**ISOLATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS IN *BEILSCHMIEDIA ROXBURGHIANA* (Lauraceae)**

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- **Premise of the study:** Although there are as many as 250 species in the genus *Beilschmiedia*, their genetic diversity has been poorly investigated. Our objective was to develop microsatellite markers for *B. roxburghiana* to study its genetic diversity for the sustainable management of this species.

- **Methods and Results:** Using the microsatellite-enriched library and PCR-based screening method, 22 microsatellite markers were developed and 10 showed high polymorphism in a population. The number of alleles per locus for these 10 microsatellites ranged from five to 19. The observed and expected heterozygosities ranged from 0.298 to 1.000 and from 0.314 to 0.878, respectively.

- **Conclusions:** Our results from the 10 highly polymorphic microsatellites indicate that the principal reproductive mode of *B. roxburghiana* is clonal in the studied population. These microsatellites will facilitate further studies on genetic diversity and structure in *B. roxburghiana*.

**Key words:** *Beilschmiedia roxburghiana*; genetic diversity; Lauraceae; microsatellite markers; population genetics; Xishuangbanna Plot.

*Beilschmiedia* Nees is one of the largest pantropical genera of the Lauraceae, with approximately 250 species (Nishida, 1999). It is best represented in tropical Asia and Africa (van der Werff, 2003). Although *Beilschmiedia* contains many ecologically and economically important species, they are still poorly investigated (Nishida, 1999) and no genetic diversity information is available for them. *Beilschmiedia roxburghiana* Nees is an evergreen small to medium-sized tree growing in tropical evergreen broadleaf forests in southeastern Xizang and Yunnan provinces, China, and in northeastern Myanmar and India. It is a forest-dwelling species that generally occupies the second and third levels of the canopy layer and can grow up to 20 m tall. Its hermaphroditic flowers form paniculate or raceme inflorescences and are pollinated by insects. Its fruits are baccate, oblong, and 2–3 cm long (Li, 1982), with seeds that are dispersed by gravity and vertebrates, such as birds and small mammals. Its wood is hard and has multiple usages in construction and in the boat and paper industries. The terpenoid compound α-amirin from *B. roxburghiana* stem bark exhibits insecticidal and cytotoxic activities (Zetra and Prita, 2007).

Populations of *B. roxburghiana* have become increasingly fragmented in recent years due to the deforestation and environmental deterioration caused by economic development in China. Therefore, to design efficient conservation programs for the species, it is necessary to study its genetic diversity and population structure. To explore this, we developed 10 polymorphic microsatellite loci for *B. roxburghiana*. To our knowledge, this work is the first to report microsatellite loci in this valuable genus.

**METHODS AND RESULTS**

Total genomic DNA was extracted from one dry leaf of *B. roxburghiana* (Appendix 1) using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). Approximately 250 ng of genomic DNA was digested into 300–1000-bp fragments using the restriction enzyme *Msel* (New England Biolabs, Ipswich, Massachusetts, USA). The fragments were ligated to *Msel* adapters (*Msel* F: 5′-TACTCAAGGACTCAT-3′ and *Msel* R: 5′-GACGATGAGTCCTGAGT-3′) using T4 DNA ligase (New England Biolabs) overnight at 16°C. The digestion-ligation mixture was then diluted 10×, and 2 µL of the diluted mixture was used for PCR amplification using *Msel*-adapter-specific primers (5′-GATGAGTCCTGAGTAAAN-3′, i.e., *Msel*-N). The amplified products were hybridized with 5′-biotinylated (AG)15 and (AAG)8 probes. The probe-bound DNA fragments were enriched for AG or AAG repeats using streptavidin-coated magnetic beads (New England Biolabs). The enriched fragments were recovered by PCR using *Msel*-N as a primer and then ligated into the pGEM-T plasmid vector (Promega Corporation, Madison, Wisconsin, USA) and transformed into *Escherichia coli* DH5α competent cells (TaKaRa Bio Inc., Otsu, Shiga, Japan). A PCR-based method (Lunt et al., 1999) was used to screen the recombinant clones. A total of 299 positive clones were identified and sequenced by Majorbio Biotech Co. Ltd. (Shanghai, China) with M13R or M13F as primers. We detected microsatellites having at least eight AG or AAG.
repeats in 208 sequences. We then used Primer3 software (Rozen and Skaltsky, 2000) to design primers for these sequences, of which 66 were discarded because repeats were too close to one end of the sequences or because Primer3 found no suitable primers. PCR amplifications were performed for the remaining 142 sequences in a 20-μL volume containing 20 mM Tris-HCl (pH 8.4), 100 mM (NH₄)₂SO₄, 3 mM MgCl₂, 0.4 mM dNTPs, 0.4 μM each primer, 50 ng of genomic DNA, and 1 U Taq polymerase (TaKaRa Bio Inc.). The amplification program was 95°C for 5 min, 35 cycles of 94°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. We checked the PCR products on 2% agarose gels and found that 41 of them could be successfully amplified with expected sizes.

Polymorphisms were initially assessed in these 41 microsatellite loci using 12 individuals that were randomly selected from 421 samples of B. roxburghiana collected from the Xishuangbanna Plot in the Mengla Nature Reserve (21°36'42"–35°58"N, 101°34'26"–47°E). PCRs were performed using the same procedure described above. Electrophoresis of the products was done on an ABI 3730 sequencer (Applied Biosystems, Carlsbad, California, USA), and fragment lengths were analyzed using ABI GeneMapper software version 3.7 (Applied Biosystems).

### Table 1. Characteristics of microsatellites developed in Beilschmiedia roxburghiana.

| Locus   | Repeat motif | Primer sequences (5’–3’) | Size range (bp) | T_a (°C) | 5’-fluorescence label | GenBank accession no. |
|---------|--------------|--------------------------|-----------------|----------|-----------------------|-----------------------|
| BR01    | (TG)₁_(AG)₁₄ | F: ATGGGAATAATCGCATCAT  | 101–115         | 50       | FAM                   | JX524138              |
|         |              | R: GTACCAAGCTTGGAGCA     |                 |          |                       |                       |
| BR02    | (CAG)₁(GA)₁₄| F: AACCGTGATTCGGGACAAT  | 131–149         | 50       | FAM                   | JX524139              |
|         |              | R: TTCGCCCTTCTTTTTTG    |                 |          |                       |                       |
| BR03    | (AG)₁₀TAAG(GA)₁₁ | F: CATTTCCTGCAATGGCGGAT  | 183–195         | 50       | FAM                   | JX524140              |
|         |              | R: ACCATTCTCATGGCGAGAG  |                 |          |                       |                       |
| BR04    | (TC)₁₆      | F: CTCAGTCCCCGGCAATC    | 177–220         | 50       | FAM                   | JX524141              |
|         |              | R: GACCAAAACCTGAAACCTAC  |                 |          |                       |                       |
| BR05    | (AC)₁₄(AG)₁₀AAG | F: CAGATTTATTATATTACT  | 212–266         | 50       | HEX                   | JX524142              |
|         |              | R: TCAGACCAAGAAGGAGAC   |                 |          |                       |                       |
| BR06    | (GA)₁₈      | F: GTCCGAGATCACCAGATA   | 166–204         | 52       | FAM                   | JX524143              |
|         |              | R: AGTGGAATACCAAGAG    |                 |          |                       |                       |
| BR07    | (CT)₁₀      | F: GCGTCGTCTTCTGAGCTT  | 174–182         | 55       | FAM                   | JX524144              |
|         |              | R: CTCGGGTGCTTCTGAGCTT |                 |          |                       |                       |
| BR08    | (TC)₁₉      | F: GAGACGGGTCTTCTCTG   | 135–178         | 55       | FAM                   | JX524145              |
|         |              | R: GGATTTTGCCAAACCTGAG  |                 |          |                       |                       |
| BR09    | (GA)₁₉      | F: TTTTGCGACAAACAGATCC | 161–185         | 55       | FAM                   | JX524146              |
|         |              | R: TGGCAAAATCTGGATCTTTAG |                 |          |                       |                       |
| BR10    | (AG)₁₆AA(AG)₁₆ATACGT(GA)₅ | F: TATGATTTCGAGGCTTTC  | 221–241         | 55       | HEX                   | JX524147              |
|         |              | R: CCCCCACCATCACCTCTTATT |                 |          |                       |                       |
| BR12*   | (CT)₁₂Cl(A)₂₇ | F: GTGAATGTAGCATTAAAGGGA | 158–164         | 50       | FAM                   | KC211772              |
|         |              | R: GATTGAAGCAGGGGTGTT  |                 |          |                       |                       |
| BR13*   | (AC)₁₄      | F: GTAGTGGTGTAGCTG    | 200–206         | 50       | FAM                   | KC211773              |
|         |              | R: TCACCTCATCTGCTGTA  |                 |          |                       |                       |
| BR15*   | (AG)₁₆      | F: ATGCCGCGCACCCTGGA   | 230–247         | 52       | FAM                   | KC211775              |
|         |              | R: CATTCACCCCTTCCTCTCT |                 |          |                       |                       |
| BR17*   | (AG)₁₅      | F: AGAAAGAGCAGACTCCA   | 161–196         | 55       | FAM                   | KC211777              |
|         |              | R: CAGTGATGGCCAAACCGGA |                 |          |                       |                       |
| BR20*   | (AG)₁₇      | F: ACCTGACCACTGGACAAAAT | 234–238         | 55       | FAM                   | KC211780              |
|         |              | R: GCATGACACATGGCCAAACAC |                 |          |                       |                       |
| BR21*   | (AG)₁₈      | F: AAGGGAAGGTGTTGGTT   | 300–310         | 55       | HEX                   | KC211781              |
|         |              | R: GTTTACAAAGATAGGAAAGA |                 |          |                       |                       |
| BR11    | (TG)₁₃(CG)₁₃(TG)₃ | F: TTTTGCTATGACCATCAC  | 150             | 50       | FAM                   | KC211771              |
|         |              | R: CACTTACCCACACTCTCTTTTA |                 |          |                       |                       |
| BR14    | (TC)₁₇      | F: TATACCGAGCCATATTTAGC | 243             | 50       | HEX                   | KC211774              |
|         |              | R: CACAGGCTGCTTTGTTGAT  |                 |          |                       |                       |
| BR16    | (AG)₁₆AAACTCTTGA(AG)₁₄ | F: GAAGGCACTGGGAACTCT  | 166             | 55       | FAM                   | KC211776              |
|         |              | R: GGAAATCTGTGGAGGAGGAT |                 |          |                       |                       |
| BR18    | (GA)₁₅      | F: TACGGATGACAGGGGTAAG  | 172             | 55       | FAM                   | KC211778              |
|         |              | R: CAGTCGCTGCCCCCTCACATCA |                 |          |                       |                       |
| BR19    | (TC)₈       | F: AGTCACATCCCTTCTCTCTC | 214             | 55       | HEX                   | KC211779              |
|         |              | R: GCAGCAATGCTGGGAGGAGA |                 |          |                       |                       |
| BR22    | (TC)₉       | F: CAATGCTCCAGACAGAGAGGATTCA | 326          | 55       | HEX                   | KC211782              |

*Allele size ranges are obtained from 421 individuals in loci BR01–10 and from 12 individuals in loci BR11–22.

Note: T_a = annealing temperature.

Among the 41 microsatellites, 16 yielded clear and stable polymorphisms, six were monomorphic, and the remaining 19 yielded multiband patterns. The 22 polymorphic and monomorphic microsatellite loci are characterized in Table 1. Of the 16 polymorphic loci, six contained two alleles in the sample and were not further evaluated. The remaining 10 polymorphic microsatellite loci were then assessed for allelic variation on all 421 individuals sampled (Table 2).

Genetic diversity parameters for 10 loci (Table 2), deviation from Hardy–Weinberg equilibrium (HWE), and genotypic linkage disequilibrium (LD) among all pairs of 10 loci were estimated using GENEPOP version 4.1.4 (Rousset, 2008). Significance levels were adjusted using the Bonferroni correction (Rice, 1989). Alleles per loci varied from five to 19, observed heterozygosity ranged from 0.298 to 1.000, and expected heterozygosity ranged from 0.314 to 0.878 (Table 2). Significant deviation from HWE was found in nine loci; this was due to heterozygote deficit for two loci (BR02 and BR03) and to heterozygote excess for the other seven (BR04–BR10). All locus pairs showed significant LD after Bonferroni correction (P < 0.05), which was unexpected and possibly due to clonal reproduction in this species, as clonal reproduction could show significant multilocus linkage disequilibria (Halkett et al., 2005). Among the
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421 individuals genotyped, 190 different multilocus genotypes were identified, and the number of individuals per genotype varied from one to 68.

CONCLUSIONS

Twenty-two microsatellites of *B. roxburghiana* were isolated and tested. Our data indicate that 10 are highly polymorphic. A large negative fixation index (*F*) estimate (−0.170, Table 2), the presence of many identical genotypes among the individuals, and the presence of significant multilocus linkage disequilibrium suggest that the principal reproductive mode of *B. roxburghiana* may be clonal for this plot, which is unexpected. Further studies are needed to confirm this.

In the future, the microsatellites developed in this study will be useful to investigate the effects of habitat fragmentation on genetic diversity and structure in *B. roxburghiana* populations. We also have plans to use them to study the fine-scale spatial genetic structures in the 20-ha Xishuangbanna Plot. The results from such studies and from corresponding studies of population dynamics will provide useful information for the sustainable management of this species.

LITERATURE CITED

DOYLE, J. J. 1991. DNA protocols for plants—CTAB total DNA isolation. In G. M. Hewitt and A. Johnston [eds.], Molecular techniques in taxonomy, 283–293. Springer-Verlag, Berlin, Germany.

HALKETT, F., J.-C. SIMON, AND F. BALLOUX. 2005. Tracking the population genetics of clonal and partially clonal organisms. *Trends in Ecology & Evolution* 20: 194–201.

LI, H. W. 1982. *Flora Reipublicae Popularis Sinicae*, vol. 31, 133–134. Science Press, Beijing, China (in Chinese).

LUNT, D. H., W. F. HUTCHINSON, AND G. R. CARVALHO. 1999. An efficient method for PCR-based isolation of microsatellite arrays (PIMA). *Molecular Ecology* 8: 891–894.

NISHIDA, S. 1999. Revision of *Beilschmiedia* (Lauraceae) in the neotropics. *Annals of the Missouri Botanical Garden* 86: 657–701.

RICE, W. R. 1989. Analyzing tables of statistical tests. *Evolution; International Journal of Organic Evolution* 43: 223–225.

ROUSSET, F. 2008. GENEPOP’007: A complete reimplementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

ROZEN, S., AND H. SKALETSKY. 2000. Primer3 on the WWW for general users and for biologist programmers. In S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

VAN DER WERFF, H. 2003. A synopsis of the genus *Beilschmiedia* (Lauraceae) in Madagascar. *Adansonia* 25: 77–92.

ZETRA, Y., AND P. PIRTA. 2007. Isolasi senyawa α-amirin dari tumbuhan *Beilschmiedia roxburghiana* (Medang) dan uji bioaktivitasnya. *Akta Kimindo* 3: 27–32 (in Indonesian with English abstract).

APPENDIX 1. Voucher specimens of *Beilschmiedia roxburghiana* deposited in the herbarium of the Xishuangbanna Tropical Botanical Garden (HITBC), Mengla, Yunnan, China.

| Voucher no. | Collector name | Collection locality |
|-------------|----------------|---------------------|
| 109113      | Shi-Shun Guo   | Menglu, Yunnan Province, China |
| 107464      | Wang Hong      | Simao, Yunnan Province, China |

Table 2. Genetic diversity of 10 loci in 421 *Beilschmiedia roxburghiana* individuals in Xishuangbanna Plot.

| Locus | A   | H_o | H_e | F     |
|-------|-----|-----|-----|-------|
| BR01  | 8   | 0.729 | 0.655 | −0.113 |
| BR02  | 7   | 0.567 | 0.666 | 0.149* |
| BR03  | 7   | 0.298 | 0.314 | 0.049* |
| BR04  | 19  | 0.995 | 0.807 | −0.233* |
| BR05  | 15  | 1.000 | 0.798 | −0.254* |
| BR06  | 17  | 0.993 | 0.878 | −0.131* |
| BR07  | 5   | 0.882 | 0.584 | −0.511* |
| BR08  | 14  | 0.911 | 0.816 | −0.016* |
| BR09  | 13  | 1.000 | 0.820 | −0.220* |
| BR10  | 13  | 0.979 | 0.804 | −0.218* |
| Mean  | 0.834 | 0.713 | −0.170* |

Note: *A* = number of alleles; *F* = fixation index; *H_e* = unbiased expected heterozygosity; *H_o* = observed heterozygosity.

* Indicates a deviation at *P* < 0.05 from Hardy–Weinberg equilibrium after Bonferroni correction.