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Development and characterization of microsatellite markers for *Panax notoginseng* (Araliaceae), a Chinese traditional herb[1]

Han Liu[2,3], Tao Xia[2,3], Yun-Juan Zuo[2], Zhong-Jian Chen[4], and Shi-Liang Zhou[2,5]

[2]State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Xiangshan, Beijing 100093, China; [3]Graduate University of the Chinese Academy of Sciences, Beijing 100039, China; and [4]Wenshan Institute of Sanqi Research, Wenshan 663000, Yunnan, China

- *Premise of the study*: Microsatellite primers were developed for a Chinese traditional herb, *Panax notoginseng*, to investigate its genetic diversity and cultivar breeding.
- *Methods and Results*: Twelve polymorphic microsatellite loci were isolated from the microsatellite-enriched genomic library of *P. notoginseng*. The polymorphisms were assessed in two populations and an assemblage containing individuals from the whole distribution area. The number of alleles per locus ranged from three to 12, with a mean of 5.8, and the observed and expected heterozygosity values ranged from 0.0411 to 0.8472 and from 0.0804 to 0.7653, respectively.
- *Conclusions*: These new microsatellite markers will be useful for investigation of the genetic diversity of cultivated *P. notoginseng* as well as assist in cultivar breeding.

Key words: cultivar breeding; genetic diversity; microsatellites; *Panax notoginseng*; sanqi.

Sanqi (*Panax notoginseng* (Burkill) F. H. Chen ex C. Y. Wu & K. M. Feng), like ginseng (*P. ginseng* C. A. Mey.) and American ginseng (*P. quinquefolius* L.), is a well-known medicinal herb that is used to synthesize both ginseng saponins and ginseng polysaccharides. Furthermore, it has the highest content of dencichine (β-N-oxalyl-L-α,β-diaminopropionic acid) among the three ginseng species (Zheng et al., 1989). Dencichine has been proven to have a strong homeostatic effect and is used for the prevention and treatment of blood loss in many hemorrhage models (Liu et al., 1982). However, due to uncontrolled harvesting, *P. notoginseng* is now completely extinct in the wild. Currently, the cultivation of this herb has been confined to a small area of Yunnan Province, China. The conflict between the great market demand for this species and the very limited land availability has necessitated the breeding of cultivars for high yields, high concentrations of active components, and high resistance to diseases. Unfortunately, no cultivar has been bred so far and selections of superior individuals meeting the requirements are now underway from field and hybrid progenies. Molecular marker–assisted selection has become routine in cultivar breeding, and great efforts have been made to develop molecular markers such as microsatellites. Here, we report a set of novel polymorphic microsatellites for *P. notoginseng* that will provide useful markers to assess the biodiversity of sanqi to improve this important herb through breeding.

METHODS AND RESULTS

The microsatellite-enriched library was constructed following the procedures described by Chen et al. (2008), Tian et al. (2008), and Wang et al. (2008). Genomic DNA was extracted from dried leaves of a single individual of *P. notoginseng* (voucher: Maguan, Yunnan, *Shiliang Zhou 2005828*, PE) according to Doyle and Doyle (1987) and purified with the Wizard DNA Clean-Up System (Promega, Madison, Wisconsin, USA). Microsatellite enrichment was completed following Glenn and Schable (2005) with some modifications. The DNA was digested with *Rsa*I (New England Biolabs, Ipswich, Massachusetts, USA) and ligated to a double-strand SuperSNX-24 linker (forward 5′-GTTTAAGGCCTAGCTAGCAGAATC, reverse 5′-pGATTCTGCTAGCTAGGCCTTAAACAAA). For the purpose of enrichment, the resulting DNA was denatured and hybridized with a mixture (1 μM each) of single-stranded biotinylated microsatellite probes $(AG)_{12}$, $(AT)_{12}$, $(CG)_{12}$, $(GT)_{12}$, $(ACG)_{12}$, $(ACT)_{12}$, $(CCA)_8$, $(AACT)_8$, $(AAGT)_8$, and $(AGAT)_8$. The fragments hybridized to the probes were captured by streptavidin-coated paramagnetic beads (M-290 Streptavidin, Dynal Biotech ASA, Oslo, Norway), and then collected with a magnetic particle collecting unit (Dynal MPC-S, Dynal Biotech ASA). The enriched DNA was amplified by PCR, using the SuperSNX-24 linker forward strand as a primer, and enriched again. The resulting samples from the second enrichment were amplified again, purified, and ligated onto pGEM-T easy vector (Promega) and transformed into competent cells of *Escherichia coli* using the Top10 cloning kit (TransGen Biotech, Beijing, China). Of 228 positive colonies screened, 132 (58%) of different insert sizes were sequenced. Sequences from both strands were assembled and edited with Sequencher v. 4.6 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Of these sequenced colonies, 52 (39%) fragments contained SSR motifs that were identified using SSRHunter 1.3 (Qian Li, Nanjing Agricultural University, Nanjing, China). Of these, only 29 unique sequences were suitable for designing SSR primers using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA) under the following criteria: (1) guanine-cytosine content between 40% and 60%; (2) melting temperature (T_m) between 52°C and 62°C; (3) not more than 2°C in annealing

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[5]Author for correspondence: slzhou@ibcas.ac.cn

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TABLE 1. The characteristics of 12 polymorphic microsatellite loci of *Panax notoginseng*.

Locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Size range (bp)	GenBank Accession No.
noto01	$(GT)_6(TA)_5(TG)_{21}$	F: TGGCATGGGACAACAATGAAG R: TCGCAAGAGATACTGGAGCTG	56	295–315	JF262618
noto02	$(CAC)_7$	F: AACATTTTGCGGCAGTTGCAA R: GCTAGTGGTCAAGTGGGCCA	58	209–216	JF262619
noto03	$(TC)_{17}$	F: TCTTTAATTCCCTTTCGTTGG R: GTAGGTTAGTAAGGAGGTGGG	58	230–236	JF262620
noto04	$(TC)_6(CA)_{10}$	F: CTTCCATTCTTATATCCATTC R: GCTGATCGAATCAAACGG	58	150–170	JF262621
noto05	$(CT)_6N_{13}(CT)_9$	F: TGGATGAGAACCTCACCAGC R: TTAATCACCCATTCATTCGCT	58	203–213	JF262622
noto06	$(TG)_6$	F: CACAATCCCAACCATCACAGA R: AATCGCCCTCGTGAGTAGAAA	58	315–335	JF262623
noto07	$(GCC)_7$	F: TTCAGAAATTACCACCAAGC R: CACATCAACTCCCTCCTCTT	58	127–133	JF262624
noto08	$(CA)_7$	F: GTGTAGTCTTTGCCTCTGTCA R: AGTGAGTCCCAAGTTTTCTAG	58	191–197	JF262625
noto09	$(TC)_{10}(CA)_9(CT)_5$	F: GCCTGATTGTGAGAATGTTAG R: TTGGGAAAGAAAGGAAAGGTA	58	160–180	JF262626
noto10	$(TGG)_5N_{77}(TGG)_5$	F: TGCTATGGGTGGGGTGTTGAG R: ATCAACCACCTCCAACCGTCG	56	190–202	JF262627
noto11	$(TG)_8$	F: GTGACTCCATGTTGTCTGAGC R: TCTATATCCTTCACGAGCACTG	56	83–89	JF262628
noto12	$(AC)_7(AT)_5(AC)_5$	F: GAGGAATAAAAGAGCCCAAAA R: GCAATTAAATCCACCAATAAC	58	286–292	JF262629

T_a = annealing temperature (°C).

temperature (T_a) between primer pairs; (4) primer size between 18 and 22 bp in length, and (5) amplicon size between 80 and 350 bp in length. The primer pair with the highest score given by Primer Premier was chosen.

Primer pairs were initially screened for amplification success using two samples. Standard PCR conditions were carried out in a 10 μL reaction containing 5–10 ng of DNA template, 150 μM of dNTPs, 1.5 mM of $MgCl_2$, 1.0 μM of each primer, 10× PCR buffer (Tiangen Biotech Ltd., Beijing, China), and 0.5 U *Taq* DNA polymerase (Tiangen Biotech Ltd.). The PCR program was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 30 s at annealing temperature (Table 1), 72°C for 1 min, and a final extension of 72°C for 10 min. Using these PCR conditions, 23 of the 29 primer pairs were successfully amplified with amplicon sizes approximately matching the expected sizes. As a result, these 23 primer pairs were chosen for polymorphism tests using samples from two populations, one in Qiubei County (24°2′31″N, 104°11′31″E) containing 24 samples, the other in Maguan County (23°0′30″N, 104°23′34″E) containing 29 samples, and a further assemblage containing 20 samples from the whole distribution area.

Forward primers of the 23 loci were labeled with fluorescent dye (6-FAM). Amplifications were carried out under the same PCR conditions given above. The PCR products were resolved by an ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA). PCR amplification failures were rerun under the same conditions, and the unique alleles were confirmed by repeating the screening experiments. Microsatellite loci were scored using GeneMapper version 4.0 (Applied Biosystems). The resulting genotype data were analyzed using POPGENE version 1.31 (Yeh and Yang, 1999) to calculate the number of alleles per locus (A) and the observed (H_o) and expected (H_e) heterozygosity values.

Twelve polymorphic markers (Table 1) were amplified consistently over two populations and the assemblage. In the Qiubei population, the number of alleles per locus varied from one to six with an average of 3.4, and the observed and expected heterozygosity per locus ranged from 0 to 0.8750 and from 0 to 0.7571, respectively (Table 2). In the Maguan population, the number of alleles per locus varied from one to eight with an average of 3.1, and the observed and expected heterozygosity per locus ranged from 0 to 0.8276 and from 0 to 0.7532, respectively (Table 2). Although one locus in Qiubei and two loci in Maguan are monomorphic, all 12 loci are polymorphic across the two populations and the assemblage (Table 2). With all samples considered together, the number of alleles per locus ranged from three to 12 alleles and the observed and expected heterozygosity per locus ranged from 0.0411 to 0.8472 and from 0.0804 to 0.7653, respectively (Table 2).

TABLE 2. Characterization of 12 primers from two populations of *Panax notoginseng* (Qiubei and Maguan) and an assemblage.

	Qiubei (N = 24)			Maguan (N = 29)			Assemblage (N = 20)			Total sample (N = 73)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
noto01	6	0.8750	0.7571	5	0.8276	0.7532	7	0.8421	0.7852	7	0.8472	0.7653
noto02	4	0.1250	0.1977	2	0	0.0678	3	0.0526	0.1522	4	0.0556	0.1333
noto03	6	0.5417	0.5727	8	0.5517	0.7084	6	0.8889	0.7016	12	0.6338	0.6790
noto04	5	0.5000	0.6055	6	0.6897	0.6195	7	0.5000	0.6128	9	0.5753	0.6320
noto05	4	0.4167	0.4016	4	0.4138	0.3938	6	0.8000	0.7513	7	0.5202	0.5198
noto06	2	0	0.2234	2	0	0.2904	8	0.9000	0.8128	8	0.2466	0.5699
noto07	3	0.0417	0.1959	2	0.0345	0.0345	2	0.0588	0.1658	3	0.0429	0.1226
noto08	2	0	0.0816	1	0	0	4	0.7000	0.6756	4	0.1918	0.3113
noto09	3	0.0417	0.1215	2	0.0345	0.0345	3	0.0500	0.0987	4	0.0411	0.0804
noto10	2	0	0.0816	1	0	0	4	0.2000	0.2333	5	0.0584	0.0935
noto11	3	0.0833	0.0824	2	0.0690	0.0678	3	0.6000	0.4974	4	0.2192	0.2221
noto12	1	0	0	2	0.0345	0.0345	2	0.2000	0.1846	3	0.0685	0.0670
Mean	3.4	0.2188	0.2767	3.1	0.2213	0.2504	4.6	0.4827	0.4726	5.8	0.2914	0.3497

A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of samples.

CONCLUSIONS

The newly developed microsatellite loci showed high levels of polymorphism in *P. notoginseng*. These markers provide a good resource to assess the genetic diversity in sanqi and would be useful in molecular marker–assisted selection of unique forms, as well as in genetic evaluations of pure lines.

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