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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITES IN *LABISIA PUMILA* (MYRSINACEAE), AN ECONOMICALLY IMPORTANT MALAYSIAN HERB[1]

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- *Premise of the study:* The exploitation of *Labisia pumila* for commercial demand is gradually increasing. It is therefore important that conservation is prioritized to ensure sustainable utilization. We developed microsatellites for *L. pumila* var. *alata* and evaluated their polymorphism across var. *alata*, var. *pumila*, and var. *lanceolata*.
- *Methods and Results:* Ten polymorphic microsatellites of *L. pumila* were developed using the magnetic bead hybridization selection approach. A total of 84, 48, and 66 alleles were observed in *L. pumila* var. *alata*, var. *pumila*, and var. *lanceolata*, respectively. The species is likely a tetraploid, with the majority of the loci exhibiting up to four alleles per individual.
- *Conclusions:* This is the first report on the development of microsatellites in *L. pumila*. The microsatellites will provide a good basis for investigating the population genetics of the species and will serve as a useful tool for DNA profiling.

Key words: kacip fatimah; *Labisia pumila*; medicinal plant; microsatellites; Myrsinaceae; tetraploid.

Labisia pumila (Blume) Fern.-Vill. (Myrsinaceae) is a small understory shrub that is widely distributed in the tropical forests of Malaysia, Indonesia, Thailand, the Philippines, and Myanmar (Sunarno, 2005). Eight varieties are recognized (Sunarno, 2005), of which *L. pumila* var. *pumila*, *L. pumila* var. *alata* (Scheff.) Mez, and *L. pumila* var. *lanceolata* (Scheff.) Mez are commonly found in Malaysia. These varieties are morphologically distinct from one another in terms of their petiole and leaf characteristics. Among the Malay communities, these varieties are collectively known as kacip fatimah, which has long been used as the traditional medicine for the treatment of pre- and postpartum complications, menstrual disorders, dysentery, rheumatism, flatulence, and gonnorrhea (Burkill, 1966; Jaganath, 2000).

To date, the exploitation of *L. pumila* for commercial demand, particularly in the pharmacological and cosmeceutical applications, is gradually increasing. Attention to conservation should therefore be prioritized to ensure sustainable utilization. Despite the importance of *L. pumila*, the availability of genetic information for the species is still very limited. Only two genetic variability studies of the species have been reported using dominant markers (Bhore et al., 2009; Farah Fazwa et al., 2013). In this study, we report the development of 10 microsatellite loci in *L. pumila* var. *alata*, and we evaluate their polymorphism across var. *alata*, var. *pumila*, and var. *lanceolata*. Microsatellites are preferred markers because of the nature of their codominant inheritance, high abundance, extent of allelic diversity, and the ease of assessing the size variation by PCR with pairs of flanking primers (Weising et al., 2005).

METHODS AND RESULTS

Leaf samples of 25, 20, and four individuals of *L. pumila* var. *alata*, var. *pumila*, and var. *lanceolata*, respectively, were obtained from Pasoh Forest Reserve (2°58′N, 102°18′E). An additional six individuals of var. *lanceolata* were obtained from the Ethnobotanical Garden of the Forest Research Institute Malaysia (FRIM). The voucher specimens of these three varieties were deposited in FRIM Herbarium (KEP; barcode numbers 223663–223665). Total genomic DNA was extracted from fresh leaves of *L. pumila* using a modified cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson, 1980) and further purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Penzberg, Germany).

A genomic library enriched for dinucleotide CT and GT was constructed following the approach of Kijas et al. (1994). Approximately 5 µg of genomic DNA was obtained from an individual of *L. pumila* var. *alata* from the Ethnobotanical Garden of FRIM. After digestion with *Nde*II (Promega Corporation, Madison, Wisconsin, USA), the digested fragments were electrophoresed on 2% agarose gels with a 100-bp DNA ladder (New England Biolabs, Ipswich, Massachusetts, USA). Fragments of 300–1000 bp were excised and ligated into *Sau*3A1 cassettes (TaKaRa Bio, Otsu, Shiga, Japan). After ligation, the nicks were repaired using DNA polymerase I (TaKaRa Bio). The cassette-ligated DNA was enriched for microsatellite repeats via hybridization to 5′-biotinylated $(CT)_{15}$ and $(GT)_{15}$ probes and retrieved using magnetic beads coated with streptavidin (Promega Corporation). The selectively recovered hybrids were reamplified using C1 cassette primers, digested with *Nde*II, cloned into pUC118 *Bam* H1/BAP vector (TaKaRa Bio), and transformed into QIAGEN EZ Competent Cells (QIAGEN Gmbh, Hilden, Germany). Insert-containing clones were selected by blue/white screening on Luria–Bertani (LB) agar plates containing 100 µg/mL ampicillin, 50 µM isopropyl-β-D-1-thiogalactopyranoside (IPTG), and 80 µg/mL X-gal. Plasmid DNAs of a total of 608 clones were amplified using the Illustra TempliPhi Amplification Kit (GE Healthcare,

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TABLE 1. Description of 10 polymorphic and three monomorphic microsatellites screened in *Labisia pumila* var. *alata*.

Locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Allele size range (bp)	Fluorescent label	GenBank accession no.
Lpu02	$(GT)_{20}$	F: GCGGAAAGAAGGTATAGTGTG	50	197–235	HEX	KF318311
		R: AAATATAATAGGCGACACGAGTTA				
Lpu08a	$(TC)_{15}$	F: ACAAATCCAAATTCGCACCT	50	103–132	HEX	KF318312
		R: AATGAAAAATAATAATAAACCCAA				
Lpu08b	$(TC)_7$	F: CTTCCTGTCCTTGTTGGTTAGAGT	50	178–211	HEX	KF318312
		R: AGAGGGAAAGTCGAAGTTAAT				
Lpu13	$(TG)_{11}$	F: CGAACTGAGAATATCCCCTTGCTA	50	303–342	HEX	KF318313
		R: TAGTTGATGTGAGTTGGCGTAAA				
Lpu15	$(TTTTC)_3$	F: GGGCACTTGTCTTGTAGGTTAGAG	50	198–213	6-FAM	KF318314
		R: TCAGCGACCGAAACATCACTACTC				
Lpu16a	$(CAC)_5$	F: GCCGAGCTACCAAGGTTAC	50	93–107	6-FAM	KF318315
		R: GCCTCTGAGGAAAGACAAGCATAA				
Lpu16b	$(GTAT)_{10}$	F: TTTCCTCAGAGGCTGGTATGTAT	50	69–112	6-FAM	KF318315
		R: TTTCAAGGGTGTCAATCAATTAAC				
Lpu21a	$(AG)_7$	F: AGAGGGAAAGTCGAAGTTAAT	50	175–207	6-FAM	KF318316
		R: CTTCCTGTCCTTGTTGGTTAGAGT				
Lpu21b	$(GA)_{15}$	F: AGGAAGAAGCTGCCGTGAGA	50	388–415	6-FAM	KF318316
		R: GGCGAAATCACCACCACAGTTAGA				
Lpu38	$(CA)_8$	F: TTCCTACTCACTGCACATAGTTGC	50	75–83	HEX	KF318317
		R: GCTGTAGCGTGTGCGAGTG				
Lpu06*	$(CA)_6$	F: TAAAGCACCACAATTCACGCACAC	50	148	HEX	KF318318
		R: CGCTTAGGATTTGAAGTGGGTAA				
Lpu20*	$(CT)_6$	F: CATGCCCATACACCGGAAAT	50	315	6-FAM	KF318319
		R: GAGGCGAAAAGTCACATAAACGTA				
Lpu24*	$(GGAATT)_3$	F: CTATTATTGTGTTGATGGATTGAG	50	159	6-FAM	KF318320
		R: GCCAATTTCATAACCAATCATA				

Note: T_a = annealing temperature.
* Monomorphic microsatellites.

Piscataway, New Jersey, USA) and sequenced using BigDye Terminator Sequencing Kit version 3.1 (Applied Biosystems, Foster City, California, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Sequence redundancy was checked using CD-HIT (Li and Godzik, 2006), and microsatellite-containing sequences were then identified from the nonredundant sequences using the MIcroSAtellite Identification Tool (MISA) (Thiel et al., 2003). A total of 56 sequences harbored at least one microsatellite motif. Forty primer pairs of 19–24 nucleotides in length were designed using OLIGO 6.67 (Molecular Biology Insights, Cascade, Colorado, USA), based on the criteria of GC content (40–60%), melting temperature (52–58°C), and the lack of secondary structures. Four unrelated individuals of *L. pumila* var. *alata* were used for initial screening of these primer pairs. The PCR amplifications were carried out in 10-µL volumes containing 10 ng of template DNA, 1× GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.3 µM of each primer, 0.2 mM of each dNTP, and 0.5 unit of GoTaq Flexi DNA polymerase (Promega Corporation). The reaction mixture was amplified using a GeneAmp PCR System 9700 (Applied Biosystems) with the following cycling conditions: 4 min at 94°C; 35 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 30 s; followed by a final extension of 30 min at 72°C. Twenty-nine primer pairs that showed specific amplification products of expected fragment size were selected for fluorescent labeling at the 5′-end of the forward primers with either 6-FAM or HEX. These primers were further screened using 25 samples of *L. pumila* var. *alata* using the same cycling conditions as above. Fragment analysis was conducted using an ABI 3130xl with GeneScan 400 ROX (Applied Biosystems) as the internal size standard. Allele sizes were assigned using GeneMapper version 4.0 (Applied Biosystems). Characteristics of the microsatellites developed for *L. pumila* var. *alata* are shown in Table 1. Thirteen loci yielded consistent and scorable genotypes; of these, 10 were polymorphic and the remaining three were monomorphic. Because the majority of the loci evaluated (Lpu02, Lpu08a, Lpu08b, Lpu13, Lpu16b, Lpu21a, and Lpu21b) exhibited up to four alleles per individual (Fig. 1), our results suggest that the species is likely a tetraploid.

To assess the degree of polymorphism across *L. pumila* var. *alata*, var. *pumila*, and var. *lanceolata*, the 10 polymorphic microsatellites were further checked for variability in 55 individuals of the three varieties using the same PCR and genotyping protocols as above. Observed heterozygosity (H_o) of each variety was calculated based on the allele phenotypes, following Bever and Felber (1992), with values being one minus the probability that any two alleles drawn at random were identical by descent (AAAA = 0, AAAB = 0.50, AABB = 0.667, AABC = 0.833, and ABCD = 1). Minimum and maximum bounds of H_o were calculated for biallelic phenotypes (AAAB and AABB) (De Walt et al., 2011). Expected heterozygosity (H_e) was calculated using ATETRA 1.2 (Van Puyvelde et al., 2010), with 10,000 Monte Carlo simulations. A total of 84, 48, and 66 alleles were observed in *L. pumila* var. *alata*, var. *pumila*, and var. *lanceolata*, respectively (Table 2). H_e ranged from 0.039 to 0.857, 0.000 to 0.793, and 0.000 to 0.874 in *L. pumila* var. *alata*, var. *pumila*, and var. *lanceolata*, respectively. Notably, locus Lpu15 was found to be monomorphic in varieties *pumila* and *lanceolata*.

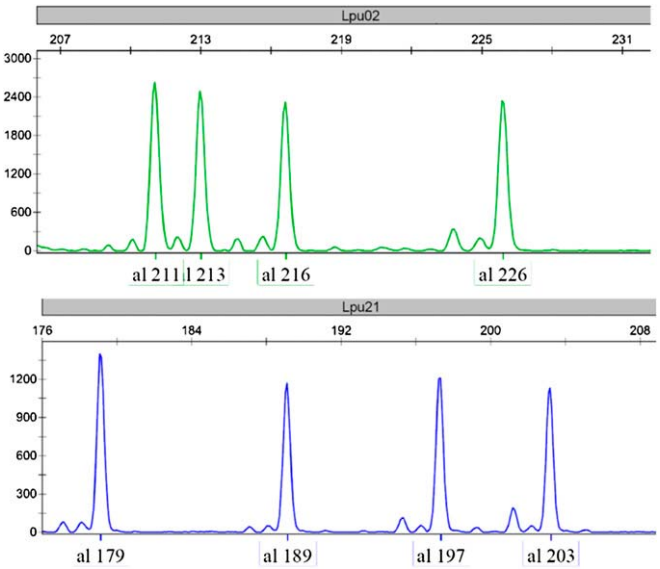

Fig. 1. Electropherogram showing four alleles amplified from an individual at loci Lpu02 and Lpu21a.

TABLE 2. Genetic properties of 10 microsatellites of *Labisia pumila* across varieties *alata*, *pumila*, and *lanceolata*.

	L. pumila var. *alata* (n = 25)				*L. pumila* var. *pumila* (n = 20)				*L. pumila* var. *lanceolata* (n = 10)			
Locus	A	H_o (min)	H_o (max)	H_e	A	H_o (min)	H_o (max)	H_e	A	H_o (min)	H_o (max)	H_e
Lpu02	11	0.940	0.953	0.857	5	0.783	0.808	0.736	9	0.483	0.584	0.806
Lpu08a	9	0.880	0.880	0.793	7	0.833	0.833	0.793	8	0.717	0.783	0.815
Lpu08b	11	0.940	0.940	0.834	6	0.833	0.833	0.763	8	0.783	0.850	0.794
Lpu13	10	0.706	0.753	0.848	6	0.533	0.684	0.695	7	0.370	0.426	0.652
Lpu15	2	0.020	0.027	0.039	1	0.000	0.000	0.000	1	0.000	0.000	0.000
Lpu16a	5	0.280	0.340	0.412	2	0.075	0.100	0.138	2	0.150	0.200	0.253
Lpu16b	10	0.973	0.973	0.853	5	0.833	0.833	0.729	9	0.816	0.833	0.852
Lpu21a	11	0.940	0.940	0.834	6	0.833	0.833	0.763	8	0.733	0.817	0.785
Lpu21b	10	0.972	0.972	0.843	7	0.833	0.833	0.777	11	0.917	0.917	0.874
Lpu38	5	0.806	0.820	0.731	3	0.125	0.167	0.405	3	0.250	0.334	0.591

Note: A = number of alleles; H_e = expected heterozygosity; H_o (min) = minimum observed heterozygosity; H_o (max) = maximum observed heterozygosity; n = number of individuals.

CONCLUSIONS

This is the first report on the development of microsatellites in *L. pumila*. The observed levels of polymorphism and genetic diversity suggest that, apart from monomorphic loci, these microsatellites can serve as useful tools for DNA profiling and population genetic studies of the species.

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