Isolation and Characterization of Microsatellite Markers in *Beilschmiedia roxburghiana* (Lauraceae)

Author(s): Lu Liu , Zheng-Feng Wang , Qiao-Ming Li , Wan-Hui Ye , Hong-Lin Cao , and Zhang-Ming Wang

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Primer Note

Isolation and characterization of microsatellite markers in *Beilschmiedia roxburghiana* (Lauraceae)[1]

Lu Liu[2,3], Zheng-Feng Wang[4,5], Qiao-Ming Li[2,5], Wan-Hui Ye[4], Hong-Lin Cao[4], and Zhang-Ming Wang[4]

[2]Laboratory of Plant Phylogenetics and Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, People's Republic of China; [3]Graduate University of the Chinese Academy of Sciences, Beijing 100049, People's Republic of China; and [4]Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, People's Republic of China

- *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 racemose 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).

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[5]Author for correspondence: wzf@scib.ac.cn; lqm@xtbg.ac.cn

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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 *Mse*I (New England Biolabs, Ipswich, Massachusetts, USA). The fragments were ligated to *Mse*I adapters (*Mse*I F: 5′-TACTCAGGACTCAT-3′ and *Mse*I R: 5′-GACGATGAGTCCTGAG-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 *Mse*I-adapter-specific primers (5′-GATGAGTCCTGAGTAAN-3′, i.e., *Mse*I-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 *Mse*I-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 Skaletsky, 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 $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 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, optimized annealing temperature 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″–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).

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 microsatellites 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 locus 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

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

Locus	Repeat motif	Primer sequences (5′–3′)	Size range (bp)[a]	T_a (°C)	5′-fluorescence label	GenBank accession no.
BR01	$(TG)_{17}(AG)_{14}$	F: ATGGGAATATCGCATCAT R: GTTACCAGACTTGGAGCA	101–115	50	FAM	JX524138
BR02	$(CAG)_4(GA)_{14}$	F: AACCGTTATCTGGACATTG R: TTCGCCTTCCTTCTTTTC	131–149	50	FAM	JX524139
BR03	$(AG)_9TAAG(GA)_{11}$	F: CATTTCCGTCAATGCCTGAT R: ACCATTTCAATGGCGAAGAG	183–195	50	FAM	JX524140
BR04	$(TC)_{16}$	F: CTCCAGTCCACGGCAATC R: GACCAAACCTGAACCTACAA	177–220	50	FAM	JX524141
BR05	$(AC)_{18}(AG)_9AAG$	F: CAATTCAAATGAAAGCGACTGA R: TCAGACCCAAAGGGAGACAC	212–266	50	HEX	JX524142
BR06	$(GA)_{18}$	F: GTCGGAGATTCAAACATA R: AGTGGGATTACCAAGAGG	166–204	52	FAM	JX524143
BR07	$(CT)_9$	F: GCTGCTGTTTCCTTGGACTT R: CCTGGGTTCTCTCAGCTTTG	174–182	52	FAM	JX524144
BR08	$(TC)_{20}$	F: GAGACGCGTTTTCTCTCTCG R: GGGATTTTGCAAACCTGAGA	135–178	55	FAM	JX524145
BR09	$(GA)_{21}$	F: TTTTGGCAACAACAGATCCA R: TGCAAAATTCGTGCATTGAT	161–185	55	FAM	JX524146
BR10	$(AG)_{10}AA(AG)_3A_4TACGT(GA)_5$	F: TATTGATTCCGAGGCTTTCG R: CCCCACCATCACCTCTATTTT	221–241	55	HEX	JX524147
BR12*	$(CT)_9C(A)_{27}$	F: GTGAAGTATGCAGTATAAAAGG R: GATTGAGCAGAGGGTGTT	158–164	50	FAM	KC211772
BR13*	$(AC)_{14}$	F: TGTATGCGTGTATGCTGT R: TCACTTCTATTCGGTTTCA	200–206	50	HEX	KC211773
BR15*	$(AG)_{16}$	F: ATGCGAGCCACTTGTGAAG R: CATCTCCATCCCCCTTCTCT	230–247	52	FAM	KC211775
BR17*	$(AG)_{15}$	F: AGAAACGACCCAGTCCCA R: CAGGTAAGGCCCAACCGA	161–169	55	FAM	KC211777
BR20*	$(AG)_7$	F: ACCTGCAATGGTGCCAAATA R: CATCACACCCACTGCAAAAC	234–238	55	FAM	KC211780
BR21*	$(AG)_9$	F: AAGGAAGGTGTTAGGGTT R: GTTTACAAAAGATGAAAGGA	300–310	55	HEX	KC211781
BR11	$(TG)_7(CG)_6(TG)_3$	F: TTGCGTATCTTGCACATC R: CACTTACTCACCACTCCTTTA	150	50	FAM	KC211771
BR14	$(TC)_{17}$	F: TATACCAGACCCTATTAGCC R: CCAACTGCCTGTTTCATT	243	50	HEX	KC211774
BR16	$(AG)_7AAATCTTGA(AG)_6$	F: GAAGGCACTGGGAACTCT R: GCAATCTGCTGAAGGGAT	166	55	FAM	KC211776
BR18	$(GA)_9$	F: TACGCGTACAAAGGGGTAGG R: CAGTCGTCTGCCCTCATACA	172	55	FAM	KC211778
BR19	$(TC)_8$	F: AGTCGATCCCCTCTTTCCTC R: GCAGCAATGCTTGTTTGAGA	214	55	HEX	KC211779
BR22	$(TC)_7$	F: GGAAGAAAGCCCCTAAAT R: CACAGTCCACGACAGTAATC	326	55	HEX	KC211782

Note: T_a = annealing temperature.

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

* Indicates locus showing only two alleles.

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	–0.116*
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.

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.

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

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

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