Conserv Genet (2009) 10:1433–1435
DOI 10.1007/s10592-008-9754-3

TECHNICAL NOTE

Isolation and characterization of microsatellite markers for *Primulina tabacum*, a critically endangered perennial herb

Zheng-Feng Wang · Hai Ren · Qian-Mei Zhang · Wan-Hui Ye · Kai-Ming Liang · Zhong-Chao Li

Received: 13 October 2008 / Accepted: 13 November 2008 / Published online: 23 December 2008

Abstract *Primulina tabacum* is a rare and endangered perennial herb with highly restricted limestone distribution in southern China. To enrich our scientific conservation for this species, we developed ten microsatellite markers using repetitive DNA enriched libraries. The number of alleles per microsatellite locus varied from two to six. The expected (H_E) and observed (H_O) heterozygosities varied from 0.4059 to 0.7294 and from 0.1364 to 0.5217, respectively. These markers will be employed in future studies of genetic structure in *P. tabacum*.

Keywords Conservation · Endangered · Limestone · Microsatellite · *Primulina tabacum*

Primulina tabacum Hance is a calciphilous perennial herb belonging to the family Gesneriaceae. It has a very restricted limestone distribution in northern Guangdong and southern Hunan, China. Because of recent climate change and increasing anthropogenic disturbances, the population size of *P. tabacum* has drastically decreased during the last three decades. It was listed among the 'first class protected key wild plants of China' in 1999 (Peng and Cheng 2002). It grows with other calciphilous and shade-tolerant plants, including bryophytes, around the entrances to karst caves, at about 300 m altitude (Flora of China Editorial Committee 1990). *P. tabacum* grow slowly, with maximum growth not exceeding 30 g year^{-1} (Ren et al. 2003). *P. tabacum* is insect pollinated (Ni et al. 2005) and seed is dispersed by wind (Li SJ, personal communication).

To enrich our scientific understanding of climate change and anthropogenic disturbances on genetic health of its species, we report here the isolation of ten microsatellite loci which will facilitate the study of mating system, gene flow and genetic structure in *P. tabacum*.

Genomic DNA was extracted from one dry leaf tissue by using CTAB method (Doyle 1991). Approximately 250 ng of the total genomic DNA was digested by a restriction enzyme *Mse*I (NEB) and the resulting fragments ligated with *Mse*I adaptor (5′-TACTCAGGACTCAT-3′/5′-GACG ATGAGTCCTGAG-3′) with T4 ligase (NEB) overnight at 16°C. The digestion–ligation mixture was subsequently diluted 10 times, and 2 μl was used for PCR amplification using adaptor-specific primers (5′-GATGAGTCCTGAGT AAN-3′, i.e., *Mse*I-N). PCR products hybridized to a 5′ biotin-labelled oligonucleotide probe $(GA)_{15}$ and $(AC)_{15}$, respectively. Subsequent probe-bound DNA fragments were enriched for GA or AC repeats using streptavidin-coated magnetic beads (NEB). Enriched fragments were recovered with PCR amplification using *Mse*I-N as primer. PCR products were size-selected (300–700 bp) using an agarose gel DNA Fragment Recovery Kit Ver. 2.0 (Takara). Purified DNA fragments were then ligated into the pGEM-T plasmid vector (Promega), and transformed into the *Escherichia coli* DH5α competent cells (Takara). The PCR-based method described by Lunt et al. (1999) was used to screen the recombinant clones. Identified positive

Zheng-Feng Wang and Hai Ren are joint first author

Z.-F. Wang · H. Ren · Q.-M. Zhang · W.-H. Ye (✉) · K.-M. Liang · Z.-C. Li
Guangdong Key Laboratory of Digital Botanical Garden, South China Botanical Garden, Chinese Academy of Sciences, XingKe Route 723, TianHe, Guangzhou 510650, People's Republic of China
e-mail: why@scib.ac.cn

K.-M. Liang
Graduate School of the Chinese Academy of Sciences, Beijing 100049, People's Republic of China

Table 1 Details of microsatellite loci in *Primulina tabacum* including locus name, forward and reverse primer sequences, repeat motif, annealing temperature (T_a), numbers of alleles (A), observed/expected heterozygosities (H_O/H_E), fixation index (F_{IS}) by Weir and Cockerham's (1984), GenBank accession number

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	A	H_O	H_E	F_{IS}	GenBank accession number
Pta01	F:GCAGGAAACAGCATCCAAGA R:TCTGCATTGGGAGGATCACTA	$(TC)_{15}$	64	4	0.3182	0.6607	0.5243**	FJ236471
Pta02	F:GCTCTCTCACAATTATGCCTT R:GAGCTTGTCTTCCGATCC	$(AT)_8(GT)_{12}$	56	4	0.1364	0.6501	0.7941**	FJ236472
Pta03	F:CCGATTCAATATCTATGCAAA R:GGTCTGGAATATGTGTGCGT	$(CT)_3(AC)_8$	52	3	0.2174	0.4435	0.5154	FJ236473
Pta04	F:CCATAAATGAACCCTAAACCC R:CTCTCGGCGGAGCAACCACTA	$(GA)_{18}$	62	5	0.3333	0.7294	0.5491**	FJ236474
Pta05	F:CGTCCAACGTAACAGTCATAA R:CGAAACGTGCGAAAGAC	$(AG)_{20}$	64	3	0.2609	0.5643	0.5433*	FJ236475
Pta06	F:CAGTGCTCAGCCTCGTTCTCA R:TGTTATCTCTTCCGTCATGC	$(CT)_3AT(CT)_6(CA)_5$ $CC(CA)_2(TG)_2$	60	2	0.3636	0.4059	0.1064	FJ236476
Pta07	F:CACAGATCGCTTCGACA R:AACAAAGGTTGAAACGAAAAG	$(GT)_4GC(GT)_4AC(GT)_3$ $(GC)_4(GT)_8(AT)_5$	56	3	0.3636	0.5539	0.3488	FJ236477
Pta08	F:GGGCAATAGAGTCATTC R:CATGAGTGCAAGAGTGATTGA	$(TC)_{17}$	54	6	0.3810	0.6678	0.4356*	FJ236478
Pta09	F:CATTCATTTGCACGCACTTTC R:ATTGATGGGCTTGCTGATCTG	$(GAGAGT)_5(GA)_6$	64	4	0.4762	0.5203	0.0868	FJ236479
Pta10	F:GGCAATGGCACTAAGGGA R:TGGCACCCAACCAAGTAACTG	$(GT)_7(GA)_2C(AG)_{22}$	64	6	0.5217	0.7014	0.2605	FJ236480

*$P < 0.05$ after Bonferroni correction

**$P < 0.01$ after Bonferroni correction

clones were sequenced by United Gene Holdings, LTD (Shanghai, China) with M13R or M13F as primer. Primers were designed using OLIGO 6.54 software (MBI) for 20 of the sequences contain microsatellite repeats.

Polymorphisms of these microsatellite loci were assessed by 23 *P. tabacum* individuals collected from Guangdong Province, China. PCR amplification were performed in 10 μl reaction mixtures, consisting of approximately 5 ng of template DNA, 50 mM KCl, 20 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.5 μM of each primer, 0.6 mM of each dNTP, and 0.5 U of *Taq* DNA polymerase (Takara). The reaction mixture was subjected to PCR amplification in a PTC-100 (MJ) using a PCR program, 4 min at 94°C, followed by 35 cycles of 94°C for 30 s, 52°C–64°C (depending on locus) annealing temperature for 30 s, and 72°C for 45 s, followed by 10 min at 72°C. PCR products were then resolved on 4% denaturing polyacrylamide gels and visualized by silver staining.

Observed heterozygosity (H_O), the unbiased expected heterozygosity (H_E) and fixation index (F_{IS}) were calculated using GDA 1.1 (Lewis and Zaykin 2001). Deviations from Hardy–Weinberg equilibrium (HWE) for each locus and genotypic linkage disequilibrium (LD) between all pairs of loci were tested using FSTAT 2.9.3 (Goudet 1995).

The number of alleles per locus varied from two to six. The expected (H_E) and observed (H_O) heterozygosities varied from 0.4059 to 0.7294 and from 0.1364 to 0.5217, respectively (Table 1). Five loci, *Pta*01, *Pta*02, *Pta*04, *Pta*05 and *Pta*08, deviated significantly from Hardy–Weinberg equilibrium after Bonferroni correction due to a deficit of heterozygosity. Linkage disequilibrium was only found between *Pta*04 and *Pta*07 after Bonferroni correction ($P < 0.05$). All these markers will enrich our scientific in situ and ex situ conservation for this species.

Acknowledgments We thank Shi-Jin Li for helping collect *Primulina tabacum* samples. This work was supported by National Basic Research Program of China (973 Program) (No. 2007CB411600), Guangdong Key Technologies R&D Program—Sci-Tech Basic Platform project (2007A060306011), Guangdong & Guangzhou Sci-Tech Planning Project (2006B60101034, 07118249, 2007J1-C0471)

References

Doyle JJ (1991) DNA protocols for plants—CTAB total DNA isolation. In: Hewitt GM, Johnston A (eds) Molecular techniques in taxonomy. Springer-Verlag, Berlin, pp 283–293

Flora of China Editorial committee (1990) Flora of China Vol 69. Science, Beijing

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. J Hered 86:485–486

Lewis PO, Zaykin D (2001) Genetic data analysis: computer program for the analysis of allelic data. Version 1.0 (d16c). Free program distributed by the authors over the internet from http://lewis.eeb.uconn.edu/lewishome/software.html

Lunt DH, Hutchinson WF, Carvalho GR (1999) An efficient method for PCR-based isolation of microsatellite arrays (PIMA). Mol Ecol 8:891–894. doi:10.1046/j.1365-294X.1999.00636.x

Ni X, Huang Y, Wu L et al (2005) Genetic diversity of the endangered Chinese endemic herb *Primulina tabacum* (Gesneriaceae) revealed by amplified fragment length polymorphism (AFLP). Genetica 127:177–183. doi:10.1007/s10709-005-3227-0

Peng SL, Cheng WC (2002) Rare and endangered plants in Guangdong. Science, Beijing

Ren H, Peng SL, Zhang DX et al (2003) The ecological and biological characteristics of an endangered plant, *Primulina tabacum* Hance. Acta Ecol Sin 23:1012–1017

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evol Int J Org Evol 38: 1358–1370. doi:10.2307/2408641