Applications in Plant Sciences

PRIMER NOTE

Development of 26 microsatellite markers in *Bupleurum latissimum* (Apiaceae), an endangered plant endemic to Ulleung Island, Korea

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PREMISE OF THE STUDY: To enhance our understanding of evolutionary consequences and to establish a suitable conservation strategy, we isolated microsatellite markers for the endangered *Bupleurum latissimum* (Apiaceae), which is endemic to the oceanic Ulleung Island. We also attempted cross-amplification in *B. euphorbioides* and *B. longeradiatum* to investigate its continental progenitors.

METHODS AND RESULTS: Using high-throughput sequencing data, we developed 26 polymorphic microsatellite loci in three multiplexes, of which 13 loci were polymorphic in the two related species. For *B. latissimum*, alleles numbered two to four and the observed and expected heterozygosity ranged from 0.000 to 0.500 and 0.061 to 0.529, respectively.

CONCLUSIONS: These developed markers will be useful for understanding evolutionary patterns of *B. latissimum* in an oceanic island system and for establishing suitable conservation strategies at the genetic level.

KEY WORDS Apiaceae; *Bupleurum latissimum*; conservation; microsatellites; speciation; Ulleung Island.

Ulleung Island, Korea, was formed by volcanic eruption approximately 1.5 million years ago (Xu et al., 1998; Kim et al., 1999; Song et al., 2006) and is located on the East Sea (Sea of Japan), 137 km from the Korean Peninsula. Most of its endemic species were derived through anagenetic speciation from continental progenitors (Korea and/or Japan) at a frequency that is the highest among the world's oceanic islands (Stuessy et al., 2006). The plants of Ulleung Island have long been of great interest to researchers who focus on aspects other than the typical cladogenetic model of evolution (Takayama et al., 2012, 2013; Stuessy et al., 2014). Although several studies were conducted using RAPDs (Ku et al., 2004) and ITS (Kim et al., 2012) for our target species, no clear information is yet available about the evolutionary history of the species on Ulleung Island, including the formation of island vegetation and patterns of speciation.

Bupleurum latissimum Nakai (Apiaceae) is a perennial herb endemic to Ulleung Island. This species is closely related morphologically to *B. euphorbioides* Nakai and *B. longeradiatum* Turcz. However, whereas the involucres and involucels are ovate or broadly ovate for *B. latissimum* and *B. euphorbioides*, they are linear or linear-lanceolate for *B. longeradiatum* (Kim and Yoon, 1990). Although we can speculate that *B. latissimum* has evolved anagenetically from the source populations, especially the Korean endemic *B. euphorbioides*, their evolutionary relationship is still unresolved, and an association with *B. longeradiatum* is also controversial. Moreover, populations of *B. latissimum* are now extremely restricted to a few habitats on the island where they are now being protected as endangered plants (Ministry of the Environment of Korea, 2012). Here, we describe the development of a set of polymorphic microsatellite markers from *B. latissimum* to enhance our understanding of evolutionary consequences in an ideal environmental model, i.e., Ulleung Island. Our goals were to establish a suitable conservation strategy at the genetic level and to attempt cross-amplification with its related species, *B. euphorbioides* and *B. longeradiatum*.

METHODS AND RESULTS

To produce high-throughput sequencing data, we obtained a fresh leaf sample of *B. latissimum* from Ulleung Island and extracted its genomic DNA with a DNeasy Plant Mini Kit (QIAGEN, Seoul, Korea) according to the manufacturer's protocol. A library was developed using the Illumina MiSeq platform (LAS Inc., Seoul, Korea)

to generate 300-bp paired-end reads. SSR_pipeline version 0951 (Miller et al., 2013) was used to screen di-, tri-, and tetranucleotide motifs with flanking regions larger than 100 bp and a minimum of 10, six, and four repeats, respectively. From the 5,702,505 paired-end reads that were sequenced, we detected 161,801 microsatellite loci. The raw reads were then deposited in the National Center for Biotechnology Information's GenBank database (GenBank BioProject number: PRJNA407690). To achieve loci with low copy numbers, we assembled the filtered reads using Geneious R 10.1.3 (Biomatters Ltd., Auckland, New Zealand) following the method of Cho et al. (2015). For the final selected reads, we designed 54 primer pairs with Primer3 in the Geneious program and added three

TABLE 1. Characterization of 26 microsatellite loci for *Bupleurum latissimum*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	*A*	Allele size range (bp)	Fluorescent label	GenBank accession no.
BuL008	F: TACCCATGAAATTCCTCTGC R: AGTCCCATTTGATTAAGAACCT	$(AC)_{13}$	2	166–170	NED	KY940221
BuL010	F: CAGCTCCCAATTGATATTTCA R: CTTACCCTTCCTACATCCCT	$(AC)_{13}$	2	122–124	6-FAM	KY940222
BuL012*	F: GGTTCAACAACTACAAATGTA R: CAGGGGATGAATAGCTCTTT	$(AG)_{15}$	3	234–240	6-FAM	KY940223
BuL015	F: CCCCTTTAATGGGTAGCCC R: CCATTTGGTAAAAGCATTCAG	$(CA)_{12}$	2	236–238	VIC	KY940224
BuL016	F: AAAAACAGCACATGCATTCA R: GCAGGATCTTTGGTCATTGT	$(GA)_{16}$	2	133–135	NED	KY940225
BuL020	F: ACTCCCTCATGGTTGACATT R: CCCATCTATCAAATCCCCAC	$(TG)_{13}$	2	166–170	6-FAM	KY940227
BuL045	F: ATACGTACCCTAGCAAATGC R: TCTCACGGATCTACCAATTG	$(TAA)_{9}$	2	219–222	NED	KY940240
BuL049	F: CTGAAGTGGTGATGGTAAGA R: ACACTAAATAGAGGATGTGGG	$(GAT)_{9}$	2	127–130	VIC	KY940242
BuL050	F: TGACAACAGAACCACTTTTT R: AGTTTGCTGAATTATGAAATCA	$(TGA)_{9}$	2	176–179	VIC	KY940243
BuL002	F: AATATGCACAATCAATATTGCA R: CAGACTGATGAGCTAGCTAC	$(GA)_{12}$	4	157–167	6-FAM	KY940219
BuL007	F: GATAGAGTTTCCACTTTACAGC R: TAGAAAACAAAAGGGTTGGC	$(AC)_{12}$	2	121–123	NED	KY940220
BuL018	F: ACACACACAAATCTGATAGT R: CATAGAGGTGGCTTCTTCAT	$(GA)_{11}$	2	239–249	NED	KY940226
BuL024*	F: CACATGTTCTTGATTCCACA R: ACTTTGTCTTTATTATTGCGTT	$(GT)_{13}$	2	220–226	VIC	KY940229
BuL026	F: GCGAAATCGTATTGCATTCT R: GGTTCAACAACTACAAATGTA	$(CT)_{15}$	3	173–179	NED	KY940230
BuL030	F: TTATCTATCTGCATGTCAAGG R: TGTCTCTAGACTCTTTTTGCA	$(CT)_{11}$	2	230–232	6-FAM	KY940232
BuL041	F: CTAATAATTTGGGCATCAGCA R: AGAAATAGGGGAGAAAACGC	$(ATT)_{11}$	3	161–167	VIC	KY940237
BuL046	F: AATTCTCTTCTCCTGTCTGC R: GGACCCAAATGATGATGATG	$(ATT)_{9}$	2	123–126	6-FAM	KY940241
BuL021	F: ATCCATGGTTTGGTGTGAAT R: AACTTGCATATACATTTGGCT	$(GT)_{12}$	2	228–230	6-FAM	KY940228
BuL027*	F: CTGACGCAAGCTGTAACA R: TCTTCCAAAATTGTCCACCT	$(CT)_{12}$	2	230–236	NED	KY940231
BuL032	F: CCTGCTCCTAAGGATAGAGT R: CTCTGCCATGTACATACCATA	$(GT)_{13}$	2	170–174	VIC	KY940233
BuL037	F: GAGAATGTGAGTGAATTTGAGA R: TGCTGATCAGACTCCTAAAC	$(GCA)_{7}$	2	110–113	6-FAM	KY940234
BuL038	F: TGGAGATGATAGTTAATCTACG R: ACTCTATTTTCTGATCCAGTTT	$(CTT)_{8}$	3	163–169	6-FAM	KY940235
BuL040	F: AGAAAGAGTTACAGAGACTTGT R: GCTTGATCAATTGCTCCAAA	$(TGA)_{9}$	2	116–119	VIC	KY940236
BuL042	F: ATTTGGGTGAAATTTGTGCA R: TCGGAATTTGGCAGAAACTA	$(CAT)_{9}$	2	212–215	VIC	KY940238
BuL043	F: GGGTTTCCGTACATCTGTAA R: TCGAAGACGAACTCTTTCAA	$(ATA)_{9}$	2	117–120	NED	KY940239
BuL053	F: TGTGATGTAATGTTTGTGTGT R: CCTTCTGGGCTACAATAACA	$(AGA)_{8}$	2	179–182	NED	KY940244

Note: A = number of alleles.
[a]The reaction concentrations in PCR for primers were 0.01 for the forward primer and 0.2 for the reverse primer. Loci marked with an asterisk had reaction concentrations of 0.02 for the forward primer and 0.4 for the reverse primer.

sets of M13 tag sequences (5′-CACGACGTTGTAAACGAC-3′, 5′-TGTGGAATTGTGAGCGG-3′, and 5′-CTATAGGGCACGCGTGGT-3′) on the forward primer with 6-FAM, VIC, and NED fluorescent dye, respectively. To assess the effectiveness of these microsatellite markers, we collected 16 individuals of *B. latissimum* from Ulleung Island. Because a few individuals remain in some continuous locations, we could not artificially subdivide the group. We considered our sample size to be sufficiently representative of all extant individuals for *B. latissimum*. Cross-species amplification was also tested by sampling 13 individuals of the related species *B. euphorbioides* from Gyeongsangnam Province and 12 of *B. longeradiatum* from Gangwon Province in Korea (Appendix 1). We then performed PCR amplifications for validation and genotyping in a final volume of 5 μL that was composed of 15 to 20 ng of extracted DNA, 2.5 μL Multiplex PCR Master Mix (QIAGEN), 0.01 μM forward primer, 0.2 μM reverse primer, and 0.1 μM of the M13 primer (fluorescently labeled). The PCR protocol consisted of an initial denaturation at 95°C for 15 min; followed by 35 cycles of denaturing for 30 s at 95°C, 1.5 min at annealing temperature of 56°C, and extension for 1 min at 72°C; and a final extension at 72°C for 10 min. The PCR products were analyzed on an ABI 3730XL sequencer with GeneScan 500 LIZ Size Standard (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Allele sizes and peaks for each sample were determined with Peak Scanner Software version 2.0 (Thermo Fisher Scientific). Overall genetic parameters, i.e., number of alleles, expected heterozygosity (H_e), and observed heterozygosity (H_o), were evaluated using GenAlEx 6.5 (Peakall and Smouse, 2006). Deviations from Hardy–Weinberg equilibrium (HWE) were estimated with GENEPOP version 4.6.9 (Rousset, 2008).

TABLE 2. Genetic diversity of 26 microsatellites developed for *Bupleurum latissimum* and cross-amplification in *B. euphorbioides* and *B. longeradiatum*.[a]

	B. latissimum (*n* = 16)			*B. euphorbioides* (*n* = 13)			*B. longeradiatum* (*n* = 12)		
Locus	*A*	H_e	H_o[b]	*A*	H_e	H_o[b]	*A*	H_e	H_o[b]
BuL002	4	0.529	0.313**	0	NA	NA	6	0.809	0.667
BuL007	2	0.492	0.125**	1	0.000	0.000	5	0.694	0.500
BuL008	2	0.469	0.125**	3	0.272	0.154	7	0.767	0.667
BuL010	2	0.451	0.438	2	0.260	0.000**	3	0.344	0.083**
BuL012	3	0.314	0.125*	4	0.521	0.154**	0	NA	NA
BuL015	2	0.469	0.375	2	0.142	0.154	8	0.833	0.833
BuL016	2	0.498	0.188*	2	0.500	1.000**	8	0.792	0.500**
BuL018	2	0.061	0.063**	2	0.426	0.308	0	NA	NA
BuL020	2	0.061	0.063**	0	NA	NA	3	0.226	0.250
BuL021	2	0.492	0.125**	1	0.000	0.000	0	NA	NA
BuL024	2	0.305	0.375	0	NA	NA	0	NA	NA
BuL026	3	0.314	0.125*	3	0.518	0.077**	0	NA	NA
BuL027	2	0.170	0.188	2	0.500	1.000**	6	0.653	0.417*
BuL030	2	0.492	0.375	3	0.500	0.769	3	0.226	0.250
BuL032	2	0.469	0.125**	3	0.328	0.231	8	0.795	0.833
BuL037	2	0.117	0.125	1	0.000	0.000	1	0.000	0.000
BuL038	3	0.486	0.313	1	0.000	0.000	0	NA	NA
BuL040	2	0.482	0.313	1	0.000	0.000	2	0.278	0.167
BuL041	3	0.521	0.500	0	NA	NA	0	NA	NA
BuL042	2	0.451	0.438	4	0.660	0.538	0	NA	NA
BuL043	2	0.404	0.313	0	NA	NA	0	NA	NA
BuL045	2	0.170	0.063	2	0.204	0.231	7	0.701	0.583
BuL046	2	0.482	0.188*	0	NA	NA	0	NA	NA
BuL049	2	0.451	0.438	3	0.531	0.333	4	0.608	0.750
BuL050	2	0.117	0.000*	1	0.000	0.000	7	0.774	0.917
BuL053	2	0.219	0.125	0	NA	NA	6	0.604	0.583

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals sampled; NA = unavailable PCR products.
[a]Locality and voucher information is provided in Appendix 1.
[b]Significant deviations from Hardy–Weinberg equilibrium (*$P < 0.05$ and **$P < 0.01$).

Using the 54 designed primer pairs, we produced 26 polymorphic microsatellite loci with clear, strong bands for each allele in the 16 individuals of *B. latissimum* (Table 1). The number of alleles per locus ranged from two to four (mean of 2.2). Values for H_e and H_o ranged from 0.061 to 0.529 and from 0.000 to 0.500, respectively. Our results from the cross-amplification indicated that 13 loci were successfully amplified and were polymorphic; they displayed one to eight alleles per locus for the two related species (Table 2). After a Bonferroni correction, we found no significant deviation in HWE from the 26 developed markers in *B. latissimum* ($P < 0.0019$). However, four of those loci (BuL012, BuL016, BuL026, and BuL027) showed a significant deviation from HWE in *B. euphorbioides* (Table 2).

CONCLUSIONS

We developed a set of 26 polymorphic microsatellite markers from *B. latissimum* and determined that 13 of the loci were transferable to the related species *B. euphorbioides* and *B. longeradiatum*. The microsatellite markers described here will be a powerful genetic tool for elucidating, across a large scale, the evolutionary pattern of an oceanic island–endemic species with its progenitors. Furthermore, these results will be beneficial for establishing suitable conservation strategies to manage *B. latissimum* as an endangered species on Ulleung Island, Korea.

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APPENDIX 1. Voucher and location information for species used in the development and evaluation of microsatellite markers for *Bupleurum* species.

Taxon	Location	Geographic coordinates	*N*	Voucher no.[a]
B. latissimum Nakai	Taeha-ri, Seo-myeon, Ulleung-gun, Gyeongsangbuk Province, Korea	37°30′25.9″N, 130°49′58.5″E	16	C. Kim 2015-37
B. euphorbioides Nakai	Mt. Namdeogyu, Sojeong-ri, Buksang-myeon, Geochang-gun, Gyeongsangnam Province, Korea	35°50′10.7″N, 127°47′25.1″E	13	KSC1408980-2
B. longeradiatum Turcz.	Mt. Daeam, Wolhak-ri, Buk-myeon, Inje-gun, Gangwon Province, Korea	38°10′14.3″N, 128°10′19.7″E	12	J. Kim 2015-14d

Note: N = number of individuals.
[a]All vouchers are stored at the Gachon University Herbarium (GCU), Seongnam, Korea.