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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS BASED ON EXPRESSED SEQUENCE TAGS IN *ASPARAGUS COCHINCHINENSIS* (ASPARAGACEAE)[1]

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- *Premise of the study:* Transcriptome-derived simple sequence repeat (SSR) markers were developed in *Asparagus cochinchinensis* (Asparagaceae). Due to its application in traditional medicine, its wild populations are threatened by over-collection even in protected areas, requiring immediate conservation efforts.
- *Methods and Results:* Based on transcriptome data of *A. cochinchinensis*, 96 primer pairs with two to seven alleles per locus were selected for initial validation; of those, 27 primer pairs amplified across all samples, resulting in 15 polymorphic and 12 monomorphic microsatellite markers. The usefulness of these markers was assessed in 60 individuals representing three populations of *A. cochinchinensis*. Observed and expected heterozygosity values ranged from 0.050 to 0.950 and 0.049 to 0.626, respectively. Cross-species amplification of the 27 markers was tested in the related species *A. rigidulus* and *A. schoberioides*.
- *Conclusions:* These polymorphic, transcriptome-derived SSR markers can be used as molecular markers to study population genetics and ecological conservation in *A. cochinchinensis* and related taxa.

Key words: Asparagaceae; *Asparagus cochinchinensis*; EST-SSR markers; genetic diversity; medicinal plant.

The genus *Asparagus* L. (Asparagaceae) comprises approximately 200 species distributed worldwide. The genus includes highly valuable plant species that have therapeutic properties and are also consumed as food (Shasnay et al., 2003). *Asparagus cochinchinensis* (Lour.) Merr. is distributed in northeastern Asia (Xiong et al., 2011) and has been used in traditional medicine in Korea and China (Lee et al., 2009). The tuberous roots of this plant have various medicinal properties including anti-inflammatory (Lee et al., 2015), antibacterial, and antipyretic qualities (Samad et al., 2013). In addition, previous research has also demonstrated that *A. cochinchinensis* has antitumor properties, particularly targeting lung cancer (Zhang and Jin, 2016). Such uses have led to a great demand for this plant, increasing the risk of extinction in this species due to over-collection of its wild populations (Jiang et al., 2010). *Asparagus cochinchinensis* is recorded in several protected areas in China (Information Center for the Environment, 2013), but information about its population size in the existing protected areas remains insufficient (International Union for the Conservation of Nature, 2016). Therefore, the genetic diversity and population structure of *A. cochinchinensis* requires immediate investigation to establish a conservation strategy.

Despite the ecological and medical importance of *A. cochinchinensis*, the genetic diversity in wild populations of this species is yet to be evaluated. Accordingly, polymorphic microsatellite markers in *A. cochinchinensis* were developed based on expressed sequence tag (EST) data obtained from Illumina paired-end sequencing. Simple sequence repeat (SSR) markers derived from ESTs are a powerful molecular tool for exploring genetic diversity and high level of transferability (Xu et al., 2014; Zhou et al., 2016). To the best of our knowledge, the current study is the first to profile the leaf transcriptome of *A. cochinchinensis* to generate EST-SSR markers. The usefulness of these markers was assessed in 60 individuals from three populations of *A. cochinchinensis* in Korea, Taiwan, and Japan. Cross-amplification of polymorphic microsatellite markers was performed in two related species (n = 8, for each species), *A. rigidulus* Nakai and *A. schoberioides* Kunth.

METHODS AND RESULTS

Sixty individuals of *A. cochinchinensis* were collected from wild populations in three countries (Korea, Taiwan, and Japan). Voucher specimens were deposited in the Herbarium of the National Institute of Biological Resources (KB) and the Herbarium of Hallym University (HHU), Republic of Korea (Appendix 1). To test cross-species amplification of the markers, we sampled eight individuals of each *A. rigidulus* and *A. schoberioides* (Appendix 1).

For RNA library construction, total RNA was extracted from a leaf of a single plant collected from Korea (voucher no: NIBRVP0000556138; Appendix 1). We constructed Illumina-compatible transcriptome libraries using a TruSeq

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TABLE 1. Characteristics of 15 polymorphic microsatellite loci developed for *Asparagus cochinchinensis* and tested for this study.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent dye	T_a (°C)	GenBank accession no.	Putative function [Organism]	*E*-value
AC008	F: GAAGCGAGGGGAGATTCTGG R: AACTGGTGAAGGTCGTGGAC	$(TC)_6$	205–213	HEX	55	Pr032824981	Predicted: uncharacterized protein LOC102705341 [*Oryza brachyantha*]	2E-35
AC011	F: TGTGCGGTCGACTGAATTGA R: GAGGCTACACACTCCCAAGG	$(TA)_7$	170–222	FAM	57	Pr032824995	Not found	—
AC014	F: CACGTTGTGAGTGGGTCTGA R: CTCAGGATGCTCTCGATGCA	$(GT)_7$	216–250	FAM	55	Pr032824986	Not found	—
AC017	F: CAAAAACCCAGCTGGCAGAC R: CCGCAAGTGAGGAGAGGTTT	$(GA)_8$	235–239	HEX	57	Pr032824988	DEA(D/H)-box RNA helicase family protein isoform 3 [*Theobroma cacao*]	9E-132
AC020	F: AATTATGAGACGCGCCGCTA R: AGCAGAACGAGCGTCAGAAA	$(GA)_{10}$	154–176	HEX	57	Pr032824993	Hypothetical protein OsI_20242 [*Oryza sativa* Indica Group]	2E-37
AC041	F: GAGAGACAGCGTGTGTGTGA R: ACCAGGGCACAAGACACAAT	$(AG)_8$	238–278	FAM	55	Pr032824994	Not found	—
AC050	F: CCTCACCTCAAAGGCCATGT R: TCCGGCATTCTGAAAAGCCT	$(TTC)_7$	184–196	FAM	57	Pr032824971	Predicted: homeobox protein BEL1 homolog isoform X3 [*Glycine max*]	8E-120
AC053	F: GGTGATGGTTCTGCTGGACA R: TCGCGCAACTGTTTCACAAG	$(TGC)_6$	336–339	FAM	55	Pr032824989	Predicted: uncharacterized protein LOC103340883 [*Prunus mume*]	0.0
AC065	F: CGCGATGATCATTTGCAGCA R: GAGCAAGGTCGAGATACCCG	$(GCA)_7$	201–216	HEX	55	Pr032824972	Predicted: myb-related protein Zm1-like [*Brachypodium distachyon*]	1E-33
AC069	F: AGGGCTAGGGTTTTGGTTCG R: TCCTTCTCTTCGCTCATGGC	$(GAC)_6$	215–236	FAM	55	Pr032824975	Not found	—
AC079	F: GCTTTCGGAGGGGGAAGAAA R: GAAGCGGCGAGAGAGAGTAC	$(CCG)_6$	222–237	FAM	55	Pr032824980	Predicted: 3-isopropylmalate dehydrogenase, chloroplastic-like [*Setaria italica*]	0.0
AC082	F: TGCCAAAGGAGAAGCTGGTT R: GGATGGAGGCCTAGACTTGC	$(CAG)_6$	357–363	HEX	55	Pr032824990	Putative adenosine kinase family protein [*Populus trichocarpa*]	2E-111
AC084	F: GACACATGTCCTCCAGCACA R: TACTCATTTCATCCCCCGCG	$(CAA)_7$	244–259	HEX	57	Pr032824969	Predicted: IST1-like protein-like [*Brachypodium distachyon*]	2E-48
AC085	F: CCTGAAGGCGAAGTTACCGA R: CTCAACGTCAGGAGCGAAGA	$(ATT)_7$	225–228	HEX	55	Pr032824991	Not found	—
AC086	F: GCAACCTCTCCTTTGACGGA R: CGAGTCTCGTTGGGGCTAAA	$(ATC)_7$	234–243	HEX	55	Pr032824970	Hypothetical protein POPTR_0015s00560g, partial [*Populus trichocarpa*]	3E-122

Note: T_a = annealing temperature.

TABLE 2. Genetic diversity data of 15 polymorphic microsatellite loci developed for *Asparagus cochinchinensis* in *A. rigidulus* and *A. schoberioides* populations.[a]

	Asparagus rigidulus ($N = 8$)		*Asparagus schoberioides* ($N = 8$)	
Locus	*A*	Allele size range (bp)	*A*	Allele size range (bp)
AC008	—	—	—	—
AC011	1	170	3	170–220
AC014	1	250	1	250
AC017	3	235–239	2	235–237
AC020	2	156–176	2	154–176
AC041	—	—	1	240
AC050	2	184–193	1	193
AC053	1	339	1	339
AC065	1	201	2	201–213
AC069	2	224–227	2	224–230
AC079	2	222–225	4	222–234
AC082	3	357–363	2	357–360
AC084	1	247	2	247–256
AC085	1	228	1	228
AC086	3	234–240	2	234–237

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals.

[a] Voucher and locality information are provided in Appendix 1.

RNA Library Preparation Kit version 2 (Illumina, San Diego, California, USA) following the manufacturer's instructions. Briefly, mRNA was purified from total RNA by polyA selection, then chemically fragmented and converted to single-stranded cDNA by random hexamer-priming. A second cDNA strand was generated to create a double-stranded cDNA for TruSeq library construction. The short double-stranded cDNA fragments were then connected using sequencing adapters. Finally, the RNA libraries were quantified using real-time PCR (qPCR), according to the qPCR Quantification Protocol Guide (Illumina), and validated using an Agilent 2200 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA).

The cDNA library was sequenced on the Illumina HiSeq 2000 platform. All raw reads have been deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; accession no. SRP100733). The de novo transcriptome assembly of these reads was performed using the short read assembling program Trinity r20140717 (Haas et al., 2013) with the default parameters. To detect SSR motifs containing two to six nucleotides, the Perl script MicroSAtellite Identification Tool (MISA) version 1.0.0 (Thiel et al., 2003) was applied with thresholds of 10 repeat units for dinucleotides, and five repeat units for tri-, tetra-, penta-, and hexanucleotides. MISA identified 20,104 microsatellite sequences, of which 96 loci were selected depending on the number of SSR repeats and primer depths for further testing of *A. cochinchinensis*. The primer sets were designed to flank the microsatellite-rich regions with a minimum of six repeats using Primer3 (Rozen and Skaletsky, 1999).

Whole genomic DNA was extracted from leaves of 60 individuals from three populations of *A. cochinchinensis* (including the specimen used to generate the transcriptome) and 16 individuals from two other *Asparagus* species (*A. rigidulus* and *A. schoberioides*) using a DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). Three individuals from each population were selected to amplify the 96 markers. To test polymorphism of microsatellite markers, PCR amplifications were performed using 2.5 μL of 10× *Ex Taq* buffer (TaKaRa Bio, Otsu, Japan), 2 μL of 2.5 mM dNTPs, 0.01 μM of each forward and reverse primers, 0.1 μL of *Ex Taq* DNA polymerase (5 units/μL) (TaKaRa Bio), 5–10 ng template DNA, and distilled water (Sigma-Aldrich Co., St. Louis, Missouri, USA) in a final volume of 25 μL. PCR was carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Carlsbad, California, USA) using the following program: initial denaturation step at 98°C for 5 min; followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55–57°C for 1 min (Table 1), and extension at 72°C for 1.5 min; and a final extension step at 72°C for 10 min. Fluorescently labeled (HEX, FAM) PCR products were analyzed by an automated sequencer (ABI 3730XL) with GeneScan 500 LIZ Size Standard (Applied Biosystems), genotyping was performed using GeneMapper version 3.7 (Applied Biosystems), and peaks were scored manually by visual inspection. The genetic diversity parameters of polymorphic loci, namely the number of alleles, observed heterozygosity, expected heterozygosity, and Hardy–Weinberg equilibrium, were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012).

The results showed that 27 markers could be successfully amplified (Table 1, Appendix 2), and size polymorphism in *A. cochinchinensis* was detected in 15 markers (Tables 1, 2). Functional annotations for these 27 markers were performed to a subset of ESTs with BLASTX score (E-value $< 1 \times 10^{-10}$) using the GO database (www.geneontology.org). Fifteen microsatellite markers were polymorphic in *A. cochinchinensis*, with the number of alleles per locus ranging from two to seven. The observed heterozygosity and expected heterozygosity ranged from 0.050 to 0.950 and 0.049 to 0.626, respectively (Table 3). Of these polymorphic loci, seven loci significantly deviated from Hardy–Weinberg equilibrium within the populations (Table 3). Transferability of microsatellite loci was tested in eight individuals each of *A. rigidulus* and *A. schoberioides* (Table 2). Of the 12 markers that were monomorphic in *A. cochinchinensis*, four loci (AC016, AC032, AC047, and AC093) were polymorphic in *A. rigidulus* and *A. schoberioides*, with the remaining loci amplifying consistently across both related taxa (Appendix 2).

TABLE 3. Genetic diversity in three *Asparagus cochinchinensis* populations[a] based on the 15 polymorphic microsatellite markers.

	Korea ($N = 20$)			Taiwan ($N = 20$)			Japan ($N = 20$)		
Locus	*A*	H_o	H_e[b]	*A*	H_o	H_e[b]	*A*	H_o	H_e[b]
AC008	3	0.100	0.486**	4	0.200	0.588**	2	0.000	0.095**
AC011	3	0.950	0.564**	3	0.950	0.599**	2	0.737	0.499*
AC014	1	0.000	0.000	2	0.105	0.100	2	0.800	0.480**
AC017	2	0.300	0.375	1	0.000	0.000	2	0.900	0.495**
AC020	2	0.250	0.219	2	0.150	0.139	1	0.000	0.000
AC041	1	0.000	0.000	7	0.650	0.524	3	0.947	0.605**
AC050	2	0.053	0.145**	1	0.000	0.000	1	0.000	0.000
AC053	2	0.050	0.049	1	0.000	0.000	1	0.000	0.000
AC065	3	0.200	0.184	3	0.400	0.331	1	0.000	0.000
AC069	2	0.313	0.498	4	0.357	0.605**	2	0.000	0.391**
AC079	3	0.400	0.339	4	0.750	0.626	3	0.579	0.589
AC082	2	0.278	0.424	2	0.125	0.117	2	0.200	0.180
AC084	2	0.500	0.480	2	0.100	0.095	1	0.000	0.000
AC085	1	0.000	0.000	2	0.150	0.139	1	0.000	0.000
AC086	2	0.211	0.188	3	0.250	0.224	1	0.000	0.000
Mean	1.842	0.190	0.208	2.368	0.220	0.215	1.526	0.219	0.175

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals.

[a] Voucher and locality information are provided in Appendix 1.

[b] Significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction for multiple tests (*$P < 0.05$, **$P < 0.01$).

CONCLUSIONS

Cross-species amplification of microsatellite markers is a time-saving as well as cost-effective approach for developing locus-specific markers for new species. In this study, a total of 27 markers were developed, of which 15 novel polymorphic markers were used for the medicinal plant *A. cochinchinensis*. These markers were successfully used for cross-amplification in *A. rigidulus* and *A. schoberioides*. These markers are an important tool for the development of effective strategies that can be used to study genetic diversity and genetic structure of *A. cochinchinensis* and related species.

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Appendix 1. Voucher information for *Asparagus cochinchinensis*, *A. rigidulus*, and *A. schoberioides* populations sampled in this study.

Species	Country	Locality	*n*	Geographic coordinates	Voucher no. (Herbarium)[a]
A. cochinchinensis (Lour.) Merr.	Korea	Yeonggwang, Jeonnam	20	35°23′29.7″N, 126°24′34.3″E	NIBRVP0000556138 (KB)
	Taiwan	Ruifang, Keelung	20	25°07′29.0″N, 121°55′17.4″E	NIBRVP0000556140 (KB)
	Japan	Kunigami, Okinawa	20	26°30′16.9″N, 127°51′03.0″E	NIBRVP0000601493 (KB)
A. rigidulus Nakai	Korea	Namhae, Gyeongnam	8	34°43′43.7″N, 127°51′35.7″E	2016ASP092 (HHU)
A. schoberioides Kunth	Korea	Goesan, Chungbuk	8	36°47′45.2″N, 128°01′44.9″E	NIBRVP0000601489 (KB)

Note: *n* = number of individuals sampled.

[a]Voucher specimens were deposited in the Herbarium of the National Institute of Biological Resources (KB) and the Herbarium of Hallym University (HHU), Republic of Korea.

Appendix 2. Characteristics of 12 monomorphic microsatellite loci developed for *Asparagus cochinchinensis* and tested in the related species *A. rigidulus* and *A. schoberioides*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent dye	T_a (°C)	GenBank accession no.	Putative function [Organism]	*E*-value
AC016[a]	F: GATCGATGTCTCCCGAACCC R: GTCGCTTCTTGACCCACAGA	$(GA)_9$	254–266	HEX	55	Pr032824977	Hypothetical protein MTR_3g065210 [*Medicago truncatula*]	3E-09
AC029[b]	F: AAATGAGCTGCCACCTCACA R: AACGGTCCCCCACTTGAAAA	$(CA)_9$	177	FAM	55	Pr032824973	Not found	—
AC030[b]	F: CCGGTGAATCTCTCCGACTG R: CTCTCGGTCCAAAGATCCGG	$(CA)_7$	245	FAM	55	Pr032824987	Unnamed protein product [*Coffea canephora*]	3E-49
AC032[a]	F: TCCATGTGCTTGTGTTTGCG R: TTGCTCTGCTCTGTGTGTGT	$(CA)_6$	262–266	FAM	55	Pr032824976	Unnamed protein product [*Vitis vinifera*]	2E-155
AC047[a]	F: AACGACGGCCACTCAAATCT R: GGATCGTGGAACTTGCATGC	$(AC)_8$	193–195	HEX	55	Pr032824983	Not found	—
AC054[b]	F: ATCATGGGTCGAGCTTTCCC R: TTCTGACCAGCCCATGTTCC	$(TGA)_6$	234	FAM	55	Pr032824985	Predicted: uncharacterized protein LOC100244334 [*Vitis vinifera*]	5E-169
AC057[b]	F: GTCGGCAGCTCATATCGACA R: GACCCAAGAGGATTTGGCCA	$(TCC)_7$	172	FAM	55	Pr032824979	E3 ubiquitin-protein ligase RING1 [*Morus notabilis*]	6E-58
AC058[b]	F: CCTTGTTGCCAGGTTTCAGC R: TTCAGAGCGGCGGTAAGTTT	$(TCA)_6$	234	HEX	56	Pr032824982	Hypothetical protein VITISV_017318 [*Vitis vinifera*]	4E-85
AC083[b]	F: AAAGAGCGAGAACATGCCCA R: GCCTGGCAGTCAAGTACAGT	$(CAC)_6$	225	FAM	55	Pr032824974	Predicted: leucine–tRNA ligase, cytoplasmic-like [*Setaria italica*]	0.0
AC088[b]	F: CTGAGAAGTGTTGGGGTCCC R: TGACGGTACTTCCAGGGAT	$(AGG)_6$	196	FAM	57	Pr032824978	Hypothetical protein POPTR_0005s17250g [*Populus trichocarpa*]	3E-122
AC090[b]	F: ACTCCTGAAGACGGCAAAGG R: ACCGCTCGCTTCTTTCTTCA	$(AGA)_6$	173	HEX	55	Pr032824992	Predicted: RINT1-like protein [*Prunus mume*]	0.0
AC093[a]	F: GCAGCAGCAACAGATTCGAG R: TGCTGTCGTAACAACGTCGA	$(AAT)_6$	204–210	FAM	55	Pr032824984	Hypothetical protein CISIN_1g0056332mg [*Citrus sinensis*]	1E-144

Note: T_a = annealing temperature.
[a]Only observed as polymorphic in *A. rigidulus* and *A. schoberioides*.
[b]Monomorphic microsatellite loci.