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Isolation and Characterization of 13 Microsatellite Loci from *Luculia pinceana* (Rubiaceae), a Typical Distylous Species

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***Abstract. Luculia pinceana* Hook. (Rubiaceae) is a typical distylous species with dimorphic and long-styled monomorphic populations. Within this study, we developed 13 microsatellite markers from *L. pinceana* using a modified biotin–streptavidin capture method. Polymorphism of each locus was assessed in 30 individuals from four dimorphic populations and one monomorphic population. The average allele number of these microsatellites was 4.153 per locus ranging from three to seven. The observed and expected heterozygosities were from 0.040 to 0.840 and from 0.571 to 0.769, respectively. Additionally, all 13 identified microsatellite markers were successfully amplified in its related species, *L. yunnanensis*, 10 of which showed polymorphism. These microsatellite markers could provide a useful tool for further study of the breeding system and the population genetic structure in this species and within other *Luculia* species.**

Luculia Sweet is a small shrubby genus containing three species and is a member of the family Rubiaceae (tribe Cinchoneae). All the species have compact pink or white tubular flowers contained in a tight inflorescence. The flowers are sweetly fragrant. It is widely cultivated in gardens as ornamentals. *Luculia pinceana* Hook. is widely distributed throughout southwest China, Myanmar, India, and Vietnam (Luo et al., 1999). It occurs on limestone mountains, open slopes, secondary shrubby woodland, and roadsides at an altitude of between 330 m and 1800 m. Our primary study showed that *L. pinceana* was a typical distylous species with reciprocally placed stigma and anthers in each floral morph. Our field investigation indicated that this species possesses two types of population with a dimorphic population sharing both short-styled (thrum flower) and long-styled (pin flower) individuals and a monomorphic population only with long-styled individuals. Furthermore, dimorphic and monomorphic populations exhibited particular geographic distribution patterns with the former occurring in the east and west zones of the latter. The polymorphism of both the sexual system and the population composition within the species provided an ideal system to study the adaptive function, evolutionary history of distyly, and its relationship with other stylar conditions (especially homostyly) (Barrett and Shore, 2008). Primary morphometric analysis on floral traits suggested that the species might address a transition of mating patterns from disassortative mating to selfing and/or intramorph crossing (W. Zhou, H. Wang and D.Z. Li, unpublished data). To further chase its population genetic structure and the evolutionary podogram of the breeding system, we developed 13 microsatellite markers from *L. pinceana* and tested their use in another *Luculia* species, *L. yunnanensis* Hu, which is also a distylous species.

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Materials and Methods

Genomic DNA samples of *L. pinceana* were extracted from silica gel-dried leaves using a CTAB methodology (Doyle and Doyle, 1990). The extracted DNA was dissolved in 30 μL TE buffer. A microsatellite enriched library was then conducted using a modified biotin–streptavidin capture method (Chen et al., 2008). Total genomic DNA (≈250 to 500 ng) was completely digested with 2.5 U of *Mse*I restriction enzyme (New England Biolabs Inc., Beverly, MA) and then ligated to an *Mse*I amplified fragment length polymorphism adaptor (5′-TACTCAGGAC TCAT-3′/5′-GACGATGAG TCCTGAG-3′) using T4 DNA ligase (New England Biolabs). The digested-ligated fragments were diluted in a ratio of 1:10, and 5 μL was used in amplification reaction with adaptor-specific primers (5′-GATGAGTCCTGAGTAAN-3′). Amplified DNA fragments, with a size range of 200 to 800 base pairs (bp), were enriched by magnetic bead selection with a 5-biotinylated $(AG)_{15}$ and $(AC)_{15}$ and $(AAG)_{10}$ probe (Zane et al., 2002). The recovered DNA fragments were reamplified with *Mse*I-N primers. Polymerase chain reaction (PCR) products were purified using the EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified DNA fragments were ligated into pBS-T II vector (Tiangen; Tiangen Biotech Co., Ltd., Beijing, China) and then transformed into DH5α competent cells (Tiangen). The positive clones were tested by PCR using T3/T7 and $(AC)_{10}$/$(AG)_{10}$/$(AAG)_7$ as primers. A total of 320 positive clones were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems Inc., Foster City, CA), of which 154 clones contained microsatellites. It was found that 73 primer pairs were suitable for primer designing using Primer 5.0 (Clarke and Gorley, 2001).

Polymorphism of all 73 microsatellite loci were assessed in 30 individuals of *L. pinceana* equal from four dimorphic populations (Yunnan, Jingdong: long. 24°47′ N, lat. 100°30′ E; Yunnan, Lushui, Pianma: long. 25°58′ N, lat. 98°48′ E; Yunnan, Yongde: long. 24°07′ N, lat. 99°43′ E; Guangxi, Tianyang: long. 23°32′ N, lat. 106°31′ E) and one monomorphic population (Yunnan, Jinping: long. 22°53′ N, lat. 103°13′ E) in southwest China. The PCR reactions were performed in 20 μL of reaction volume containing 50 to 100 ng genomic DNA, 0.5 μM of each primer, and 10 μL 2 × Taq PCR MasterMix [Tiangen; 0.1 U Taq polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$]. PCR amplifications were conducted

Table 1. Characteristics of 13 microsatellite loci developed for *Luculia pinceana* (n = 30) and cross-amplification in *L. yunnanensis* (n = 5).

Locus	GenBank accession no.	Primer sequence (5′-3′)	Repeat motif	Size range (bp)	T_a (°C)	*L. pinceana* NA	*L. pinceana* H_O	*L. pinceana* H_E	*L. yunnanensis* NA
LP 54	GU208853	F: GTCCCGCAGAAGACATCC R: AAGCTGCCTTTGGCTCAG	$(CA)_{11}(GA)_6$	233–241	63	4	0.600	0.641	P(2)
LP 45	GU208854	F: ATGGTAGCATCAAAACAA R: CATTGAACAGACACTCCC	$(GA)_{14}(GGGA)_3(GA)_5$	146–168	60	6	0.520	0.665	P(3)
LP 70	GU208855	F: ACCACTCTACAGATCGCTAC R: ATTGAAGGAATTGGGACT	$(TC)_{20}$	147–187	60	7	0.680	0.700	P(2)
LP 4	GU208856	F: CCCACATCAGTCCTCACA R: CATACATGGAATCGCAGAA	$(TTC)_8$	120–132	62	3	0.040	0.624[z]	P(2)
LP 23	GU208857	F: CCTCATTTTGACTGTCAT R: CATTATTGTAAGTTCTCG	$(AAG)_{13}$	236–248	56	4	0.160	0.744[z]	P(2)
LP 18	GU208858	F: TGCTTCTACGCACTGGTT R: ATGTGGCTTCGGATTGGT	$(GA)_7(CA)_7(GAA)_6$	169–179	63	4	0.120	0.669[z]	M
LP 154	GU208859	F: TGACAACAACATTGGAGC R:TGCGTTAGGGTAATAATAGAC	$(AG)_{12}$	177–195	63	3	0.600	0.639	P(2)
LP 107	GU208860	F: CTTTCCGTCAAGTTAGCC R: TTGCGTGATTGAACGAGTGC	$(TC)_{11}(AC)_7$	101–113	63	3	0.040	0.620[z]	M
LP 162	GU208861	F: TCGCTGCTTTCTCATTTT R: TTACGTTTCAATAGTCATCC	$(TC)_{10}(AC)_{10}$	243–255	61	4	0.560	0.740	P(2)
LP 184	GU208862	F: GGCATTGAAGATGAGAAG R: GGCAAAGAAATCGAAAGT	$(CT)_{12}$	205–211	59	3	0.160	0.571[z]	M
LP 170	GU208863	F: CTACAGGGTAAAATGGAT R: CTTGTAGACCTGGATAGA	$(TG)_{17}$	163–193	59	4	0.840	0.760	P(4)
LP 17	GU208864	F: CATTTTGCAGCAAACAAT R: TAACTCCAGCATAACCGA	$(AAG)_{14}$	87–103	59	3	0.680	0.644	P(2)
LP 198	GU208865	F: GTCCACCGATGCTTCTTA R: ATTACCCACCCAGTTTCA	$(GA)_{25}$ GG$(GA)_7$	90–121	62	6	0.520	0.769	P(4)

[z]Statistically significant deviation from Hardy-Weinberg equilibrium at $P < 0.01$.
T_a = polymerase chain reaction annealing temperature; NA = number of alleles revealed; H_O = observed heterozygosity; H_E = excepted heterozygosity; P = polymorphic amplification; M = monomorphic amplification.

on an MJ PTC-200 Thermal Cycler (Waltham, MA) under the following conditions: 95 °C for 5 min followed by 32 to 35 cycles at 94 °C for 45 s at the annealing temperature for each specific primer (optimized for each locus; Table 1) for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR products were separated on 8% polyacrylamide denaturing gels using a 20-bp or 10-bp ladder molecular size standard (O'rangeRuler™; Fermentas, Shenzhen, China) visualized by silver staining.

Results and Discussion

In all, PCR products with 13 primer pairs displayed polymorphism. Standard genetic diversity parameters, departure from Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between pairs of loci were estimated in POPGENE Version 4.0 (Raymond and Rousset, 1995). The number of alleles per locus was three to seven with an average of 4.153; values for observed and expected heterozygosities ranged from 0.040 to 0.840 and from 0.571 to 0.769 with averages of 0.424 and 0.675, respectively (Table 1). For all 13 microsatellite loci, except LP 54, LP 45, LP 70, LP 154, LP 162, LP 170, LP 17, and LP 198, the genotypic frequencies showed significant deviation from HWE ($P < 0.01$) indicating the possibility of null alleles, the Wahlund effect, and the disassortative mating in a dimorphic population of this typical distylous species. LD P values were obtained for 78 pairs of markers combinations. Of these, 20 (25.64%) pairs showed significant LD at $P < 0.01$.

Cross-species amplification in *L. yunnanensis*, which only has dimorphic populations in nature, was tested using five individuals (Yunnan, Fugong, Yaping: long. 27°01′ N, lat. 98°49′ E). All of the 13 identified microsatellite markers were successfully amplified and 10 (76.92%) of them revealed polymorphism (Table 1). The results showed that there was a high potential for transferring microsatellite markers in the genus *Luculia*. The 13 novel polymorphic microsatellite loci developed here will be useful for further understanding the differentiation of population and evolution of distyly in *L. pinceana* and its related species.

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