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

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Primer Note

# Isolation and characterization of 27 microsatellite markers for the endemic species *Diplarche multiflora* (Ericaceae)[1]

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- *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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## 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<br>R: TGAACGGAAGGGAGCAAT | 57 | 240–270 | #FJ839839 |
| DA13* | $(TC)_8$ | F: TCATCAAACTTCACGCCTCT<br>R: GCCATTGCTTCTCCTCCT | 56 | 84–102 | JQ993329 |
| DA17* | $(AC)_6$ | F: GCCAAGGTCACAGGGTAT<br>R: CAGCGTTCACCAGGGTCT | 58 | 114–122 | JQ993330 |
| DA31* | $(CT)_{13}$ | F: AAAGCAGCAATTACAGGT<br>R: CATAGGAATCCAGAAAGC | 51 | 91–105 | JQ993331 |
| DA42* | $(AG)_7$ | F: AAGGCAGCAAGGGAAACC<br>R: GTCAGCAAACGCCAACGA | 57 | 255–259 | #FJ839838 |
| DG15* | $(AG)_5(AG)_5$ | F: AGCGAGCAGGAGACGAAT<br>R: CACGACCTGTTTCAATCCAC | 57 | 180–192 | JQ993336 |
| DG18* | $(CT)_{11}$ | F: TCTCCCTCACGTCCACCT<br>R: AATCAGCGGCGATTCCTC | 58 | 156–170 | JQ993337 |
| DG34* | $(CA)_8$ | F: ACTCCCTAACCCCTATCT<br>R: AGGTGAATTACTGCCATG | 53 | 96–118 | JQ993339 |
| DG36* | $(TC)_7(TC)_{11}$ | F: TATGGACCGAGGTGAATC<br>R: GAAGTTCGCAAGAAATACC | 54 | 250–262 | JQ993340 |
| DG41* | $(AG)_9$ | F: CGCAACTTCACGCTCAAA<br>R: TAGCTGGTTTCCACAATCACAA | 57 | 134–150 | #FJ839834 |
| DG50* | $(AG)_5GCCC(AG)_4$ | F: TTTATGGACCACAACCCA<br>R: GATCCGAGGTATAATGCT | 52 | 108–120 | JQ993341 |
| DG64* | $(AC)_6$ | F: CGACAGCAACGAAACCCTA<br>R: ATCGAAACATCAATCACCACAG | 56 | 174–194 | JQ993342 |
| DG67* | $(TC)_6$ | F: CTGTAGACTTGAATAAACGAA<br>R: AGCGATTGACAAATAGAAAG | 50 | 112–124 | #FJ839832 |
| DG71* | $(TC)_{19}$ | F: CGTCAGAATACGGTCCAG<br>R: GCACAGTAGCACGAGCAG | 58 | 243–251 | JQ993344 |
| DG98* | $(TC)_{11}$ | F: GTCCGAAGCACTGAATAA<br>R: AGGGACATCATAGGGTTG | 57 | 190–218 | #FJ839831 |
| DG110* | $(AG)_9$ | F: TGACGGTCAGGATCTTCC<br>R: CAATGGCTGCTGGTTTAG | 52 | 127–131 | #FJ839830 |
| DG116* | $(TC)_{16}$ | F: CCCTGCTGTTGAGTGTTG<br>R: GAGGCATACGATGGATAAGT | 52 | 136–150 | #FJ839829 |
| DA6 | $(TC)_{13}$ | F: CGGAGTGGGAAGAAAGTA<br>R: TAGAAAGGGAAATAGAGGTT | 54 | 154 | JQ993332 |
| DC17 | $(AC)_5$ | F: CACCGACCACGTAACAAC<br>R: TGGAGGAGGAAGAGCAGT | 56 | 178 | JQ993333 |
| DC36 | $(AC)_5$ | F: AGCCACTAGATAAACCTT<br>R: GAATCCAACATACCATAAT | 46 | 123 | JQ993334 |
| DC109 | $(TC)_7(CA)_7$ | F: GTTTTGGAGTGGCTTTTG<br>R: GGTCCAACTTTCCCTTTT | 51 | 148 | #FJ839837 |
| DC114 | $(AC)_6$ | F: CCAAACCCATCTGAGACA<br>R: CTGAACACGGCGAAGGAG | 53 | 179 | #FJ839836 |
| DG2 | $(TC)_6$ | F: CCACGTTCGTCAATCTTT<br>R: GAGGGTCATACACCATTTCT | 54 | 109 | JQ993338 |
| DG20 | $(AG)_7$ | F: TGGAATTGAGTAGTGAGA<br>R: ATACCAAGTAGGTTTGTAT | 48 | 153 | #FJ839835 |
| DG70 | $(AG)_6$ | F: TAAATGCGAGTAGAGGAGG<br>R: GGGAAGGCTATGGGATAA | 54 | 121 | JQ993343 |
| DG97 | $(TC)_8$ | F: GTCCAATCCAAATCTCAA<br>R: CAAATGTCAAAAGTAAGCAA | 48 | 169 | JQ993345 |
| DG105 | $(TG)_6$ | F: CTTCCCGACTTGTTTATT<br>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 $\chi^2$ 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.

## LITERATURE CITED

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of leaf tissue. *Phytochemical Bulletin* 19: 11–15.

KALA, C. P. 2000. Status and conservation of rare and endangered medicinal plants in the Indian trans-Himalaya. *Biological Conservation* 93: 371–379.

MYERS, N., R. A. MITTERMEIER, C. G. MITTERMEIER, G. A. B. DA FONSECA, AND J. KENT. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

OFFERMAN, J., AND W. RYCHLIK. 2003. Oligo primer analysis software. *In* S. Krawetz and D. Womble [eds.], Introduction to bioinformatics: A theoretical and practical approach, 345–361. Humana Press, Totowa, New Jersey, USA.

ROUSSET, F. 2008. GENEPOP'007: A complete reimplementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

YANG, H. B., AND C. D. CHAMBERLAIN. 2005. *Diplarche. In* Z. Y. Wu, P. H. Raven, and D. Y. Hong [eds.], Flora of China, vol. 14, 259. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

YANG, H. B., R. C. FANG, AND C. L. JIN. 1999. Ericaceae. *In* R. C. Fang [ed.], Flora Reipublicae Popularis Sinicae, vol. 57(1), 1–213. Science Press, Beijing, China.

ZANE, L., L. BARGELLONI, AND T. PATARNELLO. 2002. Strategies for microsatellite isolation: A review. *Molecular Ecology* 11: 1–16.