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

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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 alloxroic 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 4; for a unit size of 3–4 bp, the repeat number was greater than 5; and for a unit size of 3–4 bp, the repeat number was greater than 4. MISA identified 27,441 sequences containing 31,776 simple microsatellite loci from *G. paucinervis*. Of these loci, 17 were polymorphic.

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.

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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 ddH2O, 1 μL of gDNA (at least 50 μg/mL), 0.2 μL of each primer (50 μM), and 10 μL of 2x 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.

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: GAGAAAATGTGTTGACAC | (TTG)_9       | 315              | 58      | KU375202             |
|       | G: TCTGCAAGATGCTACCTT   |              |                  |         |                      |
| JSL3  | F: GAATGGAATACAGAAAGG   | (AG)_10      | 236–242          | 57      | KU375203             |
|       | R: TCAAGCTTCCAACAAGGGTT | (AG)          |                  |         |                      |
| JSL5  | F: GTGAAAGCAACGACAGAAG  | (GTA)_10     | 151–160          | 62      | KU375204             |
|       | R: GACACACTCTCTCCTGATATG | (TA)          |                  |         |                      |
| JSL12 | F: TGGAACGGCTGAAAAACTCT | (GA)         | 186              | 59      | KU375205             |
|       | R: GCGGTTCTCTTCTGACCT   | (GA)         |                  |         |                      |
| JSL16 | F: CCTAATGTTGGTCTACGGGC | (TC)         | 145              | 61      | KU375207             |
|       | R: ATTTGAGACACGAGCCATCC |             |                  |         |                      |
| JSL17 | F: ATTAGGGGCTTTACGAG    | (AG)         | 262–286          | 55      | KU375208             |
|       | R: TGTCGCAACCTGCTACCT   | (AG)         |                  |         |                      |
| JSL19 | F: AGTCATTTATATGCGCTT   | (TA)         | 199–201          | 60      | KU375209             |
|       | R: GTTGTCCTCTATGACCTTT  | (TA)         |                  |         |                      |
| JSL22 | F: ATTTAGGAATGCACATTC   | (CTT)_11     | 158–176          | 58      | KU375210             |
|       | R: ACTCATAGATGAGCCCAAT  | (TA)         |                  |         |                      |
| JSL23 | F: CCAATTACAGAAGCTACCG  | (ACA)        | 213–219          | 61      | KU375211             |
|       | R: TACCCCAACACCTGAGGAG  | (ACA)        |                  |         |                      |
| JSL26 | F: AAGGAGATGTGACCATAC   | (AGA)_8      | 257–272          | 56      | KU375213             |
|       | R: CTCTACTCTTGAGTGAG    | (AGA)        |                  |         |                      |
| JSL27 | F: GCTTTAGATATCCTCACCC  | (TTT)_8      | 158–170          | 56      | KU375214             |
|       | R: GTCCAAAGCAATGAGTATG  | (TTT)_8      |                  |         |                      |
| JSL29 | F: CGTGCTCTACTACACAC    | (AT)_10      | 159–163          | 60      | KU375215             |
|       | R: AGGTCCCTGATATGCTCT   | (AT)         |                  |         |                      |
| JSL30 | F: TTGGTGCTGTGGCGGAG    | (GA)         | 190–206          | 61      | KU375216             |
|       | R: AGTTCATCTCTTCAAGGGAG | (AG)         |                  |         |                      |
| JSL32 | F: CTGAGACACTCTTTTGGG   | (AT)         | 169–187          | 57      | KU375217             |
|       | R: GAGACGAAATACACTAAGG  | (AT)         |                  |         |                      |
| JSL33 | F: CTCAAGGGGCAAGACAGAAG | (ATA)_24     | 170–194          | 62      | KU375218             |
|       | R: CTGGCAAACTCTGAGGACCT | (ATA)_24     |                  |         |                      |
| JSL34 | F: AGAAGGAGATGACAGAAC  | (AG)         | 184–198          | 60      | KU375219             |
|       | R: GATGCTCTTCTACAC      | (AG)         |                  |         |                      |
| JSL39 | F: ACAATGGTTGTTCTCTCTG  | (ACA)_9      | 188–203          | 59      | KU375220             |
|       | R: GATTTAGGGGCTTCCTTAG  | (ATA)_9      |                  |         |                      |
| JSL42 | F: TCAATCGGCGAAGACAG    | (CTC)_7      | 302              | 56      | KU375221             |
|       | R: GAATGGGAAGACCTAAGG   | (CTC)_7      |                  |         |                      |
| JSL43 | F: TAGCAATCTACAAGAGTAC  | (GAA)_12     | 127–142          | 58      | KU375222             |
|       | R: CAAGAAGACACATACACT   | (GAA)_12     |                  |         |                      |
| JSL45 | F: TGTTGCTGATAAGAAGGTTG  | (ATG)_13     | 222              | 60      | KU375223              |
|       | R: ACCCAAGCCTCTACACCACT | (ATG)_13     |                  |         |                      |
| JSL47 | F: CTGTTTATATGTTGAGATCT | (AG)         | 150–176          | 60      | KU375224             |
|       | R: CTTGGCTCTCTAGATCT    | (AG)         |                  |         |                      |
| JSL50 | F: AGGGCTGGTTGTTTGGTCT  | (AT)         | 236–258          | 59      | KU375226             |
|       | R: GGGTAGCTACATTTTGGGG  | (AT)         |                  |         |                      |

Note: T_a = annealing temperature.
Table 2. Results of initial primer screening of 17 polymorphic loci in three populations of *Garcinia paucinervis*.

| Locus | A<sub>n</sub> | A | H<sub>e</sub> | H<sub>e</sub> | P value | A | H<sub>e</sub> | H<sub>e</sub> | P value |
|-------|---------------|---|-------------|-------------|----------|---|-------------|-------------|----------|
| JSL3  | 4             | 3 | 0.391       | 0.381       | 0.894    | 4 | 0.300       | 0.501       | 0.108    |
| JSL5  | 5             | 4 | 0.522       | 0.589       | 0.182    | 2 | 0.650       | 0.489       | 0.140    |
| JSL7  | 6             | 6 | 0.478       | 0.775       | 0.092    | 2 | 0.550       | 0.558       | 0.892    |
| JSL19 | 2             | 2 | 0.478       | 0.466       | 0.899    | 2 | 0.350       | 0.439       | 0.366    |
| JSL22 | 6             | 6 | 0.739       | 0.688       | 0.221    | 5 | 0.900       | 0.784       | 0.449    |
| JSL23 | 8             | 3 | 0.565       | 0.628       | 0.929    | 3 | 0.300       | 0.374       | 0.137    |
| JSL26 | 6             | 4 | 0.652       | 0.678       | 0.502    | 6 | 0.750       | 0.745       | 0.605    |
| JSL27 | 5             | 4 | 0.391       | 0.525       | 0.117    | 5 | 0.350       | 0.533       | 0.698    |
| JSL29 | 3             | 3 | 0.565       | 0.644       | 0.415    | 1 | 0.000       | 0.000       | —        |
| JSL30 | 12            | 8 | 0.696       | 0.784       | 0.141    | 6 | 0.550       | 0.811       | 0.191    |
| JSL32 | 8             | 6 | 0.826       | 0.791       | 0.749    | 5 | 0.750       | 0.724       | 0.738    |
| JSL33 | 9             | 9 | 0.696       | 0.768       | 0.388    | 6 | 0.400       | 0.350       | 1.000    |
| JSL34 | 9             | 6 | 0.826       | 0.739       | 0.993    | 6 | 0.900       | 0.734       | 0.578    |
| JSL39 | 8             | 6 | 0.783       | 0.729       | 0.667    | 5 | 0.800       | 0.761       | 0.825    |
| JSL43 | 6             | 6 | 0.783       | 0.751       | 0.264    | 3 | 0.650       | 0.580       | 0.738    |
| JSL47 | 11            | 7 | 0.783       | 0.812       | 0.207    | 7 | 0.950       | 0.796       | 0.873    |
| JSL50 | 12            | 9 | 0.957       | 0.850       | 0.331    | 6 | 0.800       | 0.778       | 0.547    |

Note: A<sub>n</sub> = number of alleles per population; A = total number of alleles; H<sub>e</sub> = expected heterozygosity; H<sub>e</sub> = observed heterozygosity; n = sample size; P value = test for deviation from Hardy–Weinberg expectations.

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

| Locus | A | H<sub>e</sub> | H<sub>e</sub> | 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<sub>e</sub> = expected heterozygosity; H<sub>e</sub> = observed heterozygosity.

REFERENCES

Clarke, K. R., and R. N. Gorley. 2001. PRIMER v5: User manual/tutorial. PRIMER-E Ltd., Plymouth, United Kingdom.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Fan, P. F., H. L. Fei, M. B. Scott, W. Zhang, and C. Y. Ma. 2011. Habitat and food choice of the critically endangered cao vit gibbon (*Nomascus nasutus*) in China: Implications for conservation. *Biological Conservation* 144: 2247–2254.

Fu, L. G. 1992. China Plant Red Data Book—The rare and endangered plant, vol. 1.320. Science Press, Beijing, China.

Li, X. W., J. Li, N. K. B. Robison, and P. F. Stevens. 2007. Clusiaceae (Guttiferae). In Z. Y. Wu and D. Y. Hong [eds.], *Flora of China*, vol. 13, 44. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

Peakall, R., and P. E. Smouse. 2006. GenAIEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

Raymond, M., and F. Roussel. 1995. GENEPOP (version 1.2): Population genetic software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249.

Tibbits, T., W. Michael, R. K. Varshney, and A. Graner. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare*). *Theoretical and Applied Genetics* 106: 411–422.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.

Wang, S., and Y. Xie. 2004. China Species Red List, 366. Higher Education Press, Beijing, China.

Zhang, Z. H., G. Hu, J. D. Zhu, and J. Ni. 2013. Aggregated spatial distributions of species in a subtropical karst forest, southwestern China. *Journal of Plant Ecology* 6: 131–140.
**APPENDIX 1. Voucher and locality information for *Garcinia* species used in this study.**

| Species        | Population | Collection localitya | 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.*

aLocality and Chinese province.

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