Isolation and Characterization of 27 Microsatellite Markers for the Endemic Species Diplarche multiflora (Ericaceae)

Authors: Zhang, Lin, Zhang, Zhi-Rong, and Gao, Lian-Ming

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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF 27 MICROSATELLITE MARKERS FOR THE ENDEMIC SPECIES *DIPLARCHE MULTIFLORA* (ERICACEAE)[1]

LIN ZHANG[2,3], ZHI-RONG ZHANG[4], AND LIAN-MING GAO[2,5]

[2]Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, People's Republic of China; [3]Graduate School of Chinese Academy of Sciences, Beijing 100049, People's Republic of China; and [4]Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, People's Republic of China

- *Premise of the study:* Microsatellite markers from the genome of *Diplarche multiflora* were developed and characterized to investigate its genetic diversity and population structure.
- *Methods and Results:* Twenty-seven microsatellite loci were isolated from the genome of *D. multiflora* using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol. Of these markers, 17 were polymorphic, and the number of alleles for the polymorphic microsatellite markers ranged from two to four, with an average of 2.2 per allele. The observed and expected heterozygosities varied from 0.0000 to 1.0000 and from 0.0000 to 0.7826, respectively.
- *Conclusions:* These polymorphic microsatellite markers will be useful for population genetic studies and for assessing the genetic diversity of this alpine species.

Key words: *Diplarche multiflora*; Ericaceae; FIASCO; microsatellite markers; polymorphism.

Diplarche multiflora Hook. f. & Thomson (Ericaceae) is an evergreen dwarf shrub 8–15 cm tall that occurs in cold, open habitats on alpine meadows, rocky slopes, or cliffs at elevations of 3500–4800 m. This species is endemic to the eastern Himalayas and northwestern Yunnan Province, China (Yang et al., 1999; Yang and Chamberlain, 2005), one of the 25 global biodiversity "hotspots" (Myers et al., 2000). Loss of habitat by deforestation and excessive grazing pressure in high-altitude pastures threatens the survival of endemic species and landraces in this region (Kala, 2000). The wild populations of *D. multiflora* are rapidly declining, and most populations of this species are small and scattered in isolated patches throughout this region. Therefore, it is urgent to initiate and establish appropriate conservation management strategies for this species. To contribute to these strategies, we developed 27 novel microsatellite markers (simple sequence repeat [SSR] markers) using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol of Zane et al. (2002) for a conservation genetics study.

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[5]Author for correspondence: gaolm@mail.kib.ac.cn

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METHODS AND RESULTS

Total genomic DNA was isolated from silica gel–dried leaves of a single individual following the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). The microsatellite loci were isolated based on the FIASCO protocol (Zane et al., 2002). Approximately 500 ng of total genomic DNA was digested with *Mse*I (New England Biolabs, Beverly, Massachusetts, USA), and the fragments were ligated to an *Mse*I AFLP adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) at 37°C for 2 h with T4 DNA ligase (Fermentas, Burlington, Ontario, Canada). Five microliters of a diluted digestion–ligation mixture (1 : 10) was used for amplification reactions with the adapter-specific primer *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′), with the following cycle program: 95°C for 3 min, 30 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 60 s, with a final extension step of 7 min at 72°C. The amplified fragments (200–800 bp) were enriched for microsatellite repeats by magnetic bead selection with 5′-biotinylated $(AC)_{15}$, $(AG)_{15}$, and $(AAG)_{10}$ probes. These enriched fragments were amplified again with the *Mse*I-N primer. The PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified PCR products with enriched microsatellite repeats were ligated into the pGEM-T vector (Promega Corporation, Madison, Wisconsin, USA) and transformed into DH5α cells (TaKaRa Biotechnology Co., Dalian, China). Identification of recombinant clones was performed in a blue/white selection assay; positive clones were then tested for microsatellite inserts by PCR with $(AC)_{10}$/$(AG)_{10}$/$(AAG)_7$ and T7/Sp6 primers and sequenced and analyzed on an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, California, USA). A total of 292 clones with positive inserts were sequenced. Among these sequences, 155 (53%) sequences were found to contain microsatellite repeats (SSRs), and 64 of these sequences with sufficient flanking regions were suitable for designing locus-specific primers using the program Oligo 6.0 (Offerman and Rychlik, 2003).

The presence of polymorphisms for all 64 microsatellite loci was assessed in 12 individuals each from two natural *D. multiflora* populations (population LZ: Sejilashan, Linzhi County, Xizang Province, 29°36′27″N, 94°39′03″E, 4460 m; and population CWL: Zhamo Highway 30 km, Bomi County, Xizang Province, 29°46′31″N, 95°41′20″E, 3500 m) collected from southeastern Xizang Province, China. Voucher specimens were deposited in

the herbarium of the Kunming Institute of Botany (KUN), Chinese Academy of Sciences (population LZ: GLM-081271–081282; population CWL: STET1378 [CWL1–CWL12]). PCR reactions were performed in a 20 µL volume containing 30–50 ng genomic DNA, 0.6 µM of each primer, 7.5 µL 2× *Taq* PCR MasterMix (containing 0.1 U *Taq* polymerase/µL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, and 3 mM $MgCl_2$ [Tiangen, Beijing, China]). The PCR amplifications were conducted with the following cycle program: 95°C for 3 min followed by 30–35 cycles at 94°C for 30 s, an annealing temperature optimized specifically for each primer pair (Table 1) for 45 s, 72°C for 60 s, and a final extension step at 72°C for 7 min. The amplified fragments were separated on 6% polyacrylamide denaturing gels with a 20-bp molecular size standard ladder (Fermentas) and visualized by silver staining. Standard genetic diversity parameters for the polymorphic loci, i.e., the number of alleles (*A*), expected heterozygosity (H_e), and observed heterozygosity (H_o), were calculated using GENEPOP version 4.0.10 (Rousset, 2008). Deviations from Hardy–Weinberg equilibrium

Table 1. Specific primer sequences and characterization for the 27 microsatellite loci isolated in *Diplarche multiflora*.

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Allele size (bp)	GenBank accession no.
DA2*	$(CT)_{13}$	F: GCTTCAAACCTAGTAGGCACA R: TGAACGGAAGGGAGCAAT	57	240–270	#FJ839839
DA13*	$(TC)_8$	F: TCATCAAACTTCACGCCTCT R: GCCATTGCTTCTCCTCCT	56	84–102	JQ993329
DA17*	$(AC)_6$	F: GCCAAGGTCACAGGGTAT R: CAGCGTTCACCAGGGTCT	58	114–122	JQ993330
DA31*	$(CT)_{13}$	F: AAAGCAGCAATTACAGGT R: CATAGGAATCCAGAAAGC	51	91–105	JQ993331
DA42*	$(AG)_7$	F: AAGGCAGCAAGGGAAACC R: GTCAGCAAACGCCAACGA	57	255–259	#FJ839838
DG15*	$(AG)_5(AG)_5$	F: AGCGAGCAGGAGACGAAT R: CACGACCTGTTTCAATCCAC	57	180–192	JQ993336
DG18*	$(CT)_{11}$	F: TCTCCCTCACGTCCACCT R: AATCAGCGGCGATTCCTC	58	156–170	JQ993337
DG34*	$(CA)_8$	F: ACTCCCTAACCCCTATCT R: AGGTGAATTACTGCCATG	53	96–118	JQ993339
DG36*	$(TC)_7(TC)_{11}$	F: TATGGACCGAGGTGAATC R: GAAGTTCGCAAGAAATACC	54	250–262	JQ993340
DG41*	$(AG)_9$	F: CGCAACTTCACGCTCAAA R: TAGCTGGTTTCCACAATCACAA	57	134–150	#FJ839834
DG50*	$(AG)_5GCCC(AG)_4$	F: TTTATGGACCACAACCCA R: GATCCGAGGTATAATGCT	52	108–120	JQ993341
DG64*	$(AC)_6$	F: CGACAGCAACGAAACCCTA R: ATCGAAACATCAATCACCACAG	56	174–194	JQ993342
DG67*	$(TC)_6$	F: CTGTAGACTTGAATAAACGAA R: AGCGATTGACAAATAGAAAG	50	112–124	#FJ839832
DG71*	$(TC)_{19}$	F: CGTCAGAATACGGTCCAG R: GCACAGTAGCACGAGCAG	58	243–251	JQ993344
DG98*	$(TC)_{11}$	F: GTCCGAAGCACTGAATAA R: AGGGACATCATAGGGTTG	57	190–218	#FJ839831
DG110*	$(AG)_9$	F: TGACGGTCAGGATCTTCC R: CAATGGCTGCTGGTTTAG	52	127–131	#FJ839830
DG116*	$(TC)_{16}$	F: CCCTGCTGTTGAGTGTTG R: GAGGCATACGATGGATAAGT	52	136–150	#FJ839829
DA6	$(TC)_{13}$	F: CGGAGTGGGAAGAAAGTA R: TAGAAAGGGAAATAGAGGTT	54	154	JQ993332
DC17	$(AC)_5$	F: CACCGACCACGTAACAAC R: TGGAGGAGGAAGAGCAGT	56	178	JQ993333
DC36	$(AC)_5$	F: AGCCACTAGATAAACCTT R: GAATCCAACATACCATAAT	46	123	JQ993334
DC109	$(TC)_7(CA)_7$	F: GTTTTGGAGTGGCTTTTG R: GGTCCAACTTTCCCTTTT	51	148	#FJ839837
DC114	$(AC)_6$	F: CCAAACCCATCTGAGACA R: CTGAACACGGCGAAGGAG	53	179	#FJ839836
DG2	$(TC)_6$	F: CCACGTTCGTCAATCTTT R: GAGGGTCATACACCATTTCT	54	109	JQ993338
DG20	$(AG)_7$	F: TGGAATTGAGTAGTGAGA R: ATACCAAGTAGGTTTGTAT	48	153	#FJ839835
DG70	$(AG)_6$	F: TAAATGCGAGTAGAGGAGG R: GGGAAGGCTATGGGATAA	54	121	JQ993343
DG97	$(TC)_8$	F: GTCCAATCCAAATCTCAA R: CAAATGTCAAAAGTAAGCAA	48	169	JQ993345
DG105	$(TG)_6$	F: CTTCCCGACTTGTTTATT R: CCAACCATTACCTCCATA	48	171	JQ993335

Note: T_a = annealing temperature.

*Displayed polymorphisms in *Diplarche multiflora*.

#Sequences of these loci were developed and submitted to GenBank as part of an earlier study, but had not been previously published. These loci were re-evaluated and characterized in this study.

(HWE) and genotypic linkage disequilibrium (LD) between locus pairs according to χ^2 tests were estimated using the same software.

Of the 64 primer pairs tested, 27 were successfully amplified, of which 17 showed polymorphisms and 10 were monomorphic (Table 1). Of the 17 polymorphic primers, *A* was two to four, with an average of 2.2, and values for H_o and H_e ranged from 0.0000 to 1.0000 and 0.0000 to 0.7826, with averages for all samples of 0.4054 and 0.3696, respectively. Nine of the 17 polymorphic microsatellite loci deviated from the HWE ($P < 0.01$) (Table 2), most likely due to the presence of null alleles or limitations on the sample size. Four loci (2.9%) showed significant LD between the pairs of loci ($P < 0.001$).

TABLE 2. Results of 17 polymorphic microsatellite loci screened in two populations of *Diplarche multiflora*.

	Population LZ ($N = 12$)			Population CWL ($N = 12$)		
Locus	*A*	H_o	H_e	*A*	H_o	H_e
DA2*	1	0.0000	0.1594	2	1.0000	0.6775
DA13*	2	0.0000	0.2899	4	0.3333	0.2899
DA17	1	0.0000	0.0000	2	0.0000	0.4706
DA31*	2	1.0000	0.5217	2	1.0000	0.5217
DA42	1	0.0000	0.0000	2	0.4167	0.3442
DG15	1	0.0000	0.0000	2	0.4167	0.5616
DG18*	2	1.0000	0.5217	1	0.0000	0.0000
DG34*	2	0.0000	0.4638	3	0.3333	0.2899
DG36*	2	1.0000	0.5217	4	0.0000	0.0000
DG41*	4	1.0000	0.6667	3	0.0000	0.5072
DG50	1	0.0000	0.0000	2	1.0000	0.6775
DG64	2	0.5000	0.3913	2	1.0000	0.7826
DG67*	3	0.7500	0.6486	3	0.7273	0.4848
DG71	2	0.3333	0.2899	1	0.2500	0.2283
DG98*	2	0.0000	0.2899	3	0.6364	0.5065
DG110*	2	0.0000	0.5217	3	0.0000	0.0000
DG116	2	0.6667	0.4638	1	0.2500	0.4746
Mean	1.9	0.3676	0.3382	2.4	0.4332	0.4010

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = number of individuals.

* Statistically significant deviation from Hardy–Weinberg equilibrium ($P < 0.01$).

CONCLUSIONS

The 27 microsatellite markers developed in this study are the first set of such markers for *D. multiflora*. The 17 identified polymorphic SSR markers are expected to be useful tools for population genetic studies and for assessing genetic variations and population differentiation of *D. multiflora* and its allied species, which will help in the establishment of appropriate conservation and management strategies for this alpine species.

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