Development of Polymorphic Microsatellite Loci in the Perennial Herb Hepatica nobilis var. japonica (Ranunculaceae)

Authors: Kameoka, Shinichiro, Higashi, Hiroyuki, and Setoguchi, Hiroaki

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Primer Note

Development of polymorphic microsatellite loci in the perennial herb *Hepatica nobilis* var. *japonica* (Ranunculaceae)[1]

Shinichiro Kameoka[2,3], Hiroyuki Higashi[2], and Hiroaki Setoguchi[2]

[2]Graduate School of Human and Environmental Studies, Kyoto University, Yoshida Nihonmatsu-cho, Sakyoku, Kyoto 606-8501, Japan

- *Premise of the study:* Microsatellite markers were developed and characterized in the vulnerable plant *Hepatica nobilis* var. *japonica* (Ranunculaceae) to investigate its genetic diversity, population structure, and gene flow.
- *Methods and Results:* Fourteen microsatellite markers were developed. The number of alleles per locus ranged from one to 12, and the expected heterozygosity per locus ranged from 0.043 to 0.855. Eleven markers were successfully amplified in the cultivar 'Mego' from Japan.
- *Conclusions:* These microsatellite markers can be used to investigate the genetic diversity, population structure, and gene flow of *H. nobilis* var. *japonica*.

Key words: gene flow; genetic diversity; *Hepatica nobilis* var. *japonica*; microsatellite; population structure; Ranunculaceae.

Hepatica nobilis Schreb. var. *japonica* Nakai (Ranunculaceae) is a perennial herb that inhabits the Japanese archipelago. This plant characteristically exhibits polymorphisms in floral color within a population (e.g., white, pink, and purple). However, the biological background for retaining floral color variations is not fully understood. Generally, different flower colors attract different types of pollinators, and flower color polymorphisms within a population might be attributable to pollinator differentiation, resulting in genetic differentiation between individuals of distinct flower color. In other words, individuals of different flower colors would form respective genetic groups due to no mating between distinct flower colors. Microsatellite markers could be a valuable tool for testing the hypothesis by analyzing gene flow, genetic variation (e.g., AMOVA), and genetic structure (e.g., STRUCTURE analysis) among different types of flower colors.

Takayama et al. (2011) reported 24 microsatellite loci in *H. nobilis* var. *japonica*; however, more than half of these loci did not amplify in our samples. Here, we aimed to develop viable novel microsatellite markers to investigate genetic diversity, genetic structure, and gene flow.

METHODS AND RESULTS

Total genomic DNA was extracted from fresh leaves collected from two populations of *H. nobilis* var. *japonica* on Sado Island, Niigata Prefecture (38°12′N, 138°25′E), and Mt. Saru, Ishikawa Prefecture (37°19′N, 136°43′E), and from the horticultural cultivar 'Mego' (possibly derived from *H. nobilis* var. *japonica*). The voucher specimens (*S. Kameoka* JP14001 and JP14002 for the Sado Island and Mt. Saru populations, respectively) were deposited at the herbarium of Kyoto University (KYO). After removing polysaccharides and polyphenols from the leaf powder by washing in HEPES buffer (pH 8.0; Setoguchi and Ohba, 1995), total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). The isolated DNA was dissolved in 100 µL of TE buffer. We identified microsatellite loci using a method developed for isolating codominant compound microsatellite markers (Lian and Hogetsu, 2002; Lian et al., 2006). We separately digested DNA samples (50 µL, approximately 50 ng/µL of DNA) of an individual from Sado Island with the blunt-end restriction enzymes *Ssp*I, *Eco*RV, and *Alu*I. The fragments obtained were ligated to a specific blunt adapter (48-mer: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′, and the 3′-end capped the primer 5′-ACCAGCCC-NH_2-3′) using a DNA Ligation Kit (TaKaRa Bio Inc., Otsu, Shiga, Japan). The included fragments were amplified with the aid of simple sequence repeat (SSR) primers [$(AC)_6(AG)_5$ or $(GA)_5(CA)_5$] and an adapter primer (5′-CTATAGGGCACGCGTGGT-3′). PCRs were performed in 23-µL reaction volumes containing 16.375 µL of sterilized water, 2 µL of a solution of the 2.5 mM dNTP mixture, 0.625 units of AmpliTaq Gold (Applied Biosystems, Foster City, California, USA), 2.5 µL of 15 mM reaction buffer with $MgCl_2$ (Applied Biosystems), 0.5 µL of each primer (10 pM), and 1.0 µL of template DNA (approximately 50 ng/µL). The amplification profiles included an initial denaturation step at 94°C for 9 min; followed by 20 cycles of 30 s at 94°C, 30 s at 66–60°C (reduced by 0.3°C per cycle), and 1 min at 72°C; then 20 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C; with a final extension at 72°C for 6 min. After electrophoresis on a 1.5% agarose gel, the fragments (400–800 bp) were separated and purified using a GeneClean II Kit (MP Biomedicals, Santa Ana, California, USA). The purified DNA fragments were cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California, USA). Recombinant clones were isolated by blue/white screening on Luria–Bertani agar plates with ampicillin and X-gal. In total, 480 cloned fragments were amplified using T3 and T7 primers. Amplifications were performed in 6-µL reaction volumes containing 1 µL of sterilized water, 3 µL of a SapphireAmp Fast PCR Master Mix (TaKaRa Bio Inc.), 0.5 µL of each primer (10 pM), and 1.0 µL of the template DNA (approximately 50–80 ng/µL), following the standard manufacturer's protocol for use of the Master Mix (TaKaRa Bio Inc.). The amplification profiles included an initial denaturation at 94°C for 1 min; followed by 40 cycles of 5 s at 96°C, 5 s at 54°C (annealing), and 5 s at 72°C; with a final extension at 72°C for 4 min. PCR products were sequenced using a BigDye Terminator Cycle

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[3]Author for correspondence: kameoka.shinichiro.42n@st.kyoto-u.ac.jp

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TABLE 1. Characteristics of 14 microsatellite loci developed in *Hepatica nobilis* var. *japonica*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	GenBank accession no.
H5	F: GAGAGAGAGACACACACACA R: GAACAAGGTACGCTAGCTAG	$(GA)_5(CA)_5$	187–191	LC002875
H7	F: GAGAGAGAGACACACACACA R: GGGAATGATGTATTTCAAAC	$(GA)_5(CA)_{11}$	77–111	LC008360
H8	F: GAGAGAGAGACACACACACA R: AGAATGAATATTATTAGCAGTGC	$(GA)_5(CA)_6$	218	LC008361
H12	F: GAGAGAGAGACACACACACA R: GTGCACCACAACATAAAATAG	$(GA)_5(CA)_{22}$	147–193	LC002876
H13	F: GAGAGAGAGACACACACACA R: CATTAGTCATGACCAATGG	$(GA)_5(CA)_9$	137–162	LC002877
H16	F: GAGAGAGAGACACACACACA R: AGCTCTGCTCTGTTTAAGG	$(GA)_5(CA)_7$	153–157	LC008362
H20	F: GAGAGAGAGACACACACACA R: ACCAAGAGGTTAATACTGAGG	$(GA)_5(CA)_5$	163–165	LC008363
H31	F: ACACACACACACAGAGAGAGAG R: CTAGGTTTTACACATTTCTCATCTAAAATC	$(AC)_6(AG)_6$	111–133	LC002878
H32	F: ACACACACACACAGAGAGAGAG R: GAAGGTAACAAGTTCTTTCCTCCC	$(AC)_6(AG)_{15}$	93–125	LC002879
H51	F: ACACACACACACAGAGAGAGAG R: GCTAATCAATCTGTCTCCGGAG	$(AC)_6(AG)_{12}$	134–156	LC006950
H53	F: ACACACACACACAGAGAGAGAG R: CCTTGCAAGCCAAGAAATGACAC	$(AC)_6(AG)_7$	122–162	LC002880
H57	F: ACACACACACACAGAGAGAGAG R: GTGAGGAATAGGACACAACCC	$(AC)_6(AG)_5$	112–134	LC002881
H60	F: ACACACACACACAGAGAGAGAG R: GTGAGGAATAGGACACAACCC	$(AC)_6(AG)_6$	112–132	LC002882
H64	F: ACACACACACACAGAGAGAGAG R: CTTTCCAATGCTATCCTCGATAGC	$(AC)_6(AG)_{18}$	106–150	LC002883

[a]The optimum annealing temperature was 52°C.

Sequence Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Specific primers were designed for the 66 fragments containing $(AC)_6(AG)_5$ or $(GA)_5(CA)_5$ sequences. Using six individuals, PCR amplifications were performed with the aid of a Multiplex PCR Kit (QIAGEN, Hilden, Germany) in 6-μL reaction volumes containing 1.7 μL of sterilized water, 2.5 μL of Multiplex (QIAGEN), 0.5 μL of a compound SSR primer (10 pM), 0.5 μL of a designed primer (10 pM), and 0.8 μL of template DNA (approximately 50–80 μg). The fluorochromes 6-FAM or HEX were used to label compound SSR primers. The amplification profiles included an initial denaturation at 95°C for 15 min; followed by 32 cycles of 30 s at 95°C, 1.5 min at 52°C (annealing temperature), and 1.5 min at 60°C; with a final extension at 72°C for 10 min. We measured the sizes of PCR products obtained on an ABI PRISM 3130 Genetic Analyzer by referencing to the GeneScan 350 ROX Size Standard (Applied Biosystems). Microsatellite bands were scored using GeneMapper software (Applied Biosystems).

We selected 14 of 66 microsatellite loci that yielded clear and reproducible bands in the initial screening using four samples each from the two populations (Table 1). For further characterization, 50 individuals from two populations (30 from Sado Island and 20 from Mt. Saru) were genotyped. The products of locus H8 were monomorphic in both populations, whereas one locus (H5) was not

TABLE 2. Population genetic parameters estimated (per nSSR locus) in two populations and one horticultural cultivar of *Hepatica nobilis* var. *japonica*.

	Hepatica nobilis var. *japonica*								
	Sado Island population (n = 30)				Mt. Saru population (n = 20)				
Locus	A	H_o	H_e	r	A	H_o	H_e	r	cv. Mego
H5	3	0.233	0.482*	0.1871	—	—	—	—	–
H7	7	0.292	0.677*	0.2388	4	0.571	0.686	0.0905	–
H8	1	—	—	—	1	—	—	—	+
H12	12	0.633	0.754	0.0859	7	1.000	0.789	0.0000	+
H13	8	0.967	0.666	0.0000	11	0.696	0.794	0.0845	–
H16	2	0.148	0.346*	0.1671	2	0.091	0.165	0.1039	+
H20	2	0.467	0.464	0.0002	2	0.739	0.485	0.0000	+
H31	6	0.800	0.776*	0.0534	10	0.261	0.855*	0.3206	+
H32	5	0.633	0.554	0.0000	2	0.043	0.043	0.0000	+
H51	8	0.500	0.721*	0.1461	10	0.350	0.773*	0.2284	+
H53	9	0.533	0.772*	0.1570	10	0.286	0.776*	0.2715	+
H57	6	0.667	0.599	0.0213	6	0.333	0.569	0.1785	+
H60	6	0.633	0.529	0.0000	5	0.267	0.598*	0.2274	+
H64	9	0.600	0.796*	0.1268	11	0.792	0.789	0.0269	+

Note: + = successful PCR amplification; – = unsuccessful PCR amplification; A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; r = null allele frequency.

*Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

amplified in the Mt. Saru population. GenAlEx version 6.501 (Peakall and Smouse, 2006, 2012) was used to estimate the total numbers of alleles and observed and expected heterozygosities (H_o and H_e, respectively). The numbers of alleles per locus over two populations ranged from one to 12 (Table 2). H_e ranged from 0.043 to 0.855, while H_o ranged from 0.043 to 1.000. Although several loci exhibited a lower H_o than H_e in both populations, the presence of null alleles was not detected ($r < 0.2$) except for H7 in the Sado Island population and H31, H51, H53, and H60 in the Mt. Saru population using FreeNA (Chapuis and Estoup, 2007). Thus, the presence of null alleles and other biological reasons such as inbreeding and/or past genetic drift due to population size reduction could be responsible for the deviation between H_o and H_e in several loci. Deviations from Hardy–Weinberg equilibrium (HWE) were tested using FSTAT version 2.9.3.2 (Goudet, 1995). Significant deviation ($P < 0.05$) from HWE was detected in seven loci from Sado Island (H5, H7, H16, H31, H51, H53, and H64) and four from Mt. Saru (H31, H51, H53, and H60). Of the 14 microsatellite markers developed for *H. nobilis* var. *japonica*, 11 were amplified successfully in the horticultural cultivar 'Mego' (Table 2), indicating that these loci will be useful in horticultural applications of the plant.

CONCLUSIONS

The 14 microsatellite markers will facilitate genetic analyses of *H. nobilis* var. *japonica*. These markers will contribute to determining the biological background of retaining floral color variation within a population, using parameters of gene flow, genetic variation (e.g., AMOVA), and genetic structure (e.g., STRUCTURE analysis) among different types of flower colors.

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