# Development of 18 Novel Microsatellite Primers for Begonia fimbristipula (Begoniaceae), an Endangered Medicinal Plant in China

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PRIMER NOTE

# DEVELOPMENT OF 18 NOVEL MICROSATELLITE PRIMERS FOR *BEGONIA FIMBRISTIPULA* (BEGONIACEAE), AN ENDANGERED MEDICINAL PLANT IN CHINA[1]

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- *Premise of the study: Begonia fimbristipula* (Begoniaceae) is a medicinal herb distributed in the Chinese provinces of Fujian, Guangdong, Guangxi, Hainan, Hunan, Jiangxi, and Zhejiang, and it is on the verge of extinction due to habitat destruction and deterioration of its ecosystem. Here we developed a set of highly polymorphic microsatellite markers for population genetic and conservation studies of this endangered medicinal plant.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol, 18 polymorphic microsatellite markers were identified within 48 individuals from two geographic locations. The observed and expected heterozygosities ranged from 0.208 to 1.000 and from 0.291 to 0.812, respectively. These microsatellite markers were cross-amplified in five related *Begonia* species, and six loci were successfully amplified in all species.
- *Conclusions:* These 18 markers will be useful for better conservation and utilization of wild resources of *B. fimbristipula* and other *Begonia* species in the future.

**Key words:** *Begonia fimbristipula*; Begoniaceae; conservation; microsatellite markers.

*Begonia fimbristipula* Hance (Begoniaceae), a medicinal herb, is mainly distributed in the Chinese provinces of Fujian, Guangdong, Guangxi, Hainan, Hunan, Jiangxi, and Zhejiang. Its leaves, dried stems, and flowers are used in Chinese herbal medicine to reduce inflammation, eliminate phlegm, and relieve cough and asthma (Han et al., 2013). It is also used to make a cool healthy drink in Guangdong Province (Shao and Liang, 2012). Its main components include cyanidin chloride, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside (Tan et al., 2012). *Begonia fimbristipula* requires typical shade plant and acidophilic (pH 3.1–4.21) growing conditions, and its ideal temperature and humidity range is narrow. Because of its high economic value, it has been excessively exploited to a degree that the wild populations have been greatly reduced. Environmental vulnerability and human activities caused a sharp decrease of the wild populations of *B. fimbristipula*. Consequently, it has been listed as an endangered species (Wang et al., 2014).

There are no available reports on microsatellite DNA markers for *B. fimbristipula*. Thus, in view of the medicinal importance of this species, we developed a set of microsatellite markers in *B. fimbristipula* that will be a useful tool for the characterization of genetic structure of its populations and offer practical advice for its further breeding, utilization, and conservation.

## METHODS AND RESULTS

A single individual of *B. fimbristipula* from Dinghushan, Guangdong Province, China, was used to construct a microsatellite-enriched library (Appendix 1). The microsatellites were isolated using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol (Zane et al., 2002). Briefly, total genomic DNA (250–500 ng) was completely digested by 2.5 units of *Mse*I restriction enzyme and then ligated to an *Mse*I amplified fragment length polymorphism (AFLP) adapter (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) by T4 DNA ligase (New England Biolabs, Beverly, Massachusetts, USA) in a 30-µL reaction mixture. After being diluted in a ratio of 1 : 10, 5 µL of digested-ligated fragments were amplified using the adapter-specific primers MseI-N (5′-GATGAGTCCTGAGTAAN-3′) (25 µM). The amplified DNA fragments (size between 200–800 bp) were enriched for simple sequence repeats by magnetic bead selection using 5′-biotinylated $(AC)_{15}$ and $(AG)_{15}$ probes, respectively. Enriched DNA fragments were reamplified using MseI-N primers. After being purified by the Sanpre PCR Purification Kit (Sangon, Shanghai, China), the purified DNA fragments were ligated into pBS-T II vector (Tiangen, Beijing, China) and then transformed into JM109 competent cells. Two hundred and eighty-six clones with positive inserts were selected by PCR using vector primers M13+/M13− and primers $(AC)_{10}$/$(AG)_{10}$, and then sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Waltham, Massachusetts, USA). A microsatellite library was established using SSRHunter software (version 1.30) (Li and Wan, 2005) with the following criteria: all sequences containing at least six di- or trinucleotide repeats. A total of 137 primer pairs with product size range 100–350 bp, GC content 40–60%, and primer melting temperature ($T_m$) 45–60°C were designed using the program Primer version 5.0 (Clarke and Gorley, 2001).

The newly designed 137 primer pairs were used to assess genetic polymorphism of 48 individuals of *B. fimbristipula* from Guangdong and Guangxi provinces in China. Voucher and location information of *Begonia* species used in this study are given in Appendix 1. The PCR reactions were performed in 25-µL reaction volumes containing approximately 40 ng of genomic DNA, 0.3 µL

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TABLE 1. Characteristics of 18 microsatellite loci developed in *Begonia fimbristipula*.

| Locus | Primer sequences (5′–3′) | Repeat motif | $T_a$ (°C) | Allele size (bp) | GenBank accession no. |
|---|---|---|---|---|---|
| QHT01 | F: CTTTGGTAACGTGGTGTG<br>R: AGTGGGAGATTTGGAGAC | $(TC)_{16}$ | 55 | 160 | KT224486 |
| QHT02 | F: TTTATGGACGACAGACGC<br>R: GTGAAAATACAGAGGAGACG | $(CT)_{14}$ | 55 | 134 | KT224487 |
| QHT03 | F: CACTTCATTGTGCTGCTG<br>R: CACTTCATTGTGCTGCTG | $(AG)_{10}$ | 53 | 190 | KT224488 |
| QHT04 | F: TCATTGTCGAGTTCCCAT<br>R: AGAGGAGCTTAGAAGGACT | $(CT)_5...(CTT)_7$ | 53 | 159 | KT224489 |
| QHT05 | F: GACATTGTTCGCCCTTGC<br>R: GGGTATTTTGGGGATAAGAG | $(CT)_{19}$ | 55 | 208 | KT224490 |
| QHT06 | F: CGCATTTGAAACGAGGTG<br>R: GCTCTAGTGGAATGGAGACG | $(CT)_{16}$ | 56 | 190 | KT224491 |
| QHT07 | F: CTCCCTCAATATGTGTGC<br>R: AACAACGAGAAACCAACG | $(GA)_{18}$ | 53 | 166 | KT224492 |
| QHT08 | F: GAAACAAGGGCTGAAACG<br>R: AGCAAAGAATGAGCACAAG | $(CT)_{32}$ | 53 | 153 | KT224493 |
| QHT09 | F: TCCGTCTACAGTTCTCTCAC<br>R: ACTTTCTCATTGCTCATCAG | $(TC)_7TA(TC)_6$ | 54 | 119 | KT224494 |
| QHT10 | F: GTGGATGAAACTTGTCGC<br>R: GGTCTTCAATCTCACTGC | $(TC)_{14}$ | 53 | 158 | KT224495 |
| QHT11 | F: CGAGTCCCATTGAATCAT<br>R: TAACAGGGGCAAGAAGAG | $(GA)_9$ | 53 | 199 | KT224496 |
| QHT12 | F: GAGACAACACTCATAGCG<br>R: TTATCTTCTCCAGTCGTG | $(AG)_8$ | 53 | 157 | KT224497 |
| QHT13 | F: TCCCTCAATATGTGTGCC<br>R: GCCTTGCTCAATAAAACG | $(CT)_6T(TC)_8$ | 53 | 158 | KT224498 |
| QHT14 | F: TCTCTCAAATCCTAACCCAT<br>R: GATAGCCTACTTTCATCAGAC | $(TC)_{24}$ | 53 | 159 | KT224499 |
| QHT15 | F: ATCGCAGTTTTCCATCTC<br>R: CGCAGTAGTTGGTGAATC | $(AG)_{12}$ | 52 | 127 | KT224500 |
| QHT16 | F: GGGTTTTGTCCATACTCTTC<br>R: TTGTGGTGTCTGAGGGAG | $(TC)_{17}A(CT)_4$ | 55 | 177 | KT224501 |
| QHT17 | F: GTTTTGTCCTATCCCAGC<br>R: CATCGGTCGTTACAGTCC | $(TC)_{16}$ | 55 | 237 | KT224502 |
| QHT18 | F: TGAACCAGTGGCTTGAAC<br>R: AATCCCTCTTGATAAGTGTG | $(AG)_{17}$ | 53 | 148 | KT224503 |

*Note*: $T_a$ = annealing temperature.

dNTPs (10 mmol/L), 0.3 µmol/L of each primer, 2.5 µL of 10× PCR buffer, and 0.6 units of *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China). PCR amplifications were conducted using an initial step of 95°C for 3 min; followed by 35 cycles of 94°C for 30 s, at the annealing temperature for each specific primer (optimized for each locus) for 30 s, and 72°C for 45 s; and a final extension of 72°C for 7 min. PCR products were separated by 8%

TABLE 2. Genetic properties of 18 newly developed microsatellites for *Begonia fimbristipula*.[a]

| | Dayeshenjing population ($n$ = 24) | | | | Dinghushan population ($n$ = 24) | | | |
|---|---|---|---|---|---|---|---|---|
| Locus | $A$ | $H_o$ | $H_e$ | $P$ value | $A$ | $H_o$ | $H_e$ | $P$ value |
| QHT01 | 3 | 0.292 | 0.291 | 0.508 | 4 | 0.375 | 0.533 | 0.153 |
| QHT02 | 3 | 0.417 | 0.344 | 1.000 | 4 | 0.417 | 0.576 | 0.062 |
| QHT03 | 4 | 0.708 | 0.658 | 0.985 | 6 | 0.583 | 0.715 | 0.050 |
| QHT04 | 3 | 0.583 | 0.667 | 0.121 | 2 | 0.208 | 0.305 | 0.152 |
| QHT05 | 2 | 0.500 | 0.497 | 1.000 | 2 | 0.458 | 0.353 | 0.291 |
| QHT06 | 4 | 0.542 | 0.659 | 0.237 | 5 | 0.542 | 0.545 | 0.088 |
| QHT07 | 3 | 0.417 | 0.452 | 0.495 | 2 | 0.292 | 0.492 | 0.05 |
| QHT08 | 5 | 0.833 | 0.689 | 0.360 | 5 | 0.792 | 0.716 | 0.023* |
| QHT09 | 4 | 0.667 | 0.506 | 0.470 | 4 | 0.375 | 0.653 | 0.000* |
| QHT10 | 5 | 0.792 | 0.716 | 0.967 | 8 | 0.708 | 0.759 | 0.027* |
| QHT11 | 5 | 0.708 | 0.653 | 1.000 | 6 | 0.792 | 0.812 | 0.036* |
| QHT12 | 4 | 0.583 | 0.541 | 0.587 | 5 | 0.667 | 0.575 | 0.310 |
| QHT13 | 5 | 0.708 | 0.705 | 0.341 | 5 | 1.000 | 0.761 | 0.152 |
| QHT14 | 6 | 0.625 | 0.526 | 0.116 | 4 | 0.667 | 0.668 | 0.016* |
| QHT15 | 5 | 0.708 | 0.660 | 0.639 | 5 | 0.667 | 0.745 | 0.915 |
| QHT16 | 4 | 0.583 | 0.617 | 0.440 | 7 | 0.583 | 0.768 | 0.043* |
| QHT17 | 6 | 0.625 | 0.720 | 0.706 | 5 | 0.708 | 0.723 | 0.669 |
| QHT18 | 4 | 0.75 | 0.643 | 0.748 | 7 | 0.792 | 0.792 | 0.324 |

*Note*: $A$= number of alleles; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity.
[a]Geographic coordinates and voucher information are provided in Appendix 1.
* $P < 0.05$.

TABLE 3. Cross-species amplification of 18 microsatellite loci in five closely related species of *Begonia fimbristipula.*

| Locus | *Begonia palmata* (*n* = 5) | *Begonia crassirostris* (*n* = 5) | *Begonia handelii* (*n* = 5) | *Begonia cathayana* (*n* = 5) | *Begonia edulis* (*n* = 5) |
|---|---|---|---|---|---|
| QHT01 | — | + | — | + | — |
| QHT02 | — | — | + | — | — |
| QHT03 | + | + | + | — | — |
| QHT04 | + | + | + | + | + |
| QHT05 | + | + | + | + | + |
| QHT06 | + | — | — | — | — |
| QHT07 | + | + | + | + | + |
| QHT08 | — | — | + | — | — |
| QHT09 | + | + | + | + | + |
| QHT10 | + | + | + | + | + |
| QHT11 | + | — | — | + | — |
| QHT12 | + | — | — | — | — |
| QHT13 | — | — | + | — | — |
| QHT14 | + | + | + | + | + |
| QHT15 | — | + | — | + | — |
| QHT16 | — | — | — | + | — |
| QHT17 | — | + | + | + | — |
| QHT18 | + | — | — | + | — |
| No. of loci | 11 | 10 | 11 | 12 | 6 |

*Note*: + = successful PCR amplification; — = unsuccessful PCR amplification.

nondenaturing PAGE gel and stained with a silver-staining method. The number of alleles, polymorphic information content (PIC), and observed ($H_o$) and expected ($H_e$) heterozygosity were calculated using GenAlEx 6 (Peakall and Smouse, 2006). Linkage disequilibrium (LD) and deviation from Hardy–Weinberg equilibrium (HWE) were calculated using GENEPOP version 4.2 (Rousset, 2008). The possible presence of null alleles was tested at a 95% confidence interval using the program MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004).

All of the 137 primer pairs successfully amplified in all samples. Only 18 primer pairs displayed polymorphism. All amplification products matched the expected lengths (Table 1). The mean numbers of alleles per locus in each population were 4.1 and 4.7, respectively, and the observed and expected heterozygosities per locus within populations varied from 0.208 to 1.000 and from 0.291 to 0.812, respectively. Six loci deviated from HWE ($P < 0.05$) in the Dinghushan population (Table 2), revealing significant heterozygote deficiencies.

Five closely related species of *B. fimbristipula* were selected for cross-amplification testing according to Tian et al. (2014). Cross-species amplification of the 18 polymorphic microsatellite markers was performed with five individuals for each of five closely related species (Table 3). DNA extraction and PCR amplification were performed as described above for *B. fimbristipula*, except for the different annealing temperature for each locus. The allele sizes of five closely related species were all similar to those found in *B. fimbristipula*.

The results of cross-species amplification are shown in Table 3. Six loci (QHT4, QHT5, QHT7, QHT9, QHT10, and QHT14) amplified in all five related *Begonia* species (Table 3), while the other 13 loci amplified in fewer than five species. These results suggest that these 18 novel microsatellite markers could also be useful for genetic studies of other related *Begonia* species.

## CONCLUSIONS

The 18 polymorphic microsatellites developed in this study will be useful for investigating genetic diversity and population structure, and helpful for better conservation and utilization of wild resources of *B. fimbristipula* and other *Begonia* species in the future.

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Appendix 1. Voucher and location information of *Begonia* species used in this study. All vouchers were deposited in the herbarium of the Guangxi Institute of Botany (IBK), China.

| Species | Locality | Geographic coordinates | Herbarium ID |
|---|---|---|---|
| *Begonia fimbristipula* Hance | Dayeshenjing, Guangxi, China | 23.16667°N, 112.51667°E | Twxqht1–24 |
| | Dinghushan, Guangdong, China | 25.14271°N, 110.59242°E | Twxqht25–48 |
| *Begonia palmata* D. Don | Jingxi, Guangxi, China | 22.99806°N, 106.67579°E | Twxqht49–53 |
| *Begonia crassirostris* Irmsch. | Jingxi, Guangxi, China | 23.02437°N, 106.64925°E | Twxqht54–58 |
| *Begonia handelii* Irmsch. | Napo, Guangxi, China | 23.38049°N, 105.83390°E | Twxqht59–63 |
| *Begonia cathayana* Hemsl. | Jingxi, Guangxi, China | 22.88777°N, 106.32221°E | Twxqht64–68 |
| *Begonia edulis* H. Lév. | Jingxi, Guangxi, China | 22.99774°N, 106.67557°E | Twxqht69–73 |