAAGCCTCTC				
maackii-027	F: GCATGTACTTGCTGAATCAGTTGAAGA	$(AG)_{17}$	PET	1	LC020528
	R: AATTCGAGCTAGGATTATCACTCCAGAG				
maackii-044	F: GAGCGAGTAAGGGTTAGGGTTT	$(CAG)_8$	VIC	2	LC020529
	R: AGGCGGATTAGGGTGAGAGTA				
maackii-056	F: CGCAGGTACACCATGCTGAACAAG	$(CTT)_8$	PET	3	LC020530
	R: AGACCCGTATCTTCTCGCAGATTTCC				
maackii-088	F: GGTTCAATGGCTCGATGGGTGGTT	$(AG)_{12}$	FAM	3	LC020531
	R: GGAGATGGACATTGGTCAAGGAGGG				
maackii-135	F: AGCTCGGAGCCGTCATCAATGG	$(GA)_{14}$	VIC	3	LC020532
	R: GACCTCTTGTAGACCCACCGATGC				
maackii-148	F: CGTATCGTTAGTGTGTCCGTTTCAGTGT	$(AG)_{12}$	PET	2	LC020533
	R: GCCCTCTCTGTTCTCTATCTAGGTTCCA				
maackii-340	F: GCACGATGAGGCTTATGAGTATCC	$(CT)_{20}(CA)_{10}$	NED	2	LC020535
	R: CTCCTCCTCGGTACAGAACTTGAT				

[a]Annealing temperature (T_a) for all loci was 60°C.

distribution in Japan (see Appendix 1; a northern population: Tanesashi; a central population: Mitsuke; and a southern population: Kirishima-dake) were used. PCR reactions were performed separately for each locus in 3-µL volumes containing approximately 60 ng of genomic DNA, 1× Type-it Multiplex PCR Master Mix (QIAGEN), 0.075 µM tailed forward primer, 0.1 µM fluorescently labeled 454A primer, and 0.25 µM reverse primer. The reaction parameters involved an initial denaturation step at 95°C for 5 min; followed by 32 cycles at 95°C for 30 s, 60°C for 90 s, and 72°C for 30 s; and a final step at 60°C for 30 min. PCR products were run with a GeneScan 600 LIZ internal size standard (Applied Biosystems, Foster City, California, USA) on an ABI 3100 Genetic Analyzer (Applied Biosystems).

After excluding loci that showed unclear amplification patterns (no monomorphic markers were detected), we selected 10 polymorphic primer pairs, which were combined into three multiplex reactions using Multiplex Manager 1.0 (Holleley and Geerts, 2009). Of the 10 primer pairs, eight came from the 454 sequencing and two came from the modified biotin-capture method. The forward primer was labeled using one of the fluorochromes 6-FAM, VIC, NED, or PET (Life Technologies, Tokyo, Japan; Table 1). Preliminary population genetic analyses were performed on individuals from Tanesashi (n = 20), Mitsuke (n = 14), and Kirishima-dake (n = 19). Cross-species amplification was performed in *V. album* L. subsp. *oxysepalum* (Turcz.) Hultén, *V. stamineum* Maxim. var. *stamineum*, and *V. stamineum* var. *micranthum* Satake (Appendix 1). Multiplex PCRs were performed in 3-µL volumes containing approximately 30 ng of genomic DNA, 1× Type-it Multiplex PCR Master Mix (QIAGEN), and 0.2 µM forward and reverse primer, respectively. The reaction parameters were an initial denaturation step at 95°C for 5 min; followed by 28 cycles at 95°C for 30 s, 60°C for 90 s, and 72°C for 60 s; and a final step at 60°C for 30 min. PCR products were run with a GeneScan 600 LIZ internal size standard (Applied Biosystems) on an ABI 3100 Genetic Analyzer (Applied Biosystems). Allele sizes were scored using GeneMapper version 3.7 software (Applied Biosystems).

The number of alleles per locus ranged from three to 19. The observed heterozygosity and expected heterozygosity ranged from 0.143 to 0.929 and 0.269 to 0.904, respectively (Table 2). The tests for deviation from Hardy–Weinberg equilibrium (HWE) at each locus and the linkage disequilibrium (LD) of all combinations of loci were conducted using GENEPOP 4.2 (Rousset, 2008). No significant departures from HWE were detected in loci among populations Tanesashi and Mitsuke of *V. maackii* ($P > 0.01$) (Table 2). One significant deviation ($P < 0.01$) from HWE was detected in the population Kirishima-dake (maackii-056) (Table 2). No combination of loci was in LD after Bonferroni correction ($P > 0.05$) (Rice, 1989). Of the 10 microsatellite markers developed for *V. maackii*, three, two, and three were successfully amplified for samples in each of three congeneric taxa, namely, *V. album* subsp. *oxysepalum*, *V. stamineum* var. *stamineum*, and *V. stamineum* var. *micranthum*, respectively (Table 3).

TABLE 2. Characteristics of the 10 polymorphic microsatellite markers for *Veratrum maackii.*

	Tanesashi (n = 20)					Mitsuke (n = 14)					Kirishima-dake (n = 19)				
Locus	A	H_o	H_e	P	Allele size range (bp)	A	H_o	H_e	P	Allele size range (bp)	A	H_o	H_e	P	Allele size range (bp)
maackii–003	19	0.750	0.901	0.020	284–354	8	0.786	0.755	0.271	282–308	13	0.842	0.832	0.406	294–332
maackii–011	12	0.900	0.868	0.683	215–239	10	0.857	0.862	0.365	204–229	8	0.789	0.821	0.678	208–233
maackii–015	12	0.900	0.869	0.740	212–239	9	0.786	0.814	0.675	207–233	8	0.737	0.735	0.702	204–243
maackii–027	18	0.900	0.891	0.510	291–337	9	0.929	0.811	0.762	293–339	12	0.737	0.799	0.096	287–337
maackii–044	4	0.250	0.269	0.354	174–194	4	0.500	0.579	0.191	176–185	3	0.684	0.661	0.673	176–188
maackii–056	9	0.750	0.830	0.263	273–320	10	0.500	0.681	0.091	276–309	5	0.143	0.755	0	287–300
maackii–088	12	0.850	0.854	0.111	125–155	4	0.929	0.712	0.303	133–149	7	0.722	0.795	0.051	129–151
maackii–135	17	0.900	0.904	0.156	180–230	8	0.929	0.837	0.925	186–222	9	0.895	0.848	0.077	178–271
maackii–148	6	0.550	0.668	0.319	214–282	6	0.714	0.768	0.245	222–234	3	0.526	0.422	0.749	222–226
maackii–340	11	0.750	0.803	0.198	217–250	10	0.714	0.829	0.399	219–267	6	0.895	0.807	0.267	180–224

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals genotyped; P = probability of departure from Hardy–Weinberg equilibrium.

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TABLE 3. Cross-species amplifications of 10 microsatellite markers in three congeneric species of *Veratrum maackii*.

	V. album subsp. *oxysepalum* (n = 20)				*V. stamineum* var. *stamineum* (n = 20)				*V. stamineum* var. *micranthum* (n = 20)			
Locus	A	H_o	H_e	Allele size range (bp)	A	H_o	H_e	Allele size range (bp)	A	H_o	H_e	Allele size range (bp)
maackii-003	—	—	—	—	—	—	—	—	—	—	—	—
maackii-011	4	0.600	0.651	216–232	10	0.750	0.845	223–249	6	0.850	0.763	226–240
maackii-015	4	0.353	0.625	232–272	—	—	—	—	8	0.550	0.796	227–250
maackii-027	—	—	—	—	—	—	—	—	—	—	—	—
maackii-044	4	0.250	0.423	173–190	7	0.471	0.671	176–191	4	0.450	0.536	176–188
maackii-056	—		—	—	—	—	—	—	—	—	—	—
maackii-088	—	—	—	—	—	—	—	—	—	—	—	—
maackii-135	—	—	—	—	—	—	—	—	—	—	—	—
maackii-148	—	—	—	—	—	—	—	—	—	—	—	—
maackii-340	—	—	—	—	—	—	—	—	—	—	—	—

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals genotyped.

CONCLUSIONS

The novel microsatellite markers showed high levels of polymorphism and will be useful for describing the genetic diversity, population structure, and demographic history of *V. maackii* across its distribution range.

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APPENDIX 1. Voucher and locality information of *Veratrum* samples used in this study.

Taxon	Locality	Geographic coordinates	Voucher no.
V. maackii Regel	Sendai, Miyagi, Japan	38°16′49″N, 140°48′49″E	*Maki 14073*[a]
V. maackii Regel	Tanesashi, Iwate, Japan	40°33′7″N, 141°35′56″E	—
V. maackii Regel	Mitsuke, Niigata, Japan	37°31′1″N, 138°57′22″E	—
V. maackii Regel	Kirishima-dake, Kagoshima, Japan	31°54′0″N, 130°53′24″E	—
V. album L. subsp. *oxysepalum* (Turcz.) Hultén	Tsurugi-san, Tokushima, Japan	33°51′13″N, 134°06′41″E	—
V. stamineum Maxim. var. *stamineum*	Amou-shitsugen, Gifu, Japan	36°15′36″N, 136°57′47″E	—
V. stamineum Maxim. var. *micranthum* Satake	Tomika, Gifu, Japan	35°30′32″N, 136°58′15″E	—

[a]Voucher specimen deposited at the herbarium of Tohoku University (TUS), Sendai, Japan.

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