Development of 18 microsatellite markers for *Atractylodes japonica*

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PREMISE: *Atractylodes japonica* (Asteraceae) is endemic to East Asia, where its rhizomes are used in traditional medicine. To investigate the genetic diversity of this species, we developed polymorphic microsatellite markers.

METHODS AND RESULTS: We obtained a total of 175,825 simple sequence repeat (SSR) loci using the Illumina HiSeq 2500 system. Eighteen polymorphic SSR primer pairs were selected to determine heterozygosity levels and allele numbers in 80 individuals from four *A. japonica* populations. The levels of observed and expected heterozygosity ranged from 0.000 to 1.000 and from 0.133 to 0.892, respectively. Cross-amplification in the related species *A. macrocephala* and *A. lancea* was successful in 15 and 14 of the 18 markers, respectively.

CONCLUSIONS: These microsatellite markers will be useful for future studies involving *A. japonica* population genetics and breeding.

KEY WORDS Asteraceae; *Atractylodes japonica*; genetic diversity; microsatellite markers; population genetics; simple sequence repeat (SSR) .

The genus *Atractylodes* DC., which consists of important medicinal plants in northeastern Asia, belongs to the Asteraceae and is generally known to consist of five species: *A. japonica* Koidz., *A. macrocephala* Koidz., *A. lancea* (Thunb.) DC., *A. koreana* (Nakai) Kitam., *A. chinensis* Koidz., and *A. carlinoides* (Hand.-Mazz.) Kitam. (Shi, 1981; Kunio et al., 1997; Peng et al., 2012). There is considerable taxonomic debate regarding this; *A. japonica* has been treated as a synonym for *A. lancea* in some reports (Shi and Greuter, 2011; Peng et al., 2012). However, the Korean and Japanese Pharmacopoeia not only distinguish the two species but also treat them as distinct herbal medicines (Ministry of Food and Drug Safety, 2008; Pharmaceuticals and Medical Devices Agency of Japan, 2016). Among these, *A. japonica* and *A. macrocephala* produce the "white *Atractylodes* rhizomes" used in traditional medicine in Korea and Japan (Lee et al., 2002). *Atractylodes japonica* is a perennial herb that reaches 30–100 cm in height. Unlike *A. macrocephala* and *A. lancea*, which are native to China, *A. japonica* grows naturally in the Republic of Korea (Lee, 2006). Although the two species are similar in appearance, *A. japonica* is monoecious and produces a white flower, whereas *A. macrocephala* is gynodioecious and produces a claret flower (Peng et al., 2012; Jeong et al., 2018). The mass of a seed of *A. japonica* is half of that of *A. macrocephala*; thus, it takes longer for *A. japonica* seeds to germinate (Rural Development Administration, 2018). Despite challenges in its propagation and cultivation, *A. japonica* is of particular interest in medical applications. It has a high content of sesquiterpenoids, including atractylon and atractylenolides (Yun et al., 2013; Jeong et al., 2018), which are useful in the treatment of stomach disorders, inflammation, and obesity (Kim et al., 2011; Chen et al., 2016).

Compound simple sequence repeat (SSR) markers were previously developed from a related species, *A. macrocephala* (Zheng et al., 2012). However, this earlier study did not test the cross-amplification in *A. japonica*. We initially tested these markers in *A. japonica* samples, but without much success. In this study, we developed 18 polymorphic SSR markers from the genome of *A. japonica* and tested them for analysis of genetic diversity in various populations and related species.

METHODS AND RESULTS

We sampled four natural populations (80 individuals total) of *A. japonica* in Korea: Jeol Mountain (n = 21), Sageum Mountain (n = 20), Jiri Mountain (n = 18), and Cheonbul Mountain (n = 21)

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(Appendix 1). Voucher specimens were deposited at the Korea Medicinal Resources Herbarium, Eumseong, Korea (Appendix 1), and DNA was extracted from fresh leaves using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA).

Four *A. japonica* individuals collected from four different farms in Korea were used for sequencing. Sequencing libraries for genomic DNAs were prepared using a TruSeq Nano DNA Sample Prep Kit according to the manufacturer's instructions (Illumina, San Diego, California, USA). Sheared DNA fragments were subjected to end-repairing, A-tailing, adapter ligation, and amplification with clean-up. The libraries were subjected to paired-end sequencing with a 150-bp read length using the Illumina HiSeq 2500 platform. The sequencing reads of the four plants, with sizes of 54.9 Gbp, 11.3 Gbp, 12.0 Gbp, and 11.5 Gbp, respectively, were deposited in the National Agricultural Biotechnology Information Center (NABIC) Sequence Read Archive (BioProject ID: NN-5968, NN-5970, NN-5971, and NN-5972). Short-read correction was done with the SOAPec package of SOAPdenovo2 (version 2.04) (Luo et al., 2012), and genome assembly of the four plants was performed separately using SOAPdenovo2 (Luo et al., 2012) with the following parameters: pregraph -K 71 –d 0, contig –M 1, map –k 41, and scaff –F –b 1.5.

A total of 175,825 SSRs were identified using the stand-alone version of SSRIT (Temnykh et al., 2001) with the following parameters: SSRs were defined as di-, tri-, tetra-, penta-, and hexanucleotide repeats with ≥4 repeats; and no variation (mutation) in repeat motifs was permitted. Forty-eight polymorphic SSR loci with at least a 4-bp motif containing a minimum of four repeats were selected by the comparison of the specific SSR loci of the four sequenced individuals using CLC Main Workbench (version 6.8.4, QIAGEN) according to Gil et al. (2017). SSR primers were designed using Primer3 (Untergasser et al., 2012) using the following conditions: length 18–26 bp, GC content 50–70%, and melting temperature 55–62°C. The PCR products ranged between 150 and 300 bp.

Preliminary PCR analysis of the 48 primers was performed on one *A. japonica* individual collected from the Jeol Mountain population. Forty-four pairs of primers amplified the targets successfully. Four individuals per population were then tested with the selected primer sets and analyzed with the Fragment Analyzer Automated CE system (Advanced Analytical Technologies, Ankeny, Iowa, USA). Eighteen pairs of primers were selected based on the amplification efficiency and the number of alleles, and the forward primer of each set was labeled (Table 1). The PCR reaction mixture (total

TABLE 1. Characteristics of the 18 SSRs developed for *Atractylodes japonica*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label[b]	GenBank accession no.
AJSSR001	F: AACATCGATGAGTTGGACCA R: ATAGCAGGCTTTCGGAAAGA	$(ATGT)_5$	157–189	VIC	MN107252
AJSSR002	F: AGGAGGTAGGAGGCTTGTTA R: GGCAATTGAGCATGCACATA	$(ACCAA)_5$	216–281	FAM	MN107253
AJSSR003	F: CAAACTCCGTTGCATTTTGC R: GAAGAGCGGAGTCGAGTTTA	$(GGTTT)_6$	261–291	VIC	MN107254
AJSSR004	F: CAGGTTACGCCTCATAGTGA R: ACCTTCTCCCTGTAATCAACC	$(GAGAGG)_5$	118–161	FAM	MN107255
AJSSR005	F: ATGTGGGACAAGTTGGAAGT R: GGGGTAGAGGTAAAGGTGTG	$(AAACC)_5$	220–250	FAM	MN107256
AJSSR006	F: TTCAGCCGACCACATCAATA R: GGCACCCAAGTTTGTCATTT	$(GTTTG)_5$	293–318	VIC	MN107257
AJSSR007	F: TCTACGGACTCAGTCTCCTT R: TGACCTACCCAACAAACTTGT	$(GGTTT)_7$	270–295	NED	MN107258
AJSSR008	F: TGCTGTACCACCAACTTCAT R: CTGGTGTTGGTTGTTGTTGT	$(AGGAGT)_6$	344–404	VIC	MN107259
AJSSR009	F: TTTCCTTCGACTCCAACACA R: CAACCCAGATGCCAAAAACA	$(CTCTT)_6$	195–240	PET	MN107260
AJSSR010	F: CCTGTTGGTTTTCACAAGGT R:TGTAGTTTGACATTACGAGGGA	$(GGTTT)_5$	217–259	NED	MN107261
AJSSR011	F: GTCAGAACTTCCATGTCATGC R: TAAGGCTGCGTACATCCTAC	$(AAACC)_8$	148–178	PET	MN107262
AJSSR012	F: TGAGTGTTATACGCGGTTCA R: TCCTGCACTTTACGGACAAT	$(AATAAA)_4$	208–230	FAM	MN107263
AJSSR013	F: GCAATGGAGGCACTACTAGT R: AGCGTTCTCTCTACAAAGGG	$(GTTTG)_5$	380–405	PET	MN107264
AJSSR014	F: ATGTGATTGTGCTCCATCCT R: GTTTTACTTGCTGGAGCTGG	$(GGAA)_4$	238–262	PET	MN107265
AJSSR015	F: GGCTATTAGCATCTTCCCCA R: CTCTGCCCTGTGACCTAAAA	$(CATA)_6$	228–263	VIC	MN107266
AJSSR016	F: GATGCATTTTGCCCGTATCA R: ATGTAATGGGAAGGTCGGTC	$(CTTCTC)_5$	303–327	NED	MN107267
AJSSR017	F: GAGAATGATTCTGCTTCGGC R: TTTCACTGCATCCCAGGAAT	$(ATGT)_{10}$	294–334	FAM	MN107268
AJSSR018	F: TGAGTAGGTGGTTAAATGGCA R: GAGATGAGGCCCATGCTTT	$(AAACC)_5$	147–192	NED	MN107269

[a]Annealing temperature was 56°C for all loci.
[b]Fluorescent labeling was applied to forward primers.

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TABLE 2. Genetic properties of 18 polymorphic SSR markers in four *Atractylodes japonica* populations.[a]

	Jeol Mountain (*n* = 21)			Sageum Mountain (*n* = 20)			Jiri Mountain (*n* = 18)			Cheonbul Mountain (*n* = 21)		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
AJSSR001	3	0.476	0.381	3	0.333[b]	0.634	5	0.571	0.511	3	0.191	0.176
AJSSR002	4	0.619[b]	0.552	5	0.471	0.543	4	0.381	0.354	4	0.750	0.529
AJSSR003	8	0.714	0.796	6	0.529	0.630	7	0.810	0.726	7	0.810	0.727
AJSSR004	5	0.905[b]	0.638	4	0.500[b]	0.532	5	0.952[b]	0.720	5	0.952	0.736
AJSSR005	5	1.000	0.728	5	0.944	0.674	6	0.952[b]	0.689	6	1.000[b]	0.694
AJSSR006	5	0.333	0.434	3	0.278	0.332	3	0.316	0.277	3	0.150	0.185
AJSSR007	5	0.235[b]	0.786	5	0.267[b]	0.684	3	0.444[b]	0.656	3	0.222[b]	0.607
AJSSR008	8	0.810	0.813	7	0.500	0.730	9	0.895	0.769	11	0.619	0.858
AJSSR009	11	0.905	0.829	9	0.833	0.798	14	0.857	0.892	13	0.857	0.835
AJSSR010	7	0.762	0.621	6	0.529[b]	0.505	8	0.700	0.734	8	0.667	0.720
AJSSR011	6	0.667	0.734	5	0.611	0.679	6	0.571	0.630	5	0.700	0.749
AJSSR012	5	0.333[b]	0.736	3	0.083[b]	0.531	5	0.000[b]	0.703	3	0.066[b]	0.354
AJSSR013	6	0.810	0.659	6	0.667	0.688	5	0.667	0.612	4	0.476	0.659
AJSSR014	2	0.143	0.133	7	0.444	0.426	6	0.429	0.438	5	0.571	0.492
AJSSR015	8	0.450[b]	0.836	3	0.222[b]	0.569	9	0.286[b]	0.730	6	0.191[b]	0.756
AJSSR016	4	0.154[b]	0.524	7	0.143[b]	0.765	7	0.200[b]	0.729	8	0.688[b]	0.809
AJSSR017	6	0.381	0.528	5	0.444[b]	0.651	5	0.550	0.669	7	0.429[b]	0.721
AJSSR018	6	0.667	0.692	7	0.563	0.647	7	0.524[b]	0.780	7	0.619[b]	0.812
Mean	5.78	0.576	0.634	5.33	0.465	0.612	6.33	0.561	0.646	6.00	0.553	0.634

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals.
[a]Locality and voucher information are provided in Appendix 1.
[b]Significant deviation from Hardy–Weinberg equilibrium at $P < 0.05$.

volume 25 μL) comprised 6 μL of distilled water, 12 μL of Inclone Exel TB 2× Taq Premix with dye (Inclone Biotech, Yongin, Korea), 1 μL of 10 μM of each forward and reverse primer, and 5 μL of gDNA. The PCR reaction conditions were: initial denaturation at 95°C for 5 min; 34 cycles at 95°C for 30 s, 55°C for 25 s, and 72°C for 1 min; and a final extension at 72°C for 30 min. The amplified DNA product (0.2 μL) was mixed with 9.8 μL of Hi-Di formamide (Applied Biosystems, Foster City, California, USA) and 0.2 μL of GeneScan 500 LIZ size standard (Applied Biosystems). The mixture was denatured at 95°C for 5 min and kept on ice before being separated by capillary electrophoresis on an ABI 3730 DNA analyzer (Applied Biosystems). The amplified fragments were analyzed by size using GeneMapper version 4.1 software (Applied Biosystems). The allele count, levels of expected and observed heterozygosity, and Hardy–Weinberg equilibrium (Emigh, 1980) of each locus were calculated using PowerMarker software (version 3.23) (Liu and Muse, 2005).

The 18 SSR primer pairs were then tested in all collected *A. japonica* individuals, and the genetic diversity was calculated for each, as described above. The number of alleles per locus varied from two to 14 (Table 2). The levels of observed and expected heterozygosity per locus ranged from 0.000 to 1.000 and from 0.133 to 0.892, respectively. Some markers showed significant deviation from Hardy–Weinberg equilibrium (Table 2). For the applicability test of the developed markers, we applied the markers to five individuals each of the related species *A. macrocephala* and *A. lancea* (Appendix 1), and 15 and 14 markers were successfully amplified, respectively (Table 3).

TABLE 3. Cross-amplification of microsatellite loci developed for *Atractylodes japonica* in two related species.[a]

	A. macrocephala (*n* = 5)		*A. lancea* (*n* = 5)	
Locus	*A*	Allele size (bp)	*A*	Allele size (bp)
AJSSR001	2	185–189	1	185
AJSSR002	1	217	1	277
AJSSR003	3	276–291	1	286
AJSSR004	4	123–142	2	123–130
AJSSR005	2	221–231	2	231–236
AJSSR006	2	303–313	1	302
AJSSR007	—	—	—	—
AJSSR008	—	—	—	—
AJSSR009	—	—	—	—
AJSSR010	1	217	1	217
AJSSR011	1	153	1	153
AJSSR012	1	218	2	208–214
AJSSR013	3	385–400	2	395–410
AJSSR014	1	239	2	242–250
AJSSR015	1	237	1	242
AJSSR016	2	313–319	1	323
AJSSR017	3	310–320	1	295
AJSSR018	2	172–177	—	—

Note: — = unsuccessful amplification; *A* = number of alleles; *n* = number of individuals.
[a]Locality and voucher information are provided in Appendix 1.

CONCLUSIONS

We developed 18 polymorphic SSR markers from *A. japonica* and successfully used them to analyze genetic diversity in different populations and in two related species. These markers will be useful for the development of *A. japonica* cultivars and potentially for differentiation among *Atractylodes* species.

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DATA AVAILABILITY

Sequencing reads have been deposited in the National Agricultural Biotechnology Information Center (NABIC) Sequence Read Archive (BioProject ID: NN-5968, NN-5970, NN-5971, and NN-5972). Primer sequences have been deposited to the National Center for Biotechnology Information's GenBank database; accession numbers are listed in Table 1.

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APPENDIX 1. Locality and voucher information for *Atractylodes japonica* and the two related species used in this study.

Species	Collection locality[a]	Voucher no.[b]	Geographic coordinates	*n*
A. japonica Koidz.	Jeol Mountain, Hwacheon-gun, Gangwon-do	MPS005754	38°05′57.1″N, 127°44′57.1″E	21
A. japonica	Sageum Mountain, Samcheok-si, Gangwon-do	MPS005755	37°10′22.3″N, 129°10′56.6″E	20
A. japonica	Jiri Mountain, Gurye-gun, Jeollanam-do	MPS005756	35°20′14.4″N, 127°29′28.5″E	18
A. japonica	Cheonbul Mountain, Naju-si, Jeollanam-do	MPS005757	34°55′30.8″N, 126°52′11.0″E	21
A. macrocephala[c] Koidz.	Eumseong-gun, Chungcheongbuk-do	MPS004740	36°56′34.6″N, 127°45′02.5″E	2
A. macrocephala[c]	Mungyeong-si, Gyeongsangbuk-do	MPS004741	36°37′05.3″N, 127°59′54.3″E	3
A. lancea[d] (Thunb.) DC.	Eumseong-gun, Chungcheongbuk-do	MPS000723-1	36°56′34.6″N, 127°45′02.5″E	5

Note: n = number of individuals sampled.
[a]Locality and Korean province.
[b]Vouchers deposited at the Korea Medicinal Resources Herbarium, Eumseong, Republic of Korea.
[c]These samples represent cultivated materials in Korea without known geographical sources from China.
[d]The sample was collected in a cultivation area.

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