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DEVELOPMENT OF MICROSATELLITE MARKERS FOR *Balfourodendron riedelianum* (Engl.) Engl. (PAU MARFIM - IVORY TREE): AN ENDANGERED HIGH VALUE TROPICAL TREE (SCIENTIFIC NOTE)[1]

DESENVOLVIMENTO DE MARCADORES MICROSSATÉLITES PARA *Balfourodendron riedelianum* (Engl.) Engl. (PAU-MARFIM): UMA ESPÉCIE ARBÓREA TROPICAL AMEAÇADA E DE ALTO VALOR (NOTA CIENTÍFICA)

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ABSTRACT - Pressure on tropical forests by agriculture and livestock expansion, frequently leads to highly fragmented and isolated populations. Limited gene flow drives increased drift and genetic differentiation among populations, ultimately reducing the overall genetic diversity of forest tree species. *Balforoudendron riedelianum* (Engl.) Engl., commonly known as pau marfim (ivory tree), a valuable Brazilian hardwood tree used in carpentry and building, is currently endangered due to logging and forest fragmentation. Information on genetic diversity and structure of remnants populations is necessary to support its conservation and sustainable management. Microsatellite markers are an effective tool for understanding and quantifying the effects of fragmentation on genetic diversity. Seven microsatellite markers were developed and validated using a sample of 98 individuals. The number of alleles per locus ranged from 3 to 25, the observed and expected heterozygosities from 0.051 to 0.909 and 0.050 to 0.930, respectively, the fixation index corrected for null alleles from 0.036 to 1.0 and all markers were found in linkage equilibrium. This microsatellite marker set is suitable to estimate population genetic parameters in support of sustainable management and conservation, and to assess relatedness and parentage in breeding populations.

Keywords: Genetic Conservation; Genetic Diversity; Population Genetics, Simple Sequence Repeat; SSR.

RESUMO - A pressão sobre as florestas tropicais devido à expansão da agricultura e pecuária frequentemente resulta em populações fragmentadas e isoladas. Fluxo gênico restrito aumenta a deriva e a diferenciação genética, reduzindo a diversidade genética das espécies arbóreas. *Balforoudendron riedelianum* (Engl.) Engl., popularmente conhecida como pau-marfim, é uma árvore brasileira com madeira valiosa utilizada na carpintaria e construção civil, mas está em perigo de extinção devido à exploração madeireira e fragmentação florestal. Informações sobre a diversidade genética e estrutura das populações existentes são necessárias para delinear estratégias para a conservação e manejo sustentável. Marcadores microssatélites são uma ferramenta eficaz para entender e quantificar os efeitos da fragmentação na diversidade genética. Sete marcadores microssatélites foram desenvolvidos e validados usando uma amostra de 98 indivíduos da espécie. O número de alelos por locos variou de 3 a 25, as heterozigosidades observada e esperada variaram de 0,051 a 0,909 e 0,050 a 0,930, respectivamente, o índice de fixação corrigido para alelos nulos variou de 0,036 a 1,0 e todos os marcadores estão em equilíbrio genotípico de ligação. Este conjunto de marcadores microssatélites é adequado para estimar parâmetros de genética de populações e contribuir para o manejo sustentável, conservação e avaliar o parentesco e a paternidade de populações no melhoramento.

Palavras-chave: Conservação Genética; Diversidade Genética; Genética de Populações; Sequências Simples Repetidas; SSR.

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1 INTRODUCTION

Around the world, natural forests have been threatened by deforestation and fragmentation. However, tropical forests lose more area when compared to boreal forests (Song et al., 2018). Among the tropical forests, the Atlantic Forest is one of the most intensively impacted biome, since its extension has been reduced to only 12% when compared to its original coverage (Zwiener et al., 2017). From the remaining small and isolated forest tree fragments with reduced genetic variability and increased rates of self-fertilization follows generations with a greater proportion of inbred individuals (Tambarussi et al., 2015, 2017).

Balfourodendron riedelianum Engl. (Engl.) is an outstanding tree native from Atlantic Forest. It grows up to 35 m tall and 100 cm in diameter at breast height, with hermaphrodite flowers pollinated by small insects, and anemochoric seeds dispersed by the wind (Carvalho, 2004). It is currently listed as endangered species (International Union for Conservation of Nature - IUCN, 2019). due to the intensive exploitation of its high wood value and to the overall fragmentation of the Atlantic forest biome (Carvalho, 2004). With the objective of contributing to genetic conservation and potential breeding efforts, this study reports the development of an initial set of microsatellite markers for *B. riedelianum.*

2 MATERIAL AND METHODS

2.1 Microsatellite markers development

Total genomic DNA was extracted from fresh leaves collected from a single *B. riedelianum* individual using the protocol by Inglis et al. (2018), and the voucher was deposited at Universidade de São Paulo herbarium, São Paulo, Brazil (voucher number: ESA142827). A microsatellite-enriched genomic library was constructed following the protocol by Billotte et al. (1999). The *Afa*I enzyme (Invitrogen, Carlsbad, California, EUA) was used to digest genomic DNA of *B. riedelianum* enriched using $(CT)_8$ and $(GT)_8$ motifs. Enriched fragments were cloned using *pGEM-T-Vector* (Promega Corporation, Madison, Wisconsin, EUA) and ligation products were used to transform XL1-BLUE *Escherichia coli* - competent cells (Stratagene, California, EUA). Transformed cells were cultivated in 100 µg/mL ampicillin, 50 µg/mL X-galactosidase, and isopropyl β-D-1 thiogalactopyranoside (IPTG). Ninety-six positive clones (white colonies) were obtained and sequenced with *Afa*21 (5′–CTCTTGCTTACGCGTGGACTA – 3′) and *Afa*25 (5′–TAGTCCACGCGTAAGCAAGAGCACA–3′) in 3730/3730xl Data Collection Analyzer (Applied Biosystem, Foster City, California, USA) using the BigDye v3.1 terminator kit (Applied Biosystem). Of the 96 positive clones, microsatellites loci were found in 40 of them. Dinucleotide motifs were most abundant followed by compound, tri and tetranucleotide motifs (65, 30, 2.5 and 2.5%, respectively). Twenty-two microsatellite sequences were located at the ends of respective sequences, precluding the design of the primers.

The vector segments were removed from each of the sequences by VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) and the primers were designed considering sequences with 40–60% GC content in Primer 3Plus (Untergasser et al., 2012), with final products ranging from 95 to 300 base pairs (bp) and primer size ranges from 18 to 22 bp. In order to increase specificity of allele amplification and reduce stutter, a 5’- GTTTCTT -3’ pig-tail was added to the 5’ end of the reverse unlabeled primers (Brownstein et al., 1996). PCR reactions in multiplexed systems were carried out in 10 µl volumes containing 1 µl of 10 × Qiagen Multiplex PCR Buffer (Qiagen Inc., Valencia, CA, USA), equal concentration (0.1 µM) of all primers for all microsatellite markers co-amplified, and 2.0 ng of genomic DNA. The recommended Qiagen Multiplex PCR Handbook cycling protocol was used with an annealing temperature of 60 °C and 30 PCR cycles. PCRs were carried out in triplex or duplex systems combining markers in such a way that loci whose alleles migrate in the same size range were labeled with different fluorochromes either 6-FAM (blue), NED (yellow), or VIC (green). An aliquot of 1 µl of PCR mixture was mixed with 1 µl of ROX-labeled size standard (Brondani and Grattapaglia, 2001) and 10 µl of Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was electroinjected in an ABI 3100XL genetic analyzer and data collected under dye set D spectral calibration using Genescan and analyzed with Genotyper (Applied Biosystems).

2.2 Analysis of genetic diversity, null alleles and linkage disequilibrium

A total of 98 randomly selected but unrelated individuals were sampled from a provenance and progeny trial of *B. riedelianum* located in the Experimental Station of Luiz Antonio (21°40' S, 47°49' W), São Paulo State, Brazil. Genetic diversity, fixation index (*F*) and genotypic linkage disequilibrium (LD) were estimated using FSTAT 2.9.3.2. software (Goudet, 1995). To test whether *F* values were significantly different from zero and to evaluate the presence of genotypic linkage disequilibrium, 1,000 Monte Carlo permutations (alleles among individuals) associated with Bonferroni correction (95%, $\alpha = 0.05$) were used. The INEST 2.0 software (Chibicki and Burczyk, 2009) was used to identify null alleles (*Null*), using a Bayesian approach (IIM) and the fixation index was corrected (F_{Null}) for loci showing significant frequency of null alleles.

3 RESULTS AND DISCUSSION

Suitable microsatellites for primer design were only found in 18 sequences (18.7%), eight tested microsatellite loci yielded successfully amplified fragments and their information is shown in Table 1. From the eight microsatellite markers tested one was monomorphic. For the seven polymorphic markers the number of alleles per locus (*k*) ranged from 3 to 25, the observed and expected heterozygosities ranged from 0.051 to 0.908 and 0.050 and 0.909 respectively, the corrected fixation index for null alleles ranged from 0.036 to 1.0 and all markers were found in linkage equilibrium (Table 2). Significant presence of null alleles was detected at loci Bri16 and Bri17 (*Null* = 0.129 and 0.229, respectively). However, after correction for null alleles the fixation index decreased (F_{Null} = 0.422 and 0.535 to 0.137 and 0.036, respectively), and the observed (H_o = 0.398 and 0.423 to 0.643 and 0.856, respectively) and expected heterozygosity estimates (H_e = 0.688 and 0.909 to 0.745 and 0.887, respectively) were also modified. Loci Bri4 and Bri23 showed a negative fixation index, indicating heterozygosity excess and absence of null alleles. Of the 560 permutations performed between pairs of loci, neither showed genotypic linkage disequilibrium after Bonferroni correction ($P = 0.05$).

These seven microsatellite markers also showed the Mendelian inheritance pattern and the absence of genetic linkage, proven based on 235 open-pollinated seedlings collected from 17 heterozygous mother trees, indicating that these molecular markers can be used as genetic markers (Aguiar et al., 2019). From the confirmation of linkage equilibrium and Mendelian inheritance, the seven DNA markers were used to analyze the genetic diversity, inbreeding, pollen dispersion, mating system, and inbreeding depression of 1335 individuals of *B. riedelianum* in a provenance and progeny test established at the Experimental Station of Luis Antônio (Aguiar et al., 2020, no prelo). A total of 93 alleles were identified, with high observed (H_o = 0.58) and expected (H_e = 0.58) heterozygosities and low fixation index (F = -0.01), in addition to high outcrossing rate (t_m = 0.93), which suggests that the species reproduces predominantly by crossbreeding. However, some self-fertilization (s = 0.07) and mating among related trees (t_m - t_s = 0.20) events have also been observed, suggesting that the species is self-compatible. The inbreeding depression was evaluated by comparing the growth of quantitative traits measured in seedlings originated from mating among unrelated trees with those originated from self-fertilization and mating among related trees. Seedlings originated from mating among unrelated trees showed the higher root-collar circumference (*RCC* = 3.34 mm), plant height (H = 14.6 cm) and observed heterozygosity (H_o = 0.51), and low fixation index (F = 0.06) than seedlings originated from self-fertilization (*RCC* = 3.01 mm, H = 14.0 cm, H_o = 0.24, F = 0.59) and mating among related trees (*RCC* = 3.13 mm, H = 13.78 cm, H_o = 0.38, F = 0.33). These results indicate an inbreeding depression for self-fertilization of 9.7 and 4.1% for *RCC* and H, respectively and for mating among related trees of 6.1 and 5.6% for *RCC* and H, respectively. These results were used to determine the selective logging in the provenance and progeny test, in order to minimize the inbreeding originated from mating among related trees and to produce seeds with less inbreeding to be used in environmental restorations (Aguiar et al., 2020, no prelo). Therefore, this set of microsatellite markers can be useful to estimate population genetic parameters in support of sustainable management and conservation programs and to assess the relationship and paternity in breeding populations.

Table 1. Nuclear microsatellite loci for *Balfourodendron riedelianum* with motifs, primers sequences, allelic size range in base pairs and Genbank accession numbers.

Tabela 1. Locos nucleares microssatélites para *Balfourodendron riedelianum* com motivos, sequência dos primers, amplitude alélica em pares de base e números de acesso no Genbank.

Loci	**Motifs**	**Primer sequence**	**Size (pb)**	**GenBank accession no.**
Bri4	(AC)19	F: GTCCACTGCTCATGTCAACAC R: GACAACAAGAGAGGGGATGC	145-170	MG642961
Bri6	(GA)6	F: CGGTCCGGTCTGAGTTTTAC R: CGTGGTCCAAATCTACAAAGG	96-120	MG642962
Bri10	(CA)15	F: CTCGACCCAATCTTATGCAA R: TTTTTTGGTAGCGGGGAGTA	140-190	MG642963
Bri13	(TG)6	F: TTAAATGCCCCAAAAGGATG R: TGGGAATAGACAGAGGGAAAA	119-140	MG642964
Bri16	(CT)8	F: TTCCTTATCTGGGGTGGAGA R:AAAGAACGAAGCAGCCAAAC	230-270	MG642965
Bri17	(TG)18	F: GCGATCGTAGAGAAAAACAACA R: GAGGGTCGCGATGTAAAAGA	230-274	MG642966
Bri21	(TC)9 (TA) 4 (TG)8	F: GGTAAGAGCCAAAGGCAACA R: ACGGGATCATGGCAATTTT	180-210	MG642967
Bri23	(CA)6	F: TGATAGCTCTCAGATTGATTCTGTC R: TCCAATTCGGAGGCTTACAT	97-110	MG642968

Table 2. Microsatellite characterization based on genotyping 98 unrelated individuals of *Balfourodendron riedelianum*.

Tabela 2. Caracterização dos microssatélites com base na genotipagem de 98 indivíduos de *Balfourodendron riedelianum* sem parentesco.

Loci	*N*	N_G	*k*	*R*	H_e	H_o	*F*	*Null*	F_{Null}
Bri4	98	98	4	4.00	0.551	0.908	-0.640	0.000	-0.640
Bri6	98	98	1	1.00	0.000	0.000	1.000	0.000	1.000
Bri10	98	97	18	18.00	0.903	0.794	0.121	0.017	0.121
Bri13	98	98	3	3.00	0.390	0.367	0.057	0.000	0.057
Bri16	98	98	12	11.95	0.688	0.398	0.422	0.129*	0.137
Bri17	98	98	25	25.00	0.909	0.423	0.535	0.229*	0.036
Bri21	98	98	6	6.00	0.752	0.704	0.064	0.000	0.064
Bri23	98	97	4	3.98	0.050	0.051	-0.021	0.000	-0.210
Mean	98	97.5	9.12	9.11	0.530	0.456	0.191	0.047	0.036

N = sampled individuals; N_G = genotyped individuals; *k* = number of alleles per locus; *R* = allelic richness; H_e = expected heterozygosity; H_o = observed heterozygosity; *F* = fixation index; *Null* = null alleles; F_{Null} = corrected fixation index; *$P \geq 0.01$.

N = indivíduos amostrados; N_G = indivíduos genotipados; *k* = número de alelos por locos; *R* = riqueza alélica; H_e = heterozigosidade esperada; H_o = heterozigosidade observada; *F* = índice de fixação; *Null* = alelos nulos; F_{Null} = índice de fixação corrigido; *$P \geq 0,01$.

4 CONCLUSION

Seven microsatellite loci for *B. riedelianum* showed a moderate to high level of polymorphism and no genotypic linkage disequilibrium was detected. Finally, these microsatellite markers are the first set development to the species.

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