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

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DEVELOPMENT OF MICROSATELLITE MARKERS FOR THE ENDANGERED Pedicularis ishidoyana (Orobanchaceae) USING NEXT-GENERATION SEQUENCING

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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 rhizanths, 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 water-front 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.

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 (QIA-GEN, 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 Trimomatic 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 MgCl₂, 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

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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 genotypes on an ABI 3730XL sequencer with GeneScan 500 LIZ Size Standard (Applied Biosystems). Locus Primer sequences (5′-3′) Repeat motif \(T_a\) (°C) Allele size range (bp) 5′ end-labeled dye GenBank accession no.

| Locus | Primer sequences (5′-3′) | Repeat motif | \(T_a\) (°C) | Allele size range (bp) | 5′ end-labeled dye | GenBank accession no. |
|-------|--------------------------|--------------|-------------|-----------------------|-------------------|---------------------|
| Pi005 | F: CGAAGGTTATGCGATCTGATG | \((CA)_{14}\) | 60 | 393–403 | PET | KP274932 |
| Pi033 | R: TACGGACATTCACAGTCAGTCT | \((AG)_{10}\) | 52 | 280–284 | PET | KP274933 |
| Pi034 | F: CGAATATCACTCATATCAACGA | \((CA)_{10}\) | 52 | 189–191 | NED | KP274934 |
| Pi040 | R: TTCAGAACAAGATCTGCTTAC | \((TG)_{12}\) | 52 | 318–332 | VIC | KP274935 |
| Pi043 | F: ATATATTGTGATGGATGATATA | \((TG)_{11}\) | 52 | 315–325 | PET | KP274936 |
| Pi048 | R: GCTCCACAACCTTAATTAACAC | \((GA)_{10}\) | 52 | 218–222 | PET | KP274937 |
| Pi049 | F: TTTGGCTCTCTCTTCTATGCTAC | \((GA)_{12}\) | 52 | 254–266 | NED | KP274938 |
| Pi051 | R: CATCTAATGGCCTGATATA | \((TG)_{11}\) | 52 | 220–228 | VIC | KP274939 |
| Pi053 | F: GTCTGGTCAAGACATTCTGCTGTA | \((AC)_{10}\) | 52 | 193–203 | FAM | KP274940 |
| Pi055 | R: GGACGATAGTAATGGTACGCA | \((GA)_{18}\) | 52 | 286–304 | PET | KP274941 |
| Pi056 | F: CCAGAAGATAGCATATCTTCT | \((TG)_{10}\) | 52 | 238–240 | NED | KP274942 |
| Pi057 | R: CAGTGGATGTTTGGGAAGAAA | \((TG)_{10}\) | 52 | 290–300 | NED | KP274943 |
| Pi060 | F: CAGCAGAGATTTGTTGTACAG | \((GA)_{13}\) | 52 | 231–235 | FAM | KP274944 |
| Pi061 | R: GGTATGATGGAGATAGTAAAG | \((GA)_{18}\) | 52 | 276–288 | FAM | KP274945 |
| Pi063 | F: CCAGATACCACTGACGATGA | \((AG)_{11}\) | 52 | 200–222 | FAM | KP274946 |
| Pi064 | R: GATTGGATGATACTTGAGTC | \((AC)_{10}\) | 52 | 178–186 | PET | KP274947 |
| Pi065 | F: CCAAGATTTACCTGTTGAGC | \((AC)_{13}\) | 52 | 268–284 | VIC | KP274948 |
| Pi073 | R: GGAACAAACACAAATATTGGG | \((AC)_{19}\) | 52 | 237–257 | PET | KP274949 |

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

In conclusion, we developed 18 microsatellite markers for the endangered *Pedicularis 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 distribution of this species.

CONCLUSIONS

In conclusion, we developed 18 microsatellite markers for the endangered *Pedicularis 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 distribution of this species.
history of the species based on gene flow and spatial genetic patterns.

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