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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR THE SEMI-NATURAL GRASSLAND HERB *VERONICASTRUM JAPONICUM* (PLANTAGINACEAE)[1]

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- *Premise of the study: Veronicastrum japonicum* (Plantaginaceae) grows in grasslands on Honshu Island, Japan, and is threatened by habitat loss because of rapid land development over recent decades. For the genetic characterization of the remaining populations, microsatellite markers were developed.
- *Methods and Results:* Twelve polymorphic microsatellite loci were developed using next-generation sequencing. The number of alleles per locus ranged from two to 24 (mean 7.7), and the expected heterozygosity per locus ranged from 0.35 to 0.94 (mean 0.68).
- *Conclusions:* These markers can be used for genetic studies in conservation, such as the evaluation of genetic diversity and genetic structure.

Key words: conservation; grassland herb; next-generation sequencing; Plantaginaceae; *Veronicastrum japonicum*.

Veronicastrum japonicum (Nakai) T. Yamaz. (Plantaginaceae) is a perennial plant species growing in the grasslands of mountainous regions on Honshu Island, Japan (Ohwi, 1978). In recent decades, the number of *V. japonicum* populations has rapidly decreased, and this species is now classified as near threatened or higher in five of the 47 Prefectural Red Lists of Japan (Association of Wildlife Research and EnVision, 2007). The decline of *V. japonicum* populations can be attributed to shrinking grasslands resulting from land development or the abandonment of traditional management. Furthermore, serious grazing damage by deer, which has explosively increased in the past few decades, has threatened *V. japonicum* populations (Takatsuki, 2009; Nagaike et al., 2014). *Veronicastrum japonicum* is also the main larval food plant species of *Melitaea ambigua* (Butler) (Nymphalidae), a rare butterfly species classified as endangered in the Japanese Red List (Ministry of the Environment of Japan, 2012). Accordingly, knowledge of the genetic diversity and structure of *V. japonicum* populations is important for the conservation management of *V. japonicum* and *M. ambigua*. Thus, 12 nuclear microsatellite markers for *V. japonicum* were developed using next-generation sequencing.

METHODS AND RESULTS

A fresh leaf sample of *V. japonicum* was collected from an individual plant growing in Gunma Prefecture, Japan, and genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Germantown, Maryland, USA). A DNA fragment library was constructed with an Ion Xpress Plus Fragment Library Kit (Life Technologies, Foster City, California, USA), amplified with an Ion PGM Template OT2 400 Kit, and subsequently sequenced with an Ion PGM Sequencing 400 Kit and an Ion 318 Chip v2 (both from Life Technologies), yielding 161,880 reads (size range 8–618 bp; mean 220 bp). The reads were screened using Primer3 software (Rozen and Skaletsky, 1999) embedded in MSATCOMMANDER version 0.8.2 (Faircloth, 2008) to identify dinucleotide and trinucleotide loci of at least eight and seven repeats, respectively. After screening, 284 putative microsatellite loci were identified. Using these loci, a total of 57 primer pairs with melting temperatures (T_m) of 57–61°C, GC content of 35–75%, and fragment sizes of 150–350 bp were designed.

Amplification and specificity of 34 out of the 57 primer pairs were tested using four *V. japonicum* individuals. For all confirmed microsatellite loci, the forward primers were synthesized using one of the three different M13 sequences, 5′-CACGACGTTGTAAAACGAC-3′, 5′-CTATAGGGCACGCGTGGT-3′, or 5′-TGTGGAATTGTGAGCGG-3′ (Boutin-Ganache et al., 2001) as shown in Table 1. Each PCR amplification was performed in a final volume of 5 μL, containing approximately 16 ng of template DNA, 2.5 μL of 2× Multiplex PCR Master Mix (QIAGEN, Valencia, California, USA), 0.01 μM of forward primer, 0.2 μM of reverse primer, and 0.1 μM of M13 (fluorescent-labeled) primer. The PCR thermal profile was as follows: initial denaturation at 95°C for 15 min; followed by 33 cycles of 94°C for 30 s, 57°C for 1.5 min, and 72°C for 1 min; and a final extension at 60°C for 30 min. The PCR products were detected on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). Fragment lengths were calculated using GeneMapper software (Applied Biosystems). Of the 34 primer pairs tested, 13 did not amplify specific regions and nine did not amplify in all four individuals. Twelve primer pairs amplified in all four individuals, and the lengths of amplified fragments were scored unambiguously. The 12 primers were used for analysis of 21 and 27 individuals sampled from two populations located in Gunma and Nagano prefectures, respectively (Gunma: 36°26′12″N, 138°25′27″E; Nagano: 35°57′25″N, 137°37′49″E). One specimen from each population was collected and deposited in the Kyoto University Museum herbarium (accession numbers: KYO_00019997 and KYO_00019998, respectively). Population statistics were calculated using GenAlEx version 6.4 (Peakall and Smouse, 2006). Tests for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between loci were performed using FSTAT version 2.9.3 (Goudet, 1995).

The number of alleles per locus ranged from two to 24 (mean 7.7) (Table 2). The observed and expected heterozygosities per locus ranged from 0.30 to 1.00

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TABLE 1. Characteristics of microsatellite loci for *Veronicastrum japonicum*. All values are based on 48 samples from two populations in Gunma and Nagano prefectures in Japan.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent label[b]	Allele size range (bp)	GenBank accession no.
Veja004	F: AGGAGCCCAGTTTGCCTAC	$(CT)_9$	FAM	211–221	LC030210
	R: CAAATGCTAATTGATCTCGACC				
Veja005	F: CCCTCTTTCTGGAAGTTTGAGC	$(TGT)_6$	VIC	232–237	LC030211
	R: ACGACTCCTCCTTGCTTAGG				
Veja007	F: GGGACATTGGAATGGAGGC	$(TCT)_6$	FAM	166–176	LC030212
	R: ATTGATGCTGAACCAGGGC				
Veja009	F: CATTAAATACTCTCTCCGCAGG	$(CT)_9$	NED	233–253	LC030213
	R: GCTCTTCCTCACGAGTTTCC				
Veja011	F: TGATGCTTGCCATCGTTCC	$(GCT)_9$	VIC	200–221	LC030214
	R: AACTCAGCTGTTCCTCCCG				
Veja012	F: CTTCCTTCACCAACCAAGGG	$(GT)_9$	NED	308–332	LC069371
	R: AACAGGACTCGCTCGTCAG				
Veja015	F: CTGTCCCACCTTTAGATTCCC	$(TC)_9$	NED	187–193	LC030215
	R: GTCCCTTCACAAGCTTCCC				
Veja018	F: CACCAATCATTTCCGCAAACC	$(GA)_{12}$	NED	251–321	LC030216
	R: ACAACCGCCCTTTATGATCTAC				
Veja021	F: TGGACACATCAACCAGATTTCC	$(TAT)_7$	NED	194–209	LC030217
	R: TCCTGGATTCAACGAGAACC				
Veja025	F: AGGCCAATCAAACTAATTACGC	$(CA)_9$	FAM	173–192	LC030218
	R: ACGCTTGTAGAGAGGTTAGGC				
Veja029	F: GCGCACTGTTAAGGACAGG	$(TGT)_6$	VIC	178–196	LC030219
	R: CGTGAACAACGGCATACCC				
Veja032	F: CAACGCCAGCCATCCATAC	$(GT)_{10}$	VIC	238–265	LC030220
	R: TTGGAGGAGAATGACACCCA				

[a] Annealing temperature was 57°C for all loci.

[b] Sequence of the fluorescent labels: FAM = 5′-CACGACGTTGTAAAACGAC-3′, NED = 5′-CTATAGGGCACGCGTGGT-3′, VIC = 5′-TGTGGAATTGTGAGCGG-3′.

(mean 0.66) and from 0.35 to 0.94 (mean 0.68), respectively (Table 2). Only one locus (Veja009) in the Gunma population showed significant deviation from HWE ($P < 0.05$). Significant LD was found in the Nagano population for only one pair of loci (Veja004 and Veja021; $P < 0.05$).

CONCLUSIONS

The microsatellite markers described in this study will be useful for conservation genetic studies of *V. japonicum*, such as those evaluating genetic diversity and structure within and between populations. Assessment of their genetic information will also contribute to elucidating how *V. japonicum* genetic diversity and structure has been affected by declining populations and habitat management practices. Understanding the impacts of habitat management will help the conservation of both *V. japonicum* and the endangered butterfly *M. ambigua*, because maintenance of *V. japonicum* population size is crucial for survival of this butterfly species.

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TABLE 2. Results for primer screening of all samples for 12 microsatellite loci in two populations of *Veronicastrum japonicum*.[a,b]

	Gunma ($N = 21$)				Nagano ($N = 27$)			
Locus	A	H_o	H_e	F_{IS}	A	H_o	H_e	F_{IS}
Veja004	7	0.81	0.75	–0.09	5	0.48	0.59	0.19
Veja005	2	0.43	0.39	–0.11	3	0.37	0.60	0.39
Veja007	6	0.62	0.71	0.13	6	0.70	0.65	–0.08
Veja009	8	**0.48**	0.70	0.32	8	0.70	0.80	0.12
Veja011	6	0.62	0.53	–0.17	5	0.59	0.64	0.08
Veja012	13	0.86	0.82	–0.05	12	0.78	0.77	–0.01
Veja015	4	0.52	0.67	0.22	4	0.30	0.35	0.15
Veja018	18	0.95	0.91	–0.04	24	1.00	0.94	–0.06
Veja021	7	0.62	0.73	0.15	6	0.67	0.71	0.06
Veja025	9	0.57	0.67	0.14	10	0.93	0.85	–0.09
Veja029	5	0.52	0.52	–0.01	3	0.70	0.60	–0.17
Veja032	5	0.86	0.73	–0.17	9	0.63	0.67	0.06

Note: A = number of alleles; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size.

[a] Populations: Gunma Prefecture: 36°26′12″N, 138°25′27″E; Nagano Prefecture: 35°57′25″N, 137°37′49″E. Vouchers deposited in the Kyoto University Museum herbarium (accession numbers: KYO_00019997 and KYO_00019998, respectively).

[b] Numbers in boldface show deviations from Hardy–Weinberg equilibrium ($P < 0.05$).

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