Development of Microsatellite Markers for the Endangered Pedicularis ishidoyana (Orobanchaceae) Using Next-Generation Sequencing

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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR THE ENDANGERED *PEDICULARIS ISHIDOYANA* (OROBANCHACEAE) USING NEXT-GENERATION SEQUENCING[1]

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- *Premise of the study:* Microsatellite primers were developed for *Pedicularis ishidoyana* (Orobanchaceae), an endangered and hemiparasitic plant that is narrowly endemic to Korea. Because its populations are threatened by loss of suitable habitat, conservation efforts are required.
- *Methods and Results:* We developed polymorphic microsatellite loci through reference mapping of 300-bp paired-end reads obtained from Illumina MiSeq data. In all, 74 primer pairs were designed and 32 were amplified. Of these, 18 pairs were polymorphic, with two to six alleles each occurring in 26 individual plants. Expected and observed heterozygosities ranged from 0.142 to 0.703 and from 0.077 to 0.615, respectively.
- *Conclusions:* These microsatellite markers are expected to be useful for studies of the population genetics of *P. ishidoyana*.

Key words: endangered species; microsatellite; next-generation sequencing (NGS); Orobanchaceae; *Pedicularis ishidoyana*.

The genus *Pedicularis* L. (Orobanchaceae) comprises approximately 600 species of root-hemiparasitic plants that are distributed mostly in high-latitude or alpine habitats of the Northern Hemisphere (Yang et al., 1998). Within the traditional family Scrophulariaceae, *Pedicularis* was originally placed in tribe Rhinantheae Benth. Since then, this genus, along with other hemiparasitic rhinanthoids, has been transferred to Orobanchaceae based on molecular evidence and pollen morphology (Minkin and Eshbaugh, 1989; dePamphilis et al., 1997; Young et al., 1999; Olmstead et al., 2001). It is characterized by its diversification of corolla morphology, including variations in the galea (beaked, curved, toothed, or crested) and the length of the corolla tube, as a result of adaptive radiation (Li, 1951). *Pedicularis ishidoyana* Koidz. & Ohwi is a Korean endemic that is distinguished from its congeners by its long pedicels and undeveloped stems. This species is listed as Vulnerable (VU) in the Korea Red Data Book (Ministry of the Environment of Korea, 2012). As of 2012, it is also protected under the Endangered Species Act within Korean law. Populations are restricted to fewer than 10 locations in the lowlands of cool valleys, and are threatened by anthropogenic disturbances such as waterfront development. *Pedicularis ishidoyana* is considered a potentially important natural resource for medicinal products. The genus *Pedicularis* is known as "pseudo-ginseng" and is used for traditional medicines in East Asia. New pharmaceutical iridoids have also been discovered in its congener *P. artselaeri* Maxim. (Su et al., 1998). As part of the effort to preserve these threatened plants, we developed microsatellites using next-generation sequencing technology so that they can serve as valuable molecular tools for understanding population dynamics based on genetic diversity.

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METHODS AND RESULTS

We collected 26 individuals of *P. ishidoyana* from a natural population at Mt. Geomma, Gyeongbuk, Korea, and deposited voucher specimens in the Inha University herbarium (IUI), Incheon, Korea (voucher no. Cho.105024; 36°43′27″N, 128°14′52″E). Whole genomic DNA was extracted from silica gel–dried leaf tissues by a protocol that used the DNeasy Plant Mini Kit (QIAGEN, Seoul, Korea). Measurements were made with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA). High-quality DNA (concentration 186 ng·μL^{-1}; A260/280 = 1.87; A260/230 = 2.3) was sequenced using the Illumina MiSeq platform (BML Co., Daejeon, Korea).

A total of 17,537,200 reads (300 × 300) were produced by Illumina paired-end sequencing and then trimmed and read by Trimmomatic 0.32 (Bolger et al., 2014). To identify the microsatellites from those reads, we screened them using SSR_pipeline version 0.951 (Miller et al., 2013). The parameters were set for detection of di-, tri-, tetra-, and pentanucleotide motifs with flanking regions larger than 40 bp and a minimum of 10, seven, five, and four repeats, respectively. In all, we found 63,531 microsatellite loci meeting the above criteria. Primer pairs were designed with Primer3 in Geneious R7 (Biomatters, available from http://www.geneious.com) and labeled via the M13 sequence tag method. To design primers efficiently, we attempted reference mapping of total reads to each microsatellite-containing singleton using Geneious R7. After discarding putative multicopy 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 (SNPs) in the flanking region. For the 26 tested individuals of *P. ishidoyana*, we designed 74 primer pairs and successfully amplified 32 of them.

We conducted PCR with 10-μL reaction volumes containing 5 μL of 2× PCR *Plus* Mix (400 μM dNTP, 4 mM $MgCl_2$, 0.4 units of *Taq* DNA polymerase), 10 ng of DNA, 0.01 μM forward M13(–21)-tagged primer, 0.1 μM reverse primer, and 0.1 μM M13(–21)-labeled fluorescent marker (NED, PET, VIC, 6-FAM). Reactions were performed in a GeneAmp PCR System 2700 Thermal Cycler (Applied Biosystems, Foster City, California, USA) under the

TABLE 1. Characteristics of 18 microsatellite markers developed in *Pedicularis ishidoyana*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	T_a (°C)	Allele size range (bp)	5′ end-labeled dye	GenBank accession no.
Pi005	F: CGAAGGTTAGTGCATTGCTATG	$(CA)_{14}$	60	393–403	PET	KP274932
	R: TCCGGACCATCACTGGATCT					
Pi033	F: AGATGATTATGCATGGGTAG	$(AG)_{10}$	52	280–284	PET	KP274933
	R: GTACGAACAAGTAGAAGGAT					
Pi034	F: CGAATATCACTCAATCAAGC	$(CA)_{10}$	52	189–191	NED	KP274934
	R: TTCGAACACAAGATCTCTAG					
Pi040	F: CAGTTATTCCCAATTGTAGC	$(TC)_{12}$	52	318–332	VIC	KP274935
	R: CGGAGACTTGGTTAAGAATA					
Pi043	F: AAATTTCCTCGATCTAGCC	$(GA)_{11}$	52	315–325	PET	KP274936
	R: GCTCCACAACCAATAATAAC					
Pi048	F: TTGTCCTCTCTTCCTAGTAA	$(CA)_{10}$	52	218–222	PET	KP274937
	R: GGTTGTCAACTATCCTTGTA					
Pi049	F: TTCAGAAATATGTTGACGGG	$(GA)_{12}$	52	254–266	NED	KP274938
	R: CACTTATGACCGCTGATATA					
Pi051	F: CTGTTTCCTTCATATTCAGC	$(TG)_{11}$	52	220–228	VIC	KP274939
	R: CTTAAATAAATGGGTCTCCC					
Pi053	F: GAATAGTTGGGTGGTTGAT	$(AC)_{10}$	52	193–203	FAM	KP274940
	R: CTTGAATTTGTACACAAGGG					
Pi055	F: GCGGCTATTAAAGTAAACTG	$(GA)_{18}$	52	286–304	PET	KP274941
	R: GAACACGTGTCAATCATCT					
Pi056	F: CCGAGAATAGCATCATTCTT	$(TC)_{10}$	52	238–240	NED	KP274942
	R: CGTGAGATACTTTGAGGAAA					
Pi057	F: ATACAGTCACCAAGAATAGG	$(GA)_{13}$	52	290–300	NED	KP274943
	R: TGACTCGAGGTTAATTATCC					
Pi060	F: CAGCAGAGTATTTGTGTTAC	$(CA)_{10}$	52	231–235	FAM	KP274944
	R: GGATGATCGGAGAAATGATA					
Pi061	F: AGGTTATAAAGAACGGTGAG	$(TC)_{18}$	52	276–288	FAM	KP274945
	R: CAGACTTAATCAGCAGGATA					
Pi063	F: CTAATCTGCTCATGCTTAGA	$(AG)_{11}$	52	200–222	FAM	KP274946
	R: GATATTGGATGACACACAGA					
Pi064	F: CCCAATTAAGAGTGAAGAGT	$(AC)_{10}$	52	178–186	PET	KP274947
	R: CAAGAAGAAATTGAGAGGTC					
Pi065	F: CCAGAGTAAACTTGTTTGAC	$(TC)_{13}$	52	268–284	VIC	KP274948
	R: AAATGGTAATTACGGAGCTC					
Pi073	F: GGAAACAAACACAAATGGG	$(AC)_{19}$	52	237–257	PET	KP274949
	R: TTGGTTTGAGATGGGATTC					

Note: T_a = PCR annealing temperature.
[a]All forward primers were M13-tailed (5′-TGTAAAACGACGGCCAGT-3′) at the 5′ end.

following conditions: denaturation at 94°C for 2 min; then 35 cycles at 94°C/30 s, 52°C to 60°C/1 min, and 72°C/1 min; and a final extension at 72°C for 7 min. Fluorescently labeled PCR products were resolved to genotype on an ABI 3730XL sequencer with GeneScan 500 LIZ Size Standard (Applied Biosystems). Sizes were determined with GeneMapper 3.7 (Applied Biosystems) and Geneious R7. Null allele frequencies were calculated by MICRO-CHECKER version 2.2 (van Oosterhout et al., 2004). The number of alleles plus values for expected heterozygosity (H_e) and observed heterozygosity (H_o) were determined in GenAlEx 6 (Peakall and Smouse, 2006). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were estimated with GENEPOP 4.0 (Rousset, 2008).

Finally, 18 primer pairs proved polymorphic while the remaining 14 either were monomorphic or produced inconsequential peaks (Table 1). Alleles per locus numbered two to six (average 3.72), while values for H_e and H_o ranged from 0.142 to 0.703 and from 0.077 to 0.615, respectively (Table 2). Due to heterozygote deficiencies, two loci (Pi040 and Pi073) significantly deviated from HWE values after Bonferroni correction ($P < 0.05$). No significant LD was detected among locus pairs at the population level ($P > 0.05$).

TABLE 2. Genetic properties of 18 newly developed, polymorphic microsatellites for *Pedicularis ishidoyana*.[a]

Locus	*n*	*A*	H_e	H_o
Pi005	26	4	0.643	0.615
Pi033	26	3	0.421	0.462
Pi034	26	2	0.464	0.500
Pi040	26	4	0.590	0.462*
Pi043	26	5	0.533	0.500
Pi048	26	2	0.260	0.154
Pi049	26	3	0.615	0.615
Pi051	26	3	0.452	0.423
Pi053	26	4	0.624	0.615
Pi055	26	5	0.520	0.423
Pi056	26	2	0.142	0.077
Pi057	26	3	0.144	0.154
Pi060	26	3	0.301	0.269
Pi061	26	6	0.619	0.615
Pi063	26	4	0.456	0.615
Pi064	26	3	0.530	0.462
Pi065	26	5	0.703	0.615
Pi073	26	6	0.699	0.538*

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals.
[a]Population locality: Mt. Geomma, Gyeongbuk, Korea (36°43′27″N, 128°14′52″E) (voucher no. Cho.105024 [IUI]).
*Significant deviation from HWE after correction for multiple tests ($P < 0.05$).

CONCLUSIONS

In conclusion, we developed 18 microsatellite markers for the endangered *P. ishidoyana*. These markers will be informative tools for investigating genetic structure and diversity among populations of this species, and will help facilitate effective strategies for its conservation. They will also be useful in future studies to increase understanding of the phylogeographic

history of the species based on gene flow and spatial genetic patterns.

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