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Nuclear and chloroplast SSR markers in *Paeonia delavayi* (Paeoniaceae) and cross-species amplification in *P. ludlowii*[1]

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- *Premise of the study:* Microsatellite primers were developed for *Paeonia delavayi* and *P. ludlowii* (Paeoniaceae) to study their population genetics and phytogeography.
- *Methods and Results:* Nine polymorphic nuclear microsatellite loci were isolated from an enriched library of *P. delavayi* and primers were designed. The number of alleles per locus ranged from two to 16; the observed and expected heterozygosities ranged from 0.014 to 0.687 and 0.042 to 0.875, respectively. Six polymorphic chloroplast microsatellite loci were identified in *P. delavayi* and primers were provided. The number of alleles per locus ranged from two to six and the polymorphic information content ranged from 0.08 to 0.716. Both nuclear and chloroplast primers were successfully applicable to *P. ludlowii*.
- *Conclusions:* The markers developed here will facilitate analyses of genetic diversity, population genetic structure, phytogeographical patterns, and conservation for *P. delavayi* and *P. ludlowii*.

Key words: genetic diversity; microsatellite marker; *Paeonia delavayi*; *Paeonia ludlowii*; Paeoniaceae; population genetic structure.

Paeonia delavayi Franch. and *P. ludlowii* (Stern & G. Taylor) D. Y. Hong (Paeoniaceae) are endemic to the eastern Himalayas, China (Hong and Pan, 1999). *Paeonia delavayi* and *P. ludlowii* are the only sources of yellow pigments in tree peony cultivar breeding. Being typical dwellers in the Himalayas and sensitive to temperature changes, their phytogeographical dynamics are good indicators of climate oscillations since the last ice age. However, due to relatively short population histories and clonal propagation, there are very limited sequence variations available as markers for new cultivar breeding, population genetics, phytogeographical studies, and conservation.

Microsatellites are commonly used as molecular markers for population genetic studies in plants. Combining information from nuclear simple sequence repeat (nSSR) markers with chloroplast simple sequence repeat (cpSSR) markers, the mechanisms and processes of evolution can be elucidated (Ebert and Peakall, 2009). Recently, microsatellite markers were reported for some peonies, including *P. suffruticosa* Andrews (Wang et al., 2009; Homolka et al., 2010) and *P. lactiflora* Pall. (Li et al., 2011). Here, we characterize and verify nine polymorphic nSSR and six polymorphic cpSSR markers in *P. delavayi* and test their applicability to *P. ludlowii*. These microsatellite loci will facilitate the studies of genetic diversity, population genetic structure, phytogeographical patterns, and sustainable conservation for *P. delavayi* and *P. ludlowii*.

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METHODS AND RESULTS

Leaves of *P. delavayi* were collected in Shangri-La County, Yunnan Province, China (Appendix 1). Genomic DNA was extracted and purified from silica gel–dried leaves of a single individual of *P. delavayi* using the Plant Genomic DNA Extraction and Purification Kit (Tiangen Biotech, Beijing, China). A microsatellite-enriched library was constructed to obtain nSSR regions following Glenn and Schable (2005) with minor modifications (Wang et al., 2009). Briefly, genomic DNA was digested by *Rsa*I (New England BioLabs, Ipswich, Massachusetts, USA) and a mixture of 5′-biotinylated probes containing $(AG)_{12}$, $(AT)_{12}$, $(CG)_{12}$, $(GT)_{12}$, $(ACG)_{12}$, $(ACT)_{12}$, $(CCA)_8$, $(AACT)_8$, $(AAGT)_8$, and $(AGAT)_8$ were further used for target fragment enrichment. Enriched-repeat libraries were cloned using the pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA) and transformed into Top 10 competent *E. coli* cells (TransGen Biotech, Beijing, China). Four hundred and eighty-four positive clones were screened by PCR amplifications using SP6 and T7 and finally 126 positive clones (> 200 bp) were selected and sequenced on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA). After exclusion of redundant or unsuitable sequence for designing primers, 29 unique sequences containing 31 nSSR loci were identified. In addition, the cpSSR markers for *P. delavayi* were developed by searching through the complete chloroplast genome sequence (Shiliang Zhou, unpublished) of *P. suffruticosa* using SSR Hunter version 1.3 (Qian Li, Nanjing Agricultural University, Nanjing, China). Finally, 10 cpSSR loci containing (AT)n and (TC)n were identified.

Nuclear and chloroplast microsatellite PCR primers for *P. delavayi* were designed using Primer Premier version 5.0 (Premier Biosoft International, Palo Alto, California, USA) and Oligo version 6.71 (Molecular Biology Insights, Cascade, Colorado, USA) under the following criteria: (1) guanine-cytosine content 30–60%; (2) melting temperature (T_m) 50–65°C; (3) primer size 18–25 bp in

length; (4) amplicon size 150–500 bp in length; and (5) few hairpins, false primings, or cross dimers. For nSSR markers, PCRs were carried out in 10 μL reaction volume containing ~20 ng genomic DNA, 2.5 μM of each primer, 2.0 mM dNTPs, 0.5 U *Taq* polymerase (Yuanchen, Beijing, China), and 1× PCR buffer (Yuanchen). PCRs were programmed as 3-min initial denaturation at 94°C, 30 cycles of 30 s at 94°C, 30 s at 48–56°C (Table 1), 45 s at 72°C, and a final extension of 72°C for 10 min. PCR products were resolved on 6% polyacrylamide denaturing gels and visualized by silver staining. Amplified fragments were sized and genotyped manually. Fifteen (48.4%) of 31 primer pairs showed good amplifications. The authenticity of the bands amplified from *P. delavayi* was confirmed by isolating them from agarose gels, subcloning, and sequencing eight clones per band.

For cpSSR markers, PCR amplifications were the same as for nSSR markers with the modification that the forward primers were 5′-FAM-labeled. PCRs were programmed as 3-min initial denaturation at 94°C, 25 cycles of 30 s at 94°C, 30 s at 48–54°C (Table 1), 1 min at 72°C, and a final extension of 72°C for 10 min. PCR products were resolved on an ABI 3730xl DNA analyzer with GeneScan-500 LIZ Size Standard (Applied Biosystems). Alleles were identified using GeneMapper version 4.0 (Applied Biosystems) and confirmed manually. The authenticity of the loci amplified in *P. delavayi* was confirmed by sequencing the representative PCR products.

Seventy-six *P. delavayi* individuals from 13 populations were used to characterize nSSR loci, and 23 representatives were used to characterize cpSSR loci (Appendix 1). Polymorphism parameters were calculated with PowerMarker version 3.25 (Liu and Muse, 2005), GENETIX version 4.05 (Belkhir et al., 2004), or GenAlEx version 6.1 (Peakall and Smouse, 2006). Tests for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium of nSSRs were performed using Genepop version 4.0 (Rousset, 2008).

Nine of the 15 nSSR loci and six of the 10 cpSSR loci were polymorphic with a moderate level of polymorphism (Table 2). For nine polymorphic nSSRs, the number of alleles per locus (N_a) and the number of genotypes per locus (N_g) ranged from two to 16 and from two to 24, respectively. The observed (H_o) and expected heterozygosities (H_e) ranged from 0.014 to 0.687 and from 0.042 to 0.875, respectively. The major allele frequency (*MAF*) and the polymorphic information content (*PIC*) ranged from 0.254 to 0.979 and from 0.041 to 0.865, respectively. Significant departures from HWE ($P < 0.05$) were detected at six loci (Table 2) and significant heterozygote deficits ($P < 0.01$) were detected at four loci (Pdel03, Pdel04, Pdel07, and Pdel12), likely due to distantly related samples used in this study and few variations within populations. Linkage disequilibrium was detected among pairs of Pdel01, Pdel02, and Pdel04 loci ($P < 0.05$). For six polymorphic cpSSRs, N_a ranged from two to six and the unbiased haploid diversity per locus (H) ranged from 0.087 to 0.791. *MAF* and *PIC* ranged from 0.304 to 0.957 and from 0.08 to 0.716, respectively. All six polymorphic loci are located in the intergenic spacers (Table 1) in which the mutations are usually neutral.

All the primers developed for *P. delavayi* were able to successfully amplify nuclear and chloroplast microsatellite markers in four *P. ludlowii* samples from two populations in Tibet, China. The authenticity of these amplified loci was confirmed by sequencing representative PCR products of *P. ludlowii*, and these sequences have been deposited in GenBank (see Table 1 for accession numbers). The expected loci of *P. ludlowii* were found to be homologous to those of *P. delavayi*.

CONCLUSIONS

Moderate levels of genetic diversity of *P. delavayi* based on both nuclear and chloroplast microsatellite loci suggest that the newly developed nine polymorphic nuclear and six polymorphic

TABLE 1. Characteristics of nine polymorphic nuclear and six polymorphic chloroplast microsatellite loci in *Paeonia delavayi* shown with the GenBank accession numbers of the sequences on which the primers are based for *P. delavayi* or verified for *P. ludlowii*.

Locus*	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank Accession No.	
					P. delavayi	*P. ludlowii*
Nuclear microsatellite loci						
Pdel01	F: AATACCCACGACTAACCTA	$(AC)_5$	422–428	50	HQ437318	HQ230325
	R: TTCCTTATTATCTCCCCCTA					
Pdel02	F: CCAATGTGGAAAATGAGTT	$(AG)_{15}$	210–226	48	EU375736	HQ230315
	R: CAAGCACAAGATGTAAGAA					
Pdel03	F: TATCATTCTAACGGTGGTT	$(AG)_{18}$-G-$(GA)_{12}$	180–226	52	EU375733	HQ437319
	R: GAGGTAGATACTGGAACTT					
Pdel04	F: ATAGCCTGAGCGTTATACTTT	$(CT)_9$-CATAATTTCC-$(CT)_5$	304–314	50	EU375731	HQ230326
	R: CCTCCCTATTTGAATCTGAAC					
Pdel07	F: ATGTGGACAAGGAAAATT	$(TG)_{19}$	288–304	50	EU375735	HQ437320
	R: CAGCGTACTGCTTAGAGT					
Pdel09	F: CAAAGCCTCAAGAAACTCCCTA	$(GA)_{19}$	127–131	56	HQ230312	HQ230320
	R: GATGTACAACACCAAGATGCAA					
Pdel10	F: TGGATTCTTATTTGTTTTGAG	$(AG)_8$-AA-$(AG)_{10}$	206–224	48	HQ230314	HQ437321
	R: ACACCGTGTAGCAGATGATGA					
Pdel11	F: CTGCCATTTCTTGCCTTCTTTGT	$(TGG)_6$	230–248	54	HQ230311	HQ230318
	R: TCTACCCTGCCAACAGCACATAC					
Pdel12	F: ATCAAGTCCCACAATAGGAGAAC	$(TC)_{17}$	397–439	52	HQ230313	HQ230321
	R: CTTGTATGAGTCTAAACCCAGTTA					
Chloroplast microsatellite loci						
Pdelcp01[a]	F: ATACCATCAGCATCCGTAGAAAG	$(TA)_7$	316–318	51	HQ020500	HQ025961
	R: ACAACGGGTACACTAATCAGTAA					
Pdelcp02[b]	F: GAGTACCTGGGCCGGGTTCTGTA	$(AT)_8$	176–180	54	HQ020501	HQ025962
	R: ATTATGTTACGCGGGGCGGATAG					
Pdelcp03[c]	F: GCGGTACAAAACTAATGCTGTGG	$(TA)_5$	180–190	50	HQ020502	HQ025963
	R: AAGTCTTATTCGTATTGCGGGTG					
Pdelcp04[d]	F: TTTGTTTGCTGTGGTCCCTGTCT	$(AT)_8$	164–172	48	HQ230322	HQ230324
	R: ATCCGAGGTGAAGTAAAGTGAAA					
Pdelcp05[e]	F: TGCTGCTTGTGAAGTATGGAAAG	$(AT)_5$	368–376	49	HQ020503	HQ025964
	R: TCGTGGGTCAAAACTACAGGATA					
Pdelcp06[f]	F: GCCCCAACCCTACCCATTTCTTT	$(AT)_5$	171–185	50	HQ020504	HQ025965
	R: CATCGGCGGATTCCAATCTATTT					

Note: T_a = annealing temperature.

* The positions of the intergenic spacers of each chloroplast locus are indicated as: [a] trnS-UGA-psbZ intergenic spacers; [b] psbZ-trnG-GCC intergenic spacers; [c] trnF-GAA-ndhJ intergenic spacers; [d] ndhC-trnV-UAC intergenic spacers; [e] rbcL-accD intergenic spacers; [f] psaZ-ycf4 intergenic spacers.

TABLE 2. Polymorphism of the (A) nine nuclear microsatellite loci and (B) the six chloroplast microsatellite loci assayed using samples from 13 populations of *Paeonia delavayi*.

	Nuclear microsatellite loci (N = 76)					
A. Locus	N_a	N_g	H_o	H_e	*MAF*	*PIC*
Pdel01	2	3	0.071	0.191	0.892	0.173
Pdel02	2	2	0.118	0.111	0.941	0.105
Pdel03	16	24	0.687*	0.875	0.254	0.865
Pdel04	3	4	0.353*	0.491	0.647	0.415
Pdel07	6	8	0.333*	0.693	0.433	0.643
Pdel09	2	2	0.563	0.404	0.719	0.323
Pdel10	2	3	0.014*	0.042	0.979	0.041
Pdel11	6	8	0.515*	0.643	0.493	0.585
Pdel12	9	5	0.333*	0.806	0.333	0.782
Mean	5.33	6.56	0.332	0.473	0.632	0.437

	Chloroplast microsatellite loci (N = 23)			
B. Locus	N_a	*H*	*MAF*	*PIC*
Pdelcp01	2	0.087	0.957	0.080
Pdelcp02	3	0.672	0.435	0.568
Pdelcp03	6	0.791	0.304	0.716
Pdelcp04	4	0.689	0.500	0.603
Pdelcp05	4	0.298	0.842	0.271
Pdelcp06	2	0.087	0.957	0.080
Mean	3.5	0.437	0.666	0.386

Note: H = unbiased haploid diversity per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; *MAF* = major allele frequency; N = sample size; N_a = number of alleles; N_g = number of genotypes; *PIC* = polymorphic information content.

* Deviation from Hardy-Weinberg equilibrium: $P < 0.05$.

chloroplast microsatellite markers are suitable for studies of genetic diversity, population genetic structure, phytogeographical patterns, and conservation biology. In addition, the primers designed for amplifying the loci of *P. delavayi* were applicable to *P. ludlowii*.

LITERATURE CITED

BELKHIR, K., P. BORSA, L. CHIKHI, N. RAUFASTE, AND F. CATCH. 2004. GENETIX 4.0.5.2: Software under Windows for population genetics. Laboratory genome, populations, interactions, CNRS UMR 5000. University of Montpellier II, Montpellier, France.

EBERT, D., AND R. PEAKALL. 2009. Chloroplast simple sequence repeats (cpSSRs): Technical resources and recommendations for expanding cpSSR discovery and applications to a wide array of plant species. *Molecular Ecology Resources* 9: 673–690.

GLENN, T. C., AND N. A. SCHABLE. 2005. Isolation microsatellite DNA loci. *Methods in Enzymology* 395: 202–222.

HOMOLKA, A., M. BERENYI, K. BURG, D. KOPECKY, AND S. FLUCH. 2010. Microsatellite markers in the tree peony, *Paeonia suffruticosa* (Paeoniaceae). *American Journal of Botany* 97: e42–e44.

HONG, D. Y., AND K. Y. PAN. 1999. Taxonomical history and revision of *Paeonia* sect. *Moutan* (Paeoniaceae). *Acta Phytotaxonomica Sinica* 37: 351–368.

LI, L., F. Y. CHENG, AND Q. X. ZHANG. 2011. Microsatellite markers for the Chinese herbaceous peony *Paeonia lactiflora* (Paeoniaceae). *American Journal of Botany* 98: e16–e18.

LIU, K., AND S. V. MUSE. 2005. PowerMarker: An integrated analysis environment for genetic marker analysis, 2128–2129. Oxford University Press, Oxford, United Kingdom.

PEAKALL, R., AND P. E. SMOUSE. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

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

WANG, J. X., T. XIA, J. M. ZHANG, AND S. L. ZHOU. 2009. Isolation and characterization of fourteen microsatellites from a tree peony (*Paeonia suffruticosa*). *Conservation Genetics* 10: 1029–1031.

APPENDIX 1. Geographic localities and sample sizes of *Paeonia delavayi* populations in this study.

Population code	Locality	Latitude (°N)	Longitude (°E)	Herbarium and voucher accession code	Sample size
S-LT	Litang, Sichuan	29.02111	100.63690	PE WY06078-LTP	7
S-ML	Muli, Sichuan	28.89030	100.92469	KUN 91035	7
S-XC	Xiangcheng, Sichuan	28.91417	99.56833	PE WY06074-XCP	6
T-BM	Bomi, Tibet	29.73733	96.01847	PE H06016	2
T-NC	Nyingchi, Tibet	29.59106	94.25330	PE H06012	4
Y-DD	Duoduo, Yunnan	27.73212	99.03417	KUN 82410	8
Y-DL	Dali, Yunnan	25.92557	100.03791	KUN 91027	6
Y-DQ	Deqin, Yunnan	28.49306	98.93471	PE 701	6
Y-KM	Kunming, Yunnan	24.95773	102.64323	PE WH05	6
Y-LBL	Laboluo, Yunnan	27.66513	98.82443	KUN 82507	5
Y-LJ	Lijiang, Yunnan	27.18192	100.26360	KUN 82101	7
Y-PB	Pingbian, Yunnan	22.68668	103.67170	PE 704.01	6
Y-SL	Shangri-la, Yunnan	27.95319	99.58082	PE R05	6
Total					76

Note: KUN = Kunming Institute of Botany, Chinese Academy of Sciences; PE = herbarium of the Institute of Botany, Chinese Academy of Sciences.