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A set of novel microsatellite markers developed for a traditional Chinese medicinal plant, *Sarcandra glabra* (Chloranthaceae)

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Introduction

Sarcandra glabra (Thunb.) Nakai (Chinese name: *Zhong-jie-feng* or *Cao-shan-hu*) (Chloranthaceae), an evergreen subshrub distributed in the southern parts of China and in Southeast Asia. It is an important crude herb used in traditional Chinese medicine. It has been demonstrated that herba sarcandrae, i. e. the dried *S. glabra*, has extensive pharmacological actions and is effective in the treatment of cancer, pneumonia, appendicitis, gastritis, enteritis, rheumatism, and injuries due to fall and fracture (China Pharmacopeia 2010; Xu *et al.* 2011). Due to its beneficial pharmacological effects, especially the increasing recognition of the use of Chinese medicine in cancer treatment, the wild resources of *S. glabra* dramatically reduced due to years of overharvesting and curtailment of habitat since the 1980s (Xu *et al.* 2011).

Understanding the genetic basis of germplasm resources is essential for the efficient evaluation, conservation and utilization. However, the population genetic variation in *S. glabra* has only been reported using ISSR markers (Ni *et al.* 2008). Microsatellites or simple sequence repeats (SSRs) are DNA sequence units composed of tandemly repeated 2–5 bp DNA core sequences, and the variations in the number of tandem repeats result in PCR product length differences. SSRs have proved to be useful markers for the study of genetic diversity, population structure and genetic resources assessment because of their highly polymorphic and codominant nature. Here, we have characterized a set of microsatellite markers for *S. glabra*.

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Materials and methods

Materials included 33 *G. jasminoides* plants collected from one wild population in Jiangxi province (25°30′5319″N, 115°17′44″E), China. A dinucleotide-enriched microsatellite genomic library was constructed and screened using a fast isolation by AFLP of sequences containing repeats (FIASCO) protocol (Zane *et al.* 2002) with some modifications. Approximately 250 ng of total genomic DNA was digested with *Mse*I restriction enzymve (New England Biolabs, Massachusetts, USA) at 37°C for 3 h, and then fragments of 200 to 800 bp were ligated to the *Mse*I adapters (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′). A diluted digestion-ligation mixture (1:10) was PCR amplified 18 cycles with *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′). Approximately 500 ng of amplified DNA was hybridized with 80 pmol of 5′-biotinylated $(AC)_{13}$ oligonucleotide in a total volume of 100 μL of 4.2 × SSC and 0.07% SDS. The mixture was incubated at 95°C for 5 min and cooled to room temperature for 30 min. The hybridization products containing microsatellites were selectively captured with 300 μL of streptavidin-coated beads (Promega, Madison, USA), three nonstringent and three stringent washes were carried out following Zane *et al.* (2002). DNA containing repeats was amplified 27 cycles with *Mse*I-N primers. PCR product was purified with E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, Doraville, USA) and ligated into pMD19-T plasmid vector (Takara, Dalian, China) and transformed into DH5α competent cells.

Recombinant clones were detected by PCR amplification using M13 forward and reverse primers. The positive clones with foreign inserts were sequenced using an ABI PRISM 3730XL automated sequencer (Invitrogen, Shanghai, China). Unique microsatellites with sufficient flanking regions were

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chosen to design primer pairs with PRIMER 3 software (Rozen and Skaletsky 2000) or PRIMER 5.0 (Clarke and Gorley 2001).

All SSR primers were tested using 33 individuals from one wild population of species that sampled from Jiangxi province. Amplifications of microsatellite loci were performed in a final volume of A 10 μL containing approximate 50 ng of template DNA, 0.5 μM of forward and reverse primers, 1.5 mM of $MgCl_2$, 200 μM of dNTPs, 1× *Taq* buffer (100 mM Tris-Hcl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20) (Fermentas, Shenzhen, China) and 0.5 unit of *Taq* polymerase (Fermentas, Shenzhen, China). Cycling conditions were 94°C for 5 min followed by 34 cycles at 94°C for 50 s, locus-specific annealing temperatures (43–58.5°C, table 1) for 50 s and 72°C for 1 min, with a final extension step of 10 min at 72°C. The PCR product was then separated on 6% denaturing polyacrylamide gel and visualized by silver staining. Twenty-five base pair DNA marker ladder (Promega, Beijing, China) was used as a standard for identifying allele size.

Preliminary population genetics analyses, e.g. the number of alleles, the observed and expected heterozygosity, deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD), were performed using Genepop ver. 4.0 (Raymond and Rousset 1995). The value of polymorphic information content (PIC) was calculated using Cervus ver. 3.0.3 (Kalinowski *et al.* 2007).

Table 1. Primer sequences and characteristics of 18 microsatellite loci isolated from *S. glabra*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a	Size range (bp)	N_a	H_O	H_E	PIC	GenBank no.
SG01	F: ACTCAAGTGCACCCATGGTC R: GTTGTGGCACCCAATTCTTT	$(GT)_{11}$	56	240–270	6	0.125	0.623*	0.575	JQ713100
SG02	F: CCCCTACTAAACACACACACTCA R: GTGGACCCATGATTTCCATC	$(CT)_{29}$	58	165–193	4	0.000	0.517*	0.452	JQ713101
SG03[P5]	F: ACCAGAAGTACCTGGATA R: ACACTACAAGGATGAGACA	$(GA)_{15}$	43	170–174	2	0.194	0.513*	0.375	JQ713102
SG04	F: TCACGGAATACGAGGTAAATGA R: TGGGAAGCAAAGAATAACGA	$(AC)_{11}$	56	174–178	2	0.061	0.350*	0.266	JQ713103
SG05[P5]	F: TTTTCTTGGTTGGTATCCTC R: TGTGGGCACTACTCCTATCT	$(TC)_3A(TC)_6$	45	219–225	2	0.121	0.116	0.083	JQ713104
SG06	F: GAAGTCAGGTAGCAGCCATCA R: GCGCACTCAAACAGGTACAA	$(TC)_{26}$	56	141–175	6	0.031	0.771*	0.714	JQ713105
SG07	F: CCAACCCATTGAGACCCCTA R: CGGCTTCCCTCTAGGTCTTC	$(GA)_{12}$	56	157–159	2	0.606	0.506	0.375	JQ713106
SG08	F: GCAATCAATTGGTTCGAATG R: TACTCCAGTAGCCGGAGGAG	$(TG)_{19}(TA)_2(TG)_{13}$	55	231–245	3	0.061	0.321*	0.288	JQ713107
SG09	F: GAGAGCGATGGACGACTGAT R: TTTCTCTTCCCCAAAGAGGAG	$(AG)_{30}$	56	151–175	6	0.030	0.527*	0.490	JQ713108
SG10	F: TGCTAAAGGAGCCAATGGTAA R: ACCAATACAAGGGCAAGGTG	$(TA)_8(CA)_8$	58.5	165–181	4	0.485	0.579	0.562	JQ713109
SG11[P5]	F: GAAAGACAACCTGCCACA R: ATCCCGATTCGTAAACAC	$(TC)_{26}$	45	150					JQ713110
SG12	F: CTCACTACAATCGGGGTTCG R: CAGTTTCGCCCGTGTAGG	$(GT)_{10}$	58	100					JQ713111
SG13	F: AACACCTCCAAAATGGCTTG R: TCCCGGTTTTGTACTTAGGG	$(AT)_6(AC)_{11}$	58	190					JQ713112
SG14	F: GAGCAACTACCATGGATTGTGA R: ATCAAGGATTGGGGGTTTCT	$(CT)_{23}$	57	155					JQ713113
SG15	F: ACAGGATCCATGAAGGGTCA R: TCAATCAGATGCCTGGATCA	$(CA)_8$	56	219					JQ713114
SG16	F: GCCATTTCCCCATCTAGGTC R: GCAATTGATGAAGCCATGTG	$(TG)_9$	55	250					JQ713115
SG17	F: TGGATCCGACAGTGATGATG R: TGGATTGACGAGATGTTGGA	$(AC)_9$	56	235					JQ713116
SG18	F: CAGCATCCTTGGGAGAGAAG R: AGAGGGCAGACAAGACACAAA	$(TG)_{14}$	56	263					JQ713117

N_a, the number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphism information content.
*Significant departure from HWE ($P < 0.05$).
[P5]Primer designed by PRIMER 5 and others were by Primer 3.

Results and discussion

A total of 99 positive clones were identified and sequenced, and 65 (65.6%) DNA sequences containing SSRs were obtained. Forty-two primer pairs were designed and were used for next characterization. Eighteen of the 42 primers (42.9%) successfully amplified DNA fragments with expected size, of which 10 showed polymorphism and eight yielded monomorphic products (table 1). Despite best efforts at primer design, some microsatellite loci with apparently suitable priming sites fail to amplify. Some optimization of reaction conditions can improve success, but this nevertheless represents an additional source of attrition (Squirrell *et al.* 2003). In addition, genome size may be an important factor on amplification potential. The proportion of primer sets that did not give PCR products is often strongly positively correlated with the haploid *C* value of target species (Garner 2002). But we know little about the genome size of *S. glabra*. However, obtaining a PCR product seems very difficult in some taxon. In *Epimedium koreanum* Nakai (Zhou *et al.* 2007) and *E. brevicornum* Maxim (Xu *et al.* 2008), the ratio of successfully amplified the corresponding regions was 20.4% (19/93) and 28.8% (17/59), respectively. In *Isoetes hypsophila* Palmer, 30% (9/30) successfully amplified target fragments (Li *et al.* 2012). For the *Potentilla* core group (Rosaceae), 37% (74/200) primer-to-marker conversion ratio was reported (Dobeš and Scheffknecht 2012).

The polymorphic loci had two to six alleles, with an average of 3.70 alleles, the expected heterozygosity (H_E) ranged from 0.116 to 0.771 and the observed heterozygosity (H_O) varied from 0.000 to 0.606 (table 1). Seven of the 10 polymorphic loci showed heterozygote deficiency and significantly deviated from the HWE (table 1). Two pairs of loci (SG07 and SG08, SG07 and SG10) showed significant LD (Fisher's exact test, $P < 0.01$), but it became nonsignificant after Bonferroni correction ($P > 0.00011$). Generally, the 10 polymorphic loci showed moderate polymorphism in 33 individuals from the same population. Typically a researcher will screen the candidate primer sets against a range of samples representative of the broad geographical/ecological diversity of their study taxon to evaluate whether individual loci are likely to be polymorphic (Squirrell *et al.* 2003), but only one population was used in this study. Therefore, higher genetic variation would expect when more populations and individuals detected. Moreover, the monomorphic locus of present study may be polymorphic in other populations. We are currently using these microsatellite loci together with AFLP markers to assess patterns of geographic molecular variation at the population level and across its distribution range in China. We anticipate that these data will be useful to guide the evaluating and preserving the genetic resources of this important medicinal plant.

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