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SHORT COMMUNICATION

Development of polymorphic microsatellite markers for tree peony *Paeonia delavayi* (Paeoniaceae) using ddRAD-seq data

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Abstract

Microsatellite markers were developed for the tree peony *Paeonia delavayi* to investigate fine scale population genetics of this species. Using ddRAD-seq data from twenty individuals of *P. delavayi*, we identified 529 polymorphic microsatellite loci, of which 195 were suitable for designing microsatellite primers. Of the 120 microsatellite loci selected for validation, 20 were successfully amplified with clear peaks and displayed polymorphism. Three populations were genotyped using the 20 polymorphic microsatellites. The number of alleles per locus ranged from two to thirteen. Observed and expected heterozygosity ranged from 0 to 0.941 and 0 to 0.834 respectively. The cross-species amplification test using five individuals from a population of *P. ludlowii* showed that 15 of the 20 polymorphic loci were successfully amplified, and four loci showed polymorphism. Among the 22 alleles occurring in *P. ludlowii* across fifteen loci, eight alleles across five loci were exclusive to *P. ludlowii.* The results demonstrate that ddRAD-seq is an efficient method for the development of microsatellite markers for non-model organisms with large genomes. The newly developed markers will be valuable tools to investigate the genetic diversity, genetic structure, and gene flow of *P. delavayi* from local to regional spatial scales.

Keywords Cross-species amplification · Next generation sequencing · Non-model species · Population genetics · SSR

Introduction

Microsatellites have the advantages of being co-dominant, locus-specific, hyper-variable markers, which is still a popular marker choice for a wide range of applications in population genetics [1]. The development of microsatellite markers using traditional approaches can be time consuming and expensive [2, 3]. During the last decade, next-generation-sequencing (NGS) technologies have been increasingly used for SNP discovery and genotyping in ecological and evolutionary studies, and also facilitate the development of polymorphic microsatellite markers for non-model species [3–5]. However, microsatellites are still one of the most cost-effective options for genotyping species with very large genomes and large sample sizes, even in the "genomic era" [1].

Paeonia is the only genus of the family Paeoniaceae, which diverged into the woody (sect. *Moutan*) and the herbaceous (sect. *Paeonia* and sect. *Onaepia*) lineages. *Paeonia delavayi* Franch. is a tree peony endemic to the Himalayas and Hengduan Mountains of Southwest China [6–8]. It is a diploid species ($2n=2x=10$) with a very large genome ($1C=14.68$ pg). *P. delavayi* and its closest relative *P. ludlowii* are the only yellow flowered tree peonies, making them a valuable genetic resource for breeding new cultivars [9]. To our knowledge, approximately 16 nuclear microsatellite markers have been developed for *P. delavayi* to date [8, 10, 11]. However, most of these loci showed a significant deviation from Hardy–Weinberg equilibrium (HWE) or significant linkage disequilibrium (LD). At first, we selected 159 nSSR markers from previous studies which were developed for *P. delavayi* or congeneric species for screening available markers for our study on the fine scale landscape genetics of *P. delavayi* in the Yulong Mountain, but only one marker, *Pdel29b* [12], produced clean peaks with polymorphism. Therefore, in this study, we applied double digest restriction-site-associated

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DNA sequencing (ddRAD-seq) to develop novel polymorphic microsatellite markers for population and conservation genetic studies of this valuable species.

Materials and methods

Sample collection and DNA extraction

Leaf samples of 16 natural populations of *P. delavayi* from northwest Yunnan, and one population of *P. ludlowii* from Milin County, southeast Xizang, China were collected and dried immediately in silica gel. The distance between sampled plants within each population was at least 10 m apart to avoid sampling clones. Voucher specimens were collected and deposited in the Herbarium of Kunming Institute of Botany (KUN), Chinese Academy of Sciences, China. Total genomic DNA was extracted using a modified CTAB protocol [13]. DNA quality was assessed via agarose gel (1%) electrophoresis and a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Qualified DNA was then quantified with Qubit® dsDNA Broad Range (BR) Assay kit (Invitrogen, Carlsbad, USA).

ddRAD library preparation and sequencing

Twenty samples from the 16 populations were selected for ddRAD library preparation and sequencing. A single sample per population was selected from the 14 populations in the Yulong Mountain (Lijiang), and three samples were selected from both the Cangshan Mountain population (Dali) and the Haba Mountain population (Shangri-La). The ddRAD libraries were prepared following the protocol of Yang et al. [14]. Briefly, 200 ng of DNA of each individual was digested by incubation at 37 °C for 2 h with two restriction enzymes, AvaII and MSPI (New England Biolabs, Ipswich, MA, USA), followed by enzyme inactivation at 65 °C for 20 min. Digested products were ligated with barcoded adapters. Equal amount of the ligated DNA from each of the twenty samples were pooled together and fragments of 600–700 bp were retrieved from a 2% agarose gel. The fragments were enriched by PCR amplification using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, USA), and then the purified products were quantified with the Qubit® dsDNA High Sensitivity (HS) Assay kit (Invitrogen, Carlsbad, USA). Twenty mixed samples were sequenced in a single lane of Illumina HiSeq X Ten (Illumina, San Diego, CA, USA) (150 bp, pair-end).

Microsatellites mining and primer design

We developed microsatellite markers for *P. delavayi* following the methods described by Qin et al. [5]. The program *process_radtags* from the software *Stacks* v 1.39 was used to demultiplex the ddRAD-seq data, remove adapter sequences, and to filter out reads where the scores dropped below 90% probability of being correct [15]. The program *ustacks* was used to comparing and aligning the reads. The software QDD v3 was used to detect sequences containing microsatellites [16]. The minimum repeat number for microsatellite detection was set to six repeats for di- and trinucleotide motifs and three repeats for tetra-, penta-, and hexanucleotide motifs. The program Primer v3 was used to design primers flanking the microsatellite loci [17].

Validation of microsatellite loci

Firstly, we selected 120 microsatellite loci for amplification using four samples of *P. delavayi*. PCR amplification was performed in 15 µL reactions consisting of 10 µL PCR Mix (Tiangen, Beijing, China) contained $MgCl_2$ (3 mM), KCl (100 mM), TrisCl [pH 8.3], dNTP mixture (0.5 mM), and Taq polymerase (0.1 U), 0.25 µL of forward and reverse primers (10 µM), 25 ng of DNA, and 3.5 µL ddH_2O. PCR amplification was performed with the following conditions: denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 4 min, annealing temperature according to the *Ta* value (Table 1) for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. Secondly, successfully amplified markers were then validated with 24 samples from three populations. Thirdly, the primer pairs that yielded PCR products in the expected size range with clear peaks and displayed polymorphism were selected for further validation with 61 samples from three populations. The forward primers were fluorescent labeled with either Fam or Hex [18]. PCR products were multiplexed according to dye sets and size ranges to avoid overlap, and fragment sizes were determined on an ABI 3730 (Applied Biosystems, Foster City, CA, USA). Finally, we also tested the cross-species amplification of the 20 polymorphic loci on five samples of *P. ludlowii* which were collected from the Milin County, southeast Xizang.

Data analysis

SSR data were visualized and converted to diploid genotypes using automated allele calling implemented in GeneMarker v. 2.2.0 (SoftGenetics, State College, PA, USA). All automated genotypes were rechecked manually. Linkage disequilibrium and deviation from Hardy–Weinberg equilibrium

Table 1 Characteristics of the 20 polymorphic microsatellite loci developed in *Paeonia delavayi*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	*A*	H_O	H_E	*Ta* (°C)	GenBank accession no.
PDP01	F: GTCCCATTCCGCTTCGAATT R: GGGCATTTGTATACACGGAAATT	AGG	121–142	7	0.213	0.443	59	MH894735
PDP04	F: CCAACTACAGCATGCCGAGA R: GACCCAAGACCCAACAGCTT	GGT	81–96	5	0.607	0.666	60	MK460443
PDP06	F: GTCCTTGATCTCTTGATGCACT R: AGCACAAGAACCAAGACTAACA	CTT	86–98	5	0.361	0.361	58	MH894736
PDP09	F: GGTTCAGCAATCAATATCCAATAACG R: GGGTATGTTGTGTCACATTTCACA	AAAT	89–93	2	0.1	0.095	59	MK460444
PDP16	F: CATGCTGCACTCTTGGATGC R: ATTCCAAGGGAGATGCGGAG	TTC	109–118	4	0.18	0.225	60	MH894737
PDP21	F: TCCAACTACAGCATGCCGAG R: GGACTAGGGTAAACATTACCGCA	GGT	131–161	7	0.59	0.72	60	MH894738
PDP25	F: TGCACAGGAATCAGGTAGTGT R: AGGCCCTACTTCAAATTAGGCT	TAA	100–103	2	0.016	0.048	59	MH894739
PDP30	F: TGTCCTTACCTGGTCTGTTTGA R: ACTCTCTCCCTCTCTTGCTG	AG	84–106	7	0.508	0.553	59	MH894740
PDP31	F: GTCCTGCACACCTCCAAATT R: GGTCGCCTCCTACAAAGAATG	AG	125–145	11	0.623	0.815	59	MH894741
PDP37	F: CCCACGCATAAGAAAGTAGAAGG R: CTTGATGCCTCTCCGAGTGC	AG	127–147	7	0.295	0.426	61	MH894742
PDP48	F: GACCCTATAAGCAGATAAATTGTACAT R: AGGGTTTAACTAGGGTTTCTTCTGT	CT	129–141	7	0.525	0.588	59	MK460445
PDP49	F: GACCTTCTACGTCGGCGATT R: AGAGGGAATGTTTAGATTTGAAGCT	CT	87–97	4	0.426	0.505	60	MH894743
PDP51	F: CCCATTTCATCTCATTCTTCTCCC R: AGCACCTATTTACGTCAGATATGA	CT	110–120	5	0.672	0.634	59	MH894744
PDP53	F: GGGAGGAGTATAAGCAAAGAGTGT R: TTTGCCATTTCTATTGTCGCCA	GA	80–118	13	0.672	0.805	60	MH894745
PDP71	F: GTCCCATGATATTTGGAGGGAT R: GTAGCACAATGGTCACATGCC	AG	81–99	6	0.77	0.721	59	MK460446
PDP89	F: ACAGGAAAGTCATATTGTAGACATGG R: GCATGCTTTAATTTCACAAGTGCA	AT	81–99	9	0.7	0.698	59	MK460447
PDP113	F: CCCAATCTCATCCTTGTAATATAAGTT R: AACTCCTTGTCACCAACCGT	CT	113–129	8	0.917	0.716	59	MK460448
PDP122	F: TTGCGTAAAGGTGGAGGGAG R: TCTCTAACATTGTCGACGCCT	GA	74–94	4	0.18	0.467	59	MK460449
PDP125	F: AGGGCGGTGAGTTGTTTGAT R: GCAAGTGACAAGCCTCCACA	GA	106–112	4	0.295	0.527	60	MK460450
PDP151	F: TTTCTGGTGATGGAGAGCCC R: AGCATCATTGTGGAATATCACCG	TA	89–93	3	0.115	0.193	59	MK460451

A number of alleles per population, H_O observed heterozygosity, H_E expected heterozygosity, *Ta* annealing temperature

(HWE) were carried out using GENEPOP v. 4.0 [19]. The number of observed alleles per locus (*A*), observed heterozygosity (H_O), and expected heterozygosity (H_E) were calculated using GenAlEx v. 6.5 [20]. The fixation indices were estimated using FSTAT v. 2.9.3 [21]. Evidence of scoring errors due to stuttering, large allele dropout and null alleles were evaluated using MICRO-CHECKER v. 2.2.3 [22].

Results and discussion

A total of 209,397,046 clean reads were obtained from 211,115,278 raw reads after filtering out low quality reads. The clean reads were further reduced to 4,486,256 sequences after aligning using *ustacks*, among which 97,484 contained microsatellites, corresponding to 1889 loci. Of the

529 microsatellite loci that exhibited polymorphism (about 28%), 195 were suitable for designing microsatellite primers, which including 168 perfect (uninterrupted) microsatellites and 17 compound microsatellites. We selected 120 perfect microsatellites for validation on 24 individuals from the three populations, and 20 loci yielded PCR products in the expected size range with clear peaks and displayed polymorphism (Table 1). We used 61 individuals in total from three populations to validate and characterize the 20 polymorphic microsatellite markers (Table 2). The number of alleles per locus ranged from 2 to13. Observed heterozygosity ranged from 0 to 0.941, and expected heterozygosity ranged from 0 to 0.834. No evidence of significant linkage disequilibrium was detected, and most of the loci showed HWE (Table 2). No evidence of null alleles was detected except for the following four loci, locus PDP125 in all of the three populations, locus PDP122 in the GHBI and SZB populations, locus PDP16 in the SZB population, and locus PDP25 in the MNP population.

Of the 20 markers used for cross-species amplification with five individuals of *P. ludlowii*, fifteen markers were successfully amplified, and four markers were polymorphic (Table 3). Among the 22 alleles occurring in *P. ludlowii* across the fifteen loci, eight alleles from five loci were exclusive to *P. ludlowii* (four loci with one fixed allele of each, and another locus with four alleles). The levels of genetic diversity of *P. delavayi* were relative high, while the genetic diversity of *P. ludlowii* was extremely low (Tables 2, 3). Our results are consistent with a recent study by Zhang et al. [8], and the obvious differences between the two species are likely due to their contrasting distribution range size and different evolutionary history. *Paeonia ludlowii* populations are constrained in an extremely narrow area in southeast Tibet, where the latitudes and altitudes are higher than that of the *P. delavayi* populations [7, 8]. Natural selection, genetic drift, and the small population size might account for the low level of genetic diversity of *P. ludlowii*. On the contrary, *P. delavayi* is the most widely distributed species among all of the wild tree peonies [7], and shows an astonishing morphological diversity in terms of plant height, leaf shape, flower color, and carpel number and size. However, the evolutionary history of the great morphological diversity of *P. delavayi* are remains largely unknown. To date, whether *P. ludlowii* is a distinct species from *P. delavayi* remains controversial [6, 8, 23].Our study revealed considerable genetic differentiation between these two species, although more evidences are needed to conform this issue.

Table 2 Genetic diversity tests of the 20 polymorphic microsatellite markers in the three populations of *Paeonia delavayi*

Locus	GHBI population (n = 22)				MNP population (n = 22)				SZB population (n = 17)			
	A	H_O	H_E	F_{IS}	A	H_O	H_E	F_{IS}	A	H_O	H_E	F_{IS}
PDP01	5	0.136	0.211	0.373*	4	0.136	0.282	0.533*	3	0.412	0.493	0.194
PDP04	4	0.591	0.707	0.186	4	0.636	0.638	0.026	5	0.588	0.599	0.048
PDP06	3	0.364	0.373	0.048	4	0.455	0.433	0.027	3	0.235	0.215	0.067
PDP09	2	0.136	0.127	0.05	1	0.000	0.000	–	2	0.188	0.170	0.071
PDP16	3	0.364	0.310	0.151	4	0.091	0.170	0.485	3	0.059	0.164	0.66
PDP21	6	0.545	0.682	0.222	5	0.727	0.696	0.021	5	0.471	0.657	0.312
PDP25	1	0.000	0.000	–	2	0.000	0.087	1.000	2	0.059	0.057	0
PDP30	4	0.727	0.637	0.118	5	0.409	0.447	0.108	5	0.353	0.476	0.286
PDP31	9	0.682	0.772	0.139	7	0.636	0.803	0.229	7	0.529	0.720	0.292
PDP37	4	0.273	0.247	0.082	6	0.182	0.250	0.294	4	0.471	0.548	0.172
PDP48	6	0.571	0.670	0.171	5	0.455	0.503	0.119	3	0.563	0.471	0.164
PDP49	3	0.455	0.480	0.077	4	0.591	0.657	0.124	3	0.176	0.164	0.043
PDP51	5	0.727	0.686	0.037	3	0.591	0.600	0.039	3	0.706	0.593	0.16
PDP53	11	0.773	0.834	0.096	6	0.591	0.695	0.173	5	0.647	0.749	0.166
PDP71	6	0.727	0.705	0.009	4	0.682	0.623	0.071	4	0.941	0.715	0.29
PDP89	6	0.636	0.740	0.162	6	0.636	0.629	0.012*	5	0.875	0.656	0.304*
PDP113	7	0.905	0.741	0.197	6	0.909	0.685	0.306	3	0.941	0.651	0.422
PDP122	4	0.045	0.375	0.884*	2	0.273	0.351	0.246	3	0.235	0.507	0.557
PDP125	4	0.364	0.557	0.367	3	0.182	0.447	0.608*	3	0.353	0.567	0.404*
PDP151	3	0.318	0.439	0.297	1	0.000	0.000	–	1	0.000	0.000	–
Mean	4.8	0.467	0.515	0.193	4.1	0.409	0.45	0.246	3.6	0.44	0.459	0.243

A number of alleles per population, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient, n number of individuals sampled

*Significant deviation from Hardy–Weinberg equilibrium ($\alpha = 0.01$)

Table 3 Cross-species amplification of the 20 polymorphic microsatellites in *Paeonia ludlowii*

	P. ludlowii (*n*=5)		
	Amplification Success	Number of alleles	Allele size range (bp)
PDP01	–	–	–
PDP04	–	–	–
PDP06	+	1	92
PDP09	+	1	74
PDP16	+	1	115
PDP21	–	–	–
PDP25	+	1	118
PDP30	+	4	114–120
PDP31	+	2	135–143
PDP37	+	1	129
PDP48	–	–	–
PDP49	+	1	95
PDP51	+	1	120
PDP53	+	1	100
PDP71	+	1	93
PDP89	+	1	83
PDP113	+	3	117–123
PDP122	+	1	92
PDP125	+	2	106–108
PDP151	–	–	–

+ successful amplification, – unsuccessful amplification; n number of samples tested

The 20 novel markers developed in this study will be valuable tools to investigate the evolutionary history of the morphological diversity species *P. delavayi* across its distribution range. Moreover, the markers also enabling the assessment of genetic diversity, population structure and gene flow in *P. delavayi* from local to regional spatial scales, which will help in formulating appropriate strategies and policies for the conservation, management, and sustainable use of this valuable germplasm resource. However, more polymorphic markers are to be developed and applied for *P. ludlowii* to study the conservation genetics of the endangered species in the future. Furthermore, this work also confirms the usefulness of ddRAD-seq data for the development of polymorphic microsatellite markers for non-model species with very large genomes is efficient.

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Data availability Illumina clean reads were deposited to NCBI's Sequence Read Archive (SRA accession: PRJNA492718). Sequences for the developed primers were also deposited to NCBI (Table 1).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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