Conserv Genet (2007) 8:1499–1501
DOI 10.1007/s10592-007-9313-3

TECHNICAL NOTE

Development of ten microsatellite loci for *Gentiana crassicaulis* (Gentianaceae)

Yong Li · Lin-Feng Li · Guo-Qing Chen · Xue-Jun Ge

Received: 19 January 2007 / Accepted: 21 February 2007 / Published online: 15 March 2007

Abstract *Genetiana crassicaulis* is one of famous Chinese medicinal plant. The over-collection for its root has caused its dramatic reduction. In order to devise adequate conservation and management strategies for this species, it is important to characterize its genetic diversity and understand its population structure. Here, 10 polymorphic microsatellite markers have been developed. AC/TG microsatellite was enriched by combining biotin capture method. Polymorphism of each locus was assessed in 30 individuals from six populations. The number of alleles ranged from 2 to 9 and the expected heterozygosity ranged from 0.32 to 0.78.

Keywords *Gentiana crassicaulis* · Genetic diversity · Microsatellite markers · Medicinal plant

Gentiana crassicaulis Duthie ex Burk. (Gentianaceae) is a perennial herb which distributed in East Himalaya and Hengduan Mountains. This species is one of traditional Chinese medicinal plants to treat inflammation of liver (Zhou and Wu 2006). The natural resources of *G. crassicaulis* have been declining in recent years because *G. crassicaulis* was traditionally harvested for its roots. In order to devise adequate conservation and management strategies for this species, it is important to characterize its genetic diversity and understand its population structure. Here, 10 polymorphic microsatellite loci of *G. crassicaulis* were developed.

Genomic DNA was extracted from leaf tissues using the cetyltrimethyl ammonium bromide (CTAB) method (Milligan 1992). About 300 ng genomic DNA was completely digested with *Mse*I (NEB). The double-stranded adaptor molecular A/B was prepared by mixing equal mole amount of oligonucleotides A (5′-TAC TCA GGA CTC AT-3′) and B (5′-GAC GAT GAG TCC TGA G-3′), heating to 95°C 5 min, 65°C 10 min, 37°C 10 min, 25°C 10 min, stored at 4°C. The DNA fragments were suspended in a 30 μl ligation reaction which containing 3 μM of A/B double-stranded adaptors and 2U of T4 DNA ligase (Fermentas) for 2 h at 37°C. Two microliter of the adapter-ligated fragments acted as templates to perform PCR in a volume of 20 μl, using the adaptor-specific primer (5′-GAT GAG TCC TGA GTA AN-3′) and following the program: 4 min at 94°C; 30 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 1 min; followed by 5 min at 72°C.

For enrichment, the PCR products were denatured at 95°C for 5 min, then hybridized with a 5′-biotinylated probe $(AC)_{15}$ in 250 μl hybridization solution (4 × SSC, 0.1% SDS, 0.5 μmol/l probe) at 48°C for 2 h. The DNA hybridized to the probe was separated and captured by streptavidin-coated magnetic beads at room temperature for 20 min, followed by two washing steps, including three times in TEN_{100} for 15 min, three times in TEN_{1000} for 24 min. The separated single-stranded DNA was subjected to a second round of PCR according to the same procedure as the first round of PCR.

The PCR products, after purified with E.Z.N.A® Gel Extraction Kit (Omega Bio-Tek), were ligated into pMD18-T vector (Takara) according to the manufacturer's instructions, then transformed into *E. coli* strain JM109

Y. Li · L.-F. Li · X.-J. Ge (✉)
State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Xingang W Road 125, Guangzhou, Guangdong 510275, China
e-mail: xjge@scbg.ac.cn

G.-Q. Chen
South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China

Table 1 Characteristics of 10 polymorphic microsatellite loci for *Gentiana crassicaulis*

Locus	Repeat	Primers sequence (5′–3′)	Ta (°C)	*N*	Size range (bp)	No. of alleles	H_o	H_E	GenBank accession no.
Gcr001	$(TC)_9(AC)_{11}$	F:GGTACAACCTTCCGACGAC	56	28	153–183	9	0.61	0.77	EF371444
		R:TTTACTCCCTTTCACATAAT							
Gcr023	$(AC)_{20}$	F:TGGAAAATGGAAACTACAC	56	28	220–245	4	0.21	0.37	EF371445
		R:CATCAGACTGACCAAAGAA							
Gcr059	$(AC)_{15}$	F:CTGCCACTGTTCCGGTAAG	60	30	158–176	4	0.47	0.70	EF371446
		R:GTTTAGAGGCTTTGGAGGG							
Gcr074	$(AG)_7(TG)_8$–$(TG)_5$	F:GGAGGAGGACCTGATGAAC	58	29	410–470	4	0.24	0.57	EF371447
	$(AG)_{12}(TG)_5(AG)_5$	R:AAGTCGTCGGCTATTTTAG							
Gcr128	$(CACCCA)_5$–$(CACCCA)_4$	F:TCAGCCTCATTTACGACAAC	59	28	200–245	6	0.32	0.73	EF371448
		R:AAGGTGAAGTGGTTAGGGTAG							
Gcr138	$(AC)_{14}$–$(AC)_{14}$	F:CTTAGTTTCTGGCATTGTAT	52	28	268–278	2	0.39	0.32	EF371449
		R:AGATTTTACTTGGACGATTT							
Gcr169	$(TG)_9G_{13}$	F:AGCTTATGTTGGACGTTTATG	52	28	160–170	5	0.04*	0.74	EF371450
		R:TTTATGATTGCACGATGAGAC							
Gcr186	$(TG)_{17}$	F:TCTACAGTTTCTGCACCGAC	60	30	218–230	4	0.20	0.53	EF371451
		R:CCCAAAGACTACATCCTTCT							
Gcr197	$(AC)_{19}$	F:GATGCAAGATCCAATAGGG	52	30	201–205	2	0.37	0.44	EF371452
		R:TCAAGTGAGTTTTCACAGA							
Gcr231	$(ACA)_6A_7CA_8(AC)_8$	F:CAAATCTAATCCCCCTAAA	52	30	200–260	5	0.67	0.78	EF371453
		R:AATGCAAATGGTATCTAAC							

Ta. annealing temperature of primer pair; *N*, number of individuals genotyped; H_o: observed heterozygosity; H_E, expected heterozygosity. Statistically significant deviation from Hardy–Weinberg expectation is indicated by * ($P < 0.01$)

(Takara). Transformants were plated, then insert-containing clones were selected by blue–white screening. The positive clone was picked out and tested by PCR using $(AC)_8$ and $M13^+/M13^-$ as primers, respectively. Seventy-six out of 196 screened clones contained potential microsatellite motifs. Sequence analysis was performed on an ABI 3700 DNA Sequence Analyzer. The sequences containing motifs repeating more than five times were regarded as microsatellites.

A total of 26 (34.2%) sequences were found to contain simple sequence repeats and then subjected to primer designing using the Primer 5.0 (Clarke and Gorley 2001). These primers were tested for polymorphism in *G. crassicaulis* population. PCR reaction was done in 20 µl volume using PTC-200 thermal cycler (MJ Research). Each reaction was performed using 20 ng of genomic DNA, 0.2 mM of each dNTP, 0.3 µM of each primer, 2 µl *Taq* buffer and 0.4 unit of *Taq* polymerase (Takara). The PCR protocols included: initial denaturation of 4 min at 94°C followed by 35 cycles with 40 s at 94°C for, 45 s at 50–60°C, 50 s at 72°C and a final extension step of 8 min at 72°C. The PCR products were resolved on 6% polyacrylamide denaturing gel and visualized by silver staining. The band size is reported using a 10 bp DNA ladder (Invitrogen) as the reference.

Table 2 Cross-species amplification of *Gentiana crassicaulis* microsatellites in other species, *G. dahurica, G. macrophylla, G. straminea and G. tibetica*

Species	Locus									
	Gcr001	Gcr023	Gcr059	Gcr074	Gcr128	Gcr138	Gcr169	Gcr186	Gcr197	Gcr231
G. dahurica	+	+	±	+	±	±	–	+	+	+
G. macrophylla	+	–	+	+	–	+	+	+	+	+
G. straminea	+	–	+	+	+	–	+	+	+	–
G. tibetica	+	+	+	+	+	–	+	+	+	–

Two individuals of each species were screened. +: expected size band amplification. ±: unexpected size band amplification. –: no amplification

A total of 30 individuals from six populations from Yunnan, Sichuan and east Tibet were used for genotyping. Preliminary population genetics analyses were performed using GENEPOP version 3.4 (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset 1995). In total, 10 primer pairs yielded polymorphic and single-locus amplification products (Table 1). The number of alleles per locus ranged from 2 to 9. The observed and expected heterozygosity ranged from 0.04 and 0.67, and from 0.32 and 0.78, respectively. One locus (Gcr169) deviated significantly from Hardy–Weinberg equilibrium (HWE) ($P < 0.01$), which was due to excess of homozygotes. No significant genotypic disequilibrium was detected for any pair of loci. For those alleles with size range exceeding 30 bp in the PCR products of Gcr001, Gcr074, Gcr128 and Gcr231, the alleles were sequenced and verified to be the target sequence. These polymorphic microsatellite loci presented here would be useful for assessing the population genetic structure of *G. crassicaulis*.

Cross-priming tests were performed in *G. dahurica*, *G. macrophylla*, *G. straminea* and *G. tibetica* using two individuals each (Table 2). Nine loci were successfully amplified in *G. tibetica*, eight in *G. macrophylla* and *G. straminea*, six in *G. dahurica.*

Acknowledgements This work was financially supported by the ''Talented People Project of the Sun Yat-Sen University''.

References

Clarke KR, Gorley RN (2001) PRIMER v5: user manual/tutorial. PRIMER-E Ltd., Plymouth, 91 pp

Milligan B (1992) Plant DNA isolation. In: Hoelzel AR (ed) Molecular genetic analysis of populations: a practical approach. IRL Press, Oxford, UK, pp 59–88

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248–249

Zhou LG, Wu JY (2006) Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinal in China. Nat Prod Rep 23:789–810