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Paeonia (Paeoniaceae) expressed sequence tag-derived microsatellite markers transferred to *Paeonia delavayi*

D.X. Wang[1], H. Ma[2], Y.L. Zhang[3], A.A. Duan[4], W.J. Li[5] and Z.H. Li[2]

[1]State Key Laboratory of Tree Genetics and Breeding,
Forestry College, Northeast Forestry University, Harbin, Heilongjiang, China
[2]Research Institute of Resource Insects, Chinese Academy of Forestry,
Kunming, Yunnan, China
[3]Zhejiang Hongyue Seed Co. Ltd., Jiaxing, Zhejiang, China
[4]Science and Technology Department, Southwest Forestry University,
Kunming, Yunnan, China
[5]Yunnan Reascend Tobacco Technology (Group) Co. Ltd.,
Kunming, Yunnan, China

Corresponding authors: Z.H. Li / A.A. Duan
E-mail: lzh4949@163.com / duananan@gmail.com

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ABSTRACT. *Paeonia* Franchet contains approximately 30 species of perennial herbs and is the only member of the family Paeoniaceae. *Paeonia delavayi* is an endangered ornamental plant that has a compound flower color and is endemic to southwest China. Its optimal habitats have been fragmented and its population size has greatly decreased as a result of human activities. Using a modified biotin-streptavidin capture method, 51 primer sets were identified in 3 wild populations of *P. delavayi* in this study. Ten primers displayed polymorphisms and 41 amplified no products, gave weak or indistinct bands, or gave larger sizes than expected. The number of alleles per locus ranged from 1 to

3, and the values for observed and expected heterozygosities ranged from 0.000 to 0.571 and from 0.000 to 0.509, with averages of 0.113 and 0.295, respectively. These markers will be useful for further investigations in resource conservation, selection of parental types in cross-breeding, the molecular evolution of this species, and for related research in *Paeonia* species.

Key words: Population structure; *Paeonia delavayi*; Microsatellite; Expressed sequence tags

INTRODUCTION

Paeonia Franchet, the only genus in the flowering family Paeoniaceae, is one of the most famous ornamental flowers in the world. The peony (the common name for plants in *Paeonia*) has a long history of use in Eastern cultures and has been represented as a national emblem in China. *P. delavayi* (section *Moutan*) is endemic to southwest China, being mainly distributed in the Yunnan and Sichuan Provinces, and has the most southern distribution among all *Paeonia* species in the region (Pan, 1979; Hong and Pan, 1999). Owing to its rich diversity in flower color, ranging from deep purple to white or yellow (green in 1 population), *P. delavayi* is regarded as ideal parental material for controlled hybridization breeding. However, the species has been placed under state protection and is currently listed in the vulnerable phase (Feng, 1992; Li et al., 2012a). Our continued field surveys have further confirmed the threatened status of *P. delavayi*, as its appropriate habitats have increasingly been fragmented and its population sizes are greatly decreasing due to human activities, such as use in traditional Chinese medicine. Following the IUCN Red List criteria (IUCN Red List Categories and Criteria, Version 3.1, 2001), *P. delavayi* can be provisionally assigned to the "endangered" category [(EN) B2b (v) c (iv)], as its area of occupancy is less than 500 km^2, and the number of mature plants are likely to fluctuate greatly in the future. Therefore, effective long-term conservation strategies are urgently needed to protect this species.

Simple sequence repeat (SSR) markers, such as microsatellites, have proven to be a powerful tool for investigating genetic diversity, mapping, and cultivar identification (Li et al., 2012b). Expressed sequence tag (EST)-derived SSR markers show a higher level of transferability to closely related species and fewer null alleles than genomic-derived SSRs, and provide an efficient means for SSR screening (La Rota et al., 2005; Cristancho and Escobar, 2008; Li et al., 2012b). In light of its high ornamental value, endemism, and endangered status, we developed 10 microsatellite markers of *P. delavayi* from peony ESTs. The genetic information obtained herein will be informative for further studies on the conservation of *P. delavayi* resources, selecting parental types in cross-breeding, as well as for the molecular evolution of this species (Ma et al., 2012).

MATERIAL AND METHODS

Fifty individuals in 3 wild populations of *P. delavayi* from Yunnan Province (population 1: Deqin County, 28°29'N, 98°46'E, 2589 a.s.l.; population 2: Shangri-La County,

25°58'N, 99°30'E, 1884 a.s.l.; population 3: Yulong County, 26°55'N, 100°11'E, 3309 a.s.l.) were collected to screen for the transferability and polymorphism of *Paeonia* EST-derived SSR markers. Plant genomic DNA from young leaves was extracted using a modification of the hexadecyltrimethylammonium bromide method (Doyle and Doyle, 1987) and stored at -20°C.

We obtained 2024 publicly available *Paeonia* ESTs from the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/nucest), which were saved as Fasta-formatted files. A total of 324 ESTs containing SSRs were identified using the SSRIT tool (http://www.gramene.org/db/searches/ssrtool). The minimum length of SSRs was defined as di-, tri-, tetra-, penta-, and hexanucleotide for 5 subunits each. Primer pairs were designed with the help of Serafer 1.3 (Rozen and Skaletsky, 2000), using the following settings: optimal primer length of 20 bp, optimal annealing temperature of 57°C (maximum, 63°C; minimum, 52°C; maximum temperature difference, 5°C), and product size of 120-500 bp. The CG content varied from 20 to 80%.

The polymerase chain reaction (PCR) was performed in a 20-µL volume containing 30 ng genomic DNA, 1 U Taq DNA polymerase (TransGen, Beijing, China), 2.0 µL 10X PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl), 2.0 mM $MgCl_2$, 0.25 mM dNTPs (Takara), and 5 µM of each forward and reverse primers. The PCR was carried out using a touchdown program: initial denaturation at 95°C for 7 min; followed by 18 cycles of denaturation at 95°C for 15 s, annealing at decreasing temperatures of 62.5° to 67°C for 30 s, and extension at 72°C for 1 min; followed by 30 cycles at 95°C for 15 s, 30 s at 51.5° to 57°C, 72°C for 1 min; and a final extension step at 72°C for 10 min. PCR products were separated on 8% non-denatured polyacrylamide gels and visualized with silver staining.

The data were analyzed with GENEPOP 4.0 (Raymond and Rousset, 1995), which included tests for observed heterozygosity (H_O) and expected heterozygosity (H_E) for the 10 polymorphic microsatellite loci.

RESULTS AND DISCUSSION

Ten of the 51 *Paeonia* EST-derived SSR primers successfully amplified PCR products at sizes close to those expected for *P. delavayi*. The remaining primer pairs amplified no products, gave weak or indistinct bands, or gave larger sizes than expected. Therefore, 10 polymorphic EST-SSR primer pairs were identified in total (Table 1). The number of alleles ranged from 1 to 3 in the 3 populations. Values for H_O and H_E ranged from 0.000 to 0.571 and from 0.000 to 0.509, with averages of 0.113 and 0.295, respectively (Table 2). The microsatellite markers developed in this study will provide useful tools for further studies of conservation genetics and will increase the understanding of the genetic structure of *P. delavayi*, so as to develop an effective conservation strategy for this endangered plant.

In summary, 10 microsatellite markers were specifically developed for *P. delavayi* in this study. These markers will facilitate further studies related to the population genetics of *P. delavayi* and its allied species. They are also expected to be useful for parental selections in controlled hybridization breeding programs, such as the development of new cultivars with yellow or green flowers, and enable us to protect germplasm sources through genetic diversification via *ex situ* nurseries and reintroduction programs.

Table 1. Characteristics of 10 microsatellite loci successfully amplified in *Paeonia delavayi*.

Locus	Primer sequence (5'-3')	Repeat motif	Size (bp)	Ta (°C)	GenBank accession No.
WD 01	F: TGGCGCTCCACACGGTATCTT R: ATCGTGTTCGGTTCGCCGGT	$(CTC)_4$	228	59	Contig65
WD 02	F: TCGATGCCGGCAGGGTTTCT R: ACAGGTCTTGTCCGGCCTTGT	$(GCA)_4$	381	58	FE528170
WD 03	F: TCCCAAAAGAGGGCGGTGCT R: TCCCCTCCGAAACGGCATGT	$(AAG)_4$	306	58	Contig34
WD 04	F: ATGGCGAGATTGCCCCGTCT R: TGCAGTCACCACCATCCGTCA	$(TTAA)_3$	228	58	Contig97
WD 05	F: CGTCATCTGAACGCCGCCGA R: TCACGTTGGATGTTCACTGCCCA	$(TCTCGC)_3$	208	59	FE528527
WD 06	F: CGGCGTGCCTTTGAGACGTG R: TGCCTCGAAAAACTCGCCTTCTCC	$(AGA)_5$	157	59	FE528179
WD 07	F: AGCGTGAAGCAACAAGCCGTG R: ACTGCGTTTCACGGCGAGGA	$(CATTT)_3$	167	59	FE528335
WD 08	F: AGCAGAGAAGGTAGGCGGCG R: AGCGCTGAAGGCCAGTCATGG	$(ACTGA)_3$	171	59	FE528943
WD 09	F: GGGGACTCAAATCCTTGCGAAAACCA R: AGGCCTAGTTTTGGTCTGGGCG	$(CAC)_4$	189	59	FE528256
WD 10	F: CAGCTGGCTTCCCACGCAGT R: TAGCCATGCCGCCTCCACCT	$(GGT)_4$	177	59	Contig225

Ta = annealing temperature.

Table 2. Results of 10 polymorphic microsatellite loci screening in 3 populations of *Paeonia delavayi*.

Locus	Population 1 (N = 50)			Population 2 (N = 30)			Population 3 (N = 20)		
	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
WD 01	2	0.000	0.075	2	0.000	0.072	2	0.270	0.027
WD 02	2	0.229	0.489	2	0.035	0.436	2	0.000	0.344
WD 03	2	0.000	0.329	2	0.000	0.486	2	0.000	0.237
WD 04	2	0.000	0.058	1	0.000	0.000	1	0.000	0.000
WD 05	2	0.378	0.494	2	0.571	0.494	2	0.297	0.387
WD 06	3	0.022	0.509	2	0.000	0.504	3	0.027	0.380
WD 07	2	0.010	0.396	2	0.000	0.408	2	0.000	0.237
WD 08	2	0.000	0.057	2	0.129	0.202	2	0.081	0.079
WD 09	3	0.049	0.431	2	0.328	0.277	2	0.297	0.494
WD 10	2	0.078	0.317	2	0.375	0.362	2	0.216	0.276

N = population sample size; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity.

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