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Isolation and characterization of ten microsatellite loci from Korean *Astragalus mongholicus* (Fabaceae)

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Introduction

Astragalus L. (Fabaceae), the largest angiosperm genus in the world, comprises about 3000 species. Members are found primarily in cold to warm arid and semiarid mountainous regions in the northern hemisphere and in South America (Chaudhary *et al*. 2008). *Astragalus mongholicus* Bunge is a perennial herb growing on mountainous grasslands in east Asia. Plants show great morphological variation and have been separated into geographical varieties (Chen and Zhu 1990; Xu and Podlech 2010). Although it has been known as *A. membranaceus* Bunge in regional flora (Iwatsuki *et al*. 2001; Li *et al*. 2001; Lee 2003), this name is illegitimate. Instead, it is now treated as a synonym, *A. mongholicus* (Xu and Podlech 2010). As an important pharmacological plant resource, roots from this species (Radix Astragali) contain various beneficial compounds, with properties that are immuno-modulating, memory-improving, anti-ageing, antiviral, anti-rhinoviral, and anti-tumour (Yip and Kwan 2006). In the inland regions of Korea, it is widely cultivated for medicinal purposes (Kim *et al*. 2000).

A. membranaceus var. *nakaianus* Y. N. Lee is an endemic wild plant growing on the subalpine grasslands of Mt Halla on Jeju island, south Korea (Lee 2003). Because its area of distribution at Mt Halla is narrowly limited to only a few individual plants, this species is listed as 'critically endangered' in the Rare Plants Data Book in Korea (Korea National Arboretum 2008). It was previously described as a distinct species (*A. nakaianus* Y. N. Lee), separated from *A. membranaceus* because of its tufted stems and very small leaflets (Lee 1981). After that, it has now been merged into *A. membranaceus*, i.e., *A. membranaceus* var. *nakaianus* (Lee 2006). However, this name is also illegitimate, since a new name is not yet given, we use *A. nakaianus* as the valid name for this plant.

Most studies about this species have focussed on distinguishing it from its adulterants, using methods such as morphological comparisons (Lee and Chung 2004), ribosomal DNA sequences (Ma *et al*. 2000; Yip and Kwan 2006; Gao *et al*. 2010), sequence characterized amplified regions (SCAR) markers (Lim *et al*. 2007; Yang *et al*. 2011), and chemical compound analysis (Ma *et al*. 2002). The power of these approaches to detect differences is low. By contrast, microsatellite markers are widely used in genetic studies to measure intraspecific variability and differentiation due to their high degree of polymorphism (Bowcock *et al*. 1994; Estoup *et al*. 1995). Thus, the aim of our research was to develop a set of polymorphic microsatellite markers for detecting geographical variations by analysing the genetic diversity among or within populations of *A. mongholicus* in east Asia, and for establishing a suitable strategy for conserving wild populations of *A. nakaianus* at Mt Halla.

Materials and methods

Materials included 30 wild *A. nakaianus* plants collected from Mt Halla (33°21′36′N, 126°31′14′E) on Jeju island. In addition, 13 individuals of *A. mongholicus* were sampled inland at Bukdongri of Gangwon-do (37°23′15′N, 128°47′12′E) from field-grown plants of a propagated cultivar.

Using the G-spin™ IIp kit for plants (iNtRON, Seongnam, Korea), we extracted genomic DNA from wild-type plants in Mt Halla population. Microsatellite loci were isolated as per the published enrichment protocol (Hammond *et al*. 1998), but with minor modifications (Lee *et al*. 2010). Briefly, genomic DNA was digested with *Mbo*I (Promega, Madison, USA) and the resulting fragments were ligated to SAUL linkers (Hammond *et al*. 1998) by using T4 DNA ligase (Promega, Madison, USA). Further these fragments were enriched for microsatellites with a cocktail comprising seven biotinylated probes ($(AG)_{15}$, $(AT)_{15}$, $(AC)_{15}$, $(ATG)_{10}$,

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Table 1. Characteristics of 10 microsatellite markers developed in Korean *Astragalus mongholicus*. Wild type was *A. nakaianus*.

Locus	Primer sequences (5′ → 3′)	GenBank accession no.	Repeat motif	T_a (°C)	Size range (bp)	Total N_a	Wild type from Mt Halla ($n = 30$)				Bukdongri cultivar ($n = 13$)			
							N_a	H_o	H_e	P value	N_a	H_o	H_e	P value
Astna1	F: GGAATCTGAATAGACGGGAAA R: CCAGCACTCGTACGCTTTTT	JX507740	$(ATG)_{13}$	54	184–223	10	7	0.433	0.444	0.623	6	0.846	0.638	0.897
Astna2	F: CGAAGGTAGGGGAAACATGA R: AAAAACGAAGCTCCCACTTG	JX507741	$(AG)_{23}$	55	246–286	18	17	0.700	0.935	0.000*	9	0.846	0.843	0.612
Astna3	F: GCGAACTAGAGAGGGGGTTG R: ATGTAGGCACACTGTTCATC	JX507742	$(GA)_{28}$	57	249–283	16	16	0.815	0.930	0.033	–	–	–	–
Astna4	F: CTACACCATCCTTAGACA R: AAGAACAAGACACAGTGC	JX507743	$(TC)_{22}$	60	222–270	16	16	0.867	0.905	0.290	2	0.417	0.432	1.000
Astna5	F: CGCCAGTGTGAGCAAAAG R: CACGCATTCCATCATGTTTC	JX507744	$(GA)_{30}$	60	267–309	19	16	0.519	0.942	0.000*	9	0.385	0.814	0.001*
Astna6	F: CGAGCAGTCTGACCAGGTG R: TCACACTCCCTTTCGCTCTC	JX507745	$(GA)_{27}$	58	186–266	22	16	0.900	0.924	0.326	6	0.462	0.744	0.002*
Astna7	F: ACATGTGCCGTTTGGTCTC R: CCTCTCCATCTCCGAAAACTT	JX507746	$(GA)_{28}$	55	102–134	15	15	0.893	0.923	0.101	9	0.846	0.872	0.146
Astna8	F: GACAGTTCTGACCGCTTGAC R: CTGGTATTCCCGTTGCACAC	JX507747	$(TG)_{26}$	61	337–391	18	16	0.633	0.851	0.000*	3	0.400	0.622	0.089
Astna9	F: TGGTGGATACTCACGTATCCTG R: ATGCAGGCAGTTGACTTTGGGT	JX507748	$(TG)_{25}$	55	222–270	18	17	0.800	0.942	0.046	5	0.385	0.740	0.001*
Astna10	F: TTTGATGGACGCGAAAGGTT R: TGCCACAAAGAGCAACTCAA	JX507749	$(GA)_{23}$	55	232–256	12	11	0.800	0.897	0.301	6	0.385	0.574	0.059

All forward primers were M13 (5′-TGTAAAACGACGGCCAGT-3′)-tailed at the 5′ end. *Significant deviation from Hardy–Weinberg equilibrium (HWE) after correction for multiple tests ($P < 0.005$). P values for HWE tests are given for each marker. T_a, PCR annealing temperature; N_a, number of alleles at population level; H_o, observed heterozygosity; H_e, expected heterozygosity.

$(AGC)_{10}$, $(CAA)_{10}$, and $(ACAT)_7$) that were bound to streptavidin-coated magnetic beads (Promega). This enrichment process was performed twice. The fragments were amplified and cloned using the PCR® 2.1-TOPO® vector (Invitrogen, Carlsbad, USA). After which the clones with 400 to 800 bp inserts were selected for sequencing. In all, 196 clones were subjected to double-stranded DNA sequencing that utilized BigDye Terminator ver. 3.1 and an ABI 3730 xl sequencer (Applied Biosystems, Foster City, USA). We excluded the primers with short repeat regions or short flanking regions, or those that were close to the vector, because they did not fit our criteria. Primer pairs were designed with FastPCR ver. 5.4.51 software (Kalendar *et al*. 2009) from 46 sequences that had microsatellite repeats and sufficient flanking regions. The M13 (-21) (5′-TGTAAAACGACGGCCAGT-3′) sequence-tag method was used to label the primers (Schuelke 2000).

The loci were initially screened using 30 individuals of *A. nakaianus*, and then applied to the *A. mongholicus* cultivar. DNA was extracted according to the method described above, and PCR was performed with a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems). Each reaction mixture contained 200 μM dNTPs (GeneCraft, Lüdinghausen, Germany), 1× PCR buffer with 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (TaKaRa, Seoul, Korea), 10 ng of DNA, and an appropriate concentration of primers in a total volume of 30 μL. The mixture also contained a 0.08 μM forward M13 (-21)-tagged primer, 0.3 μM reverse primer, and 0.3 μM M13 (-21)-labelled 6-FAM fluorescent dyes. PCR conditions included an initial denaturation at 94°C for 2 min; followed by 38 cycles of 94°C for 30 s, 54–61°C for 45 s (annealing temperature depending upon locus; see table 1), and 72°C for 1 min; with a final extension at 72°C for 10 min. Fluorescently labelled PCR products were electrophoresed concurrently with the GeneScan™-500LIZ™ Size Standard (Applied Biosystems) on an ABI 3730xl sequencer. Sizes were determined with GeneMapper ver. 3.7 (Applied Biosystems). Diversity statistics, deviations from Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) were estimated with GenePop ver. 4.0 software (Rousset 2008). Null allele frequencies were calculated by Micro-Checker ver. 2.2.3 (Van Oosterhout *et al*. 2004).

Results and discussion

Of the 46 primer pairs, 36 were excluded because of the PCR amplification failures or difficulties in data interpretation. We produced 10 polymorphic microsatellite loci with clear and strong bands for each allele from the wild *A. nakaianus* plants at Mt Halla. Genotypic data were obtained for the 43 individuals from *A. nakaianus* and the *A. mongholicus* cultivar. Parameters for genetic diversity in each population are presented in table 1. Among these, Astna3 could not be amplified from the inland cultivar. Overall, the alleles numbered 10 to 22 (average of 16.4); their observed and expected heterozygosity (H_o and H_e) ranged from 0.385 to 0.900 and from 0.432 to 0.942, respectively (table 1). Three of the 10 loci (Astna2, Astna5, and Astna8) showed significant deviations within the Mt Halla population, based on the corrected HWE after a Bonferroni correction was made ($P < 0.005$; table 1). All deviations from HWE were due to a heterozygote deficiency. It is plausible that the restricted and fragmented population structure led to a high rate of inbreeding. No significant LD was detected among locus pairs. Detailed loci characterizations and GenBank accession numbers are listed in table 1. These markers will be valuable tools for improving our understanding of the processes for differentiation and discrimination among members within the *A. mongholicus* complex in east Asia. In addition, we have obtained genetic information that will be critical to the conservation of wild-type *A. nakaianus* at Mt Halla.

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