Development of Microsatellite Markers in Garcinia paucinervis (Clusiaceae), an Endangered Species of Karst Habitats

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Primer Note

Development of microsatellite markers in *Garcinia paucinervis* (Clusiaceae), an endangered species of karst habitats[1]

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- *Premise of the study:* Microsatellite markers were developed for *Garcinia paucinervis* (Clusiaceae), an endangered and endemic tree species of karst habitats, to analyze its genetic diversity and genetic structure.
- *Methods and Results:* Using shotgun sequencing on an Illumina MiSeq platform, a total of 22 microsatellite primer sets were characterized, of which 17 were identified as polymorphic. For these polymorphic loci, the total number of alleles per locus ranged from two to 12 across 54 individuals from three populations. The observed and expected heterozygosities ranged from 0.000 to 1.000 and from 0.000 to 0.850, respectively. No pair of loci showed significant linkage disequilibrium. Three loci in one population deviated significantly from Hardy–Weinberg equilibrium ($P < 0.05$). Seven loci (JSL3, JSL5, JSL22, JSL29, JSL32, JSL39, and JSL43) were successfully amplified in *G. bracteata*.
- *Conclusions:* These markers will be useful in studies on genetic diversity and population structure of *G. paucinervis*.

Key words: Clusiaceae; *Garcinia paucinervis*; genetic diversity; microsatellite marker; population structure.

Garcinia paucinervis Chun & F. C. How (Clusiaceae) is an evergreen tree that grows only in the dry sparse or dense forests of the limestone mountains in southwestern China and northern Vietnam, at elevations between 300 and 800 m above sea level. This karst endemic tree species is valuable and used for shipbuilding, construction, quality furniture, and in the military industry (Li et al., 2007). Given the economic benefits of this species, since the 20th century, the wild populations of *G. paucinervis*, especially the older age-class individuals, have declined drastically because of overcutting (Fu, 1992). Moreover, karst landforms have been shown to lead to poor seed germination and to limit seed dispersal (Fu, 1992; Zhang et al., 2013), thus most species living in karst environments demonstrate deficient population regeneration ability, especially after populations have been destroyed (Fan et al., 2011). Therefore, according to the IUCN Red List Categories and Criteria, *G. paucinervis* has been recorded as "endangered" in the China Species Red List (Wang and Xie, 2004). To protect this species effectively and analyze the genetic diversity, genetic structure, and gene flow between populations, we developed and characterized 22 microsatellite loci from *G. paucinervis*. We selected *G. bracteata* C. Y. Wu ex Y. H. Li, another *Garcinia* L. species found in karst environments that has an overlapping geographic distribution with *G. paucinervis* (Li et al., 2007), for detection of cross-species amplification.

METHODS AND RESULTS

Fifty-four individuals of *G. paucinervis* were sampled from two natural populations and one cultivated population in southwestern China, and five individuals of *G. bracteata* were collected for detection of cross-species amplification. Voucher and locality information for both species are provided in Appendix 1. All samples were stored in allochroic silica gel (Sangon Biotech, Shanghai, China) for drying. The cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA (gDNA) from the dried leaves (Doyle and Doyle, 1987). We mixed the gDNA of all individuals from population LZ (Appendix 1) for shotgun sequencing. This procedure was entrusted to Sangon Biotech and was carried out using an Illumina MiSeq platform (San Diego, California, USA).

After sequencing, 1,325,041 reads and a total of 625,940,647 bases were obtained. All raw reads have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; accession no. SRR5026097). MISA (Thiel et al., 2003) was then used to detect microsatellite motifs using the following settings: for a unit size of 2 bp, the repeat number was greater than 5; for a unit size of 3–4 bp, the repeat number was greater than 4. MISA identified 27,441 sequences containing 31,776 simple

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sequence repeats (SSRs), including 23,522 dinucleotide, 7060 trinucleotide, and 1194 tetranucleotide repeats. Sequences with at least 10 repeats for dinucleotides and seven repeats for trinucleotides and sufficiently long flanking regions were selected to design primers using Primer Premier 5.0 (Clarke and Gorley, 2001). Only 5889 sequences contained the required number of repeats, and in most sequences the flanking regions were not sufficiently long to allow proper primer design. The specific Primer Premier criteria were as follows: (1) primer length between 17 and 25 bp; (2) CG content of each primer between 40% and 60%; (3) annealing temperature between 50°C and 65°C, and maximum temperature difference between the upstream and downstream primers less than 4°C; and (4) PCR product size between 100 and 350 bp. Finally, a total of 65 primer pairs were successfully designed, and primers were synthesized by Sangon Biotech.

Ten samples from population LZ were chosen for initial testing of these 65 primers. PCR was carried out in 20-μL reactions consisting of 8.6 μL of sterilized ddH_2O, 1 μL of gDNA (at least 50 μg/mL), 0.2 μL of each primer (50 μM), and 10 μL of 2× *Taq* PCR MasterMix (Tiangen Biotech, Beijing, China). The PCR cycle parameters were as follows: an initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation (94°C, 45 s), annealing (at the temperature for each specific primer as listed in Table 1, 45 s), and extension (72°C, 45 s); followed by a final extension (72°C, 5 min). Using a 10-bp or 25-bp DNA ladder (Invitrogen, Carlsbad, California, USA) as a reference, PCR products were resolved on 6% polyacrylamide denaturing gel and visualized by silver staining. Suitable primers were those that exhibited fragments of the expected size, clear banding patterns, and no more than two alleles per locus. Suitable primers were taken for further amplification across population LZ. The result of this procedure was used to detect null alleles by MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004). The primer sets without null alleles were preserved to scan all samples.

We used GenAlEx 6 (Peakall and Smouse, 2006) to calculate the total number of alleles per locus, the number of alleles per population, and the observed and expected heterozygosities of *G. paucinervis* from three populations. The linkage disequilibrium and deviation from Hardy–Weinberg equilibrium (HWE) were tested using GENEPOP version 4.2 (Raymond and Rousset, 1995).

A total of 22 primer pairs were successfully amplified in *G. paucinervis*. The characterization of the 17 polymorphic loci is presented in Table 2, including nine dinucleotide and eight trinucleotide repeats. Among the three populations of *G. paucinervis*, the total number of alleles per locus varied from two to 12, and a total of 121 alleles were scored with a mean of 7.1 alleles per locus. The observed and expected heterozygosity values in the survey populations ranged from 0.000 to 1.000 and from 0.000 to 0.850,

Table 1. Characteristics of 22 microsatellite markers developed for *Garcinia paucinervis*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
JSL2	F: GAGAAAGTGGTTGTAGCAC R: GTCCAAGAGAGTGACCTT	$(TTG)_9$	315	58	KU375202
JSL3	F: GAATGGAATTACAGAACCG R: TCACCTTCCCAAATGGTT	$(AG)_{10}$	236–242	57	KU375203
JSL5	F: GTGAAGAGAACGAGAGCAAG R: GACCACTCCTCCTGCTATTG	$(GTA)_{10}$	151–160	62	KU375204
JSL12	F: TGGAAACGGTCAACAATCT R: GCGGTTCGTTCTTGACTC	$(GA)_{10}$	186	59	KU375205
JSL16	F: CTCTAATTGGGTACTCAGGC R: ATTGGACACTTGGGGACTC	$(TC)_{11}$	145	61	KU375207
JSL17	F: ATTAAGGGGTCTATCGAG R: TGTCCCAACTGAACTCTT	$(AG)_{11}$	262–286	55	KU375208
JSL19	F: AGTCACTTATTTACGCCGT R: GTGTTGCCTCTATGACCTT	$(TA)_{10}$	199–201	60	KU375209
JSL22	F: ATTGGAGGAGTCAAATCTGG R: TACTCATAGGTAGCCGCAAT	$(CTT)_{11}$	158–176	58	KU375210
JSL23	F: CCATTATCACAGAACCTACG R: TACTCCACAACTCTGAAAGG	$(ACA)_9$	213–219	61	KU375211
JSL26	F: AAGGGATAGTGTCCATAC R: CTCCTCTTGATGAGTTGA	$(AGA)_9$	257–272	56	KU375213
JSL27	F: CGTTTTGATTATCTCCACC R: GTCGCAAGCAATGAGTAGT	$(TTC)_8$	158–170	56	KU375214
JSL29	F: CGTGCCTCTACAAATCAAC R: ACGCTCTCGTATATGCTCT	$(AT)_{10}$	159–163	60	KU375215
JSL30	F: TTGTTGGATGTGCCGAG R: AGTTTCACTTTCTAAGGAGG	$(GA)_{16}$	190–206	61	KU375216
JSL32	F: CTGAGACACTCTTTTTGG R: GGAGCAGAATAACTAAGG	$(AT)_{11}$	169–187	57	KU375217
JSL33	F: CTCAAGGGAACACATGGAAG R: CTGGGCAAACTTGTAACACC	$(ATA)_{24}$	170–194	62	KU375218
JSL34	F: AGAGAAATGAACAGGAACC R: GATGCTTGATTCTACCACC	$(AG)_{16}$	184–198	60	KU375219
JSL39	F: ACATAGTGGTGTTTCCTCG R: GATAATACGGGAGAAGAGACT	$(ACA)_9$	188–203	59	KU375220
JSL42	F: TCATACCGACAAGACAG R: GAAGTGGAAGAACTAAGAG	$(CTC)_{10}$	302	56	KU375221
JSL43	F: TAGCAAGTACCCTAGAGATC R: CAAAGACAACCCCAACT	$(GAA)_{12}$	127–142	58	KU375222
JSL45	F: TGTGCTGATAAAGAAGGTGT R: ACTTTAGGGCTCATAACCAC	$(ATG)_{13}$	222	60	KU375223
JSL47	F: CTGGTTTATATGTTGGAGGT R: CCTGGGTCATCCTAGACTC	$(AG)_{15}$	150–176	60	KU375224
JSL50	F: AGGTGCTGTTTGTGTTTTCT R: GGTACGGTACATTTTTGTGG	$(AT)_{20}$	236–258	59	KU375226

Note: T_a = annealing temperature.

TABLE 2. Results of initial primer screening of 17 polymorphic loci in three populations of *Garcinia paucinervis*.[a]

		LZ (natural, $n = 23$)				CZ (natural, $n = 20$)				ZWS (cultivated, $n = 11$)			
Locus	A_T	A	H_o	H_e	P value	A	H_o	H_e	P value	A	H_o	H_e	P value
JSL3	4	3	0.391	0.381	0.894	4	0.300	0.501	0.108	2	0.455	0.434	0.875
JSL5	5	4	0.522	0.589	0.180	2	0.650	0.489	0.140	4	0.455	0.541	0.094
JSL17	7	6	0.478	0.775	0.092	4	0.550	0.558	0.892	3	0.364	0.376	0.475
JSL19	2	2	0.478	0.466	0.899	2	0.350	0.439	0.366	1	0.000	0.000	—
JSL22	6	6	0.739	0.668	0.221	5	0.900	0.784	0.449	4	0.273	0.500	0.038*
JSL23	8	3	0.565	0.628	0.929	3	0.300	0.374	0.137	7	0.636	0.806	0.121
JSL26	6	4	0.652	0.678	0.502	6	0.700	0.745	0.605	4	0.727	0.612	0.266
JSL27	5	4	0.391	0.525	0.117	5	0.350	0.533	0.698	4	0.727	0.657	0.219
JSL29	3	3	0.565	0.644	0.415	1	0.000	0.000	—	2	0.364	0.463	0.477
JSL30	12	8	0.696	0.784	0.141	6	0.550	0.811	0.191	7	0.545	0.785	0.016*
JSL32	8	6	0.826	0.791	0.749	5	0.750	0.724	0.738	6	0.909	0.789	0.761
JSL33	9	9	0.696	0.768	0.388	6	0.400	0.350	1.000	5	0.636	0.698	0.146
JSL34	9	6	0.826	0.739	0.993	6	0.900	0.734	0.578	4	0.727	0.682	0.490
JSL39	8	6	0.783	0.729	0.667	5	0.800	0.761	0.825	5	0.818	0.628	0.646
JSL43	6	6	0.783	0.751	0.264	3	0.650	0.580	0.738	4	1.000	0.698	0.020*
JSL47	11	7	0.783	0.812	0.207	7	0.950	0.796	0.873	6	0.818	0.760	0.184
JSL50	12	9	0.957	0.850	0.331	6	0.800	0.778	0.547	7	0.727	0.769	0.726

Note: A = number of alleles per population; A_T = total number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = sample size; P value = test for deviation from Hardy–Weinberg expectations.

[a]Voucher and locality information are provided in Appendix 1.

*Significant deviation from HWE.

respectively. No pairs of loci showed significant linkage disequilibrium. The P value of tests for HWE ranged from 0.016 to 1.000. In population ZWS, three loci (JSL22, JSL30, and JSL43) deviated significantly ($P < 0.05$) from HWE, which could be due to admixture or insufficiency of sample size. Finally, primer transferability was also tested in the sympatric related species *G. bracteata*, and only seven loci were able to be amplified successfully (Table 3).

CONCLUSIONS

A total of 22 nuclear microsatellite markers were developed for *G. paucinervis*. These markers may also be useful for assessing and analyzing the genetic diversity and population structure in *G. paucinervis*, as well as to assess genetic diversity in other species in the genus *Garcinia*, such as *G. bracteata*.

TABLE 3. Cross-amplification results of microsatellite markers developed in *Garcinia paucinervis* as detected from five individuals of *G. bracteata.*

Locus	A	H_o	H_e	Product size (bp)
JSL3	1	0.000	0.000	224
JSL5	2	0.400	0.320	148–151
JSL22	3	0.600	0.580	158–164
JSL29	3	0.400	0.460	180–186
JSL32	1	0.000	0.000	195
JSL39	2	0.200	0.180	194–197
JSL43	3	0.600	0.620	118–127

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

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Appendix 1. Voucher and locality information for *Garcinia* species used in this study.

Species	Population	Collection locality[a]	Geographic coordinates	*n*	Voucher specimen accession no.[b]
G. paucinervis Chun & F. C. How	LZ	Longzhou County, Chongzuo City, Guangxi Zhuang Autonomous Region	22°26′55.78″N, 106°57′17.48″E	23	Gp-001-ZQW
G. paucinervis	CZ	Longzhou County, Chongzuo City, Guangxi Zhuang Autonomous Region	22°27′58.47″N, 106°57′50.11″E	20	Gp-002-HG
G. paucinervis	ZWS	Guangxi Institute of Botany, Guilin City, Guangxi Zhuang Autonomous Region	25°04′41.29″N, 110°18′19.73″E	11	Gp-002-ZQW
G. bracteata C. Y. Wu ex Y. H. Li	GB	Napo County, Baise City, Guangxi Zhuang Autonomous Region	22°58′45.44″N, 106°00′37.57″E	5	Gb-001-ZQW

Note: *n* = number of individuals.

[a] Locality and Chinese province.

[b] Voucher specimens were deposited in the Guangxi Institute of Chinese Medicine and Pharmaceutical Science herbarium (GXMI). ZQW = Qi-Wei Zhang, collector; HG = Gang Hu, collector.