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Microsatellite markers for *Duperrea pavettifolia* (Rubiaceae)[1]

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- *Premise of the study:* The development of microsatellite primers for *Duperrea pavettifolia* will be the foundation for mating system analysis and conservation research.
- *Methods and Results:* Nineteen microsatellite markers were developed and characterized in two wild populations by using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol. Polymorphisms were evaluated in 24 individuals from two natural populations. Eleven of these primers generated polymorphic loci.
- *Conclusions:* These microsatellite markers will be useful in future investigations into the population genetics and mating system of *D. pavettifolia*.

Key words: *Duperrea pavettifolia*; microsatellite marker; Rubiaceae.

Duperrea Pierre ex Pit., a small genus of the Rubiaceae with only two species, is characterized by a long exserted style and large stigma, and is widely distributed in the tropical areas of Southeast Asia. Only one of these species, *D. pavettifolia* (Kurz) Pit., is found in China (Chen et al., 2011). It is a small tree, 1.5–6 m tall, and usually grows in moist valleys. It has long been used as a folk medicinal plant by the Dai people. However, with the development of *Amomum villosum* Lour. (Fructus amomi) plantations, most *D. pavettifolia* individuals have been eliminated, and wild populations of *D. pavettifolia* have been seriously reduced in Xishuangbanna.

Duperrea pavettifolia has prominent long exserted styles and swollen stigmas with abundant pollen. It is recognized as a plant with secondary pollen presentation: (1) the pollen is deposited on the style (pollen presenter) from the anthers at the late bud stage; (2) at anthesis, the style is elongated, thereby presenting pollen to the pollinators; and (3) the stigma lobes are fused during the functional male stage, and the receptive furrows on the stigma become obvious during the functional female stage (De Block and Robbrecht, 1998; Puff, 2005). This mechanism has been hypothesized to increase the accuracy of pollen transfer (Yeo, 1993; Ladd, 1994). Nevertheless, this spontaneous sex interference also affects plant fitness (Barrett, 2002). Our previous work determined that in this self-compatible species, there is an overlap between functional male and female phases in the inflorescence; however, sex interference and scarcity of pollinators induced a very low natural fruit set (unpublished work). We developed a range of microsatellites to investigate whether *D. pavettifolia* are predominantly self-pollinating or outcrossers. In this study, we used microsatellite markers to characterize *D. pavettifolia* to further study genetic diversity, population structure, mating system, and conservation.

METHODS AND RESULTS

Genomic DNA was extracted from a single individual using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The microsatellite loci were isolated using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol developed by Zane et al. (2002). Total genomic DNA (~500 ng) 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 (Fermentas, Burlington, Ontario, Canada) in a 30-μL reaction mixture. A diluted digestion-ligation mixture (1 : 10) was amplified with the adapter-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′; 25 μM) in 20-μL reaction volumes under the following conditions: 3 min of denaturation at 95°C; followed by 26 cycles of 30 s of denaturation at 94°C, 1 min of annealing at 53°C, and 1 min of extension at 72°C; with a final extension of 72°C for 5 min. The final concentrations were 10 ng of genomic DNA, dNTPs (0.2 mM each), primer (0.1 μM each), 1× PCR buffer, 1.5 mM Mg^{2+}, and 0.4 U DNA *Taq* polymerase (Sangon, Shanghai, China). Amplified DNA fragments, with a size range of 200–800 bp, were enriched for repeats by magnetic bead selection with 5′-biotinylated $(AC)_{15}$ and $(AG)_{15}$ probes. Enriched fragments were amplified again with adapter-specific primers for 30 cycles as described above. PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified DNA fragments were ligated into the pGEM-T vector (Promega, Madison, Wisconsin, USA) and transformed into DH5α cells (TaKaRa Biotechnology Co., Dalian, China). Positive clones were tested by PCR using $(AC)_{10}$/$(AG)_{10}$ and T7/Sp6 as primers. The clones with positive inserts were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Carlsbad, California, USA).

For the microsatellite sequences containing adequate flanking regions, PCR primers were designed using the Oligo 6.0 program (Rodriguez et al., 2003). From these primers, 97 produced consistent amplification of the loci in the 24 tested individuals from two populations in Nong Gang National Nature Reserve, Guangxi Province (22°33′16.25″N, 106°48′18.92″E), and Xishuangbanna

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TABLE 1. Characteristics of 19 microsatellite loci in *Duperrea pavettifolia.*

Locus	Primer sequences (5′–3′)	Repeat motif	Size (bp)	T_a (°C)	GenBank accession no.
*DP002**	F: ATGTATGGAGAGAGGGAAAT	$(AC)_{11}$	183	53	JN896688
	R: AGGGGAAGAAAGTGTTGATA				
*DP005**	F: CGGTGGCTTTTTGTGTCTAC	$(AC)_9$	164	62	JN572123
	R: GTGTTGCGTTGTCGAGTTTC				
DP011	F: CCAAAACTTAGGCTTTATGA	$(AC)_6$	212	53	JN572125
	R: ATAGAAGTACCTGGGTATGC				
DP020	F: ATTTCTACACCTTCATCTT	$(TG)_7$	105	48	JN572126
	R: TAACTTATCAAAACTGGAA				
DP022	F: ATGCTTCCTCTCTTTCGTA	$(TG)_7$	172	53	JN572127
	R: GACACAACAAAACCGTTCA				
DP025	F: ACGTGACAGGAGGGTCAAAT	$(TG)_8$	161	57	JN572129
	R: TGGGGTGGAAAGAGAAAGGA				
DP026	F: TAGTTGCTGACACAGTTGGA	$(AC)_8$	166	55	JN572130
	R: GGCATCAAGTAGAACCAGAA				
DP031	F: TGGGATTGTCATTCACCGTA	$(TG)_{10}$	145	55	JN572131
	R: CGCAAACCTGGATTAGTAAG				
*DP032**	F: TGACGATTGGATGTATGGTA	$(AC)_7$	151	51	JN572132
	R: ACACCCTAAAATAAGAAAAC				
*DP036**	F: ATTAGAGTGACTTGGATGAA	$(TC)_{15}$	217	58	JN572133
	R: GCTGGGGTTGGCTTGTGGAA				
*DP039**	F: ATTTTACTTGGCTCTTTTAT	$(AG)_{15}$	152	51	JN572134
	R: AAACCTGATGAACTCTTCTC				
*DP061**	F: GTATCAATGGTCCGATGGTG	$(AG)_{13}$	202	53	JN896689
	R: ATGACTTTGAACTTTTGCTA				
*DP066**	F: GTTGCTACTGCTTCTTTTTC	$(AC)_{12}$	234	53	JN896690
	R: ACCAAATCAAGTCAGGACAG				
DP074	F: CCCTCCTTTCATTGGTCTA	$(AC)_8$	160	53	JN572140
	R: GGTCTGACTCTCAAGTTTTT				
*DP076**	F: AATGTTGAATGTGGTTATGG	$(TG)_8$	200	51	JN896692
	R: TCAAGAAAAACTCAAGCAGC				
DP077	F: CGTTATTTCTTCAGCCAGTG	$(TG)_7$	160	53	JN572141
	R: CAAAAGGGATTAGTTATGTC				
*DP085**	F: TGTCTCCCAACCCCCTTTATC	$(TG)_7$	161	58	JN572142
	R: GATTGAGATGATTTTTGAAGC				
*DP087**	F: ATCCAAACCACACAAAACTC	$(AC)_{10}$	180	57	JN572143
	R: TGGGATGAGATGGGATTACT				
*DP089**	F: AGGTGAGGTAAAATGTAATC	$(TG)_9$	96	55	JN572144
	R: GGACACCCGTTAGAAAAATC				

Note: T_a = PCR annealing temperature.

* Displayed polymorphisms in *Duperrea pavettifolia*.

National Nature Reserve, Yunnan Province (21°55′45.6″N, 101°14′28.6″E), separately. PCR reactions were performed in 15-μL reactions containing 30–50 ng of genomic DNA, primer (0.6 μM each), 7.5 μL of 2× *Taq* PCR MasterMix (Tiangen Biotech, Beijing, China), 0.1 U *Taq* polymerase/μL, dNTP (0.5 mM each), 20 mM Tris-HCl (pH 8.3), 100 mM KCl, and 3 mM $MgCl_2$. PCR amplifications were conducted under the following conditions: 95°C for 3 min followed by 30–36 cycles at 94°C for 30 s, at the optimized annealing temperature (Table 1, each primer pair was tested separately) for 30 s, 72°C for 1 min, and a final extension step at 72°C for 7 min. PCR products were separated and visualized using the QIAxcel capillary gel electrophoresis system (QIAGEN, Irvine, California, USA). The number of alleles at each polymorphic locus, their size range, and the observed (H_o) and expected heterozygosities (H_e) were calculated using CERVUS 3.0 software (Walling et al., 2010). The data were analyzed by GENEPOP 4.0 (Raymond and Rousset, 1995), which included testing H_o, H_e, and departure from Hardy–Weinberg equilibrium (HWE) for the 16 polymorphic microsatellite loci.

TABLE 2. Results of initial primer screening in *Duperrea pavettifolia.*

	Population 1 (N = 12)			Population 2 (N = 12)		
Locus	A	H_e	H_o	A	H_e	H_o
DP002	3	0.685	0.833	3	0.520	0.833
DP005	3	0.236	0.250	2	0.228	0.250
DP032	2	0.513	1.000*	1	0.000	0.000*
DP036	3	0.540	0.333	3	0.282	0.000*
DP039	2	0.228	0.250	5	0.509	0.667
DP061	2	0.228	0.250	2	0.431	0.583
DP066	2	0.507	0.833	2	0.159	0.167
DP076	4	0.612	0.917	3	0.230	0.250
DP085	1	0.000	0.000*	2	0.267	0.333
DP087	3	0.562	0.583	1	0.000	0.000*
DP089	4	0.634	0.917	5	0.662	0.583

Note: A = number of alleles revealed; H_e = expected heterozygosity; H_o = observed heterozygosity.

* Statistically significant deviation from Hardy–Weinberg equilibrium at $P < 0.01$.

A total of 193 (67.2%) sequences were found to contain microsatellite repeats, and 97 primer sets were identified in two wild populations (Guangxi and Yunnan provinces). These primer sets were suitable for designing locus-specific primers, as they were >300 bp and had appropriate microsatellite sequences with at least five tandem repeats and enough flanking regions. Eleven of the identified 97 primers displayed polymorphisms and eight showed similar genetic diversity (Table 1). The number of alleles per locus ranged from one to five, with a mean of 2.6. The H_e and H_o ranged from 0.000 to 0.685 and from 0.000 to 1.000, with average values of 0.365 and 0.520, respectively (Table 2).

CONCLUSIONS

The microsatellite markers developed in this study are suitable for the fine-scale understanding of population genetic structure.

They will be useful to further study the mating systems of this secondary pollen presenting plant, and to establish a conservation strategy.

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