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Use of the Internal Transcribed Spacer (ITS) Regions to Characterize Macaw Progenies

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FCV and MLL designed the study and wrote the protocol. Author MLL performed the statistical analysis and managed the analysis of the study. Authors FCV and AM wrote the first draft of the manuscript. Authors AM and JFG reviewed all manuscript before submitted. Author RVSV reviewed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study aimed to investigate the genetic structure of *A. aculeata* (macaw palm) groups by analyzing individual polymorphism of different ITS regions.
Study Design: Experimental design study.
Place and Duration of Study: Department of Forest Engineering (Universidade Federal dos Vales do Jequitinhonha e Mucuri/MG) and Germoplasm Bank of macaw (Universidade Federal de Viçosa/MG), between February 2014 and December 2015.
Methodology: DNA extracted from 42 leaf samples of Macaw palm. Five universal oligonucleotide primer pairs specific for different internal transcribed spacer (ITS) regions of nuclear ribosomal DNA of Macaw (ITS 1, ITS 2, ITS 3, ITS 4, and ITS 5) used for PCR amplification.
Results: Of 42 analyzed samples, 20 resulted in reproducible bands for primers ITS 1 and ITS 2, 21 for ITS 3 and ITS 4, 24 for ITS 2 and ITS 5, and 22 for ITS 4 and ITS 5. Samples were grouped based on these results.
Conclusion: Therefore, it was possible to identify genetic differences among Macaw progenies based on the quality of ITS sequences.

Keywords: Acrocomia aculeate; genetic variability; phylogeography; ITS regions.

1. INTRODUCTION

Macaw palm (*Acrocomia aculeata, Arecaceae*) is a wild species of palm with exceptional bioenergetic potential that can produce ~25 t/ha of fruits under natural conditions, which could be further processed to produce 4000 kg of vegetable oil with biochemical properties suitable for the cosmetic industry and biodiesel production [1].

The species occurs in the savannahs (Cerrado) and open forests of tropical America, distributed from Central America to southern South America. It is considered one of the most widespread palm trees in Brazil and is found in virtually all regions of the country with various regional names [2]. It has a broad geographic distribution in regions with different soil and climatic characteristics. [3] studied species diversity among natural populations, which further validates the need for specific studies to better characterize *A. aculeata.*

The macaw palm, *A. aculeate* (Jacq.) (Lood. Ex Mart.) – *Arecaceae* (2n = 2x = 30) [4] is an emerging perennial palm native to South America. It is monoecius and self-compatible with androgynous inflorescence, with bears a mixed reproductive system, with a predominance of out-crossing [5-6]. The combination of two pollination strategies (entomophily and anemophily) with flexible reproductive systems (cross-and self- pollination) suggest that A. aculeate can be highly successful in the colonization of new areas, as evidenced by its ample distribution in Brazilian biomes and in the rest of the Neotropics [5-7].

Despite advances in macaw palm research, phylogenetic studies to support a more refined classification of the genus *Acrocomia* are lacking, which could explain the broad classification of all macaw palm ecotypes into a single species, *A. aculeate* [3]. The natural populations of macaw found in Brazil present great phenotypic and genotypic variability. These differences may cause doubts in the taxonomic classification of this plant. From the agronomic point of view, there is consensus among researchers that there are at least three macaw ecotypes with distinct morphological characteristics. The plants that occur mainly in the Center-West and North, classified as *Acrocomia totai*, the plants found in the northeastern states, classified as *Acrocomia intumescens* and the plants found in the southeast, mainly in the state of Minas Gerais, which present larger fruits and larger oil content (up to 65% oil in the dry matter of the pulp). These are classified as *Acrocomia aculeata* or *Acrocomia sclerocarpa* [3].

The genetic structure of a species is directly related to the occurrence of factors such as mutation, selection, genetic drift, inbreeding, and gene flow, which change the distribution and frequency of alleles in space and time. Such changes might promote the formation of different groups within and among populations, depending on characteristics such as population size, reproductive system, and habitat [8-12].

Phylogeography studies analyzing intraspecific patterns of genetic variability distribution based on the relationship between genealogy and geographical distribution of species have advanced evolutionary biology research. These studies provide new insights into the role of gene flow in the genetic structure of plant populations [13] and how populations behave over time, perceive variation patterns in their effective size (Ne), and interpret them from an evolutionary and biogeographic point of view [14].

Access to data on the intra- and interpopulation genetic variability has increased considerably, mainly through DNA [15] and protein polymorphisms [16]. Molecular tools such as dominant and codominant markers, and microsatellites, which are highly polymorphic and have a high mutation, are invaluable for population and phylogeographic studies.

Sequencing of plastid and nuclear regions are examples of analysis that use molecular markers for studying the phylogeny, phylogeography, genetic diversity, and adaptive variation. Among the various nuclear markers in use, the ribosomal internal transcribed spacer (ITS) regions are unique [17-20] and most commonly used in molecular systematics of plants at lower taxonomic levels because of their rapid evolutionary rate and resolution of phylogenetic relationships at various taxonomic levels, in addition to the ease of PCR amplification [21].

Despite being a powerful tool for understanding species population dynamics in the binomial space *versus* time, relatively few phylogeographic studies of Brazilian native plants, especially those of the family *Arecaceae*, have been conducted so far [22]. With this background, the present study aimed to investigate the genetic structure of *A. aculeata* (macaw palm) groups by analyzing individual polymorphism of different ITS regions.

2. METHODOLOGY

2.1 Plant Material and DNA Extraction

Leaf samples of 42 *A. aculeata* progenies were provided by the coordinator of the Active Germplasm Bank of Macaw Palm (BAG-Macaw Palm repository #:084/2013/CGEN/MMA), Prof. Sérgio Yoshimitsu Motoike. Leaf samples were collected in appropriately labeled plastic bags placed inside a container with ice and later stored in a freezer at -80ºC.

The BAG has a collection of *ex situ* plants and is located at the experimental farm of the Federal University of Viçosa in the city of Araponga (20º40′1″ S, 42º31′15″ W), Minas Gerais, Brazil. The progenies originated from seeds collected from the States of Minas Gerais, São Paulo, Mato Grosso do Sul, Paraíba, and Rio Grande do Norte, and have been cultivated since February 2009, with a spacing of 5.0 m × 5.0 m (Table 1).

Table 1. Identification, provenance, latitude, longitude, altitude (Alt.) and number of plants per progeny (Nip) collected germplasm macaw bank

Identification	Provenance	Latitude	Longitude	Alt. (m)	Nip
BGP9	Piranga	20º39.3’34”S	043º18,8’02”W	679	3
BGP10	Montes Claros	-	-	-	3
BGP17	Pereirinhas – Entre Rios de Minas	20º40’32,0”S	044º12’09,6”W	995	3
BGP22	Abaeté – Cedro do Abaeté	19º07’15.9”S	045º51’40.0”W	991	3
BGP47	São Paulo	22º29,2’30”S	050º46,3’36”W	399	3
BGP49	Bocaiúva	-	-	-	3
BGP53	SJDR – Lavras antes trevo de Itumirim	21º17’20.2”S	044º49’15,8”W	930	3
BGP71	Antes de Ibiá – Serra do Salitre	19º19’39.8”S	046º38’14.8”W	861	3
BGP74	Ponte na BR 040 perto do acesso 62	17º57’39.7”S	045º42’11.9”W	712	3
BGP77	Entre Nova Serrana – Conceição do Pará	19º48’05.5”S	044 55 06.0”W	647	3
BGP102	Mato Grosso do Sul	21º42’04.8”S	057º53’39.0”W	-	3
BGP105	Mato Grosso do Sul	21º28’42.3”S	556º10’03.6”W	-	3
BGP112	Macaíba	-	-	-	3
BGP123	Paraíba	-	-	-	3
Total					**42**

Per Koppen's classification, the region where the progenies are cultivated has a rainy temperate climate, with high rainfall in November, December, and January, and is drier in June, July, and August. The mean annual temperature and precipitation are 18ºC and 1,339 mm, respectively. Plants were fertilized based on the soil analysis and recommendations by [2].

DNA was extracted from the leaf samples as described by [23]. Leaves were macerated in liquid nitrogen, transferred to 1.5-mL Eppendorf microtubes, to which 1000 µL extraction buffer (CTAB 2.8%; NaCl 1.4 M; Tris-HCl pH 8.0, 110 mM; EDTA pH 8.0, 55 mM) was added. Next, 100 µL of 20% SDS was added, homogenized lightly, and incubated in a water bath at 65ºC for 60 min. Microtubes were manually inverted every 10 min, and subsequently placed at room temperature (~25ºC) for 5 min. Immediately thereafter, 450 µL of a chloroform:isoamyl alcohol mixture (24:1) was added to the microtubes, which were inverted for 10 min for complete and careful homogenization. The homogeneous solution was centrifuged for 10 min at 13,000 rpm. Approximately 900 µL of the supernatant from each sample was transferred to fresh 1.5-mL microtubes, and the previous step was repeated. Approximately 600 µL of the each resultant supernatant was transferred to fresh 2.0-mL microtubes and 700 µL of ice-cold isopropanol was added.

Samples were stored in a freezer at -20ºC for 2 h for nucleic acid precipitation after which, they were centrifuged at 13,000 rpm for 10 min, and the supernatant of each sample was discarded. The precipitate (pellets) obtained was washed with 300 µL of 70% ethanol (v/v), followed by 300 µL of 95% ethanol (v/v). The pellet was dried at room temperature for approximately 60 min, resuspended in 70 µL of 0.1× TE plus RNase (10 µg/ml) solution, and incubated at 65ºC for 10 min. At the end of the extraction process, samples were stored at -20ºC until further use.

DNA samples were quantified by LAMBDA XLS Spectrophotometer (Perkin Elmer, Waltham, MA) using the absorbance values measured at 260 nm and 280 nm and the quality was assessed by electrophoresis in 0.8% agarose gel stained with ethidium bromide and run at 120 V for approximately 1 hour. Samples were diluted in working solutions to a concentration of 25 ng/µl.

2.2 Condition of Polymerase Chain Reaction (PCR) and Electrophoresis

ITS 1–5 regions of nuclear ribosomal DNA 18S–26S region [24] were amplified. The amplification reaction was conducted in 25 µL volume with 4.0 µL DNA, 2.0 mM $MgCl_2$, 0.5 µL deoxynucleotides, 1.0 µL primers, 10X buffer, and 0.5 U of Taq DNA polymerase enzyme. The PCR reaction was performed in an thermocycler My Cycle (BioRad Laboratories, USA) with an initial step at 94ºC for 5 min followed by 35 cycles, each of 30 s, at 94ºC for 45 s; 61ºC for pairing the primers ITS 2, ITS 4, and ITS 5; 65.6ºC for ITS 1 and ITS 2; 64.2ºC for ITS 3 and ITS 4; and 72ºC for 2 min for copy extension. A final step at 72ºC for 7 min was added to the program.

2.3 Data Analyses

For the analysis of the data, a binary matrix (presence and absence) and the UPMGA (Unweighted Pair Group Method with Arithmetic) method for the construction of the dendrogram were used with the help of Statistical program R [25].

3. RESULTS AND DISCUSSION

Extraction of total genomic DNA from the leaves of 42 progenies of *A. aculeata* was successful. The absorbance analysis, purity, and yield performance by spectrophotometry generated A260/A280 values within the optimum range of 1.8–2.1 [26,27] which indicated the integrity and absence of proteins and other potential contaminants (salts, polysaccharides, and organic compounds such as phenols).

The qualitative analysis of samples by agarose gel electrophoresis indicated that most samples remained intact with no significant signs of degradation, and showed clear bands and small fragmentation, which is normal in "miniprep" extractions (Fig. 1).

DNA integrity is essential for the PCR amplification process, as well as the reproducibility of the obtained products [28]. Therefore, the obtained results of the qualitative analysis of genomic DNA allowed for further molecular analysis. Of the 42 samples analyzed, 20 resulted in reproducible bands for primers of ITS 1 and 2, 21 for ITS 3 and 4, 24 for ITS 2 and 5, and 22 for ITS 4 and 5.

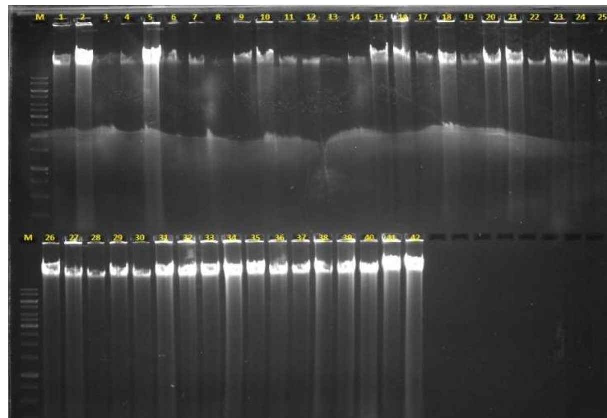

Fig. 1. Quantification of DNA extracted from leaves of 42 *A. aculeata*. The column labeled M refers to the molecular weight marker ladder with 6000, 3000, and 1000 bp. The other gel columns refer to the extracted DNA of 42 *A. aculeata* sample leaves (1–42)

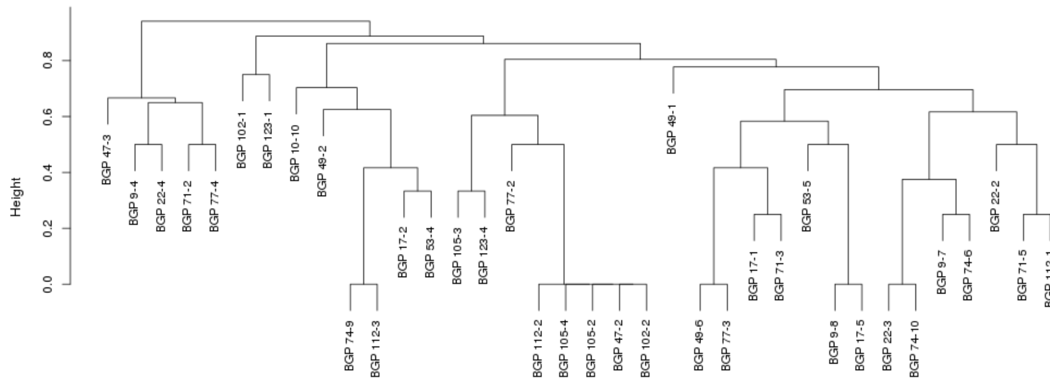

Fig. 2. Dendrogram drawn from the statistical software R of 36 samples of the Germplasm Bank of UFV

Based on the quality of obtained ITS sequences, it was possible to determine genetic differences among the matrices studied. However, no amplification was observed in six Macaw individuals (BGP 10-8, BGP 10-9, BGP 47-5, BGP 53-6, BGP 102-4, and BGP 123-2) with any of the tested primers. This observation indicates there were considerable differences between these individuals.

The dendrogram showed divergent grouping in the geography of the analyzed individuals, with the formation of 20 genetically distant groups. The molecular dissimilarities observed might justify the morphological differences among individuals, and could have contributed to them, which further divides *A. aculeata* species into subspecies. Individuals were grouped according to the botanical descriptors proposed by [3] (Fig. 2).

Some individuals formed less divergent groups than those observed among individuals of the same population, a phenomenon observed in different populations. The cluster formed by matrices BGP47-3, BGP9-4, BGP22-4, BGP71-2, and BGP77-4 showed greater genetic variability in ITS sequences among its members than other groups. However, given its origin in distant geographical regions under different evolutionary forces, the grouping also indicates a genetic similarity that generates closeness. Owing to the similarity and shared morphological characteristics such as compression and height of the arches among the members of this cluster, its distance from other clusters indicates that

such individuals are an ecotype or belong to the same species.

Clusters with a lower genetic distance were formed between BGP74-9 and BGP112-3; BGP112-2, BGP105-4, BGP105-2, BGP47-2, and BGP102-2; BGP49-6, and BGP77-3; BGP9-8 and BGP17-5; and BGP22-3 and BGP74-10. Despite the similarity in these clusters, there is heterogeneity among them. On the other hand, the individual with the highest genetic distance in comparison to others was BGP49-1.

Fewer differences were observed between individuals belonging to populations of different regions perhaps because of ecological issues, particularly where the regions are geographically close. In contrast, natural geographical barriers such as hills can lead to an increased genetic distance among populations [29]. Previous studies demonstrate that climate changes in the past, although milder in the tropics than in temperate regions, had a significant impact on the genetic diversity and geographic distribution of neotropical species [30].

Phylogenetic patterns observed in the dendrogram show that for some populations and/or individuals, the geographical distance is not sufficient for the occurrence of monophyletic groups among them. The low genetic divergence among the clusters is an indication that the separation among them is relatively recent. Further, we can infer that a secondary contact occurred among populations that have remained isolated in the past, as genetically distant individuals exist in the same geographic area (region or access). In addition, the behavior observed for BGP49-1 might be related to a low contemporary gene flow among populations that are historically linked and geographically distant [31].

In summary, the results confirm the existence of a high genetic diversity among and within populations of *A. aculeata*, strengthening the existence of a greater genetic diversity within regions than among them, validating the results reported by [29] in his study of population genetics of Macaw palm.

It is noteworthy that the classification and detailed knowledge of a species allow the exploitation of their potential by man. In the case of plants in the domestication process, such as Macaw palm, this knowledge is extremely important, as it could initiate breeding programs in the species.

4. CONCLUSION

From the results obtained in this study it was possible to conclude that:

- Molecular dissimilarities have been observed that may have contributed to the morphological differences between individuals.
- Less divergent groups were formed among individuals from populations of different regions than observed among individuals from the same population, this may have occurred due to ecological issues.
- Although they have formed clusters with matrices of distance geographical regions can indicate that they have genetic similarity.
- There was low genetic divergence between clusters, which may indicate a relatively recent separation.

Thus, these results confirm the existence of high genetic diversity among and within populations of *A. aculeata*. However, a possible occurrence of natural hybrids among the subspecies found in this study makes it difficult to correctly classify the individuals evaluated.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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