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ONLINE RESOURCES

Isolation and characterization of twenty-two polymorphic microsatellite markers from *Gardenia jasminoides* (Rubiaceae)

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Introduction

Gardenia jasminoides Ellis (Zhi Zi in Chinese) (Rubiaceae), an ancient medical herb, is noted for its medicinal properties in Chinese, Korean, Japanese and Vietnamese pharmacopoeias. It has been demonstrated that Fructus Gardeniae (FG), namely the dried ripe fruits of *G. jasminoides*, has extensive pharmacological activities, such as protective activity against oxidative damage, cytotoxic effect, antiinflammatory activity and fibrolytic activity (Koo *et al.* 2004). FG has been widely used in traditional Chinese medicine for centuries. It is proved to be effective in the treatment of jaundice, inflammation, headache, oedema, fever, hepatic disorders and hypertension (China Pharmacopeia 2010). In addition, it is reported that the extract and constituents are useful for the treatment of gastritis (Lee *et al.* 2009). Gardenia yellow from the fruit is a natural pigment which can be transformed to blue pigment by modification (Lee *et al.* 2003). Both the pigments have wide applications as food colourant in products such as noodles and confectioneries in Asian countries (Ichi *et al.* 1995). Moreover, *G. jasminoides* also has potential horticultural value for its large, attractive and fragrant flowers (Watanabe *et al.* 1993). It is cultivated in China for over 2000 years, since the Han Dynasty (Han *et al.* 2007a).

It is well known that the genotypic and phenotypic variability, heritability and some special characters, such as variations of the active component contents or pigments, and the fragrance, shape, colour and size of flower are important for further genetic improvement of the species. Collection, evaluation and conservation of germplasm are prerequisites for cultivating high-yield and better quality of *G. jasminoides*. Moreover, accurate assessments of both the amount and distribution of genetic variation are necessary to evaluate and utilize the plant genetic resources (Han *et al.* 2007b). However, the genetic diversity and biogeography of *G. jasminoides* has been evaluated only using RAPD and AFLP markers (Han *et al.* 2007a, b). Microsatellites or simple sequence repeats (SSRs) would be of greater value for population genetics studies because of their highly polymorphic and codominant nature. Here, we characterized a set of novel microsatellite markers for use in genotyping and future investigations of genetic diversity and population structure in *G. jasminoides*.

Materials and methods

Thirty-six *G. jasminoides* plants collected from one wild population in Jiangxi province (28°45′53″N, 115°43′14″E), China. A dinucleotide-enriched microsatellite genomic library was constructed and screened using the modified fast isolation by AFLP of sequences containing repeats (FIASCO) protocol as described by Zane *et al.* (2002) with minor modifications (Xu *et al.* 2008). Approximately 250 ng of the total genomic DNA obtained from a single individual was digested with *Mse*I restriction enzyme (New England Biolabs, Beijing, China) by incubating at 37°C for 3 h, then the fragments were ligated to *Mse*I adapters (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGT CCTGAG-3′). A diluted digestion–ligation mixture (1:10) was PCR amplified in 16 cycles with *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′). The number of cycles (14, 16, 18, 20 and 22 cycles) was optimized to produce a smear with the size range from 200 to 800 bp and mainly concentrated in 500 bp. Approximately 500 ng of amplified DNA was hybridized with 80 pmol 5'-biotinylated

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Table 1. Primer sequences and characteristics of 22 polymorphic microsatellite loci isolated from *G. jasminoides*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a	Size ranges (bp)	N_a	H_O	H_E	PIC	GenBank no.
GJ01	F: GTGGTATTGCCCTCCTCTT R: AACCTATGTCCTTGCCTCA	$(TG)_4(AG)_{10}(TG)_8$	49.5	190–210	5	0.171	0.596*	0.507	JQ750620
GJ02	F: GGCTCTACAATCTGATTATCTT R: ACCTCTAGCAATTCTCCAT	$(TC)_{12}$	47	118–130	6	0.667	0.682	0.615	JQ750621
GJ03	F: CCTTTCTACCTCCTCCATA R: ATCTGACAAGTTCCACCAA	$(TC)_{12}$	50	146–174	6	0.676	0.650	0.601	JQ750622
GJ04	F: GTCCAACTATCCATAAACAT R: AGAAGAAAGAAGGAAACAGA	$(GA)_9(GT)_8$	49.2	205–245	7	0.800	0.829	0.793	JQ750623
GJ05	F: CGTGCATTTAGATCCTTCA R: CGTCCACATACGGTTGATA	$(AC)_8$	49	181–193	5	0.389	0.532	0.541	JQ750624
GJ06	F: AAACTTATCCTGGTCCTTT R: AATCTATTAGTGAGATGTCTGG	$(AC)_9A(AAG)_6$	48	257–261	2	0.083	0.081	0.077	JQ750625
GJ07	F: TGATTTCCGTCTCCAAGAT R: TGATTTCCGTCTCCAAGAT	$(GA)_8 \ldots (GA)_8$	49	251–255	2	0.229	0.440	0.338	JQ750626
GJ08	F: GGAGCTGAGACTAAAGTAAG R: ATCCAGAATCTAAAGCAGT	$(TC)_{14}$	47	239–269	7	0.833	0.844	0.813	JQ750627
GJ09	F: CGGACCCAGTTCGAGAAGC R: ATCCATCGCCTGAGCAACC	$(TC)_{14}$	52	224–254	7	0.786	0.851	0.802	JQ750628
GJ10	F: TCACCTTTATCACTACCAT R: GTTGACAAGTGTTGAGAATA	$(TG)_{10}(CG)(TG)_7$	47	244–300	7	0.559	0.785	0.783	JQ750629
GJ11	F: ACTGAGACTCGGAAACTGA R: TCTTTGAAACTTTGCCTGAA	$(TG)_8$	48.6	274–276	3	0.083	0.082	0.079	JQ750630
GJ12	F: CGTGTATCTGACGATAACT R: ATCCATATCTAACACTCCA	$(CA)_4\ A_2\ (CA)_9$	45	234–246	3	0.355	0.444	0.395	JQ750631
GJ13	F: ATCTGTTCACTCATCCTTT R: AGGTTATAGATTGAGCATTG	$(AC)_8$	47	191–201	4	0.167	0.449	0.397	JQ750632
GJ14	F: TTCCATACAGAAGACAAATC R: ACTACTGCATCTTGGCTAT	$(CT)_9$	48	232–250	4	0.343	0.305	0.283	JQ750633
GJ15	F: GGAACAATGAATAAATATG R: ATGAGAATAGATGAAACCT	$(TATG)_4\ (TG)_8$	45	233–241	3	0.032	0.156	0.146	JQ750634
GJ16	F: ATGGAATATCATTTGAGCT R: GTAGACGATGTCAGAAACC	$(GT)_6(CT)_5C_3(TA)_7$	46.7	191–263	8	0.629	0.858	0.823	JQ750635
GJ17	F: GAGATTGGAAATATGAACAC R: CAACTCTAGGAACAAGGTA	$(CT)_{13}$	48	170–208	9	0.861	0.883	0.857	JQ750636
GJ18	F: AGAGTGCATGAAACCATTT R: TCTTTCCTTTGTTGACCAG	$(TC)_4(TG)_8$	49	208–220	5	0.548	0.775	0.717	JQ750637
GJ19	F: TTCCATTCGGCATTCAAAA R: GCGTTAGGCACCTCACAAG	$(GA)_{10}$	52	276–280	3	0.303	0.524	0.444	JQ750638
GJ20	F: TCAGTTTTGGTGATTCCCT R: GAAGTAGAAATCGGACGCT	$(GT)_{10}$	52.4	290–298	4	0.371	0.429	0.394	JQ750639
GJ21	F: TGTTTCTCGTCTAATCCAG R: TCATTGTTCACATAATCCC	$(AC)_8(TC)_{13}$	48	250–276	5	0.486	0.590	0.547	JQ750640
GJ22	F: GAAACTTATGACGCTCCTT R: AACCTTCTTTTCCACTACA	$(CT)_{15}$	45	153–173	4	0.417	0.437	0.429	JQ750641

N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphism information content.
*Significant departure from HWE ($P < 0.05$).

$(AC)_{13}$oligonucleotide in a total volume of 100 μL 4.2× SSC and 0.07% SDS. The mixture was incubated at 95°C for 5 min and cooled to the room temperature. Then, 300 μL streptavidin-coated beads (Promega, Madison, USA) were used to separate and capture DNA fragments hybridized to the probe at room temperature for 30 min, followed by three nonstringent and three stringent washes. The PCR was performed to amplify DNA-containing repeats in 25 cycles with *Mse*I-N primers. PCR products were purified with E.Z.N.A.® gel extraction kit (Omega Bio-Tek, Doraville, USA) and ligated into pMD19-T plasmid vector (Takara, Dalian, China). The cloned plasmids were transformed into *Escherichia coli* DH5α competent cells and the recombinant clones were screened by PCR amplification with M13 forward and reverse primers. Sequencing of positive clones was conducted using an ABI PRISM 3730XL automated sequencer (Invitrogen, Shanghai, China). For further characterization, unique microsatellites with sufficient flanking regions were chosen to design primer pairs with PRIMER 5.0 (Clarke and Gorley 2001). For each microsatellite, the primer pairs with 45–53°C melting temperature, 20–24 nucleotides length,

45–55% GC content and product size range from 100 to 300 bp were selected.

Each primer pair was screened for reliable amplification, and polymorphisms of all isolated loci were investigated in the 36 *G. jasminoides* individuals from one wild population. Approximately 50 ng template DNA was used in each 10 μL reaction, containing 0.5 μM forward and reverse primers, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1× *Taq* buffer (100 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20) (Fermentas Life Sci, Shenzhen, China) and 0.5 U *Taq* polymerase (Fermentas Life Sci, Shenzhen, China). The amplification conditions were as follows: denaturation at 94°C for 5 min, 34 cycles at 94°C for 50 s, 45–52.4°C for 50 s and 72°C for 1 min, with a final extension step of 10 min at 72°C. The annealing temperatures were locus specific (table 1). PCR products were then resolved on 6% denaturing polyacrylamide gel and visualized using silver staining. The sizes of product were standardized with 25-bp DNA marker ladder (Promega, Beijing, China).

The number of alleles, 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). CERVUS ver. 3.0.3 (Kalinowski *et al.* 2007) was employed to calculate the value of polymorphic information content (PIC).

Results and discussion

A total of 104 positive clones were identified and sequenced, and 54 (51.9%) DNA sequences containing simple sequence repeats were obtained. Thirty-seven unique microsatellites with sufficient flanking regions were chosen to design primer pairs. Twenty-two out of 37 primers successfully amplified DNA fragments with expected size and yielded polymorphic products (table 1). The number of alleles per locus (N_a) was 2 to 9 with an average of 4.95. The expected heterozygosity (H_E) and the observed heterozygosity (H_O) ranged from 0.081 to 0.883 and from 0.032 to 0.861, respectively (table 1). Only one locus (GJ01) showed obvious heterozygote deficiency and significantly deviated from HWE. No significant LD between pairs of loci was detected.

In conclusion, the microsatellite markers described here will be useful for further investigations of genetic diversity, genetic structure and germplasm evaluation of this edible and medicinal plant.

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