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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE PRIMERS FOR *ZANTHOXYLUM SCHINIFOLIUM* (RUTACEAE)[1]

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- *Premise of the study:* Polymorphic microsatellite markers of *Zanthoxylum schinifolium* (Rutaceae), a promising medicinal plant with effective antibacterial, anticancer, and anti-inflammatory compounds, were developed and evaluated for further genetic studies based on genetic variation among individuals or populations.
- *Methods and Results:* Following the selective hybridization method, microsatellite-enrichment libraries were constructed. Using these libraries, we obtained 15 polymorphic and three monomorphic microsatellite markers for *Z. schinifolium*. The number of alleles observed in each of the 15 polymorphic loci ranged from two to eight, and the observed and expected heterozygosities ranged from 0.070 to 0.677 and from 0.093 to 0.688, respectively. Eleven of these developed markers were successfully amplified for *Z. piperitum*, a related species.
- *Conclusions:* These microsatellite markers can be valuable tools for further genetic studies of *Z. schinifolium*, such as genetic resource conservation for maintaining breeding material and individual identification for breeding program improvement and variety management.

Key words: economically important plants; microsatellite; Rutaceae; *Zanthoxylum schinifolium*.

The genus *Zanthoxylum* L. (Rutaceae) includes approximately 200 or more species around the world (Wu et al., 2008), but only about six species occur in Korea. Two of these species, *Z. schinifolium* Siebold & Zucc. and *Z. piperitum* (L.) DC., are the most representative common species in this genus in Korea. *Zanthoxylum schinifolium* is a deciduous shrub distributed in China, Japan, Taiwan, and Korea (Hassler, 2016). This species has a unique aromatic flavor on its fruits and leaves and has therefore been traditionally used as an edible or medicinal plant. Recently, significant effectiveness in the antibacterial and anticancer activity of some of the compounds of this species has been scientifically confirmed (Choi et al., 2008; Li et al., 2013). Consequently, this species is attracting attention as a promising medicinal plant.

In many cases, the increasing economic value of a plant species can lead to drastic reductions in wild populations because of extensive use; thus, conservation efforts of genetic resources as breeding materials should be increased (Shippmann et al., 2003). *Zanthoxylum schinifolium*, a medicinal species that breeders have recently started to cultivate, still has ample natural populations. Therefore, it can be used as a model species to identify the impact of harvest pressure on the genetic diversity patterns of wild populations of medicinal plants. However, most studies on this species have focused on the identification of medicinal compounds. In our review of the literature, the only genetic studies found for the species were studies of molecular identification based on ribosomal DNA sequence information (Sun et al., 2010) and phylogenetic relationships with cpDNA markers (Feng et al., 2016). However, these markers are unsuitable for analyzing the genetic variation of the species for conservation and management (Wan et al., 2004). Microsatellite markers are preferred in studies of genetic variation of individuals or populations due to their high level of polymorphism, codominance, biparental inheritance, and reproducibility of results (Varshney et al., 2005).

We have developed and evaluated microsatellite markers for further genetic studies of *Z. schinifolium*, and tested their cross-amplification in the related species *Z. piperitum*.

METHODS AND RESULTS

Sample collection and DNA extraction—A total of 102 samples of *Z. schinifolium* from three natural populations were collected to develop and validate new microsatellite primers for this species. To identify cross-amplification of the markers to other related species in the same genus, 30 samples of *Z. piperitum* were collected from one population. Detailed information on all of the samples collected for this study is provided in Appendix 1. Total genomic DNA (gDNA) was isolated from fresh leaves using Biomedic Plant gDNA Extraction Kit (Biomedic, Bucheon, Gyeonggi, South Korea). The DNA and leaf samples collected for this study were stored in the Gene Bank of the National Forest Seed and Variety Center (NFSV, Chungju, South Korea).

Construction of a microsatellite enrichment library—A microsatellite enrichment library was constructed according to the magnetic bead hybridization method of Glenn and Schable (2005) using one sample of *Z. schinifolium* collected from Chungju-si, Chungcheongbuk-do, South Korea. To obtain DNA fragments ranging from roughly 0.3 to 1 kbp, total gDNA was digested using the restriction enzyme *Rsa*I and then ligated with SuperSNX linkers containing a GTTT PIG-tail. DNA molecules in the ligation products were hybridized with

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3′-biotinylated microsatellite probes and then subsequently isolated using streptavidin-coupled (M-280) Dynabeads (Invitrogen, Carlsbad, California, USA). PCR amplification was performed on the collected DNA molecules with SuperSNX-24 primers. This enrichment step was repeated once. The DNA fragments highly enriched with microsatellites were cloned into pGEM-T vectors (Promega Corporation, Madison, Wisconsin, USA) using *Escherichia coli* DH5α-competent cells. Recombinant clones were identified by colony PCR using M13 forward and reverse primers. The PCR products were purified, and then directly sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). After the trimming of vector and linker sequences, 182 nonredundant contig sequences (GenBank accession no. KU884701–KU884883) were obtained from the assembly process using Lasergene SeqMan (version 7.0.0; DNASTAR, Madison, Wisconsin, USA).

Microsatellite primer design and validation—Putative microsatellites were mined using MISA software (Thiel et al., 2003) based on the following criteria: more than three repeats for dinucleotides to hexanucleotides and a gap within 100 bp in composite types. Amplicon size (85–350 bp) and annealing temperature (57–60°C) were the main consideration in primer design. A total of 104 primer sets were synthesized by Biomedic Co. Ltd. (www.ibiomedic.co.kr; Bucheon, South Korea) and used for preliminary screening. The preliminary screening of markers was performed by conventional PCR using the gDNA from eight samples of *Z. schinifolium* as templates to identify putative loci. Then, these PCR products were separated on a 2% agarose gel.

Finally, 20 microsatellite marker candidates, which produced amplicons at putative single loci, were selected to validate polymorphism and cross-amplification. PCR was performed using an ABI 2720 Thermal Cycler (Applied Biosystems) in a 11-μL reaction volume containing 3 μL of template DNA (3 ng/μL), 1.1 μL of 2 mM dNTPs, 0.22 μL of 10 μM 6-FAM fluorescent dye–labeled forward primer and reverse primer, 0.15 μL of NeoTherm *Taq* DNA polymerase (5 U/μL; GeneCraft, Köln, Germany), 1.1 μL of 10× reaction buffer (containing 25 mM $MgCl_2$; GeneCraft), and 5.21 μL of distilled water. The PCR was performed with an initial denaturation at 94°C for 5 min; followed by 34 cycles of 94°C for 30 s, 57–64°C for 1 min (Table 1), and 72°C for 1 min; and a final extension at 72°C for 10 min. After PCR amplification, 0.2 μL of the fluorescent PCR products were mixed with 9.8 μL of Hi-Di Formamide (Applied Biosystems) and 0.2 μL of GeneScan 500 ROX Size Standard (Applied Biosystems). The mixture was denatured at 95°C for 5 min and placed on ice. The amplified fragments were separated by capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems). Each of the individual genotypes was scored using GeneMapper 4.1 software (Applied Biosystems).

Of the 20 candidate primers, 18 (90%) were successfully amplified for *Z. schinifolium.* Of these 18, 15 produced polymorphic DNA fragments, and the remaining three primers produced monomorphic amplicons (Table 1). The percentage of amplification was 61.1% (11/18) for *Z. piperitum.* Out of these 11, six primers showed polymorphism with two or more alleles, but the remaining five primers had only one allele in the 30 samples analyzed. In most primers, the size range of alleles overlapped between the two species, but three primers (Zs3027, Zs3038, and Zsm3029) showed completely different size ranges between the two species, so these markers could be used to distinguish between the two species.

Evaluation of genetic properties for use as polymorphic markers—The genetic properties of the 15 polymorphic primers for *Z. schinifolium* were evaluated using 102 samples from three populations (Table 2). Population genetic

Table 1. Characteristics of 15 polymorphic and three monomorphic loci developed for *Zanthoxylum schinifolium* and cross-amplified in *Z. piperitum*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)		T_a (°C)	GenBank accession no.
			Z. schinifolium	*Z. piperitum*		
Zs3069*	F: `CACGTTCACCTTCATAACCCA` R: `GGCTTCAGGCACACTGACTT`	$(TTGT)_4$	282–360	—	62	KU884789
Zs4034	F: `TTGACTTCCCAGAGCTTCACT` R: `GTCATTGTATTGTCGCCTCAAA`	$(ATGT)_4$	188	—	62	KU884813
Zs3005*	F: `GGAGATCAAGGTTGGTTGGTT` R: `CACTTCTGTCAAATTCCTCGCT`	$(AAGA)_{10}$	222–250	222–226	62	KU884748
Zs3006-1*	F: `TGCATCTCTGTTTTCGCAAC` R: `TCAATCAACTTCCCGTTTCA`	$(TGTT)_4$	314–323	—	58	KU884749
Zs3006-2*	F: `TGGTCTGGGTTTGTGTATGTTT` R: `AGCAGAGTCCAAAAGAAGGC`	$(TTGA)_5$	196–202	202	62	KU884749
Zs3026*	F: `TTTGAGGACCCTGCAGAACT` R: `TGCAACAACCCCAACATAAA`	$(TGTC)_5$	186–190	174–190	57	KU884763
Zs3027*	F: `TTGGGACTAAGCAAAGTGGG` R: `GGAAGCCATAGCCCTGATCT`	$(GATT)_5$	318–322	341–349	62	KU884764
Zs3035*	F: `CTCCTCCTCCATTCACTCACTC` R: `TCAATCACTGTAGCTCGCTTTC`	$(GGAATC)_4$	164–188	182–188	64	KU884769
Zs3038*	F: `ACAAACCCAGAAACCTTGTGAT` R: `ATCGTGGCTCAACAACTTACCT`	$(TTTC)_6$	163–183	128	62	KU884770
Zs4007*	F: `TTTAGGAGGATCCAGCCAAGT` R: `AATCCCAGTTCGTGAAGCAG`	$(GACA)_4$	207–211	—	62	KU884793
Zsm2010*	F: `GCTTTCTCTAATGTGGAATGTG` R: `CAAGTTCAATCCAACCCTAA`	$(TTG)_6$	223–251	—	62	KU884844
Zs3042	F: `CACGCATCAAGTAAATCAGTGC` R: `GCCGCTAGTATAAAATGTGTTGC`	$(TCAC)_5$	177	—	64	KU884774
Zsm3011*	F: `GCGAAGAAAAGGGGAAATAA` R: `CCATAGAAGCATAATTGAAGCC`	$(AGTG)_4$	193–197	189–193	62	KU884856
Zsm4032	F: `GCCGAATAAAAGCCTCTCCT` R: `ATCGGGAAGTGATTGTTTGC`	$(AAAC)_5$	146	146	62	KU884883
Zsm3029*	F: `CCATCGTTACCCCCAATAAA` R: `TCATCGAATGGCTTCAACAA`	$(TCAA)_5$	251–259	155	58	KU884866
Zsm4023*	F: `AGAAATAGAACCCTAGCCCCTG` R: `AAAGATGACGCAGAGGAAAATG`	$(TTG)_4$	109–118	118	64	KU884878
Zs2011*	F: `CCAAGAAACATGATAAGAGGGG` R: `GGGCCTAACAACAGAAGACAGA`	$(CT)_{12}$	232–250	233–246	64	KU884709
Zs2032*	F: `CAGCCCTAGTTAGTTTTCCGAC` R: `CACAGAACTCATCAACATAGACAGG`	$(TC)_{12}$	161–183	—	64	KU884722

Note: — = information not available; T_a = annealing temperature.
* Polymorphic microsatellite loci.

TABLE 2. Genetic properties of 15 polymorphic microsatellite loci of *Zanthoxylum schinifolium* and *Z. piperitum*.[a]

	Z. schinifolium																					*Z. piperitum*				
	Chungju (N = 36)					Jincheon (N = 34)					Namyangju (N = 32)					Total (N = 102)						Youngcheon (N = 30)				
Locus	n	A	A_e	H_o	H_e	n	A	A_e	H_o	H_e	n	A	A_e	H_o	H_e	n	A	A_e	H_o	H_e	PIC	n	A	A_e	H_o	H_e
Zs3069	33	3	2.1	0.394	0.509	32	4	2.1	0.375*	0.515	30	3	2.2	0.300*‡	0.555	95	4	2.1	0.358	0.532	0.486	—	—	—	—	—
Zs3005	34	4	2.6	0.706	0.617	32	6	3.2	0.688	0.690	30	5	2.8	0.633*	0.640	96	8	3.1	0.677	0.681	0.626	26	4	1.3	0.077*	0.211
Zs3006-1	36	3	1.1	0.083	0.081	32	2	1.1	0.125	0.117	32	3	1.2	0.156	0.147	100	3	1.1	0.120	0.114	0.108	—	—	—	—	—
Zs3006-2	36	3	1.2	0.056*‡	0.156	33	3	1.3	0.091*‡	0.244	32	2	1.1	0.094	0.089	101	3	1.2	0.079	0.166	0.157	20	1	—	—	—
Zs3026	35	2	1.7	0.457	0.408	32	2	1.2	0.094*	0.144	30	2	1.3	0.300	0.255	97	2	1.4	0.289	0.289	0.249	26	3	2.2	0.962*	0.536
Zs3027	35	2	1.4	0.200	0.265	33	2	1.2	0.152	0.190	32	2	1.2	0.156	0.144	100	2	1.3	0.170	0.204	0.186	25	2	1.7	0.480	0.403
Zs3035	31	2	1.9	0.419	0.481	32	3	2.2	0.563*	0.541	32	3	1.8	0.438	0.439	95	4	2.1	0.474	0.520	0.404	30	2	2.0	1.000*	0.500
Zs3038	36	4	2.3	0.556*	0.564	34	3	2.2	0.618	0.538	32	3	1.9	0.438	0.480	102	5	2.2	0.539	0.540	0.501	24	1	—	—	—
Zs4007	36	2	1.4	0.333	0.278	34	1	1	0.000†	0.000	32	1	1	0.000†	0.000	102	2	1.1	0.118	0.111	0.103	—	—	—	—	—
Zsm2010	36	4	3.3	0.528*‡	0.699	33	5	2.7	0.545*	0.634	32	3	2.3	0.438	0.565	101	5	3	0.505	0.667	0.618	—	—	—	—	—
Zsm3011	36	2	1.1	0.083	0.080	34	2	1	0.029	0.029	32	2	1.2	0.188	0.170	102	2	1.1	0.098	0.093	0.087	23	3	1.5	0.391	0.334
Zsm3029	36	3	1.7	0.278*	0.392	32	2	1.8	0.375	0.451	32	3	1.8	0.313*	0.432	100	3	1.8	0.320	0.430	0.364	30	1	—	—	—
Zsm4023	31	3	1.4	0.156*‡	0.293	30	3	1.5	0.267	0.346	31	3	1.7	0.161*‡	0.414	92	3	1.5	0.194	0.354	0.305	30	1	—	—	—
Zs2011	36	6	3.7	0.056*‡	0.711	34	5	3	0.118*‡	0.667	30	4	2.9	0.033*‡	0.659	100	6	3.2	0.070	0.688	0.650	28	4	1.9	0.607	0.480
Zs2032	36	5	1.5	0.306	0.318	29	5	2.4	0.241*‡	0.580	32	6	2.6	0.281*‡	0.616	97	8	2.1	0.278	0.518	0.494	—	—	—	—	—

Note: A = number of alleles per locus; A_e = number of effective alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; n = number of individuals genotyped; PIC = polymorphism information content.

[a] Locality and voucher information are available in Appendix 1.

* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

† Monomorphic microsatellite loci within each population.

‡ Significant possibility of presence of null alleles detected by MICRO-CHECKER (van Oosterhout et al., 2004).

diversity parameters (i.e., number of alleles [A], number of effective alleles [A_e], and observed [H_o] and expected [H_e] heterozygosities) were estimated using GenAlEx version 6.41 software (Peakall and Smouse, 2006). The Hardy–Weinberg equilibrium (HWE) at each locus for each population was tested based on χ^2 tests using GenAlEx version 6.41 software (Peakall and Smouse, 2006). Polymorphic information content (PIC) and nonexclusion probability (NEI; identity) were calculated by CERVUS version 3.0.3 (Kalinowski et al., 2007). The test for null allele presence was performed using MICRO-CHECKER (van Oosterhout et al., 2004). Over all samples, A ranged from two to eight, A_e ranged from 1.1 to 3.2, and PIC values were calculated as 0.087 to 0.650. H_o and H_e ranged from 0.070 to 0.677 and 0.093 to 0.688, respectively. The near-zero NEI value (0.0000005) indicated that the developed markers in this study are useful for individual identification. A ranged from one to four for *Z. piperitum*, and H_o and H_e at six polymorphic loci were in the respective ranges 0.077–1.000 and 0.211–0.536. Significant deviations ($P < 0.05$) from HWE were detected for some primers within each population. Because agreement with HWE depends on certain assumptions including an infinite population size, simple Mendelian inheritance in a diploid organism, discrete generations, and random mating, the test results could not be interpreted without information such as the mating system in the tested populations. Unexpected genotype patterns in the microsatellite data set were reported, of which null allele presence has been frequently mentioned as an explanatory cause (Dakin and Avise, 2004). The null test results indicated a significant possibility of the presence of null alleles at some loci in some populations (Table 2). In particular, Zs2011 showed a significant possibility of the presence of null alleles in all three tested populations; this locus should be used carefully in further genetic studies. Additional testing of known parent–offspring relationships should be used to confirm these results, as they might be affected by the presence of null alleles.

CONCLUSIONS

In this study, 15 polymorphic and three monomorphic microsatellite markers were developed for *Z. schinifolium*. In the cross-amplification test of the developed markers for *Z. piperitum*, a related species in the same genus, 61% (11/18) were successfully amplified. Three of the 11 cross-amplified primers could be useful for distinguishing between the two species because the amplified fragments have completely different size ranges. These developed markers can be useful for individual identification within species as well as for conservation and management of the genetic resources of *Z. schinifolium*.

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APPENDIX 1. Locality information and accession numbers of *Zanthoxylum schinifolium* and *Z. piperitum* samples used in this study.[a]

Species	Locality	Geographic coordinates	*N*	Accession no. (DNA)	Voucher accession no.
Zanthoxylum schinifolium Siebold & Zucc.	Chungju-si, Chungcheongbuk-do, South Korea	36°52′22.86″N, 127°58′17.34″E	36	0300-13-070792–0300-13-070827	0300-06-04679–0300-06-04681
	Jincheon-gun, Chungcheongbuk-do, South Korea	36°49′22.07″N, 127°29′46.14″E	34	0300-13-070828–0300-13-070861	0300-06-04682–0300-06-04684
	Namyangju-si, Gyeonggi-do, South Korea	37°43′50.00″N, 127°10′21.00″E	32	0300-13-070862–0300-13-070893	0300-06-04685–0300-06-04687
Zanthoxylum piperitum (L.) DC.	Youngcheon-si, Gyeongsangbuk-do, South Korea	35°59′41.49″N, 128°46′34.86″E	30	0300-13-070894–0300-13-070923	0300-06-04688–0300-06-04690

Note: *N* = number of samples.

[a] All DNA, leaf samples, and plant vouchers were deposited in the Gene Bank of the National Forest Seed and Variety Center (NFSV), Chungju, South Korea.