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# Isolation of 27 polymorphic nuclear microsatellite markers for *Roupala montana* var. *brasiliensis* (Proteaceae)

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**Abstract:** Microsatellite primers pairs were developed for the Neotropical tree *Roupala montana* var. *brasiliensis* for use in studies on genetic diversity, mating system, and gene flow. Forty-two primer pairs were developed, resulting in 27 polymorphic loci, with two to 27 alleles per locus. The primer pairs were validated against 34 *R. montana* var. *brasiliensis* adult trees from four populations. The observed ($H_o$) and expected ($H_e$) heterozygosities ranged among loci from 0.061 to 0.930 (mean of 0.544) and from 0.116 to 0.950 (mean of 0.700), respectively. Null alleles were observed for ten loci. No genotypic linkage disequilibrium was detected in any pair of loci. This set of loci is suitable for population genetic studies of the species.

**Key words:** Brazilian lacewood, Brazilian oak, High-throughput sequencing, SSR markers.

## INTRODUCTION

Twenty-one species and variations of the *Roupala* (Proteaceae) genus have been reported in Brazil (Prance et al. 2007), including lacewood (*Roupala montana* var. *brasiliensis* (Klotzsch) K.S. Edwards). Although non-endemic, this species occurs from the south of Bahia State to Rio Grande do Sul State, mainly on wet slopes and in small depressions (Rego 2009, GBIF 2019). Classified as secondary species (Sawczuk et al. 2012) or light-demanding climax species (Seubert et al. 2017), lacewood presents hermaphrodite flowers that are pollinated by small insects, and seeds are dispersed by wind (Carvalho 2003, Boeger et al. 2006). The trees can reach heights of 20 to 30 m and diameters at the breast height (dbh) between 30 and 100 cm (Boeger et al. 2006).

Used for furniture, and civil and naval construction due to its high-quality wood (Carvalho 2003, Prance et al. 2007), lacewood replaced the wood of *Cardwellia sublimis* (Proteaceae) when it became scarce in Australia (World Timbers Inc 2019). Despite its importance as a wood species, there is a lack of knowledge about genetic diversity, reproductive system, gene flow and conservation status in their natural populations. Previous genetic studies have been conducted using molecular markers of chloroplast and ribosomal DNA and have focused on understanding the phylogeny and phylogeography of the Proteaceae family (Hoot & Douglas 1998, Hoot et al. 1999, Barker et al. 2007).

Microsatellite loci or Simple Sequence Repeats (SSR) are highly informative molecular markers because of the differences in the number of repeated units; they are also

codominant, multiallelic, and abundant in the genome, which makes them useful for a wide range of applications such population genetic studies (Govindaraj et al. 2015, Vieira et al. 2016). Thus, the limiting factor in the use of microsatellites is obtaining primers to amplify the SSR loci. Here, we describe the isolation of 27 nuclear microsatellite markers for *R. montana* var. *brasiliensis* that provide the foundation for further research on genetic diversity, population structure, mating system, conservation genetics, and possibly assist breeding programs.

## MATERIALS AND METHODS

We used two methods to develop SSR markers. Total genomic DNA was extracted from leaves of a single *R. montana* var. *brasiliensis* individual (Supplementary Material - Table SI, using the protocol based on Doyle & Doyle (1990) proposed by Faleiro et al. (2003), with modifications. A microsatellite-enriched genomic library was constructed following Billotte et al. (1999). The genomic DNA was digested using the *Rsa*I enzyme (Invitrogen, Carlsbad, California, USA), enriched in microsatellite fragments using $(CT)_8$ and $(GT)_8$ motifs. The enriched fragments were cloned into pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA); ligation products were used to transform Epicurian Coli XL1-Blue *Escherichia coli*-competent cells (Stratagene, Agilent Technologies, Santa Clara, California, USA) that were cultivated on plates with LB medium containing 100 µg/mL ampicillin, 100 µg/mL tetracycline, 2% X-galactosidase, and 20% of isopropyl β-D-1-thiogalactopyranoside (IPTG). Ninety-six recombinant colonies were sequenced using the adapters *Rsa*21 (5'-CTCTTGCTTACGCGTGGACTA-3') and *Rsa*25

(5'-TAGTCCACGCGTAAGCAAGAGCACA-3') in a 3730xl DNA Analyzer sequencer (Applied Biosystems, Foster City, California, USA) and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Vector segments from each sequence were removed by VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Pairs of primers were designed using Primer3Plus (Untergasser et al. 2012) (parameters: primer size of 18–25 bp; annealing temperature ($T_a$) between 52–65 °C; GC content between 40–60%; amplified fragment size of 100-300 bp). A ThermoFisher Multiple Primer Analyzer (ThermoFisher Scientific) was used to verify primer pair quality.

The second method used was based on High-throughput Sequencing (MiSeq Sequencing System, Illumina), with a Nextera DNA Flex Library Prep kit (Illumina, Inc). Total genome DNA extracted from five individuals Table SI was used in a paired-end sequencing run performed in MiSeq Reagent Nano Kit, v2 (500 cycles), with a low coverage approach. We used the SSR_pipeline software (Miller et al. 2013) to verify sequence quality and contigs and identify the SSR loci (parameters: motifs of two to six nucleotides and 40 bp flanking regions). The primer pairs were designed using the BatchPrimer3 v1.0 software (You et al. 2008).

All primer pairs were synthesized with the M13 tail (5'-TGTAAAACGACGGCCAGT-3') (Shuelke 2000). The temperature gradient test (52.0, 53.7, 56.1, 57.3, 59.9, 61.9 °C) was applied to choose the best $T_a$ for each primer pair and those that did not work were discarded. The microsatellite loci were amplified by PCR in a final volume of 10 µL (5 µL GoTaq Colorless Master Mix (2×) (Promega Corporation), 0.6 µL of primer mix (F+R), 0.5 µL bovine serum albumin (Thermo Fisher Scientific), 0.3 µL fluorescent dyes (6-FAM, NED, VIC, PET), 2.6 µL Nuclease-Free Water, and 1.0 ng template DNA). The amplification program for all primers was: i) initial denaturing step at 94 °C for 5 min; ii) 35 amplification cycles (94 °C for 30

s, 1 min at the specific $T_a$, 72 °C for 1 min); iii) 12 cycles of M13 tail incorporation (94 °C for 30 s, 53 °C for 1 min 30 s, 72 °C for 1 min 30 s); iv) a final elongation step at 72 °C for 20 min. Amplifications were performed with a Mastercycler (Eppendorf, Hamburg, Germany). The amplification product (1 µL) of each reaction was separated on an ABI 3500 DNA analyzer (Applied Biosystems) and the GeneMapper v5 software (Applied Biosystems) was used to analyze the genotypic data.

For primer validation, we sampled a total of 34 *R. montana* var. *brasiliensis* adult trees from four South Brazilian populations Table SI. The number of alleles per locus (*k*), observed ($H_o$) and expected ($H_e$) heterozygosities, and the genotypic linkage disequilibrium (LD) were estimated using the FSTAT software (Goudet 2002). The statistical significance of LD was tested using 1000 Monte Carlo permutations (alleles among individuals) and a Bonferroni correction (95%, α= 0.05). We tested deviation from expected heterozygosity (α = 5%) based on the fixation index (*F*) for each locus for the largest population (FLONA-Irati, *N*=13), using the functions *basicStats* and *divBasic* in the *diveRsity* package (Keenan et al. 2013) implemented in the R software environment (R Core Team 2018). We also estimated null allele frequencies for each locus using a Maximum Likelihood approach (PIM) implemented in INEST 2.1 program (Chybicki & Burczyk 2009).

## RESULTS AND DISCUSSION

After the initial test of primer pair amplification, we discarded those that did not successfully yield amplified fragments. From the first method to detect SSR, a total of 22 simple sequence repeat (SSR) markers were selected for primer synthesis, but only 16 markers (Rmb4 to Rmb20) were useful (Table I). Based on the second method of the 20 initially synthetized markers, only 11 were useful (Rmb23 to Rmb44). The number of alleles per locus (*k*) ranged among loci from 2 to 27 (total = 338; mean = 12.5). Observed heterozygosity ($H_o$) ranged from 0.062 to 0.930 (mean = 0.544) and expected heterozygosity ($H_e$) ranged from 0.116 to 0.965 (mean = 0.700). No genotypic linkage disequilibrium was detected in any pair of loci (data not shown). The presence of null alleles was observed at ten of the 27 loci. Loci Rmb6 and Rmb19 showed the lowest polymorphism (*k*= 2).

Polymorphic markers are fundamental tools to obtain accurate estimates of genetic diversity parameters and the structure of populations (Rossini et al. 2018). The 27 loci are suitable for use in further studies of *R. montana* var. *brasiliensis*, including genetic diversity, genetic structure, mating system, gene flow, and parentage analysis. Furthermore, considering that many species are located in fragmented ecosystems that are rapidly and constantly being diminished due to human activities, including logging or urban and agricultural expansion, and that these species can be studied in a comparative way (Barbará et al. 2007), the potential for cross-species microsatellite transferability is key to facilitating studies and reducing costs (Hoebee 2011, Forti et al. 2014). A large number of suitable loci increases the probability of success of cross-species transferability. Thus, the SSR primer pairs developed herein may be useful in determining the conservation status of the studied species and inform research about other *Roupala* species, particularly those that are endangered, which in turn contributes to a greater understanding of the Proteaceae family and forest conservation genetics.

**Table I.** Microsatellite primers developed for *Roupala montana* var. *brasiliensis*.

| Locus | Primer sequences (5'-3') | | Repeat motif | Size range (bp) | $T_a$ (°C) | *n* | *k* | $H_o$ | $H_e$ | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|
| | Forward | Reverse | | | | | | | | |
| Rmb4 | GACAGCTAAATGTAGGTCTCTTTG | GCCTGCAATACTACAAGCAG | $(AG)_{16}$ | 144 - 202 | 59.9 | 33 | 27 | 0.752 | **0.965*** | MN961599 |
| Rmb5 | CATGTCAGTTGCCAAACAAC | CAGCACAGGATACCAGTGTTTA | $(AG)_{12}$ | 143 - 197 | 57.4 | 34 | 21 | 0.737 | 0.930* | MN961600 |
| Rmb6 | GTTTGAGCATTTCCTTCACC | CTACAAAAACGAACCACCAT | $(GT)_{6}$ | 139 - 141 | 57.4 | 34 | 2 | 0.170 | **0.163** | MN961601 |
| Rmb7 | GTCACGGAAAGAAGGAAAAG | AGGGATAGGTGGGGATAGAG | $(CT)_{8}(CT)_{12}$ | 193 - 247 | 53.7 | 34 | 26 | 0.899 | 0.962 | MN961602 |
| Rmb8 | GAACAGCTCAAAAGGAAAGG | CTCCTGTGTCCTGTGAGAGA | $(GA)_{17}$ | 228 - 266 | 53.7 | 31 | 13 | 0.347 | **0.883*** | MN961603 |
| Rmb9 | CGTCCTTAAGTTGGTCAAGC | GGAACAAAGGAGAAAAATGG | $(GA)_{9}$ | 149 - 165 | 59.9 | 33 | 5 | 0.334 | **0.618*** | MN961604 |
| Rmb10 | TAAAGGGGACAAGCTACCAG | ATGACACGAAGAATGCCATA | $(GA)_{10}(AG)_{13}$ | 302 - 358 | 53.7 | 33 | 21 | 0.818 | 0.950 | MN961605 |
| Rmb11 | GTCAGGGAGAGAGAACAGGA | CTCCCAGTTTGTGGAAAGAC | $(CT)_{21}$ | 166 - 204 | 57.4 | 34 | 14 | 0.906 | **0.887** | MN961606 |
| Rmb13 | CCACCTCTTCCACTACCACT | GAAGAGGAGTGGATCTTAAATAGC | $(AG)_{10}$ | 143 - 195 | 53.7 | 34 | 14 | 0.856 | **0.794** | MN961607 |
| Rmb14 | GGTGAGACCTCAATTCCATC | CTGCGTCAATACCATCTGAG | $(TG)_{6}(TC)_{8}$ | 182 - 218 | 57.4 | 34 | 16 | 0.693 | 0.876 | MN961608 |
| Rmb15 | GGCTCATGCGTAATGAAATAG | ATGACACTTGCTCAGGGAAC | $(CT)_{9}(CT)_{22}$ | 219 - 315 | 59.9 | 30 | 19 | 0.334 | **0.951*** | MN961609 |
| Rmb16 | AATATTGAGGCATGGTTGCT | GGTATGTTTCAGGGCAAAAG | $(GT)_{7}$ | 300 - 316 | 53.7 | 34 | 6 | 0.552 | **0.471** | MN961610 |
| Rmb17 | ATCAAATCCCAACTCTCAAG | GGCCACCTACATAGTGATCC | $(CT)_{10}$ | 123 - 227 | 57.4 | 34 | 17 | 0.378 | **0.925*** | MN961611 |
| Rmb18 | GTTGTTGCCTGTCACAGATG | GGGGGATTTCATTCTAAGGT | $(CT)_{8}$ | 247 - 253 | 53.7 | 34 | 5 | 0.372 | 0.431 | MN961612 |
| Rmb19 | CAGACAATGCCTTCAAGCTA | GATTGATCCGTGGAATATGAG | $(TC)_{8}$ | 256 - 258 | 53.7 | 33 | 2 | 0.077 | **0.116** | MN961613 |
| Rmb20 | CCTTGCAAAGATGAAGCAAT | GGAATCGAAATCGAAACTACC | $(CT)_{8}$ | 119 - 179 | 56.1 | 34 | 22 | 0.930 | 0.949 | MN961614 |
| Rmb23 | TCCACAGCAATATCAAAATGT | TTTGAAAGATTTGTAACGTGTC | $(GT)_{8}$ | 133 - 175 | 59.9 | 34 | 14 | 0.849 | 0.896 | MT001925 |
| Rmb24 | GTAACCTCAAACAATGCAGAA | CCAACGCAACATATATTGTAA | $(AT)_{8}$ | 098 - 158 | 59.9 | 33 | 11 | 0.642 | 0.787 | MT001926 |
| Rmb28 | ACTCAACTTCAAGAACCCATT | AGGTCTGAATTTTCAGGAGAC | $(ATC)_{6}$ | 106 - 124 | 53.7 | 34 | 5 | 0.271 | 0.340 | MT001927 |
| Rmb29 | AAGATGGCCAAGATTAATGA | CCCATTAACTGATAATTGAGTC | $(ATT)_{7}$ | 123 - 155 | 57.4 | 32 | 13 | 0.609 | **0.843*** | MT001928 |
| Rmb33 | TGTAAATAAACTTGGGTTGGT | AAAGACAGCTCCTACCCATAA | $(TTG)_{6}$ | 116 - 130 | 56.1 | 33 | 5 | 0.577 | 0.510 | MT001929 |
| Rmb34 | ACAAAAACTACCGAGGCTAGA | AAAGCTCATAAACTGCTGAAA | $(TA)_{7}$ | 100 - 174 | 52.0 | 28 | 4 | 0.256 | **0.257** | MT001930 |
| Rmb35 | CACTGCCAAATCAAAACTTAG | GAATTGTTGATGGCTTCTTTT | $(CT)_{8}$ | 116 - 120 | 59.9 | 32 | 3 | 0.519 | 0.556 | MT001931 |
| Rmb36 | CAAGACTTCTTTGAGCTTTGT | TTGTTTTGGCTAGTGAAATTG | $(TC)_{9}$ | 115 - 167 | 59.9 | 27 | 4 | 0.061 | **0.212** | MT001932 |
| Rmb40 | CTTCACTGATATCGTCTCTGG | TTCTTTGATCTGAAATCTGGA | $(TC)_{21}$ | 101 - 155 | 53.7 | 32 | 19 | 0.628 | **0.940*** | MT001933 |
| Rmb43 | ACATACAACACACCACATGC | CAGAAGGAATGAAAATGAAGA | $(TC)_{8}$ | 099 - 195 | 53.7 | 34 | 13 | 0.515 | 0.753* | MT001934 |
| Rmb44 | CAGCAAGAATAACAAAGGATG | TAGCTTAGCTTGTTTCATGGT | $(AG)_{16}$ | 082 - 114 | 53.7 | 33 | 17 | 0.603 | 0.929* | MT001935 |
| Overall | | | | | | | 12.5 | 0.544 | 0.700 | |

**Note:** $T_a$ is the annealing temperature when run individually; $n$ is the sample size; $k$ is the number of alleles per locus; $H_o$ is the observed heterozygosity; $H_e$ is the expected heterozygosity. Bold $H_e$ values indicate significant departure from Hardy-Weinberg Equilibrium (α = 95%) based on the "FLONA-Irati" population. * indicates loci with significant frequenci of null alleles.

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## SUPPLEMENTARY MATERIAL

**Table SI.**

**How to cite**

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