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Genetic and population diversity of bacuri (*Platonia insignis* Mart.) in Chapada Limpa extractive reserve, Maranhão State, Brazil

Edyane Moraes dos SANTOS^{1*}, Carlos Alberto de Sampaio MONTEIRO NETO², Claudio Adriano de Jesus NASCIMENTO², Francisca Helena MUNIZ³ and José de Ribamar Silva BARROS³

¹Genetics and Molecular Biology Laboratory, Maranhão State University, LabWick, Paulo VI University Campus, P. O. Box 09, CEP: 65055-970, São Luiz, Maranhão, Brazil.

²Agronomic Engineering, Maranhão State University, Paulo VI University Campus, PO Box 09, CEP: 65055-970, São Luiz, Maranhão, Brazil.

³Chemistry and Biology Department, Maranhão State University, Paulo VI University Campus, P. O. Box 09, CEP: 65055-970, São Luiz, Maranhão, Brazil.

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Landscape studies illustrate how the structure and configuration of ecological/agroecological zones influence the genetic diversity of a species. Thus, the aim of the present study is to assess how landscape structure and conservation influence the genetic diversity indices of *Platonia insignis* Mart. (bacuri) in Chapada Limpa Extractive Reserve, Chapadinha County, Maranhão State, Brazil. The study was carried out in an extractive reserve, whose surroundings are mapped for land use and occupation, as well as for the collection of bacuri biological materials used in genetic analysis with ISSR markers. The study shows there is prevalence of good preservation state and secondary vegetation, as well as a well-preserved Cerrado area, *lato sensu*. Genetic differences were higher within populations (85.2%) than between populations (14.7%). According to the genetic diversity index, there was high genetic diversity and heterozygous allele richness. This may ensure population viability. Based on the present results, it is possible to say that the genetic differentiation of *bacuri* may have suffered direct consequences from the intensive wildfire events and/or from primary vegetation reduction.

Key words: *Platonia insignis*, mapping, landscape, genetic diversity.

INTRODUCTION

Bacuri (*Platonia insignis* Mart.), belonging to the division Magnoliophyta, Class Magnoliopsida, order Malpighiales and family Clusiaceae, is the only species of the genus *Platonia* (Cavalcante, 1996). It is a fruitful tree distributed in Maranhense Amazon Region (Souza et al., 2000) and natural to the Amazon (Cavalcante, 1996; Moraes et al., 1994). It is also naturally found in other Brazilian Amazonian states (Loureiro et al., 1979; Cavalcante,

1996). It stands out for its economic relevance in the Amazonian region due to its management and sustainable development (Alvarez and Potiguara, 2013).

Bacuri trees are found in areas with intense agricultural activities in Maranhão State, where deforestation and wildfire events are common. Consequently, the number of plants has been rapidly reducing, a fact that leads to decreased genetic variability (Carvalho et al., 2009). The

aforementioned species went from an unimportant fruit tree to a timber tree of interest. Besides, its fruit became valuable. Leafy Bacuri trees were cut down over centuries for wood extraction, for use in the construction industry, and for mainly artisanal shipbuilding in the Amazon (Homma et al., 2007). Therefore, gradual Cerrado vegetation losses and bacuri clearing led to great damage to the species genetic diversity.

Inter Simple Sequence Repeats (ISSR) (Reddy et al., 2002; Zietkiewicz et al., 1994) molecular marker was adopted in a microsatellite-based method. It does not require having prior knowledge about the genome or about the first clone design. Although ISSRs are dominant markers, they are useful in multiple loci analysis applied to a single reaction (Goulão and Oliveira, 2001). They have also been used to estimate the inter- and intra-specific genetic diversity of a wide variety of species (Zietkiewicz et al., 1994). ISSR molecular marker appears as a power tool for analyzing genetic diversity, as well as characterizing many species (Charters and Wilkinson, 2000; Isshiki et al., 2008).

Bacuri species diversity in forests is relevant to local communities that make their living on selling bacuri fruit pulp. Such scenario is one of the herein assessed study objects, since the aim of the present research is to investigate the conservation status of Cerrado areas wherein bacuri trees are distributed, as well as Cerrado surrounding areas and bacuri genetic diversity rates. Chapadinha County is surrounded by the Amazonian forest and large soybean plantations (Nogueira et al., 2012; Loch and Muniz, 2016). The conservation unit was launched in 2007 for sustainable use (Filho, 2014).

The diversity of bacuri species in the forests is of great importance to the local communities that live on selling the fruit pulp. The main goal of the present study is to set the genetic structure of bacuri trees and the species' distribution pattern at Chapada Limpa Extractive Reserve, Chapadinha County, East Maranhão State.

MATERIALS AND METHODS

P. insignis leaf tissue samples were collected. Samples from two subpopulations were divided into two groups (15 individuals per group) (Figure 1). The minimum space between individuals in each group was 15 m; ten leaves were collected from each individual. The samples were kept in silica gel until the DNA was extracted and the genetic analysis was performed. The research was authorized by Sisbio (no. 50329-1) and by the competent ICMBio-MA authority responsible for the extractive reserve.

Landscape analysis

Each collected individual was georeferenced for further landscape

OpenLayers plug-in, at high resolution images (1×1 m) and 10% cloud coverage, on average, in all the study site fractions. The composition analysis. The extractive reserve mapping was performed based on the coordinates in the Quantum GIS Software 1.8 (QGIS) (Sherman et al., 2011). This was done with the aid of the mapping scale was defined in the QGIS and set at 1:10.000. The mapping aimed at finding the land use and occupation state of bacuri trees in different areas, as well as in Chapada Limpa Extractive Reserve surroundings.

DNA extraction, polymerase chain reaction (PCR), ISSR markers and data analysis

Leaf tissue DNA samples were extracted from 30 *P. insignis* samples. Tissue maceration was performed in liquid nitrogen. The genomic DNA extraction was conducted based on the protocol by Doyle and Doyle (1987), using approximately 1 g of dry leaves from each sample. After the extraction procedure was over, the DNA was stained in ethidium bromide, and subjected to electrophoresis in 1% agarose gel. Next, DNA was diluted to 5 ng/μl, in 100 μl for further PCR, according to the method of Mullis and Faloona (1987) and Saiki et al. (1985).

Twelve ISSR marker loci were amplified and the ISSR primers were previously selected and developed according to Souza et al. (2013) (Table 1). The amplification products were separated through horizontal electrophoresis, in 1.5% agarose gel, for 90 min; subsequently, they were stained in ethidium bromide.

The PCR reactions were performed in final volume (20 μL) of a mixture containing the following components: PCR buffer [13.5 mM Tris-HCl (pH 8.3); 67.5 mM KCl; 2.0 mM MgCl₂], 200 μM of each one of the four deoxyribonucleotides (dATP, dCTP, dGTP and dTTP), 0.7 μM primers, 1 U Taq DNA polymerase, 5 ng of genomic DNA and water were amplified in Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems), which was programmed as follows: 95°C for 10 min, 35 cycles [95°C for 1 min; 50 to 62°C (depending on the used primer) for 45 s and 72°C for 2 min]; and 72°C for 5 min.

The amplified products were designated as a single character, which had its presence represented by "1" and absence by "0". The ISSR markers were converted into a binomial matrix (0/1). Assumingly, all loci were dominant in all the performed analyses and in the Hardy-Weinberg equilibrium. The genetic relation between genotypes was estimated through the Jaccard's coefficient, which resulted in a similarity matrix. The compliance between the similarity matrix and the dendrogram was estimated through the cophenetic correlation coefficient (*r*), according to Sokal and Rohlf (1962). This dendrogram was found through the Unweighted Pair Group Method by using arithmetic means (UPGMA) to illustrate the relation between the two collected populations. All calculations were made in the PAST software, version 1.34 (Hammer et al., 2001).

The genetic structure of the population (Heterozygosity-He), as well as the genetic differentiation values of a single population or between populations (Fixation index-FST) (Weir and Cockerham, 1984) were analyzed in the Arlequin 3.11 software (Excoffier et al., 2005). The genetic bottleneck test was run in the Bottleneck 1.2 software (Cornuet and Luikart, 1997) in order to check whether there was recent or effective decrease in the populations through the infinite allele models (IAM) and step-wise mutation model (SMM). It is recommended to apply these analyses to ISSR marker

*Corresponding author. E-mail: edyanemoraes@hotmail.com. Tel: +55 (98) 8117 5376.

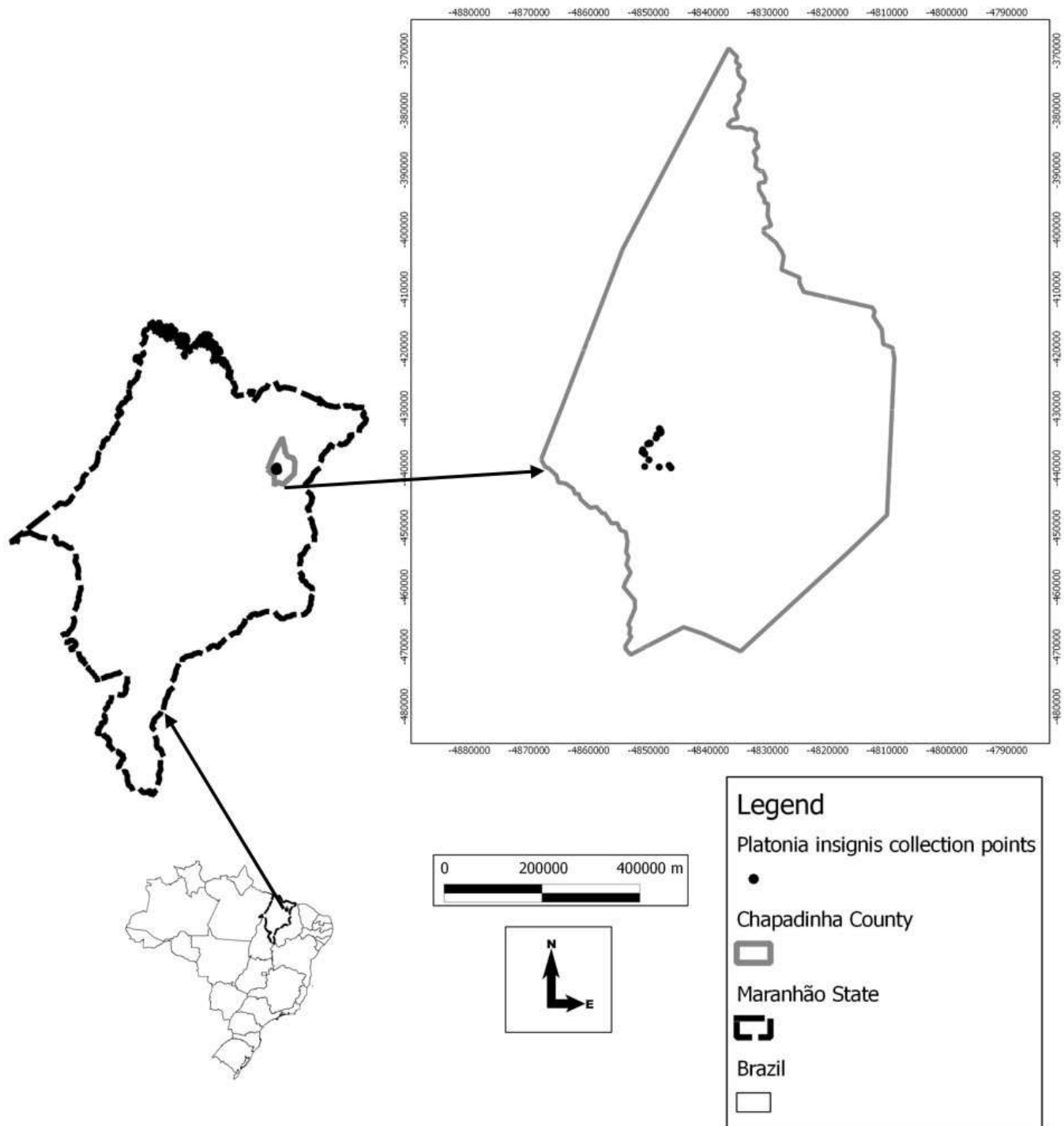


Figure 1. Chapadinha and Chapada Limpa Extractive Reserve location in the Maranhão State and *Platanus insignis* collection points.

data. $H_e > H_{eq}$ hypothesis was tested; wherein: H_e is the expected heterozygosity under the Hardy-Weinberg equilibrium; and H_{eq} is the expected heterozygosity under balance of mutation-drift ($P < 0.0001$).

The Shannon index analyzed through Fingerprint Analysis with Missing Data 1.31 (FAMD) was another diversity measure used in the present study (Schlüter, 2013). It measures the uncertainty

degree of predictions made to the species of a randomly chosen individual belonging to a sample with S species and N individuals. The analysis of molecular variance (AMOVA) was also used to show the genetic diversity distribution within and between populations. The total genetic diversity recorded through the analysis was split into two distinct hierarchical levels, namely, differences between populations and between individuals within a

Table 1. List of the 15 primer loci, their sequences and amplification conditions tested in *Platonia insignis*.

Primer	Annealing temperature (°C)	Sequence 5'-3'
UBC 807	53	(AG)7GT
UBC 808	54	(AG)8C
UBC 809	54	(AG)8G
UBC 810	53	(GA)8T
UBC 811	54	(GA)8C
UBC 817	53	(CA)8A
UBC 825	54	(AC)7A
UBC 826	59	(AC)8C
UBC 828	54	(TG)8A
UBC 829	52	(TG)8C
UBC 834	53	(AG)8YT
UBC 840	54	(GA)8YT

single population. AMOVA was performed according to Excoffier et al. (1992), with the aid of Arlequin and FAMD software.

All the steps in the bacuri genetic diversity analysis were carried out at Warwick Kerr (LabWick) Genetic and Biology Laboratory at Maranhão State Univesity.

RESULTS

Land use and occupation

The 67.3% prevalence of secondary vegetation was followed by 10.29% advanced vegetation and 9.21% initial vegetation (Figure 3). The Cerrado and bacuri forest area covered 4.59% of the map. This category can be classified as "Cerrado *stricto sensu*". The expectation was to have 9.21% of the entire mapped area to be covered with "advanced vegetation", which consisted of Cerrado areas, with high conservation degree. The "cultivation/agricultural area" accounted for only 0.08% of the total area (54,471 thousand hectares), although Maranhão State Cerrado is currently one of the most used biomes for extensive agriculture (Table 2).

Some of these land use and occupation formations were herein described. The analysis of classes followed the description by Ribeiro and Walter (2008) due to Resex landscape heterogeneity. The main formations found in the mapping process are as follows:

(1) Forest formations: This included vegetation types dominated by tree species of continuous canopy formation known as "advanced vegetation". It is possible to include the "secondary" and "shrub" vegetation classification in the phytophysiological category.

(2) Dry Forest: Formation type is defined as "initial vegetation" according to the present classification. The "pasture + shrub vegetation" and "pasture" (predominantly formed by low growing vegetation such as grass) classes were also included in the phytophysiological category.

(3) Cerrado *stricto sensu*: It is characterized by the presence of low, tilted, tortuous trees with irregular and twisted branches, often showing signs of burning.

Genetic analysis of the ISSR markers

AMOVA results (Table 3) of the genetic structure of the population showed high genetic diversity within populations (82.2%) and low diversity between populations (14.7%). The ϕ_{ST} and F_{ST} indices were used to measure the genetic distance in the population; they were run in different software (FAMD and Arlequin). Significant genetic differences were found through the FAMD calculation: $\phi_{ST}=0.17$, $P < 0.001$; and $F_{ST}=0.147$ (analyzed in the 3.5 Arlequin software). The genetic distance index calculated in the Arlequin was 0.17 for ϕ_{ST} and 0.28 for F_{ST} .

With regard to the genetic diversity index, heterozygosity variation (H_e) was found to be 0.133 to 0.533 (mean - 0.333) in population 1 and 0.133 to 0.514 (mean: 0.388) in population 2. The index showed variation from 0.163 to 0.393 in the comparison between the two populations. As for the arboreal species, this genetic diversity index set by Nei remains high; however, it can indicate small population difference and diversity loss in the short-term. When it comes to the bottleneck, H_e higher than H_{eq} in most of the loci was recorded in both models (I.A.M. and S.M.M.), except for loci UBC 817 and UBC 825, which presented higher H_{eq} values. The over heterozygosity in both models showed possible population decrease in the short-term (Table 4).

The Shannon H' diversity index calculated in the FAM software is commonly used in ecological studies to indicate species diversity per area. The Shannon index of the general population in the current study was $0.011 \leq 0.106$. This index was $0.030 \leq 0.175$ in population 1 and $0.041 \leq 0.204$ in population 2. Besides, the heterozygosity variation in the population ranged from

Table 2. Chapada Limpa Extractive Reserve land use and occupation classes and its surrounding areas, Chapadinha County, Maranhão State, Brazil.

Class	Hectares	%
Water	5.032,4	0.09
Growing area/agriculture	4.851,9	0.08
Babaçu (<i>Attalea speciosa</i>) + shrub vegetation	14.833,10	2.72
Cerrado/Bacuri forest	24.964,20	4.59
Local roads (dirty or not)	10.177,79	1.96
Pasture	74.290	0.13
Pasture + shrub vegetation	9.630,21	1.78
Residences	4.076,9	0.07
Exposed soil	15.499,9	0.28
Shrub vegetation	81,572.6	1.5
Advanced vegetation	56,043,11	10.29
Initial vegetation	50,205,07	9.21
Secondary vegetation	36.701,580	67.3
Total	54.471.555	-

Table 3. AMOVA values for *Platonia insignis* populations at Extractive Reserve Chapada Limpa, Chapadinha County, Maranhão State.

Variation source	Square sources	Variance components	Variation rate
Between populations	0.800	0.37	14.77273
Within populations	60.667	2.16667	85.22727
Total	68.467	2.54222	-
FST	0.14773	-	-
φST	0.17192	-	-

0.133 to 0.533 in population 1, but from 0.133 to 0.514 in population 2. However, the index ranged from 0.163 to 0.393 in the comparison between the two populations. The similar Jacard coefficient values calculated in the distance matrix ranged from 0.818 to 0.111, thus indicating high and low correlations between individuals. The dendrogram showed 10 small clusters demonstrating clusters comprising individuals belonging to populations 1 and 2 in separate groups, as well as clusters comprising individuals belonging to both populations in a single group (Figure 2).

DISCUSSION

Land use and occupation

Well preserved natural classes were found in Chapada Limpa Extractive Reserve; however, the frequent wildfire events are an eminent danger for the region. Morelli et al. (2009) emphasized that the use and occupation restrictions promoted by protection area (PA) implementation do not prevent wildfire to occur within these areas. Gerude (2013) stated that, despite the

protective nature of these spaces, protected areas (UCSf, USEs and TIs) account for 19.5% of the wildfire outbreaks in Maranhão State. According to Gerude, there were 29 annual wildfire outbreaks in the reserve, between 2008 and 2012. Primary and secondary vegetation replacement has become a major factor, mainly in areas of agricultural vocation of mechanized or family-based nature. Areas holding regenerating vegetation (secondary vegetation) were found in the mapping performed by Sano et al. (2008), mainly, in transition regions of the Amazonian biome; the region mapped in the present study is located right in this ecotone area.

Landscape heterogeneity at Extractive Reserve Chapada Limpa presents different phytophysiognomies, including the “Cerrado *lato sensu* class”, as well as secondary vegetation and coconut groves. According to Conceição and Castro (2009), Maranhão State has approximately 10.000.000 hectares of Cerrado area, and it represents 30% of its territorial extension, and 5% of the Brazilian total Cerrado area. The area has low-fertility and great soil variation, as well as high acidity; it has little water content. It is predominantly mechanized due to agricultural purposes.

Table 4. Bottleneck Test and Nei's diversity indices for *Platonia insignis* populations at Extractive Reserve Chapada Limpa, Chapadinha County, Maranhão State.

Locus	Bottleneck Test			Nei's diversity indices				
	He	I.A.M.	S.M.M.	He	He	Mean	s.d.	Tot. Het.
		Heq	Heq	Pop 1	Pop 2			
UBC 807	0.480	0.254	0.286	0.533	0.342	0.438	0.134	0.480
UBC 808	0.370	0.249	0.293	0.247	0.476	0.161	0.161	0.370
UBC 809	0.515	0.245	0.287	0.533	0.514	0.523	0.013	0.514
UBC 810	0.517	0.246	0.285	0.533	0.533	0.533	0.000	0.517
UBC 811	0.370	0.235	0.283	0.476	0.247	0.361	0.161	0.370
UBC 817	0.067	0.250	0.289	0.000	0.133	0.066	0.094	0.066
UBC 825	0.067	0.248	0.284	0.133	0.000	0.066	0.094	0.066
UBC 826	0.460	0.245	0.284	0.000	0.476	0.238	0.336	0.459
UBC 828	0.480	0.243	0.282	0.247	0.514	0.380	0.188	0.480
UBC 829	0.370	0.251	0.285	0.247	0.476	0.361	0.161	0.370
UBC 834	0.508	0.230	0.287	0.514	0.419	0.466	0.067	0.508
UBC 840	0.517	0.249	0.292	0.533	0.533	0.533	0.000	0.517
Mean	0.393	0.245	0.286	0.333	0.388	0.361	0.039	0.393

He, heterozygosity variation; Heq, expected heterozygosity; IAM, infinite allele models; SMM, step-wise mutation model.

Genetic analysis of ISSR markers

There are different ways to quantify genetic diversity, namely, number of alleles per locus, polymorphic loci percentage; expected heterozygosity (Nei's diversity indices), which is the proportion of heterozygotes expected for a population in Hardy-Weinberg equilibrium (Freeland, 2005) and Shannon index. Souza (2011) recorded F_{ST} difference 0.28 in his studies about bacuri populations in Maranhão and Piauí states. Pontes et al. (2015) found ϕ_{ST} 0.05 ($P \geq 0.001$), 6% variation between progenies and 94% (AMOVA) genetic variation within progenies of bacuri tree populations collected in Marajó Island, Pará State. The Nei (1973) gene diversity index represents population heterozygosity (He); it generates values between 0 and 5 for each locus. Souza (2011) compared results of Nei index and found genetic diversity (He) values ranging from 0.082 to 0.323 between populations; the rate was 0.335 at species level. According to Hamrick and Godt (1989), the mean genetic diversity in perennial arboreal species was 0.140, and the population heterozygosity in the species was consistent with "sapucaia" (*Lecythis pisonis*), which recorded 0.32 (Borges, 2015); however, the Shannon index was $H = 0.47$ in Borges' study. These results suggest that, although the genetic distance value indicated significant differences in the populations, they show high genetic diversity.

The excess of heterozygotes in the population of 10 and 12 loci in the Bottleneck results show recent genetic bottleneck, according to Luikart et al. (1998). The natural or generated wildfires are the main threats for Resex areas, as evidenced by data from our mapping, because fire leads to the loss of individuals and alleles in the

population. Deshpande et al. (2001) assure that the explanation for the large number of loci presenting over heterozygosity lies on ISSR markers that tend to generate more polymorphism than other loci. Thus, according to Luikart et al. (1998), recent genetic bottleneck processes, such as that in Resex populations may have been caused by constant wildfires that reduce the effective population.

Theoretically, F_{ST} (ϕ_{ST}) values may range from 0 (no genetic divergence) to 1 (allele fixation); however, the herein observed value was much lower than 1. Wright (1965), for example, suggested the following values in F_{ST} interpretation guideline: from 0 to 0.05, little genetic differentiation; from 0.05 to 0.15, moderate genetic differentiation; from 0.15 to 0.25, great genetic differentiation; and values above 0.25 mean excellent genetic differentiation. The ϕ_{ST} and F_{ST} results (Table 3) indicate that the geographic isolation of bacuri populations in Chapada Limpa Extractive Reserve may be due to constant wildfire events and to loss of individuals in the population, as well as to the species' biology, which has led the population to great genetic differentiation.

The F_{ST} value represents accelerated genetic differentiation process in *P. insignis* populations, and it may lead to genetic isolation in the short-term. Another factor possibly contributing to these results was the use of different bacuri varieties at harvest time. However, some subpopulations may have had their effective population size (N_e , which is defined by the size of an idealized population able to generate the same amount of inbreeding or allele variation in the same frequencies observed in the assessed population) reduced

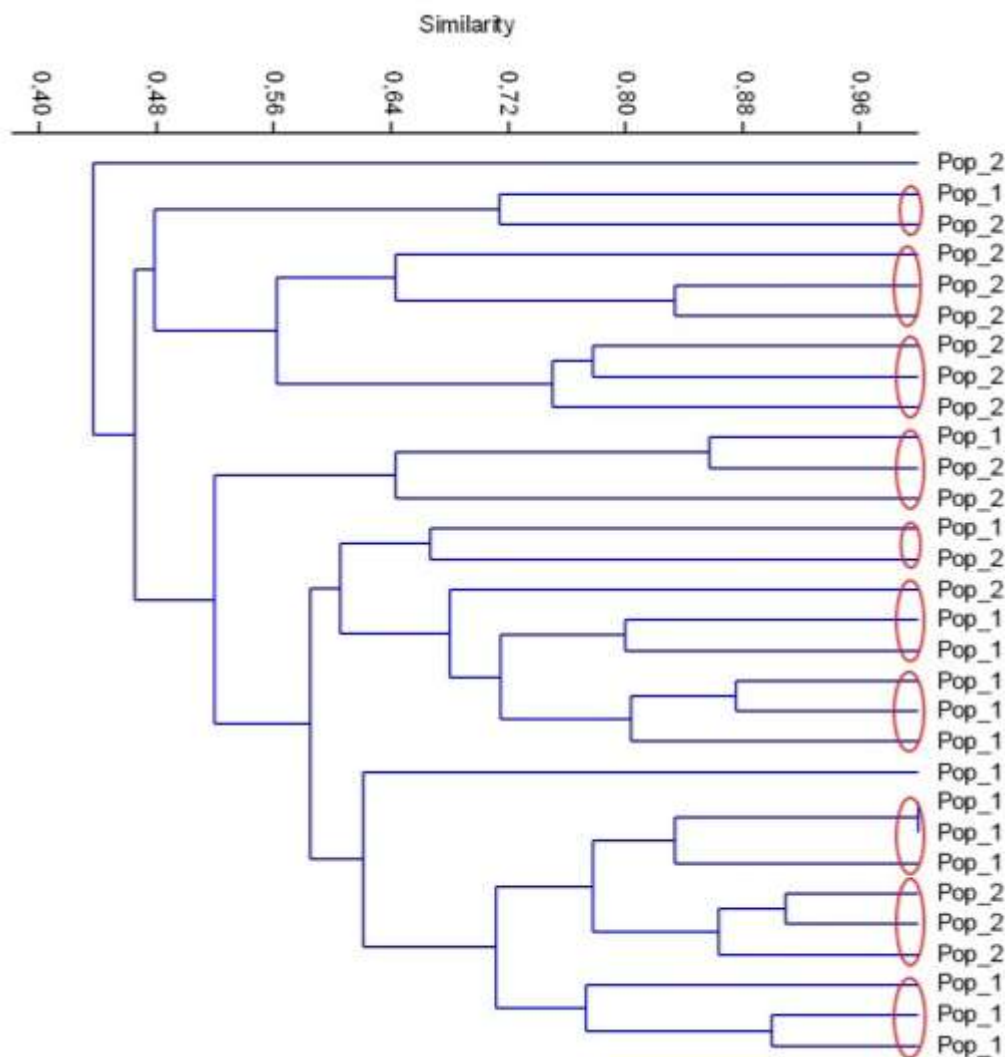


Figure 2. Dendrogram generated in the Past software showing the similarity between individuals belonging to the *Platonia insignis* populations at Chapada Limpa Extractive Reserve, Chapadinha County, Maranhão State.

(Caballero, 1994; Crow and Kimura, 1970; Kimura and Crow, 1963). Therefore, the effective population mainly consists of individuals in reproductive maturation process; hence, the greater the genetic distance, the lower the effective population and the greater the change in the population inbreeding. According to Falconer (1960) and Mettler and Gregg (1973), inbreeding means the mating between kin individuals; however, it depends on the size of the population. Thus, the smaller the population and more isolated it is, the greater the population's possibility to inbreed.

The Shannon index is not based on the population heterozygosity, but on the phenotypic frequency of the amplified fragment (presence or absence of the band) in the population (Yeh et al., 1995; Moura, 2003; Goulart et al., 2005). This index generates values from 0 to 0.73 in a logarithmic scale (Lowe et al., 2004). The present data

corroborate those found by Souza (2011), who estimated the Shannon diversity index (H') and found variation from $0.120 \leq H' \leq 0.480$ and classified the population as presenting high diversity indices.

The Jacard's similarity values which are similarity indices for binary data generated a similarity matrix and a dendrogram by comparing individuals in a single population to each other. The dendrogram (Figure 2) revealed 10 small clusters. The cluster analysis is an efficient method to measure bacuri forest clusters, because it illustrates similarities and/or differences between half-sibling progenies and different provenances. Souza (2011) also found genotype sub-clusters wherein half-sibling genotypes were grouped into one subgroup, or into a small number of subgroups. However, Sanches et al. (2015) found Jacard's genetic similarity values between 0.33 and 0.93 in Pará State

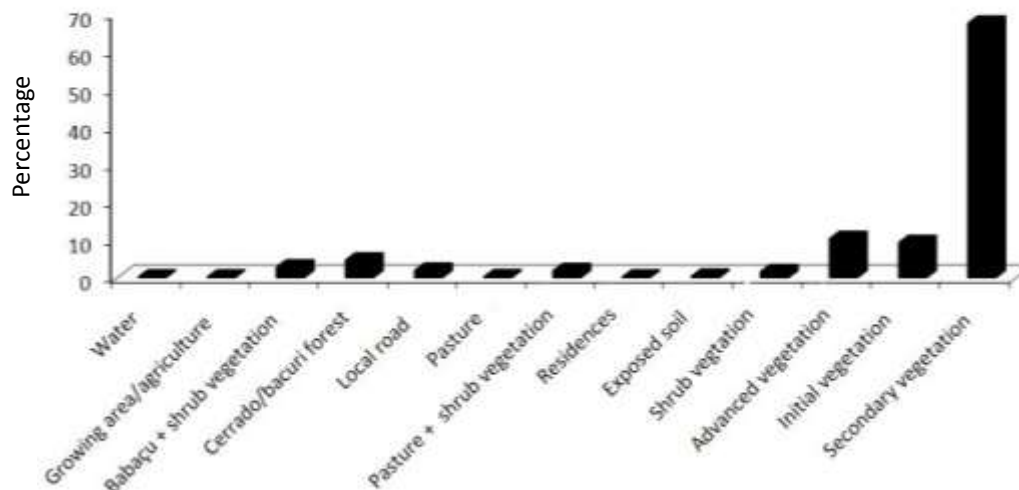


Figure 3. Land use and occupation percentage in Chapada Limpa Extractive Reserve – Chapadinha County, Maranhão State.

progenies. The study by Almeida et al. (2007) stands out among the few on the RAPD molecular marker of bacuri populations in Northern and Northeastern Brazil selecting germplasm genotypes from populations in Pará, Maranhão and Ceará states. These authors evidenced that Maranhão State has the highest degree of polymorphism among other states (93.5%) and indicated the high genetic diversity degree of the species.

Results evidenced that bacuri gradual genetic differentiation at Chapada Limpa Extractive Reserve, even at high genetic diversity may have suffered direct consequences from the intense wildfire events and/or from primary vegetation reduction. The loss of individuals in the population may lead to the loss of heterozygous alleles and to population inbreeding processes that may create genetic bottlenecks. Although Cerrado is a fire-resistant biome, the constant wildfire events of unknown cause may affect soil quality, genetic variability, as well as bacuri production and reproduction in the forest.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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