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Isolation of microsatellite markers for *Bletilla striata* and cross-amplification in other related species

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Bletilla **is a temperate, terrestrial genus of orchids containing 6 species. For the species whose whole genome is unknown, we used magnetic bead hybridization method to develop microsatellite Simple Repeat Polymorphoresis (SSR) for *Bletilla striata* and 9 primer sets were characterized in two wild populations of *B. striata* and one wild population of *Bletilla ochracea.* The number of alleles per locus ranged from 1 to 12. The expected and observed heterozygosities ranged from 0 to 0.7646 and 0 to 0.950 in *B. striata*, respectively. In *B. ochracea*, the expected and observed heterozygosities ranged from 0.296 to 0.871 and 0.05 to 1, respectively. The 9 pairs of primers we designed can be used to distinguish different ecotypes and species, and might be used for other subspecies or species in genera *Bletilla.***

Key words: *Bletilla striata*, cross-species amplification, simple repeat polymorphoresis (SSR).

INTRODUCTION

The genus *Bletilla*, composting about six species, is endemic to Asia with a distribution pattern from N Myanmar and Indochina through China to Japan. The usage of the plants of *Bletilla*, mainly *B. striata* (Thunb.) Reichb. f., includes art (Chinese painting and writing), the production of porcelain, and medicine (Lawler, 1984), as well as vegetable dyes for dyeing cloth in some minority people in Guizhou and Yunnan Province, China (Luo, person observation). Those species are also commonly used as a horticultural subject covering both indoors and out cultivation in North American and European areas (Pridgeon, 2005). Recently, *B. striata* has been reported to be able to suppress various weed species (Sakuno et al., 2010). As the traditional herb medicine using more than 1500 years, pseudobulbs of *Bletilla* have been widely used in Eastern Asian countries to treat alimentary canal mucosal damage, ulcer, bleeding, bruises and burns. The pseudobulbs also show antibacterial, anti-inflammatory, antiphlogistic and demulcent properties, and thus use in treating pneumonophthisis, pneumonorrhagia, tuberculosis and haemorrhage of the stomach or lung (Wang et al., 2006). Moreover, it is suggested that cationic polysaccharide from *B. strata* can serve as a non-viral nucleotide drug delivery vehicle for oligonucleoide or siRNA targeting to immunology system (Dong et al., 2009). Apparently, there is a huge potential demand for the pseudobulbs of *Bletilla* in future. At present, the cultivation of *Bletilla* is only limited to the temperature areas of North America and Europe for the horticultural purpose (Pridgeon, 2005), and the using of

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pseudobulbs of *Bletilla* for other purpose especially for medicinal purpose is basically based on wild resources.

Undoubtedly, the wild resource of *Bletilla* will rapidly decrease in Eastern Asian Countries due to over collections as well as habitat destruction and fragmentation (Chun and Chun, 2005). The conflict between the great market demand for the pseudobulbs of *Bletilla* and the decreased wild resource has necessitated the breeding of cultivars for high yields, high concentrations of active components and high resistance to diseases. Unfortunately, no cultivar has been bred so far and selections of superior individuals meeting the requirements are now underway from field and hybrid progenies. Molecular marker – assisted selection has become routine in cultivar breeding, and great efforts have been made to develop molecular markers such as microsatellites. More important, microsatellite markers have often been used for genetic diversity studies due to their desirable genetic attributes like hyper-variability, wide genomic distribution, co-dominant inheritance, reproducibility, multi-allelic nature and chromosome specific location (Singh et al., 2010; Tang et al., 2012). Here, we describe microsatellite loci which developed from *B. striata* and test the transferability of those markers to other related species. These loci were useful for further breeding superior cultivars and studies of genetic diversity, and contribute to knowledge on conservation of genus *Bletilla.*

MATERIALS AND METHODS

Genomic DNA of leaves of *B. striata* sampled from valley in Guiyang City, Guizhou Province, China (26° 29′ 46″ N, 106° 39′ 39″ E) was extracted using a plant genomic DNA Kit (Tiangen, Beiing, China). First, total genomic DNA was completely digested with the *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA), and then ligated to an *Mse*I adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (New England Biolabs) in a 30 µL reaction mixture. Tenfold diluted digestion–ligation mixture was amplified with adaptor-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) in 20 µL reactions with the following conditions: 3 min denaturation at 95°C; followed by 26 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C; with a final extension of 72°C for 5 min and then were enriched for microsatellites with a 5′-biotinylated $(AG)_{15}$ probe. Thirdly, using adaptor-specific primers amplified enriched fragments again and using a PCR Purification Combo Kit (BioTeke, Beijing, China) purified it. Finally, the cleaned DNA fragments were ligated into the pEASY-T1vector (Promega, USA), and transformed into competent cells of trans1-T1 Phage Resistant Chemically (Transgen, Beijing, China). Hundreds of positive clones amplified by PCR, and 95 of these, with a size range of 500 to 1000 bp, were sequenced with an ABI PRISM 3730 DNA sequencer (Applied, Biosystems, USA). The sequences which contained SSR were designed specific primers using primer primer 5.0 (Lalitha, 2000) and thirty had sufficient regions to design primer.

All primer pairs were assayed in individuals PCR and run on 1% agarose gels. Thirteen primer pairs have specific and right loci. Then, to utilize fluorescently labeled M13 primer for sequencing, the forward primer of each pair added an M13 sequence (5′-TGTAAAACGACGGCCAGT-3′) to its 5′ (Schuelke, 2000). Using the fluorescently labeled primer for amplification, nine primer pairs showed clear bands and expected size. The 25 µl volumes PCR mix contained 4 pmol reverse primer and FAM/ HEX/ TAMRA-M13(-21) primer, 1 pmol of the forward primer, 12.5 mm Tris-HCl (pH 8.3), 62.5 mm KCl, 1.875 mm $MgCl_2$, 0.25 mm of each dNTP, 0.75 U DNA polymerase (TakaraTaq) and 50 ng template DNA. PCR amplification were done as follows: 94°C for 5 min, then 30 cycles at 94°C for 30 s, specific annealing temperature (Table 1) for 45 s, 72°C for 45 s, followed by 8 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The nine microsatellite loci were characterized using 54 wild samples were collected from three Sites in China: 14 samples of *B. striata* from valley in Guiyang, Guizhou, POP- *GY*: 26° 29′ 46″ N, 106° 39′ 39″ E; 20 samples of *B. striata* from valley in Xinning, Hunan, POP-*XN*: 26° 25′ 42″ N, 110° 50′ 30″ E; 20 samples of *B. ochracea* from valley in Qingzhen, Guizhou, POP-*QZ*: 26° 36′ 2″ N, 106° 28′ 16″ E. The PCR amplification was same to the aforementioned described. Nine primer pairs tested for polymorphism on an ABIPRISM 3730 Genetic Analyzer and using GeneMapperv4.0 software (Applied Biosystems) analyzed the result.

The numbers of alleles per locus, observed and expected heterozygosities, and deviations from Hardy–Weinberg equilibrium (HWE) were estimated using FSTAT version 2.9.3 (Gouldet, 2001).

RESULTS

The newly developed microsatellite loci showed high levels of polymorphism in *B. striata*. The microsatellite markers can be used to evaluate the genetic diversity and infer evolutionary processes in natural populations, which should be useful for developing the appropriate conservation strategies for *B. Striata*.

DISCUSSION

Microsatellite markers are based on the amplification of internal sequences between microsatellites [intersimple sequence repeat (ISSR) markers] using PCR and then looking for microsatellite loci contained within these sequences, taking into account that microsatellites are generally clustered within the plant genome, and microsatellite markers have been proven useful in assessing genetic diversity of populations in different species (López-Roberts et al., 2012; Caitlin et al., 2013). In the present study, the result can draw that BJ67 were monomorphic in these two *B. striata* population, but polymorphic in *B. ochracea.* BJ311 and BJ120 failed to amplify in *B. ochracea*. In *B. striata* and *B. ochracea*, the number of alleles per locus ranged from 1 to 12 and the mean number of alleles per species was 4.33 and 4.14, respectively (Table 2). The expected and observed heterozygosities ranged from 0 to 0.7646 and 0 to 0.950 in *B. striata*, respectively. In *B. ochracea*, the expected and observed heterozygosities ranged from 0.296 to 0.871 and 0.05 to 1, respectively. The 9 pairs of primers we designed can be used to distinguish different ecotypes and species, and might be used for other subspecies or species in genera *Bletilla*, which would provide for implementing concrete protection strategy and/or tagging the very right medical herbs.

Table 1. The characteristics of nine microsatellite loci in *B. striata.*

Locus	Primers sequences (5-3′)	Repeat motif	Size range (bp)	Ta (°C)	GenBank accession No.
BJ109	F: CTATTATTCCTCCTCGTTTG R: CTAGCCTACCAAGTAGTTCC	$(GA)_{13}$	160-174	54	JQ965919
BJ98	F: GGCTAACCCATAATTGATC R: CTTTCATTGAGGTGGACTT	$(GA)_{23}$	258-310	58	JQ965917
BJ70	F: CACGAACAGCCACTATCA R: TTACAAGCCTCCCAATCT	$(GA)_{8}$	271-301	58	JQ965914
BJ120	F: CCATTACCAACCGTGGAG R: GTCGGACGAAAGTGAGCC	$(GA)_{10}$	112-126	58	JQ965916
BJ311	F: CCAAAGTGATAACGGAAGG R: TTGAATCCAAGAAGTGCC	$(GA)_{11}$	343	54	JQ965913
BJ303	F: TCAGTTTGTCGCTTCTATG R: ATCCTGAATCTGGGGCTA	$(TC)_{8}$	111-131	54	JQ965918
BJ67	F: CCGATGTGGAGGTAGAGC R: CGGAAACGGAAGAAGAAG	$(TTC)_{5}$	237	56	JQ965915
BJ68	F: CAAAGCAAACTGGACGAA R: CCATAATCACTTGGAACCC	$(GA)_{8}$	129-133	54	JQ965921
BJR100	F: GCTGAGGACAGAAGGGAG R: AGTAGAAATCATCGCACAA	$(AG)_{10}$	298-304	58	JQ965920

Table 2. Results of initial primer screening in two populations of *Bletilla striata* and one populations of *Bletilla ochracea*. Shown for each primer pair are the number of Alleles (A), average observed (H_o) and expected heterozygosity (H_e).

Locus	*POP-GY (N = 20)*			*POP-XN (N = 14)*			*POP-QZ (N = 20)*		
	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
BJ68	2	0.300	0.262	2	0.0714	0.0714	3	0.700	0.555
BJ98	6	0.950	0.751	7	0.5714	0.6799	12	0.800	0.871
BJ109	3	0.450	0.676	6	0.5714	0.7646	3	0.050	0.432
BJ303	2	0.350	0.296	2	0.1429	0.1376	5	0.450	0.706
BJ311	1	0	0	1	0	0	na		
BJ67	1	0	0	1	0	0	2	0.350	0.296
BJ70	2	0.550	0.409	4	0.3571	0.5608	2	0.500	0.431
BJ120	2	0.750	0.481	5	0.7143	0.7249	na		
BJR100	2	0.850	0.512	4	0.8571	0.5767	2	1.000	0.513

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