American Journal of Botany

American Journal of Botany: e173–e175. 2011.

*AJB* Primer Notes & Protocols in the Plant Sciences

# A set of novel microsatellite markers developed for the traditional Tibetan medicinal plant *Halenia elliptica* (Gentianaceae)[1]

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- *Premise of the study:* Microsatellite primers were developed in the traditional Tibetan medicinal plant *Halenia elliptica* D. Don to investigate its genetic diversity and population genetic structure.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences Containing (FIASCO) repeats protocol, 24 primer sets were identified in two wild populations. Of these primers, 12 displayed polymorphisms and 12 were monomorphic. The number of alleles per locus ranged from 2 to 6, with a mean of 3.9. The expected ($H_E$) and observed ($H_O$) heterozygosities ranged from 0.191 to 0.784 and from 0.417 to 0.917, respectively. All these primers successfully amplified in two close relatives of *H. elliptica*, *Swertia bimaculata* (Siebold & Zucc.) Hook. f. & Thomson ex C. B. Clarke and *S. tetraptera* Maxim.
- *Conclusions*: These markers will facilitate further studies on the population genetics of *Halenia elliptica* and its allied species.

**Key words:** Gentianaceae; *Halenia elliptica*; microsatellite marker; population genetics.

*Halenia elliptica* D. Don (Gentianaceae) is an annually growing herb in bosks, meadows, and damp hillsides at an altitude of 700–4100 m. The plant is distributed in China, India, Nepal, Bhutan, and Sikkim (Ho et al., 1988). In China, it is a famous Tibetan folk medicine herbal known as “Ji de he.” It possesses the ability to reduce fever, detoxify, and act as choleretic and liver tonics; it has been used mainly for the treatment of hepatic and choleric and inflammatory diseases, such as hepatitis and cholecystitis (Guo, 1987; Yang, 1991). *Halenia elliptica* has an extensive distribution in China, and its medicinal efficacy varies across the species distribution. For identification, differentiation of its geographic origins, and quality control, we have developed and characterized 24 microsatellite markers for *H. elliptica,* which will be used for further studies of genetic diversity, population structure, and molecular identification.

## METHODS AND RESULTS

A genomic DNA sample was extracted from a single individual from population BS (Beishan, Qinghai: 36°57.091′N, 102°29.153′E, *Xuechy 0112*, KUN) with CTAB methods (Doyle and Doyle, 1987). The fast isolation by AFLP of sequences containing repeats (FIASCO) (Zane et al., 2002) was performed in this study. Total genomic DNA (ca. 500 ng) was completely digested with *Mse* I restriction enzyme(New England Biolabs, Beverly, Massachusetts, USA), and then ligated to an *Mse* I adaptor pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a 30-μL reaction mixture. A diluted digestion-ligation mixture (1:10) was amplified with the adaptor-specific primers *Mse* I-N (5′-GATGAGTCCTGAGTAAN-3′) (25 μM). Amplified DNA fragments, with a size range of 200–800 bp, were enriched for repeats by magnetic bead selection with a 5′-biotinylated $(AC)_{15}$, $(AG)_{15}$, and $(AAG)_{10}$ probe, respectively. Polymerase chain reaction (PCR) products were purified using an EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified DNA fragments were ligated into the pGEM-T vector (Promega, Madison, Wisconsin, USA), and transformed into DH5α cells (TaKaRa, Dalian, Liaoning, China). Positive clones were tested by PCR using $(AC)_{10}$/$(AG)_{10}$/$(AAG)_7$ and $T_7$(5′-TAATACGACTCACTATAGGGCGA)/Sp6(5′-CATACGATTTAGGTGACACTATAG) as primers, respectively. In other words, a set of tested PCR included three reactions was performed using $T_7$ and $Sp_6$, T7 and $(AC)_{10}$, $(AC)_{10}$ and $Sp_6$ as primers, respectively. The second set of tested PCRs was done using $T_7$ and $Sp_6$, T7 and $(AG)_{10}$, $(AG)_{10}$ and $Sp_6$ as primers, respectively. The last set of tested PCRs was done using $T_7$ and $Sp_6$, T7 and $(AAG)_7$, $(AAG)_7$ and $Sp_6$ as primers, respectively. All these PCR reactions had the same conditions. One hundred sixty-two clones with positive inserts were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, California, USA). A total of 108 sequences were found to contain microsatellite repeats, and 35 of them that had appropriate microstellite and enough flanking regions were suitable for designing locus-specific primers, using the primer 5.0 program (Clarke and Gorley, 2001).

Polymorphisms of all 35 microsatellite loci were assessed in 24 individuals of *Halenia elliptica* from two natural populations: BS (Beishan, Qinghai: 36°57.091′N, 102°29.153′E) and ZBS (Zibenshan, Yunnan: 25°44.83′N, 99°04.19′E, voucher: *Xuechy090059*, KUN). PCR reactions were performed in 15-μl reaction containing 30–50 ng genomic DNA, 0.6 μM of each primer, 7.5 μl 2×Taq PCR MasterMix [Tiangen (Tiangen Biotech, Beijing China); 0.1 U Taq polymerase/μl, 0.5 mM dNTP each, 20 mM Tris-HCl (PH 8.3),

[1]Manuscript received 27 December 2010; revision accepted 27 February 2011.

The authors thank Jun-Bo Yang, Hong-Tao Li, and Chun-Xia Zeng for help with laboratory work and data analyses. This study was supported by the Research Fund for the Large-scale Scientific Facilities of the Chinese Academy of Sciences (grant number 2009-LSF-GBOWS-01) and the Fund of State Key Laboratory of Systematic and Evolutionary Botany.

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doi:10.3732/ajb.1000534

100 mM KCl, 3 mM $MgCl_2$]. PCR amplifications were conducted under the following conditions: 95°C for 3 min followed by 32 cycles at 94°C for 30s, at the optimized annealing temperature for each specific primer (Table 1; each primer pair was tested separately) for 30 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. PCR products were separated and visualized using QIAxcel of capillary gel electrophoresis system (QIAGEN, Irvine, California, USA).

Of the 35 primers, 11 did not successfully amplify in all samples. Of the remaining 24 primers that could be amplified, 12 primers showed monomorphism and 12 primer pairs displayed polymorphisms. The genetic statistics were calculated using the package GENEPOP (version 4.0; Raymond and Rousset, 1995), including the number of alleles per locus (A), observed heterozygosity ($H_O$), and expected heterozygosity ($H_E$). A was 2–6 with an average of 3.9; $H_E$ and $H_O$ ranged from 0.191 to 0.784 and from 0.417 to 0.917, with averages of 0.639 and 0.590, respectively (Table 2).

Viewed in a phylogenetic context, *H. elliptica* is closely related to some *Swertia* species in the Gentianaceae (Chassot et al., 2001). The marker transferability of the 12 primer pairs was tested in two allied species of *H. elliptica*, *Swertia bimaculata* (Siebold & Zucc.) Hook. f. & Thomson ex C. B. Clarke (Lushui, Yunnan: 26°01.00′N, 098°38.806′E, voucher: *Xuechy090054*) and *S. tetraptera* Maxim. (Pingan, Qinghai: 36°19.526′N, 101°54.190′E, voucher: *Xuechy0012*) using the same PCR conditions as previously described. The voucher specimens were deposited at the herbarium KUN. All these primers successfully amplified in one sample of *Swertia bimaculata* and an individual of *S. tetraptera.* These primers are universal in these species.

## CONCLUSIONS

These polymorphic microsatellite markers could facilitate population genetics and population genetic structure studies of *Halenia elliptica*, as well as in its allied species.

TABLE 1. Characteristics of 24 microsatellite loci in *Halenia elliptica.*

| Locus | Primer sequence (5′–3′) | Repeat motif | Size range (bp) | *Ta* (°C) | GenBank Accession No. |
|---|---|---|---|---|---|
| X5* | F: TCAGGAGGGTTCTAATCG<br>R: GGTGGTAGCGTAGTGTTTA | (AC)7 | 156 | 48 | HQ732229 |
| X6 | F: ACACCACGGCCAACACTT<br>R: ATTTGGATTGGGATAGGG | (CA)6 | 141 | 49 | HQ732230 |
| X7* | F: TGTAACAGCAAAGTTGAG<br>R: TTTAGTTTTAGATCCCATC | (GA)7 | 201 | 47 | HQ732231 |
| X11 | F: ATTTGAGACCGCTTGACA<br>R: ACTGAGACCCGAGCACTA | (GAA)5 | 272 | 49 | HQ732232 |
| X12* | F: ACTCTGACTCAACGACAA<br>R: CAGTGATTTGGAAGTTTT | (AC)8 | 150 | 47 | HQ732233 |
| X13 | F: AGGGCTACAACACCCATCT<br>R: GCGGCACTCTTTCACTCTAT | (GA)3G(GA)5 | 231 | 52 | HQ732234 |
| X14 | F: GAAACTAAATCTACCACCTT<br>R: CTCACCCTTTACTCCATA | (GT)8 | 146 | 48 | HQ732235 |
| X15 | F: AATCAATGCCTTCAACAAAC<br>R: GCAACCTAATACGCCAAG | (GT)3AT(GT)5 | 216 | 48 | HQ732236 |
| X17* | F: TCCAAAGTTTGAAGAAAG<br>R: CCACTAAAAGTCAGCAAC | (AG)3AT(AG)9A(TGG)3 | 99 | 45 | HQ732237 |
| X20* | F: AAACATCAACACCCAAGA<br>R: GGCTACCTCCATGCAACA | (AT)5GG(GT)4CT(GT)4ATGG(AT)5 | 128 | 46 | HQ732238 |
| X21* | F: TTCTTCGCAAAAGGTAAT<br>R: AAGACGCTGTCATCCATA | (TG)7 | 125 | 46 | HQ732239 |
| X22* | F: ACGGATTCATCATTACCG<br>R: AAGTACCTGCCATCAAAA | (GTT)3(GT)5(GTT)3 | 136 | 46 | HQ732240 |
| X23* | F: TACTGTAAGCGGCGGATGA<br>R: AGAAGCTCGGGAGCGAAG | (CTT)5 | 152 | 51 | HQ732241 |
| X24 | F: GACGACCGTGAACTACAT<br>R: TGTGACCGGACTAGATGG | (AC)29 | 242 | 49 | HQ732242 |
| X25 | F: AATCCAATGCACCTAATACA<br>R: CCATTACCGATTACCACA | (GT)2(GTT)3(GT)4 | 174 | 48 | HQ732243 |
| X26 | F: TAGCCGTCTCCGAGTGTT<br>R: CTCTTGCAGCCATCTCAA | (GAA)6 | 128 | 48 | HQ732244 |
| X30* | F: TTTTGTTCCTGGTATTGTC<br>R: GATCGGAGCAGTTTGATA | (AGAAAG)2(AG)8 | 163 | 48 | HQ732245 |
| X31* | F: GCTGCTATGAGACAACCT<br>R: CATTGAGCAATTTTCAGTA | (CT)11 | 121 | 45 | HQ732246 |
| X34* | F: ATCGAATCAAAACACCCT<br>R: TGGCGTCTCATACCTAAA | (AC)10 | 93 | 47 | HQ732247 |
| X35 | F: AAGCAGCCTGAGGAGTAAC<br>R: GCTGGTTTGCCTAATCTC | (AAG)5 | 144 | 48 | HQ732248 |
| X40 | F: GTGTAGGATGGGTTGGAT<br>R: CACTGTCTTTGACCGTAT | (GT)6 | 122 | 47 | HQ732249 |
| X42* | F: TTTGTGGGTTTCTCGTAA<br>R: TACACCGAGGGTTCTTTT | (TG)6 | 125 | 48 | HQ732250 |
| X43 | F: TTGTTTACACTCCCACTG<br>R: AGATAAGCCGATTACCTG | (TG)7 | 199 | 49 | HQ732251 |
| X46 | F: GATAAGCCGATTACCTGG<br>R: AATTGTTTACACTCCCACT | (CA)7 | 300 | 49 | HQ732252 |

*Displayed polymorphisms in *Halenia elliptica*; Ta, PCR annealing temperature.

Table 2. Results of initial primer screening in *Halenia elliptica.*

| Locus | BS ($N$ = 12) | | | ZBS ($N$ = 12) | | |
|---|---|---|---|---|---|---|
| | $N_A$ | $H_E$ | $H_O$ | $N_A$ | $H_E$ | $H_O$ |
| X5 | 4 | 0.784 | 0.667 | 4 | 0.773 | 0.667 |
| X7 | 3 | 0.643 | 0.542 | 3 | 0.665 | 0.542 |
| X12 | 4 | 0.675 | 0.417 | 4 | 0.675 | 0.417 |
| X17 | 4 | 0.728 | 0.625 | 4 | 0.728 | 0.625 |
| X20 | 5 | 0.772 | 0.708 | 5 | 0.783 | 0.708 |
| X21 | 4 | 0.726 | 0.708 | 4 | 0.726 | 0.708 |
| X22 | 2 | 0.191 | 0.208 | 2 | 0.191 | 0.208 |
| X23 | 2 | 0.422 | 0.500 | 2 | 0.422 | 0.500 |
| X30 | 4 | 0.760 | 0.917 | 4 | 0.760 | 0.917 |
| X31 | 6 | 0.734 | 0.458 | 6 | 0.712 | 0.458 |
| X34 | 5 | 0.629 | 0.667 | 5 | 0.629 | 0.667 |
| X42 | 4 | 0.602 | 0.667 | 4 | 0.615 | 0.667 |

$N_A$, number of alleles revealed; $H_E$, expected heterozygosity; $H_O$, observed heterozygosity.

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