Development and Evaluation of Microsatellite Markers for the Gynodioecious Shrub Daphne jezoensis (Thymelaeaceae)

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Primer Note

Development and evaluation of microsatellite markers for the gynodioecious shrub *Daphne jezoensis* (Thymelaeaceae)[1]

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- *Premise of the study:* Ten microsatellite markers were developed and characterized in a gynodioecious summer-deciduous shrub, *Daphne jezoensis*, to facilitate studies of the evolution of gynodioecy in the species.
- *Methods and Results:* We used a next-generation sequencing approach with the Ion Personal Genome Machine (PGM) system to identify and develop microsatellite markers with perfect di- and trinucleotide repeats. These markers were tested with 47 samples from two natural populations. The mean observed and expected heterozygosities per population ranged from 0.40 to 0.46 and 0.60 to 0.66, respectively.
- *Conclusions:* The developed markers will be useful to study the mating system, gene flow, and population genetic structure of *D. jezoensis*.

Key words: *Daphne jezoensis*; gynodioecy; microsatellite; Thymelaeaceae.

Daphne jezoensis Maxim. (synonym of *Daphne kamtschatica* Maxim. var. *jezoensis* (Maxim.) Ohwi) (Thymelaeaceae) is a gynodioecious summer-deciduous shrub that grows on floors of deciduous forests in northern Japan. This species produces pale yellow flowers soon after snowmelt (late April) until canopy closure in early June (Lei and Koike, 1998). Flowers are commonly visited by small insects, such as flies and thrips, with low pollination efficiency. Kikuzawa (1989) proposed that gynodioecy might have evolved in this species to reduce the interference effect between male and female functions within hermaphrodite flowers. When inevitable self-pollen deposition on the stigma (pollen clogging) interferes with outcross pollen receipt, production of male-sterile flowers can increase female success. To test this hypothesis, microsatellite markers will be an important tool for the clarification and estimation of mating system (e.g., selfing ability and the levels of pollen limitation), gene flow (e.g., pollen and seed dispersal), and population structure (e.g., spatial genetic structure). For this purpose, next-generation sequencing with the Ion Personal Genome Machine (also known as PGM; Life Technologies, Carlsbad, California, USA) was used to develop 10 microsatellite markers.

METHODS AND RESULTS

Microsatellite markers were developed for *D. jezoensis* using the Ion PGM system (Life Technologies). Library preparation and PGM sequencing were conducted at the Sugadaira Montane Research Center, University of Tsukuba, Japan. Total genomic DNA was extracted from fresh leaves of a single *D. jezoensis* individual (unfortunately, no voucher was collected at this time) using a QIAGEN DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The concentration of genomic DNA was quantified by a Qubit 2.0 Fluorometer (Life Technologies), and 75 ng of DNA was used for the following processes. The genomic DNA was sheared to approximately 350–450 bp by Ion Shear Plus Reagents (Life Technologies), and the adapter ligation, nick-repair, and purification of the ligated DNA was conducted using an Ion Plus Fragment Library Kit (Life Technologies). After size selection (target insert sizes 300–400 bp) was performed by an E-Gel Agarose Gel Electrophoresis System (Life Technologies), library amplification was conducted using an Ion Plus Fragment Library Kit (Life Technologies). The library was assessed and quantified using a Bioanalyzer (Agilent Technologies, Palo Alto, California, USA), and then diluted to 8 pM for template preparation using an Ion PGM Template OT2 400 kit (Life Technologies) and enriched. Sequencing was performed by an Ion PGM Sequencing 400 kit (Life Technologies) using 850 flows on the Ion 314 Chip V2 (Life Technologies) according to the manufacturer's protocol. After sequencing, single processing and base-calling were performed using TorrentSuite 3.6 (Life Technologies), and a library-specific FASTQ file was generated. We obtained a total of 362,653 reads with a total of 81.6 Mbp (available from the DNA Data Bank of Japan [DDBJ] Sequence Read Archive: DRA001272).

We directly screened the sequences for perfect di-, tri-, and tetranucleotide repeats using MSATCOMMANDER 1.0.8 (Faircloth, 2008). The 'Design Primer' option was selected in MSATCOMMANDER, in which Primer3 (Rozen and Skaletsky, 2000) searches for microsatellite repeats and identifies possible primer annealing sites according to the following criteria: amplification products within the size range of 100–500 bp, optimal melting temperature range 58–62°C, optimal GC content of 50%, possession of at least 1-bp GC clamp, low levels of self- or pair-complementarity, and maximum end stability of 8.0 (Faircloth, 2008). A total of 98 sets of primers were designed from 437 di-, 100 tri-, and 19 tetranucleotide microsatellites with at least eight, eight, and six repeats, respectively. From these, we selected 24 microsatellites, which showed at least 10 repeats (13 dinucleotides and 11 trinucleotides) for further analyses.

Initial primer screening was conducted using six samples of *D. jezoensis* collected from two populations (three individuals from Chitose, Japan, and three individuals from Nopporo, Japan; Appendix 1). PCRs were performed

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TABLE 1. Characteristics of 10 microsatellite markers developed for *Daphne jezoensis*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_m (°C)	Fluorescent dye[a]	DDBJ accession no.[b]
Dp171	F: GGAGAAGCTGTTGGAGTTGG	$(AAG)_{10}$	300–334	58	FAM	AB897817
	R: TGGAGTATTTGGATGCGGAG					
Dp180	F: GAAGGGATCCAAATGTCCGG	$(AT)_{11}$	187–215	59	FAM	AB897818
	R: CTCTGCAAGGTTTAGGTAAGGG					
Dp214	F: ATAAACAACGCGGAACCTGC	$(AT)_{12}$	87–135	60	VIC	AB897819
	R: GCGCCGGATTACATTATGGG					
Dp238	F: ACGCCCTGTACTCGAATCAC	$(AAG)_{10}$	217–253	59	PET	AB897820
	R: TCTCGAACTGATTTGCCTGTG					
Dp251	F: GCCTTGACTAGTTACACGGG	$(AAT)_{13}$	192–195	59	NED	AB897821
	R: AACGGATGGGTCTAGGTTCC					
Dp258	F: TCCTCCGTCACTCAAGCATC	$(AG)_{18}$	150–176	60	FAM	AB897822
	R: TACGAGTCAGAATACGCCGC					
Dp315	F: CCTGGCAGAAGTGATAGTGAC	$(AAG)_{10}$	160–191	59	VIC	AB897823
	R: TTCTTGTGCTTGGATTGGCG					
Dp336	F: CGCGTATAAAGATGACATGCAG	$(AT)_{10}$	144	59	FAM	AB897824
	R: GAAGCGTTTACCACTTAAGCTG					
Dp504	F: GCAAAGAAGGTCAATCAAGCTC	$(AAG)_{10}$	136–163	59	PET	AB897825
	R: TTGCACATTGGGTTTGGTCC					
Dp506	F: TCTCCATTGCCTTCACTTGC	$(AG)_{11}$	227–241	59	NED	AB897826
	R: CGTAATTGTTGGCCGAATGTG					

Note: DDBJ = DNA Data Bank of Japan; T_m = melting temperature.

[a] Fluorescent label used for multiplex PCR.

[b] All reads obtained by an Ion PGM system are available from the DDBJ Sequence Read Archive (DRA001272).

with the TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa Bio Inc., Otsu, Shiga, Japan) in a final volume of 10 µL, which contained 5 ng of extracted DNA, 0.2 mM of each dNTP, 1× PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, California, USA), 0.25 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.5 µM of each primer. The PCR conditions were 9 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at the annealing temperature, and 1 min at 72°C, followed by 72°C for 7 min. PCR products were labeled with ChromaTide Alexa Fluor 488-5-dUTP (Invitrogen, Carlsbad, California, USA) according to the method of Kondo et al. (2000), and analyzed using an Applied Biosystems 3500 Genetic Analyzer with GeneScan 500 LIZ Size Standard (Applied Biosystems). Sixteen of 24 primers that produced clear bands were selected for further analyses using 47 samples from two populations (24 from Chitose and 23 from Nopporo). Forward primers were labeled with fluorescent dye (6-FAM, NED, PET, or VIC) and multiplexed using a Type-it Microsatellite PCR Kit (QIAGEN). PCRs were performed with the TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa Bio Inc.) in a final volume of 10 µL, which contained 5 ng of extracted DNA, 1× Master Mix, and 0.2 µM of each primer. The PCR conditions were 5 min at 95°C, 35 cycles of 30 s at 95°C, 1 min at 60°C, and 30 s at 72°C, followed by 60°C for 30 min. PCR products were analyzed using an Applied Biosystems 3500 Genetic Analyzer with GeneScan 500 LIZ Size Standard (Applied Biosystems). Because six of 16 primers showed multiple or unclear bands in several samples, we report here the remaining ones as useful markers.

Ten microsatellite markers showed clear and consistent bands by multiplex PCR (Table 1). BLAST searches using read sequence data found no known gene around the markers. The mean number of alleles per locus ranged from 6.2 to 7.1 between populations (Table 2). The mean observed and expected heterozygosities per population ranged from 0.40 to 0.46 and 0.60 to 0.66, respectively (Table 2). Deviations from Hardy–Weinberg equilibrium were tested using GENEPOP 4.2 software (Raymond and Rousset, 1995). Significant deviations after Bonferroni corrections ($P < 0.05$) were detected for five (Dp180, Dp214, Dp251, Dp258, and Dp315) and three (Dp180, Dp251, and Dp315) loci in Chitose and Nopporo, respectively (Table 2). Null allele frequencies estimated by CERVUS 3.0 (Marshall et al., 1998; Kalinowski et al., 2007) were positive for most loci in both populations, which might contribute to the excess of homozygosity (Table 2). However, the same pattern could be observed to result from different factors, such as inbreeding and the Wahlund effect. The controlled crossing and paternity analysis are required to reveal the causes of Hardy–Weinberg disequilibrium.

TABLE 2. Genetic diversity of 10 microsatellite loci in two *Daphne jezoensis* populations.

	Chitose ($N = 24$)				Nopporo ($N = 23$)			
Locus	A	H_o	H_e	Null	A	H_o	H_e	Null
Dp171	7	0.500	0.772	0.212	5	0.304	0.531	0.207
Dp180	7	0.375	0.818*	0.292	9	0.174	0.818*	0.529
Dp214	14	0.625	0.901*	0.178	10	0.696	0.676	–0.031
Dp238	8	0.833	0.768	–0.043	8	0.739	0.828	0.058
Dp251	2	0.083	0.288*	0.442	2	0.087	0.340*	0.592
Dp258	11	0.625	0.824*	0.106	8	0.696	0.798	0.061
Dp315	6	0.375	0.800*	0.221	5	0.174	0.573*	0.246
Dp336	1	0.000	0.000	—	1	0.000	0.000	—
Dp504	6	0.583	0.810	0.165	6	0.522	0.614	0.077
Dp506	5	0.583	0.605	0.008	5	0.652	0.780	0.081

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; Null = null allele frequency estimate.

* Indicates significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.05$).

CONCLUSIONS

We developed 10 microsatellite markers for *D. jezoensis* using a next-generation sequencing approach with the Ion PGM system. The developed markers will contribute to studies of the evolution of gynodioecy by facilitating estimation of the mating system (e.g., selfing ability and levels of pollen limitation), gene flow (e.g., pollen and seed dispersal), and population structure (e.g., spatial genetic structure) of this species.

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Appendix 1. Voucher information for *Daphne jezoensis*. All vouchers are deposited at the herbarium of Sugadaira Montane Research Center, University of Tsukuba, Japan.

Population	Location	Geographic coordinates	Voucher no.
Chitose	Uenae, Chitose City, Hokkaido, Japan	42.717°N, 141.733°E	00697–00699
Nopporo	Nopporo Forest Park, Ebetsu City, Hokkaido, Japan	43.017°N, 141.517°E	00700–00702

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