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Original Paper

Development of microsatellite markers in *Pterodon pubescens* and transferability to *Pterodon emarginatus*, two Brazilian plant species with medicinal potential

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Abstract

Pterodon pubescens and *P. emarginatus* (Leguminosae) are native medicinal plants of Brazil. Extractivism due to its therapeutic properties threatens populations of both species. Studies of genetic diversity is a way to reason the use and promote conservation. We developed microsatellite markers for *P. pubescens* and transferred them to *P. emarginatus* to further genetic diversity investigation of these species. From genomic sequences of *P. pubescens*, obtained via the Illumina MiSeq platform, it was possible to identify 6,514 microsatellite regions, to design 5,419 primer pairs, and to test 30 markers amplification. We provide 26 polymorphic microsatellite markers, 10 of which were genotyped in 48 individuals per species. The number of alleles per locus range from 3 to 16, with high average genetic diversity (*P. pubescens* H_E = 0.753; *P. emarginatus* H_E = 0.691). The genotyped markers have a high paternity exclusion probability (Q values greater than 0.99) and low probability of identity, indicating that set of loci is capable of individual discriminating in *P. pubescens* and *P. emarginatus*. Microsatellite markers provided in this study are a tool for population genetics studies and conservation of the two species and can be applied to closely related non-model species.

Key words: genetic diversity, Illumina MiSeq, molecular markers, neotropical tree, "sucupira-branca".

Resumo

Pterodon pubescens e *P. emarginatus* (Leguminosae) são duas espécies de plantas nativas medicinais do Brasil. As populações têm sido ameaçadas pelo extrativismo para uso de suas propriedades terapêuticas. Estudos de diversidade genética ajudam a racionalizar o uso e a promover a conservação dessas espécies. Nós desenvolvemos marcadores microssatélites para *P. pubescens* e transferimos para *P. emarginatus* com intuito de permitir investigações futuras da diversidade genética das espécies. A partir de sequências genômicas de *P. pubescens*, obtidas via plataforma Illumina MiSeq, foi possível identificar 6.514 regiões microssatélites, desenhar 5.419 pares de *primers*, e testar a amplificação de 30 marcadores. Nós fornecemos 26 marcadores moleculares, 10 dos quais foram usados para genotipar 48 indivíduos de cada espécie. O número de alelos por locus variou de 3 a 16, com alta diversidade genética média para ambas as espécies (*P. pubescens* H_E = 0,753; *P. emarginatus* H_E = 0,691). Os dez marcadores apresentaram boa probabilidade de exclusão de falsa paternidade (com valores acima de 0,99) e baixos valores de probabilidade de identidade, indicando que esse conjunto é adequado para discriminar indivíduos em *P. pubescens* e *P. emarginatus*. Os marcadores

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microssatélites desenvolvidos neste trabalho representam uma ferramenta promissora para estudos de genética de populações e conservação das duas espécies e podem, eventualmente, ser aplicados a espécies não-modelos filogeneticamente próximas.

Palavras-chave: diversidade genética, Illumina MiSeq, marcadores moleculares, árvores neotropicais, "sucupira-branca".

Introduction

Pterodon pubescens Benth. (Bentham 1860) and *P. emarginatus* Vogel (Vogel 1837) (Leguminosae) are allogamous tree species popularly known in Brazil as "sucupira-branca". Both species are diploid with 16 chromosomes (Bandel 1974; Coleman & DeMenezes 1980; Albernaz 2020). They occur in phytogeographic domains of the Amazon, Caatinga, Cerrado, Atlantic Forest and Pantanal (Lima & Lima 2015; Carvalho *et al.* 2020). In regions of both species contact natural hybrids arise. Despite that, hybridization seems to be a rare event. Morphological and molecular traits data support two clearly distinct species. The species identification is performed mainly by leaflets shape and flowers color (Rocha 2006). The species names are *Pterodon pubescens*, for pink flowers trees, and *P. emarginatus* for purple flowers trees. In addition, *P. polygalaeflorus* (Benth.) Benth. is a synonym of *P. emarginatus* (Carvalho *et al.* 2020).

Both species are economically important due to their medicinal use. They have pharmacological properties such as anti-inflammatory, analgesic, anti-tumor, and antimicrobial (Araújo *et al.* 2015; Santos *et al.* 2018). Herbal medicine laboratories growing interest in *P. pubescens* and *P. emarginatus* contributes to increased extractivism, predisposing these species to genetic diversity loss. Despite that, these species were not evaluated for extinction threat degree by Flora do Brasil 2020 (Carvalho *et al.* 2020), only *P. emarginatus* was evaluated by IUCN as Least Concern (IUCN 2021). Thus, the study of the genetic diversity of uncultivated medicinal species has become indispensable for their conservation (Rao & Hodgkin 2002; Hodel *et al.* 2016).

Genetic diversity can be assessed by different genetic approaches, such as single nucleotide polymorphism (SNPs) and simple sequence repeats (SSR), also known as microsatellite markers. Few genetic studies involving species of *Pterodon* can be found in the literature, as the transferability of a few microsatellite markers from *Phaseolus vulgaris* L. and *Dipteryx alata* Vogel to *Pterodon* (Soares *et al.* 2012; Santana 2014; Araújo *et al.* 2015). Furthermore, there are no specifically developed microsatellite markers for *Pterodon*.

Specific microsatellite markers are necessary to better investigate genetic diversity and population structure of *P. pubescens* and *P. emarginatus*. High-Throughput Sequencing technologies (HTS) have facilitated the development of molecular markers for non-model species (Metzker 2010; Zalapa *et al.* 2012). These sequencing platforms allow the generation of large amounts of DNA sequences that can be used for assembling genomic sequences and identifying microsatellite regions. This strategy allows relatively fast, low cost and efficient development of microsatellite markers (Taheri *et al.* 2018).

Efficient assessment of microsatellite regions using HTS has been applied to non-model Leguminosae plants for molecular markers generation. For example, Guimarães *et al.* (2017) identified 11 microsatellite loci for *Dipteryx alata* Vog. (Leguminosae), an important genetic food resource in the Brazilian Cerrado. Beyer *et al*. (2019), developed nine polymorphic microsatellite markers for *Stizophyllum riparium* (Kunth) Sandwith (Bignoniaceae), which were successfully transferred to two species of the same genus, *S. inaequilaterum* (Kunth) Sandwith and *S. perforatum* (Cham.) Miers. The aim of the present work was to assess *P. pubescens* genome through HTS of DNA, to develop and characterize microsatellite loci as markers and test their transferability to *P. emarginatus*. In doing so, SSR markers and molecular information will be made available to help expand knowledge about the genetic diversity and conservation of these species of the genus *Pterodon*.

Materials and Methods

Leaves were collected from an adult individual of *P. pubescens* in municipality of Goiânia, state of Goiás (GO), Brazil (LAT -16.577, LON -49.273), and deposited in the herbarium of the

Universidade Federal de Goiás (Voucher: 61013). DNA was extracted using the CTAB 2% protocol (Doyle & Doyle 1987) and genomic libraries were constructed following the Nextera DNA Library Preparation protocol (Illumina). Sequencing was performed with Illumina MiSeq using Miseq Reagent kit v3 (600 cycles) and sequences of low quality were removed using Trimmomantic (Bolger *et al.* 2014). Clean reads were assembled using Dipspades software (Safonova *et al.* 2014).

Identification of suitable microsatellite regions and primer design were performed using QDD software (Meglécz *et al.* 2009). The minimum number of repeats was ten for dinucleotides, six for tetranucleotides and five for pentanucleotides; tri- and hexanucleotide regions were not identified here. Microsatellite regions identified in unique genomic sequences (singletons) were used to design pairs of primers. The parameters used for primer design were amplicon size of 150–400 bp, GC ratio of 30–60%, melting temperature value (Tm in ºC) between 56 and 62 °C and primer lengths of 22–25 bp. After designing primers, regions associated with transposable elements were identified by QDD software and excluded from the analysis. Microsatellite regions with AT motifs were not selected for the development of markers. After these initial filters, 30 pairs of primers designed for microsatellite regions with the highest number of tandem repetitions were chosen for development of markers.

The 30 developed primer pairs were tested for effectiveness of amplification and polymorphic loci identification by polymerase chain reaction (PCR). We tested the amplification at different annealing temperatures in six individuals from six different populations of each species (see Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.21355416.v1>). PCR reactions were prepared with a final volume of 10 µL, using 3 µL of DNA (2.5 ng/µL) and 3 µL of primers (forward + reverse at 0.9 µM), 0.9 µL of dNTPs (2.5 µM), 1.3 µL of BSA (bovine serum albumin, 25 mg/mL), 1 µL buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 0.15 µL Taq polymerase (5 U; Phoneutria) and sterile milli-Q water to complete the reaction volume. The amplification reaction followed the steps: (1) DNA denaturation by heating at 94 ºC for five minutes; (2) 30 cycles of denaturation at 94 ºC for one minute; primer annealing (ranging from 54 ºC to 62 ºC, increasing 2 ºC in each PCR test amplification) for one minute and extension at 72 ºC for one minute; and (3) final extension at 72 ºC for 30 minutes. Polymorphic loci were identified by electrophoresis on 6% polyacrylamide gels, visualized by silver staining procedures (Creste *et al.* 2001). Allele sizes were determined using the 10 bp DNA Ladder and the 50 bp DNA Ladder (Invitrogen™).

Polymorphic markers with great polyacrylamide gel amplification profiles were selected for fluorescent labeling to develop multiplex genotyping panels. These panels were applied to 96 individuals of *Pterodon*, 48 per species. Forty-eight individuals of *P. pubescens* were sampled from two populations: twenty-four from Perdizes, Minas Gerais (MG; LAT -47.367, LON -19.351) and twenty-four from Caldas Novas, GO (LAT -48.62528, LON -17.74528). Forty-eight individuals of *P. emarginatus* were also sampled from two populations: twenty-four from Santa Rita do Novo Destino, GO (LAT -49,052, LON -14,812) and twenty-four from Porangatu, GO (LAT -49.19576, LON -13.51403). The individuals were genotyped in an ABI3500 Genetic Analyzer (Applied Biosystems) using the GeneScan 600 LIZ marker (Applied Biosystems). Genotypes were determined using GeneMapper software (Applied Biosystems). Micro-Checker software (Oosterhout *et al.* 2004) was then used to detect errors due to stuttering, allele dropout, and null allele frequency for each locus.

Loci were characterized by estimating the average number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E) under Hardy-Weinberg equilibrium and the intrapopulation fixation index. These parameters were estimated using Genetic Data Analysis software version 1.0 (GDA) (Lewis & Zaykin 2001). The maximum diversity H_{max} for each population was calculated from the average number of alleles (A) per population as $H_{max} = (A–1) / A$, and the proportion of maximum diversity as H_E / H_{max} (Hennink & Zeven 1991). Linkage disequilibrium was evaluated using the program FSTAT 2.9.3.2 (Goudet 2002) with Bonferroni correction. The probability of paternity exclusion (Q) and the probability of genetic identity (I) were acquired with IDENTITY 1.0 software (Wagner & Sefc 1999).

The genomic sequence data from *P. pubescens* used in development of microsatellite markers (accession number: MN822196–MN822225) and information from designed primers were deposited at the National Biotechnology Information Center (NCBI).

Results

A total of 9.45 million paired-end reads (4.8 Gb) were generated in the Illumina MiSeq platform. After quality control, 3.4 Gb of data were assembled on 138,319 scaffolds (117 Mb) with N50 equal to 870 bp. The assembled sequences were searched for microsatellite loci. A total of 1,511 microsatellite sequences were identified, with the vast majority being di- (1,154 or 76%) followed by tetra- (212 or 14%) and pentanucleotides (145 or 10%). The number of tandem repetitions in the microsatellite regions varied from five to 24, in which the maximum number of repetitions in tandem was 24 for di-, 10 for tetra- and eight for pentanucleotides. A total of 5,419 primer pairs were designed, of which 78% were for di-, 13% for tetra- and 9% for pentanucleotide microsatellites. Thus, an average of three pairs of primers per microsatellite region were designed. Thirty pairs of primers were selected for molecular markers containing di- and tetranucleotide repeats (Tab. S2, available on supplementary material <https://doi.org/10.6084/m9.figshare.21355416.v1>).

From the 30 primer pairs tested, 27 were successfully amplified, with a good shape of bands in the gels and alleles identified easily, for *P. pubescens* 26 loci showed positive cross-amplification to *P. emarginatus*. Annealing temperatures ranged from 54 to 60 ºC for *P. pubescens* and 50 to 59 ºC for *P. emarginatus*. Among the selected primers, only one locus (Pem08) was monomorphic in a sample of six *P. pubescens* individuals. All loci (26) were polymorphic for *P. emarginatus* using the same sample size of six individuals (Tab. S2, available on supplementary material <https://doi.org/10.6084/m9.figshare.21355416.v1>).

To improve the cost-benefit of genetic analysis, 10 of the 27 microsatellites loci that showed the best amplification patterns were combined into two multiplex sets (Tab. 1). For the 10 markers, the number of alleles per loci in both species ranged from 3 to 16 (Tabs. S3; S4, available on supplementary material <https://doi.org/10.6084/m9.figshare.21355416.v1>), with a total number of 99 alleles for *P. pubescens* and 97 for *P. emarginatus*. The average number of alleles per population was similar between the two *Pterodon* species (9.90 for *P. pubescens* and 9.70 for *P. emarginatus*) (Tabs. S3; S4, available on supplementary material <https://doi.org/10.6084/m9.figshare.21355416.v1>).

The average expected heterozygosity (H_E) of the markers was high for both *P. pubescens* (H_E = 0.753) and *P. emarginatus* (H_E = 0.691), which represents 83% (H_{max} = 0.898) and 77% (H_{max} = 0.896) of the maximum heterozygosity within these species (Hennink & Zeven 1991). No significant linkage disequilibrium ($p > 0.05$) was identified. Significant deviation from HWE ($p < 0.05$) was observed for the Pem10 locus for both species, Pem22 for *P. emarginatus* and Pem25 and Pem18 for *P. pubescens*. No errors were detected due to stuttering and allele dropout. Null allele analysis indicated heterozygote deficiencies in *P. pubescens* for Caldas Novas - GO population (loci Pem10 and Pem25) and for *P. emarginatus* Porangatu - GO (loci Pem23, Pem10 and Pem22) and Santa Rita do Novo Destino - GO populations (loci Pem26 and Pem22) (Tabs. S3; S4, available on supplementary material <https://doi.org/10.6084/m9.figshare.21355416.v1>).

The 10 developed markers showed low values for the combined probability of genetic identity (I) in *P. pubescens* (1.40x10-9 and 6.86x10-12) and *P. emarginatus* (3.72x10-9 and 9.47x-10). Paternity exclusion (Q) was greater than 0.99 for both species (Tabs. S3; S4, available on supplementary material <https://doi.org/10.6084/m9.figshare.21355416.v1>).

Discussion

Sequencing and assembling the partial genome of *P. pubescens* allowed the identification of many di-, tetra- and pentanucleotide microsatellite regions, of which 30 were selected for development of specific microsatellite markers. Of these, ten markers successfully genotyped individuals of *P. pubescens* and transferred to *P. emarginatus*, and can be used for population genetics studies within the genus.

Tri- and hexanucleotide regions that are frequently found in coding regions of genomes were not identified in this study (Ellegren 2004; Song *et al.* 2021). Pentanucleotide microsatellite regions of *P. pubescens* were not selected for markers development because they had repetition motifs rich in AT nucleotides and low numbers of tandem repetitions. Also, microsatellite regions rich in AT motifs can form a hairpin and decrease amplification efficiency during PCR and microsatellite regions with the highest numbers of tandem repetitions are more likely to be associated with genotypic variation (Zalapa *et al.* 2012). Thus, these criteria described in

Table 1 – Panels of microsatellite markers for *Pterodon pubescens* and *P. emarginatus*, arranged in multiplex for genotyping in capillary electrophoresis.

Multiplex	Locus	Dye	Ta (^{0}C)	Allelic amplitude
1	Pem05	6 FAM	54	141–157
	Pem21	6 FAM	56	250–308
	Pem15	PET	54	166–192
	Pem24	NED	54	172–198
	Pem26	NED	56	342–392
2	Pem23	6 FAM	54	316–344
	Pem10	6 FAM	54	196–220
	Pem18	NED	54	196–224
	Pem22	VIC	54	218–304
	Pem25	PET	54; 56*	282–358

Ta = annealing temperature; Dye = fluorescent dyes; * = Ta in *Pterodon emarginatus*.

the literature were followed to select the 30 microsatellite regions with high potential for development of markers.

Most of the tested primer pairs showed a good amplification pattern for *P. pubescens* and were successfully transferred to *P. emarginatus*. Successful transfer of microsatellite markers among evolutionarily closely-related species can be explained by the presence of conserved regions flanking the microsatellites (FitzSimmons *et al*. 1995). Successful transferability was also verified by Garcia *et al*. (2011) when evaluating transferability between species of the family Leguminosae, with the highest rates of amplification occurring with species of the same genus. This explains the successful transferability found in the present study, considering that *P. emarginatus* and *P. pubescens* are evolutionarily closely-related species. Soares *et al.* (2012) found different results when testing cross amplification from *Dipteryx alata* to *P. emarginatus*, with a transferability rate of only 50%. The successful transfer of microsatellite markers from *Phaseolus vulgaris* to *P. emarginatus* was even lower: 539 pairs of primers were evaluated, of which 23 markers were transferred and only seven were polymorphic (Santana 2014).

The high average number of alleles per locus identified in *P. pubescens* and *P. emarginatus* suggests that this set of markers was able to detect polymorphisms and, therefore, is a useful tool for genetics studies of these species. The number of alleles observed in the present study for *Pterodon* is greater than that reported by Guimarães *et al.* (2017), who developed microsatellite markers using HTS for one species (*Dipteryx alata*) of the same family, which also has a mixed reproductive system (Guimarães *et al.* 2019). The estimated maximum genetic diversity for *P. pubescens* (83%) and *P. emarginatus* (77%), are similar to other native Leguminosae, such as *Dimorphandra mollis* Benth. (79%) (Souza *et al*. 2012) and *Plathymenia reticulata* Benth. (85%) (Gong *et al.* 2012). This high genetic diversity represents the variety of alleles and genotypes found in this group of individuals evaluated for *P. emarginatus* and *P. pubescens*, showing that this set of available microsatellite markers can detect genetic variation, making it a promising tool for future population genetic studies.

The presence of null alleles, attributed to heterozygote deficiencies for some allele size classes, might explain the observed deviation from HWE in some loci. The null alleles represent the failure to amplify during PCR reaction caused by mutations in flanking primer regions (Pompanon *et al.* 2005). However, another explanation for null alleles is the mating system, which can lead to inbreeding and heterozygote deficiencies (Dakin & Avise 2004). Analysis with a larger number of populations and individuals may confirm this result.

Estimated combined probability of identity showed the ten markers suitable for discriminating individuals of these species. Detected values of paternity exclusion (Q) for both species of *Pterodon* represent high probabilities of exclusion of false paternity (Weir & Evett 1998). These results, combined with the high values of genetic diversity and absence of linkage disequilibrium between loci, demonstrate the quality of the microsatellite markers set developed in the study.

In conclusion, this study developed 26 polymorphic microsatellite markers for *P. pubescens* and showed their successful transferability to *P. emarginatus*. The strategy of developing microsatellite markers using genomic sequencing and transferability within the same genus was efficient and can be used for the study of other non-model species. The new set of microsatellite markers of *P. pubescens* and *P. emarginatus* will be a valuable tool for the evaluation of genetic diversity and population structure and other evolutionary investigations in the genus *Pterodon*. Information on the genetic diversity of *P. pubescens* and *P. emarginatus* is important for the study of flora and the development of conservation strategies and rational use of these two species.

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