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Development and characterization of 21 microsatellite markers in *Daphne kiusiana*, an evergreen broad-leaved shrub endemic to Korea and Japan

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ABSTRACT: Microsatellite markers were isolated for *Daphne kiusiana* var. *kiusiana* (Thymelaeaceae), an evergreen broad-leaved shrub endemic to Korea and Japan. Because its populations in Jeju Island are morphologically controversial, and consistently threatened by anthropogenic pressures, taxonomic delimitation and conservation effort are required at the genetic level. We developed 21 polymorphic microsatellite loci from Next Generation Sequencing data. The primer set included di-, tri-, and tetra-nucleotide repeats. Variability in the markers was tested for 80 individuals of *D. kiusiana* from three natural populations in Jeju Island and Japan. Among the 21 loci, three were unavailable for population JKJU of Japan. The Neighbor-Joining tree based on microsatellite markers described here classified the three populations into two groups according to geographical or morphological traits. These will be a powerful genetics tool for determining the taxonomic boundary and establishing suitable conservation strategies for *D. kiusiana* in Jeju Island.

Keywords: conservation, *Daphne kiusiana*, Jeju Island, microsatellite markers, taxonomic entity

Approximately 2,000 islands are found in a narrow band along the southern coastal region of Korean Peninsula including Jeju Island (Yang, 2013). This edge zone is disjunctively located on the northwestern margin of an equivalent climatic area of Japan, and is covered by various evergreen broad-leaved tree species (Satake et al., 1989; Lee and Choi, 2010). Thus, Korean populations of a given species may have an important evolutionary property (J.-H. Lee et al., 2013; Lee et al., 2014) that has guaranteed their long-term survival in East Asia (Frankham, 2005; Ouborg et al., 2006; Willi et al., 2006).

Daphne kiusiana Miq. var. *kiusiana* is an evergreen broad-leaved shrub endemic to Korea (mostly on Jeju Island) and Japan. However, plants on Jeju Island are sometimes regarded as a different species with *D. kiusiana* in Japan because of several unique morphological traits, e.g., a glabrous calyx and lobes, and elliptical leaves (c.f., *D. jejudoensis* M. Kim) (J. Lee et al., 2013). Therefore, a clearer boundary of taxonomic delimitation is needed. Moreover, natural populations in Korea are threatened by anthropogenic pressures, i.e., disturbances that have caused habitat destruction due to urbanization as well as over-collecting of specimens for commercial purposes (Ro et al., 2010). For example, because of its graceful shape (resembling a bride's bouquet) and pleasant fragrance, it is commonly used as an ornamental (Ro et al., 2010). Populations there are now extremely restricted to several islands, including Geoje and Jeju, where plants are being managed as endangered organisms (Korea National Arboretum, 2008). Despite these protective measures, human activity continues to threaten their existence. Therefore, we developed a set of polymorphic microsatellite markers from *D. kiusiana* to resolve this debate about the taxonomic entity of Jeju populations and establish a suitable conservation strategy for Korean populations based on genetic diversity and structure.

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Materials and Methods

To acquire our microsatellite library, we used a DNeasy Plant Mini Kit (Qiagen, Seoul, Korea) to extract genomic DNA from a fresh leaf of a single plant sampled at Sinpyeonggotjawal, Jeju Island, Korea (Table 1). Measurements were made with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Applying a Next Generation Sequencing (NGS) approach, we sequenced high-quality DNA (concentration: 46 ng/μL; A260/280 = 1.73; A260/230 = 1.64) using the Illumina MiSeq platform (LAS, Seoul, Korea). In all, 4,936,656 reads (2 × 300 bp) were produced by Illumina paired-end sequencing and then trimmed by Trimmomatic 0.32 (Bolger et al., 2014).

To identify the microsatellites from those reads, we screened them with SSR pipeline v. 0.951 (Miller et al., 2013). The parameters were set for detection of di-, tri-, or tetra-nucleotide motifs with flanking regions larger than 50 bp and having at least 10, 6, or 4 repeats, respectively. From this screening, we acquired 28,495 sequences that were then re-filtered in an attempt to reference-map all of the reads to each microsatellite that contained a singleton. This was accomplished with Geneious R7.1.8 (Biomatters; available from http://www.geneious.com). After discarding putative multi-copy loci with exceptionally high coverage, we selected fragments with unique patterns that had two separate alleles, few variations at the site to which a primer was attached, and no additional single nucleotide polymorphisms in the flanking region. Finally, we designed 140 primer pairs with Primer3 in Geneious R7, and synthesized with a taq sequence (5′-CACGACGTTGTAAAACGAC-3′) to enable incorporation with M13 primer labeled at the 5'-end with a 6-FAM fluorescent dye.

To test the effectiveness of the developed microsatellite loci, we collected 80 individuals of *D. kiusiana* from three natural populations in Korea and Japan (Table 1). To minimize the damage to this study species, only one plant per population was taken to prepare a voucher specimen that was then deposited in the herbarium at the Korea Institute of Oriental Medicine (KIOM). The DNA was extracted as described above, and PCR was conducted with a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), using a final volume of 10 μL that comprised 15 to 20 ng of extracted DNA, 5 μL of Solg™ 2× Taq PCR Smart-Mix I (Solgent, Daejeon, Korea), 0.01 μM forward primer, 0.2 μM reverse primer, and 0.1 μM of the M13 primer (fluorescently labeled). The PCR amplifications were performed under the following conditions: initial denaturation at 95 °C for 2 min; then 30 cycles of denaturing at 94 °C for 30 s, annealing at 53 to 58 °C for 45 s, and extension at 72 °C for 45 s; with a final extension at 72 °C for 7 min. Without dilution of fluorescently labeled PCR products, 1 μL was analyzed concurrently with the GeneScan™-500LIZ™ Size Standard (Applied Biosystems) on an ABI 3730XL sequencer (Applied Biosystems). Allele sizes were manually determined three times with GENEMAPPER 3.7 software (Applied Biosystems). The number of alleles plus values for H_E (expected heterozygosity) and H_O (observed heterozygosity) were obtained with GenAlEx 6 (Peakall and Smouse, 2006). Deviations from Hardy–Weinberg equilibrium (HWE) was estimated with GENEPOP 4.0 (Rousset, 2008).

To determine the genetic association among the three populations, we quantified their relationship using a proportion of shared alleles (D_{ps}) based on 19 loci. Only DKi134 and DKi140 were excluded. The pair-wise genetic distance (D_{ps}) was generated with MSA software (Dieringer and Schlöterer, 2003) using a bootstrap analysis of 1,000 replicates. From these distance matrices, we constructed 50% consensus trees by the Neighbor-Joining (NJ) method, as implemented in PHYLIP ver. 3.68 (Felsenstein, 2004).

Results and Discussion

Of the developed 140 primer pairs, 21 proved to be polymorphic while the rest either were insufficiently amplified

Table 1. Voucher and location information for *Daphne kiusiana* samples used in this study. One specimen per population was deposited in the herbarium at the Korea Institute of Oriental Medicine (KIOM).

Voucher no.	Locality	Geographic coordinates	No. of individuals
LJH_KIOM-2015-4	Sinpyeonggotjawal, Jeju Island, Korea (KJSI)	33°16′ N, 126°15′ E	1
LJH_KIOM-2015-5	Cheongsugotjawal, Jeju Island, Korea (KJCH)	33°18′ N, 126°16′ E	30
YSG_KIOM-2016-40	Seonheulgotjawal, Jeju Island, Korea (KJSE)	33°31′ N, 126°42′ E	28
LJH_KIOM-2015-58	Juboyama, Kyushu, Japan (JKJU)	33°28′ N, 130°04′ E	22

Table 2. Characteristics of 21 microsatellite loci developed in *Daphne kiusiana.*

Locus	Primer sequence (5′–3′)[a]	Repeat motif	*A*	T_m (°C)	Size range (bp)	GenBank accession No.
DKi019	F: CCTAATGATTTAGACCGCT	$(CT)_{12}$	5	53	206–232	KT932539
	R: TTCTGGTGAGAAACGAAC					
DKi020	F: GTACAGATCTCAATTGGTCT	$(CT)_{13}$	4	53	244–252	KT932540
	R: GATAGCAATCATCCATCAAG					
DKi021	F: CGTTTAGGGTGTAAGTCATA	$(CT)_{16}$	7	53	267–285	KT932541
	R: TACTCGATCCATCCCTATTA					
DKi022	F: CAGAGACAGGATTGAACAT	$(GA)_{13}$	6	53	197–215	KT932542
	R: ATTGGATACGTGTTAGGTC					
DKi023	F: CAGTTGAGGATGTCATATCA	$(GA)_{13}$	6	53	279–293	KT932543
	R: GCCGAATGTGTTATTTGTC					
DKi101	F: AGATGCTGGTTCATGTTCG	$(AC)_{10}$	5	53	179–193	KT932572
	R: GGAACACAGTTGAACGTTG					
DKi104	F: CCTGCGATCTGGGTAATTT	$(AT)_{10}$	4	58	239–251	KT932574
	R: AGGCCACACAACAAGAATC					
DKi062	F: ATCAACAATCTACTCCTGC	$(ATG)_8$	4	53	247–262	KT932553
	R: CACTCCTTCCAGAACTAAG					
DKi070	F: AGAAGATGATGCCAAGATG	$(GAA)_9$	4	58	245–254	KT932557
	R: GGAAGACTTAACGGCTAAT					
DKi072	F: AAGGTGGGAGATTCTGATA	$(GAT)_8$	2	53	199–205	KT932558
	R: GAGCACTAGACAATTCCTT					
DKi073	F: GTCTTAGGGCTAGTGATTC	$(GAT)_9$	3	53	248–263	KT932559
	R: GACCGTCAAGTCTTCTATT					
DKi082	F: TCAAGACCAATATTGCCAG	$(TGA)_8$	4	53	183–201	KT932564
	R: CTCCATGAGGTGATTCATT					
DKi117	F: ATAGTTCTTGTGCCACCAC	$(ATA)_6$	2	53	263–266	KT932579
	R: TAGCCAAGTCGTTGTCTTG					
DKi119	F: AAGCACAATCATGGAGCAT	$(CTT)_6$	7	53	172–208	KT932580
	R: ATCGTCAGAACTAGCCTGA					
DKi091	F: TGAGAGACTCAGTACTCTC	$(AGAA)_5$	3	53	309–317	KT932569
	R: ACTACCACTCTCTGTTTCT					
DKi097	F: ACTTGTCCTCAACTTTCTC	$(TTAA)_5$	3	53	242–258	KT932571
	R: TATTTGATGATCAGGTCGC					
DKi128	F: ATAAAGCGGAATGGAGTCG	$(TTGA)_7$	3	53	179–187	KT932585
	R: ACAGTAGTAGGTCCCACAC					
DKi129	F: AGTTCAGACGCTTCAACC	$(CATA)_6$	4	58	296–312	KT932586
	R: CCATCCACAGAGACAGATTC					
DKi131	F: GAAACTCGTCTCTCTTCCTC	$(AGGA)_5$	2	53	199–203	KT932588
	R: GACGACTACCATAGCATAGC					
DKi134	F: AGTGCTTTAATGGAAGGCT	$(CTAT)_4$	2	58	203–223	KT932589
	R: GATCACCTCCTAACCAAGC					
DKi140	F: ATTCACACCCTAGTCGGAT	$(TGTA)_4$	2	53	215–219	KT932590
	R: GAAACAAAGCTGAGCGAAG					

A, number of alleles; T_m, PCR annealing temperature.

[a]All forward primers were M13 (5'-CACGACGTTGTAAAACGAC-3')–tailed at the 5'-end.

Table 3. Genetic parameters of 21 microsatellite loci for separate populations of *Daphne kiusiana* sampled in Jeju Island (KJCH and KJSE) and Japan (JKJU).

Locus	KJCH[a] (n = 30)				KJSE[a] (n = 28)				JKJU[a] (n = 22)			
	A	H_E	H_O	*Sr*	*A*	H_E	H_O	*Sr*	*A*	H_E	H_O	*Sr*
DKi019	2	0.500	0.467	216–218	2	0.436	0.429	216–218	3	0.458	0.136*	206–232
DKi020	2	0.095	0.100	244–248	2	0.035	0.036	248–250	3	0.241	0.273	248–252
DKi021	5	0.537	0.600	275–285	4	0.198	0.143*	275–285	2	0.434	0.000*	267–269
DKi022	3	0.493	0.467	197–213	3	0.601	0.643*	197–201	2	0.351	0.091*	211–215
DKi023	3	0.443	0.300	287–293	2	0.459	0.571	287–291	3	0.507	0.091*	279–283
DKi101	3	0.259	0.200*	179–183	2	0.163	0.107	179–181	2	0.351	0.091*	189–193
DKi104	3	0.509	0.433	245–251	2	0.226	0.185	245–249	1	0.000	0.000	239
DKi062	2	0.498	0.467	259–262	2	0.219	0.250	259–262	2	0.044	0.045	247–256
DKi070	3	0.513	0.700	245–254	2	0.494	0.464	245–251	1	0.000	0.000	248
DKi072	2	0.444	0.467	199–205	2	0.293	0.286	199–205	1	0.000	0.000	205
DKi073	3	0.496	0.433*	248–263	2	0.436	0.357	248–254	1	0.000	0.000	248
DKi082	2	0.064	0.067	195–198	3	0.070	0.071	195–201	1	0.000	0.000	183
DKi117	2	0.339	0.233	263–266	2	0.484	0.464	263–266	1	0.000	0.000	263
DKi119	4	0.585	0.567	172–208	4	0.284	0.321	178–208	3	0.433	0.545	193–199
DKi091	2	0.406	0.433	313–317	2	0.357	0.393	313–317	1	0.000	0.000	309
DKi097	2	0.278	0.133*	242–250	2	0.316	0.250	242–250	1	0.000	0.000	258
DKi128	3	0.415	0.367	179–187	2	0.497	0.357	183–187	2	0.044	0.045	179–183
DKi129	2	0.206	0.233	308–312	1	0.000	0.000	308	2	0.397	0.545	296–304
DKi131	2	0.480	0.267*	199–203	2	0.499	0.464	199–203	1	0.000	0.000	203
DKi134	2	0.444	0.333	203–223	2	0.494	0.607	203–223	0	*N/A*	*N/A*	*N/A*
DKi140	2	0.095	0.100	215–219	1	0.000	0.000	219	0	*N/A*	*N/A*	*N/A*

A, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; *Sr*, size range; *N/A*, unavailable PCR products.
*Significant deviations from Hardy-Weinberg equilibrium ($P < 0.05$).
[a]Population symbols are the same as those used in parentheses for Table 1.

or else produced inconsequential peaks (Table 2). Overall, the alleles numbered 2 to 7 (average of 3.90). Values for H_E and H_O ranged from 0.000 to 0.601 and from 0.000 to 0.700, respectively (Table 3). Among the 21 loci, two and nine were monomorphic in Jeju population KJSE and Japanese population JKJU, respectively. Loci DKi134 and DKi140 were not amplified in the Japanese population. Furthermore, nine polymorphic and monomorphic loci clearly did not overlap between Jeju Island and Japan within the size range of those alleles (Table 3). Although eight loci showed significant deviations from Hardy–Weinberg equilibrium, this was not consistent across populations. The NJ tree classified the three populations into two groups according to geographical or morphological traits (Jeju Island vs. Japan) with a remarkably high bootstrap value of 100 (Fig. 1). The microsatellite markers described here will be a powerful genetics tool for elucidating the taxonomic entity of Jeju populations, and in the organization of conservation programs planned to reduce inbreeding via artificial crossbreeding and to minimize loss of genetic variability in Jeju populations. Also, we expect that

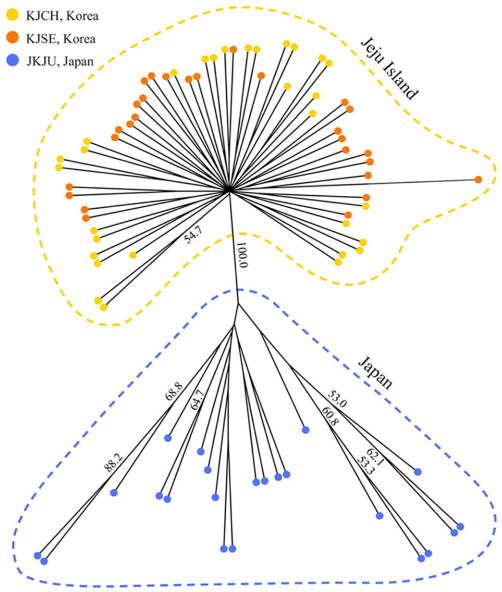

Fig. 1. Neighbor-Joining tree for 3 populations of *D. kiusiana* in Jeju Island and Japan based on proportion of shared alleles (D_{ps}). Colored circles on branch tips indicate number of individuals for each population. Bootstrap support at internodes is shown if value is >50 %.

they will also improve our understanding about historical and contemporary gene flow, not only on a fine scale within Korea but also on a larger scale across the entire range of this species.

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