Development, Characterization and Genetic Diversity of New Microsatellite Markers for *Dipteryx Alata* Vogel, A Species with Multiple Uses in the Brazilian Cerrado

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Short Report

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Abstract

Baru tree (Dipteryx alata) is an arboreal, fruitful plant native to the Cerrado Biome with an important socioeconomic impact. This study aimed to identify and characterize new microsatellite loci for *D. alata*. From the development of a genomic library enriched in microsatellites, ten pairs of primers were synthesized. Of these, seven were polymorphic, providing a total of 49 alleles, with an average of 5 to 5.57 alleles per locus. A significant content of polymorphic information was obtained, as indicated by the average expected heterozygosity (*uHE*), with a total average of 0.58 to 0.65 per locus. The average value of the observed heterozygosity (*Ho*) was also high, with a total average of 0.73 to 0.85 per locus. Some of the loci are in linkage disequilibrium, such as (Dalat G6 with Dalat B3, H3 and B4), in addition to (Dalat B4 with B5). The estimate of the combined loci for the probability of paternity exclusion obtained an average value of 1.00 for all loci, and the average combined probability of identity, the values were ($1.2^{10^{\wedge}-5}$) to ($4.4^{10^{\wedge}-6}$). The results obtained here show that the markers developed for *D. alata* are informative and suitable for studies on genetic diversity and population structure, aiming at the conservation and management of the species.

Introduction

The destruction of natural communities has represented an imminent scenario of species extinction, including the Cerrado (Brazilian savanna), which is subjected to excessive human interference due to agriculture and livestock expansion [1]. The understanding of patterns in which genetic variability is organized in populations is of fundamental importance for the development of conservation and sustainable use strategies for species and their habitats [2].

Dipteryx alata Vog., popularly known as baru tree, is a native tree species that has multiple uses ranging from human and animal food, to its pharmaceutical properties [3]. It is an endemic species to the Cerrado biome, and due to its wide distribution throughout the area and abundance in several of its habitats [4], it has potential as a model to check the impact of human intervention on the environment, through the characterization of its diversity and genetic structure and the effective size of populations [2]. For this, the microsatellite markers (SSR- Single Sequence Repeats) are considered great tools to access the genetic variability of populations [5]. SSRs have a high degree of polymorphism, the repetitive units of the sequence are flanked by unique sequences, which allow them to be amplified individually through a polymerase chain reaction (PCR), in addition to being multi-allelic and having high reproducibility [6]. Thus, this marker provides information on the long-term evolutionary history of species, mutation, isolation of the population, as well as the mechanisms of genetic drift, gene flow and selection [6].

Despite the importance of the baru tree for the Cerrado, little is known about the genetic variability existing in populations [3–7, 8]. Although specific SSR markers are already available [7–8], these studies demonstrate a low level of polymorphism in the loci, limiting the type of studies that can be carried out. Thus, in order to increase the coverage of loci representative of the genome, we developed and characterized a set of new microsatellite loci for *D. alata*. These markers have the potential to be used in population studies at various scales, in order to provide subsidies for the conservation and management of the species and its area of occupation.

Material And Methods

DNA extraction, construction of SSR-enriched genomic library and sequence analysis

For genomic DNA extraction, young leaves of *D. alata* were collected from a single individual, at the Mário Viana Municipal Park, a Conservation Unit, located in the municipality of Nova Xavantina, state of Mato Grosso, Brazil. The DNA was isolated using the commercial kit Plant DNeasy® (Qiagen), following the manufacturer's recommendations.

The DNA was used to construct a microsatellite-enriched genomic library according to the protocol adapted from [9]. Approximately 5 µg DNA was digested using the restriction enzyme *AfaI* (10u/µL) (Invitrogen) and the digested fragments were ligated to specific adapters Rsa21 (10µM) (5´ CTCTTGCTTACGCGTGGACTA 3') and Rsa25 (10µM) (5´ TAGTCCACGCGTAAGCAAGAGCACA 3'). The enrichment was based on the capture of DNA fragments containing microsatellite sequences, through the hybridization of probes with Biotin-IIIIII (CT)8 and Biotin-IIIIII (GT)8 and magnetic beads coated with streptavidin (MagneSphere Magnetic Separation Products; Promega Corporation, Madison, Wisconsin, EUA). The captured fragments were amplified by PCR, with the Rsa21 adapter (10µM), cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA) and then inserted into competent *Escherichia coli* cells 10H10b by electroporation. Competent cells were cultured in solid LB medium containing ampicillin (100 µg/mL), streptomycin (100 µg/mL), 20% IPTG (100mM) and 2% X-galactosidase (100 µg/mL).

We selected 48 positive clones using white/blue screening, which were sequenced on an ABI 3500xL Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using primers T7 ((5´-TAATACGACTCACTATAGG-3´) and SP6 (5´-ATTTAGGTGACACTATAGA-3´), and BigDye Terminator version 3.1 Cycle Sequencing Kit (Perkin Elmer – Applied Biosystems).

After obtaining the sequences of *D. alata*, chromatograms were analyzed using the software Chromas v.2.6.6 [10]. The vector sequence search was performed using the VecScreen tool (https://www.ncbi.nlm.nih.gov/tools/vecscreen/). Consensus sequences (*contigs*) were obtained using the CAP3 ("CAP3 *sequence assembly*") software [11], afterwards the *contigs* were aligned with the non-redundant NCBI database (Nr), through the BLASTN algorithm [12] with an e-value cutoff of 1e-06 using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Microsatellite regions were identified using SSRIT (The Simple Sequence Repeat Identification Tool) [13]. The primer pairs were designed using the software primer3Plus [14], with the following parameters: maximum primer size of 25 bp, annealing temperature of the primer (Tm) varying from 52°C to 65°C, minimum and maximum GC percentage of 40% and 60%, respectively, and amplification product range between 100 and 700 bp.

Validation of the SSR library

To validate the microsatellite library, a total of 90 individuals of *D. alata* were sampled from three populations in the Cerrado-Amazon transition region (Fig. 1), 30 individuals were collected from each population: 1) "BASC" population - natural population, with a history of anthropization, located in the municipality of Nova Xavantina - MT (14° 67' 78.5" S 52° 54' 36.6" W), where adult individuals are distributed in a pasture area; 2) "BAP" population - it is a conserved population, at the Bacaba Municipal Park (14° 70' 82.7" S 52° 35' 31.9" W); 3) "BAVC" population - is a young population, located in a restoration area carried out by manual direct seeding, located in the municipality of Barra de Garças - MT (14°87' 64.0" S 52°11' 81.4" W) (Fig. 1).

The total genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method [15]. PCR was performed in volumes of 20 µL using the Biocycler® thermocycler, each cocktail contained approximately 20ng genomic DNA, containing 2.0 µL 10x PCR *Buffer*, 0.60 µL $MgCl_2$ (50 mM), 1.6 µL DNTPs (0.2 mM each), 0.40 µL each *primer* (0.20 µM), 1 U Taq DNA Polymerase, 4.0 µL DNA (5ng/µL) and 10.8 µL milliQ H_2O. To optimize the annealing temperature of the primer pairs, the amplification of fragments was performed in a Biocycler® thermocycler, following the steps described by [16]. The PCR products were initially analyzed on 2% agarose gels, on TBE 1X (Tris base 0.1 M; boric acid 1 M and EDTA 0.5 M), stained with red gel (1: 500 µL) and visualized under UV light. Only the primer pairs amplified at a specific annealing temperature and with fragment sizes within the expected range were used to characterize the loci. The loci were subjected to the multiplex system, in which the forward primer was labeled with one of the following fluorescences (FAM, NED, VIC and ATTO565) and analyzed in an ABI 3500 automated sequencer (Applied Biosystems, Foster City, CA, USA) according to manufacturer's recommendations.

Statistical analysis of microsatellite loci

For genotyping the loci, alleles were scored against the GeneScan-600 (LIZ) internal Size Standard Kit (Applied Biosystems, Foster City, CA, USA) using the Geneious 8.1.6 software [17]. Descriptive statistics were run in the GenAlex 6.5 software [18], in which the estimates of number of alleles per locus (*Na*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and fixation index (*f*) and the probability of identity (*PI*) and probability of paternity exclusion (*PE*) were evaluated. The FSTAT 2.9.3.2 software [19] was used to analyze the linkage disequilibrium between the loci.

Results And Discussion

Of the 48 clones sequenced, 23% sequences contained microsatellite regions. The efficiency in the percentage of sequences obtained with the construction of the genomic library was also observed in the protocol of [9], which varied from 20 to 90% for tropical plant species. However, based on genetic diversity studies for *D. alata* [7–8], this is the first to develop SSR markers using the microsatellite-enriched genomic library technique, which produces fragments predominantly composed of repetitive sequences.

From the primer pairs designed, a set of 10 loci were selected for synthesis and validation (Table 1). After optimization, the annealing temperatures were set for the 10 loci, at 58°C or 60°C (Table 1), which presented a clear amplification pattern, with unique and well-defined bands. Of these, seven loci were polymorphic and two were monomorphic in the evaluated populations.

Table 1
Characteristics of the ten microsatellite loci developed for *D. alata*.

Loci	Primer Sequences (5'–3')	Repeat Motif	Allelic range	Ta (°C)	Dye	Multiplex	GenBank Accession Number
DalatA1	F:GCCTTATCCGCAAACTAACG	$(GA)_{10}$	406	60°C	FAM	A	MZ229345
	R: AAGCCATTTGTTGCAACTGA						
DalatB3	F:TGCAACTTGAAACACGTCTT	$(GT)_8$	528–530	58°C	VIC	A	MZ229346
	R: ACAAGTCGCAAAGGTTTCTT						
DalatB4	F:CGTTTGCACACACTTAAAAC	$(AC)_7$	554–586	60°C	NED	A	MZ229347
	R: GGGCTGAATTTGAAAGCACT						
DalatB5	F:TAGACAGAGACCCAGAGACC	$(GT)_{10}$	362–382	60°C	ATTO565	A	MZ229348
	R: TCTGGTGAGCTTTTATGTGT						
DalatC3	F:ACCTATATGCTGCTAGCTCA	$(GT)_7$	390–424	60°C	VIC	B	MZ229349
	R: GTCATGACCCTAAGCTTTAGC						
DalatD6	F:TGGGGAGTTTTGAATTTGGTG	$(TG)_7$	501	60°C	NED	B	MZ229350
	R: ACAAGACAGCTGCATAGAAAG						
DalatF3	F:AGAAAGCAACATGAGTCTGTG	$(CT)_{14}$	558	60°C	ATTO565	B	MZ229351
	R: ACATATCTGCCATTACGCCT						
DalatF6	F:ACCACCTGTTACACTTGCAA	$(AC)_{12}$	288–310	58°C	FAM	C	MZ229352
	R: TCCACTTCCATCACTTCTCT						
DalatG6	F:TTGGGTGATTTTGATGGTTGG	$(TG)_5$	480–506	60°C	VIC	C	MZ229353
	R: GTTCTACCCTCTGACACTCG						
DalatH3	F:TGGGTGATTTTGATGGTTGG	$(TG)_5$	512–538	60°C	NED	C	MZ229354
	R: CACCTCTACCTCCCCTAGAG						

Ta(°C) = Annealing temperature set after optimization tests.

A total of 49 alleles were identified at the seven polymorphic loci, with the number of alleles per locus ranging from two at the locus (Dalat B3) to twelve at the locus (Dalat B4) (Table 2). The total average of alleles in the three populations ranged from 5 to 5.57 alleles per locus (Table 2). This variation in the number of alleles per locus is common in microsatellite regions due to the high levels of polymorphism found in these markers [20], which is directly related to the high mutation rates occurring in these regions [21].

The observed heterozygosity ranged from 0.00 to 1.0 per loci depending on the sampled population, and the expected heterozygosity ranged from 0.34 to 0.74 per loci, with total averages ranging from 0.73 to 0.85 for H_O and from 0.58 to 0.65 for H_E (Table 2). The observed heterozygosity (H_O) was greater than the expected heterozygosity (H_E) for most loci, except for loci (Dalat B3 and B4). The higher value of heterozygotes observed suggests an excess of heterozygotes in relation to that expected by the Hardy-Weinberg equilibrium. This excess of heterozygotes may be occurring due to the reproductive characteristics of the species or even due to a selection in favor of heterozygotes for *D. alata* [22].

The fixation index (*f*) was negative for most loci, except for loci (Dalat B3 and B4), with total averages ranging from −0.15 to -0.28 (Table 3). When this estimate has values close to or less than zero, it indicates the occurrence of outcrossing between unrelated individuals [23].

The probability of identity (*PI*) of each locus was estimated, which ranged from 0.12 (Dalat B4, C3 and F6) to 0.75 (Dalat B3), with average estimates of the combined analysis ranging from ($1.2^{10^{\wedge}-5}$) to ($4.4^{10^{\wedge}-6}$). The probability of paternity exclusion (*PE*) based on the seven pairs of microsatellite loci ranged from 0.12 for locus (Dalat B3) to 0.70 for locus (Dalat B4), in the combined analysis, where no parent is known, the average value was 1.00 for all loci (Table 3). The set of microsatellite markers showed a low combined probability of identity (*PI*), indicating the probability of finding, by chance, two individuals from a sample with the same genotype in a given set of markers is minimal [24]. The probability of paternity exclusion through the combined analysis for the developed SSR loci was high, providing high reliability to correctly exclude an individual from paternity [25]. The seven SSR loci were sufficient to distinguish between the ninety individuals, indicating that the set of microsatellite markers is efficient in discriminating individuals in populations [24].

Table 2
Genetic parameters of microsatellite loci determined in three populations of *D. alata*.

Loci	Santa Célia Farm (BASC)				Bacaba Park (BAP)				Vera Cruz Farm (BAVC)			
	N	N_A	H_O	uH_E	***N***	N_A	H_O	uH_E	***N***	N_A	H_O	uH_E
DalatF6	29	5	0.93	0.72	30	6	0.97	0.74	30	8	0.97	0.71
DalatC3	30	7	1.00	0.72	29	4	1.00	0.67	30	7	0.97	0.73
DalatG6	30	4	1.00	0.66	30	3	1.00	0.57	30	3	1.00	0.63
DalatB3	26	2	0.00	0.40	26	2	0.00	0.14	26	2	0.00	0.46
DalatH3	29	5	1.00	0.63	29	5	1.00	0.66	30	5	1.00	0.67
DalatB4	30	8	1.00	0.71	30	9	0.83	0.66	26	5	0.31	0.34
DalatB5	30	8	1.00	0.70	30	6	0.93	0.63	29	8	0.90	0.71
Mean	29.14	5.57	0.85	0.65	29.14	5.00	0.82	0.58	28.71	5.43	0.73	0.61
SE	0.55	0.84	0.14	0.04	0.55	0.87	0.14	0.07	0.71	0.90	0.15	0.06

N = number of individuals analyzed, *Na* = number of alleles observed, H_O = observed heterozygosity, uH_E = expected heterozygosity.

Table 3
Estimates of genetic parameters of seven microsatellite loci developed for *D. alata*.

Loci	Santa Célia Farm (BASC)			Bacaba Park (BAP)			Vera Cruz Farm (BAVC)		
	f	*PI*	*PE*	*f*	*PI*	*PE*	*f*	*PI*	*PE*
DalatF6	-0.31	0.13	0.65	-0.33	0.12	0.67	-0.38	0.13	0.68
DalatC3	-0.41	0.12	0.69	-0.52	0.17	0.59	-0.34	0.12	0.69
DalatG6	-0.54	0.18	0.57	-0.79	0.29	0.39	-0.60	0.21	0.49
DalatB3	1.00	0.45	0.25	1.00	0.75	0.12	1.00	0.40	0.27
DalatH3	-0.60	0.21	0.51	-0.54	0.18	0.56	-0.52	0.17	0.59
DalatB4	-0.43	0.12	0.70	-0.28	0.15	0.66	0.09	0.45	0.33
DalatB5	-0.45	0.14	0.64	-0.51	0.20	0.54	-0.28	0.13	0.68
Mean	-0.25	$4.4^{10\text{^}-6}$*	1.00*	-0.28	$2.4^{10\text{^}-5}$*	1.00*	-0.15	$1.2^{10\text{^}-5}$*	1.00*
SE	0.21	-	-	0.22	-	-	0.21	-	-

F: fixation index; *PI*: probability of genetic identity, *PE*: probability of paternity exclusion and means of combined analysis (*)

According to the linkage disequilibrium test, most loci segregate independently, with no significant association between them, except only for pairs of loci (Dalat G6 X Dalat B3, Dalat G6 X Dalat H3, Dalat G6 X Dalat B4 and Dalat B4 X Dalat B5), for which the linkage disequilibrium was significant even after Bonferroni correction (α = 0.0023809524) (Table 4). The markers developed for *D. alata* may be used in studies on structure, genetic diversity, spatial genetic structure, mapping and kinship studies [26].

Table 4
Linkage disequilibrium between pairs of microsatellite loci of *D. alata*.

	Dalat F6	Dalat C3	Dalat G6	Dalat B3	Dalat H3	Dalat B4	Dalat B5
Dalat F6	-	0.29224	0.24833	0.88476	0.10876	0.50705	0.50376
Dalat C3	-	-	0.19157	0.48395	0.87276	0.75062	0.66348
Dalat G6	-	-	-	0.00019*	0.00043*	0.00043*	0.05476
Dalat B3	-	-	-	-	0.15795	0.09981	0.32557
Dalat H3	-	-	-	-	-	0.35319	0.07900
Dalat B4	-	-	-	-	-	-	0.00005*
Dalat B5	-	-	-	-	-	-	-

Note: p-value is the probability of genotypic disequilibrium after 21,000 permutations of alleles between individuals. Significant values (*) after applying the Bonferroni correction: p = 0.0023809524 (α = 0.05).

The developed microsatellite markers have an expressive power of discrimination and gene diversity. Therefore, these new loci added to the number of SSR markers available for *D. alata* will contribute to a greater random sampling of the genome and, consequently, estimates of genetic variability with greater precision, contributing more effectively for the conservation of this species.

Declarations

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Figures

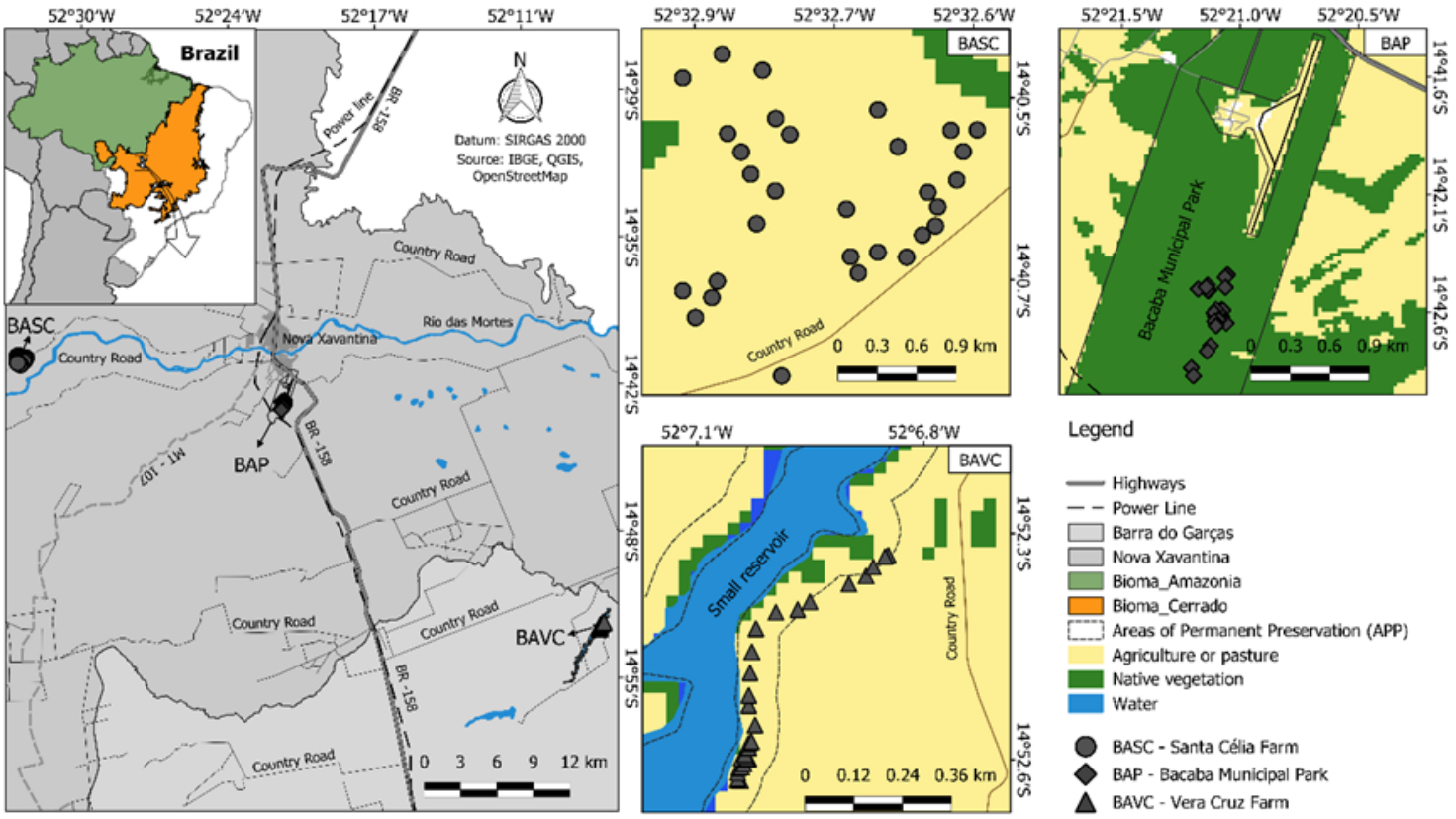

Figure 1

Distribution of the sampled populations of D. alata in the municipalities of Nova Xavantina and Barra do Garças in the state of Mato Grosso. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.