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Applications in Plant Sciences

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Primer Note

Isolation and characterization of microsatellite loci in *Rehmannia glutinosa* (Scrophulariaceae), a medicinal herb[1]

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- *Premise of the study: Rehmannia glutinosa* (Scrophulariaceae) is used in traditional Chinese medicine. Microsatellite primers were developed and characterized for this species to evaluate its genetic diversity and population genetic structure.
- *Methods and Results:* Sixteen microsatellite loci were isolated from *R. glutinosa* using an enriched genomic library, and these markers were characterized in two wild populations of this species. The number of alleles per locus ranged from two to 20. A high genetic diversity was observed in two populations, with average observed heterozygosity of 0.812 and 0.794, and average expected heterozygosity of 0.802 and 0.814, respectively.
- *Conclusions: Rehmannia glutinosa* is an important medicinal resource. The genetic markers described in our study will be useful for future population genetic studies and molecular breeding programs on this species.

Key words: genetic diversity; microsatellite; *Rehmannia glutinosa*; Scrophulariaceae.

Rehmannia glutinosa (Gaertn.) Libosch. ex Fisch. & C. A. Mey. (Scrophulariaceae) is a perennial herbaceous species of medicinal value (Zhou, 2002; Shao et al., 2008). Its tuberous roots are commonly used in Chinese traditional medicine (Wen et al., 2002). In recent decades, a significant number of chemical and pharmacological studies have been performed on *R. glutinosa* (Zhang et al., 2008; Chang et al., 2011). More than 70 compounds, including iridoids, saccharides, amino acids, inorganic ions, and other trace elements, have been found in the herb (Zhang et al., 2008). Many studies show that some active ingredients in the roots of *R. glutinosa* possess broad pharmacological actions for protecting gastric mucosa and restraining pulmonary fibrosis (Liu et al., 2009). In addition, the root has been demonstrated to improve hematopoiesis, have anti-inflammation and antitumor activities, decrease blood sugar, and promote the proliferation of vascular endothelial cells (Liu et al., 2009).

Genetic knowledge about *R. glutinosa*, such as its genetic diversity, genetic structure, and gene flow, serves as a foundation for cultivating improved varieties and exploiting and utilizing Chinese traditional medicine resources (Zhang et al., 2012). Simple sequence repeats (SSRs) are highly polymorphic, multiallelic, reproducible, abundantly distributed in the genome, and easy to interpret (Tanya et al., 2011). They are also codominant inheritance markers and can provide the amplified result of the heterozygote or the homozygote. In this study, we isolated 16 microsatellite loci from *R. glutinosa* and used these loci as markers to estimate the genetic diversity of two wild populations.

METHODS AND RESULTS

Forty-four individual leaves were collected from two wild populations of *R. glutinosa*: Hebi (HB: 35°36′00″N, 114°12′00″E) and Jiaozuo (JZ: 35°13′48″N, 113°25′48″E). The voucher specimens (*Rehmannia glutinosa* HB09001 and *Rehmannia glutinosa* JZ09001 for the HB and JZ populations, respectively) are deposited in the herbarium of Henan Agricultural University (HEAC). These leaf samples were dried quickly with silica gel and stored at −20°C. The total DNA was isolated from the dried leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Fang et al., 2009) and purified with a Universal DNA Purification Kit (Tiangen Biotech, Beijing, China). The purified DNA was digested with the enzyme *Rsa*I, and the digested DNA fragments were linked to SuperSNX-24F (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′) and SuperSNX-24R (5′-GATTCTGCTAGCTAGGCCTTAAACAAAA-3′) adapters. Using biotinylated $(AC)_8$, $(AG)_8$, and $(ATG)_{12}$ as probes (New England Biolabs, Beijing, China), the ligated fragments were hybridized and captured by streptavidin-coated magnetic beads. After purification, DNA fragments were ligated to the PMD18-T vectors (TaKaRa Biotechnology Co., Dalian, China) and transformed into DH5α cells (TaKaRa Biotechnology Co.). All white clones were tested with PCR amplification using M13F and M13R primers. Ninety-six positive clones with inserted fragments ranging from 400 to 1000 bp were selected and sequenced on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California, USA). Out of the 96 clones, 48 contained microsatellite repeats. Based on the sequences with microsatellite repeats in the middle region of the sequences, 32 primer pairs were designed using the program Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). Using 12 *R. glutinosa* individuals, PCR amplifications were performed in 10 μL of a solution containing approximately 50 ng of genomic DNA, 10 μM

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TABLE 1. Characterization of 16 microsatellite loci in *Rehmannia glutinosa.*

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank accession no.
DH-1-13	F: AAGTTGAAAGAGTGGTGG	$(CT)_{22}$	49.7	386–465	KC977459
	R: AATACAAAAGCCTCCGA				
DH-1-16	F: TGGGGTGGAGAGGTAGG	$(CAT)_6$	52.8	274–297	KC977461
	R: AAGGTTGCGTCAGGAAG				
DH-1-18	F: TTTTGGCGATGACTACGGG	$(AG)_{27}$	57.7	380–433	KC977462
	R: GATGAATGGGCTGGGCTCT				
DH-1-45	F: AAGACCCATTGCCCCGTA	$(CAT)_6$	50.6	314–344	KC977466
	R: GTTCCCATTTTCGCCTCC				
DH-1-53	F: ACGAAACGCCGACGAAT	$(GAT)_7$	51.8	343–361	KC977467
	R: CCAACAAACCCAGAGCC				
DH-1-59	F: TGAGATGTGTAGATTGCTTTTG	$(CT)_{11}(CA)_9$	52.3	275–299	KC977470
	R: GCACGCAGGGTCTTATGT				
DH-1-73	F: AGCATCATTACCGCCCAAAA	$(CAT)_7$	54.1	100–163	KC977472
	R: TCAACCGAGAATCAATAGTAG				
DH-1-94	F: TCTTATGTGGAGAATGTGTC	$(TG)_8$	52	258–298	KC977473
	R: GGGCTGATTACTGGAGAGG				
DH-1-106	F: AGCATCGTTGAATATCTGG	$(CAT)_7$	47.5	164–176	KC977475
	R: GAAAGTGTTATCTCCCCTC				
DH-1-117	F: CCATTCTCAAGCACAAA	$(CAT)_{11}$	59	107–191	KC977477
	R: AACTTCACACCACAGACC				
DH-1-118	F: TTTGCTGGTGGTTCTTCGTCC	$(GA)_{21}$	63.5	253–376	KC977478
	R: GCATTGTCGCCTCCCTCC				
DH-1-124	F: ATAAAACCTCACCTACCCGAAC	$(TC)_4A(TC)_3GAAT(TC)_4$	59.5	264–266	KC977480
	R: AAACAAGCCCTCAACCCACC				
DH-2-41	F: AGTCGTCGTCATCGGTT	$(AG)_{25}$	55.8	278–309	KC977482
	R: CCACTTGCCAGCTCTTT				
DH-2-49	F: AAGATGCTCGTCCCCCATAC	$(TCA)_3(TGA)(GAT)_3$	54	190–217	KC977483
	R: GCCGCCAAGATTCAAAAATGTC				
DH-3-43	F: CCAAGCCCAAAGCCAAA	$(CT)_{14}A(TC)_{10}(AC)_8$	55	247–291	KC977484
	R: GGAAGCATACCTAATCGCAAA				
DH-4-44	F: CACGCCAACGAAGACATA	$(AG)_{18}$	49.2	298–332	KC977488
	R: GACCCCTTGTTTGTTTCC				

Note: T_a = annealing temperature.

of each primer, and 1× PCR Mix (Tiangen Biotech). The amplification profiles included an initial denaturation at 95°C for 5 min; followed by 35 cycles of 45 s at 94°C, 30 s at 47.5–63.5°C (Table 1), and 1 min at 72°C; and a final extension at 72°C for 10 min. The PCR products were measured on 2% agarose gels.

Of the 32 primer pairs tested, 15 were polymorphic, one was monomorphic, and 16 failed to amplify the expected products. We selected the 16 scorable loci (Table 1) to determine the genotypes of the 44 *R. glutinosa* samples. According to the M13-tail PCR method (Schuelke, 2000), three primers were used in PCR, the forward primer with an M13 tail at the 5′ end, the reverse primer, and a fluorescent dye–labeled primer that was complementary to the M13 tail (FAM or HEX). The amplified fragments were subjected to capillary electrophoresis on an ABI 3730xl DNA Analyzer (Applied Biosystems), and the sizes were determined using GeneMapper ver. 4.0 (Applied Biosystems).

Arlequin suite version 3.5 (Excoffier and Lischer, 2010) was used to calculate the number of alleles per locus (A), the observed heterozygosity (H_o), the expected heterozygosity (H_e), and the deviation from Hardy–Weinberg equilibrium (HWE). All 16 loci were successfully amplified in the 44 individuals from the two populations (Table 1). The number of alleles per locus ranged from two to 20 (average = 12.0 in the Hebi population, average = 11.9 in the Jiaozuo population) (Table 2). High heterozygosity values were observed in the two populations, with an H_o of 0.812 and H_e of 0.802 in the HB population and an H_o of 0.794 and H_e of 0.814 in the JZ population. Significant deviation from HWE ($P < 0.05$) was detected in two loci from the HB population (DH-1-16, DH-1-118) and three from the JZ population (DH-1-53, DH-1-118, DH-1-124). We detected the presence of null alleles using MICRO-CHECKER (van Oosterhout et al., 2004) and had no indication of null alleles except for locus DH-1-118.

CONCLUSIONS

Sixteen microsatellite loci were isolated from *R. glutinosa*, and these loci were analyzed to estimate the genetic diversity of two wild populations. Our study observed a high genetic diversity in the two wild populations. The genetic markers described in our study will be useful for future population genetics studies and molecular breeding programs on this species.

TABLE 2. Results from the initial primer screening in two populations of *Rehmannia glutinosa.*[a]

	Hebi (N = 24)			Jiaozuo (N = 20)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]
DH-1-13	20	0.905	0.951	15	0.941	0.930
DH-1-16	10	0.625	0.834**	6	0.500	0.754
DH-1-18	18	0.870	0.947	20	0.889	0.965
DH-1-45	10	0.958	0.774	9	0.950	0.753
DH-1-53	13	0.750	0.871	13	0.650	0.878*
DH-1-59	16	1.000	0.941	16	1.000	0.909
DH-1-73	9	0.833	0.699	12	0.950	0.835
DH-1-94	18	0.917	0.944	18	0.947	0.949
DH-1-106	8	1.000	0.777	9	1.000	0.774
DH-1-117	10	0.958	0.694	11	0.950	0.838
DH-1-118	13	0.316	0.881**	16	0.400	0.929**
DH-1-124	2	0.522	0.487	3	0.474	0.553**
DH-2-41	16	0.958	0.876	15	0.900	0.885
DH-2-49	3	0.667	0.488	2	0.400	0.328
DH-3-43	14	0.750	0.777	19	0.800	0.897
DH-4-44	12	0.958	0.894	7	0.950	0.853
Average	12	0.812	0.802	11.9	0.794	0.814

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

[a]Geographic coordinates and voucher information: Hebi (HB: 35°36′00″N, 114°12′00″E), voucher *Rehmannia glutinosa* HB09001; Jiaozuo (JZ: 35°13′48″N, 113°25′48″E), voucher *Rehmannia glutinosa* JZ09001. Vouchers deposited at Henan Agricultural University (HEAC).

[b]Deviations from Hardy–Weinberg equilibrium: *$P < 0.05$, **$P < 0.01$.

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