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Genetic diversity in natural populations of mangaba in Sergipe, the largest producer State in Brazil

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ABSTRACT. Mangaba (*Hancornia speciosa* Gomes) is found in areas of coastal tablelands in the Brazilian Northeast and Cerrado regions. This species has been subjected to habitat fragmentation that is mainly due to human activity, and requires conservation strategies. The aim of this study was to analyze the structure and inter- and intrapopulation genetic diversity of natural populations of *H. speciosa* Gomes using inter-simple sequence repeat (ISSR) molecular markers. A total of 155 individuals were sampled in 10 natural populations (ITA, PAC, IND, EST, BC, PIR, JAP, BG, NEO, and SANT) in the State of Sergipe, Brazil. Fifteen primers were used to generate 162 fragments with 100% polymorphism. Genetic analysis showed that the variability between populations (77%) was higher than within populations (23%). It was

possible to identify five different groups by the unweighted pair group method with arithmetic mean and principal coordinate analysis, and only one individual (E10) remained isolated. Using ISSR markers it was possible to obtain a molecular profile of the populations evaluated, showing that these markers were effective and exhibited sufficient polymorphism to estimate the genetic variability of natural populations of *H. speciosa* Gomes.

Key words: Genetic variability; *Hancornia speciosa* Gomes; ISSR marker; Species preservation

INTRODUCTION

Mangaba (*Hancornia speciosa* Gomes, Apocynaceae) is a fruit tree species that is native to Brazil, and has social, economic, and cultural importance in the areas where it occurs. Sergipe State is the largest producer of this fruit, which is found in natural populations and is exploited almost entirely in an extractive way.

The fruit of this plant is popular due to its organoleptic characteristics and high nutritional value, being rich in the vitamins A, B1, B2, and C, as well as phosphorus, calcium, and proteins (Barros et al., 2006). Its pulp is the main product, and it is consumed *in natura* or as raw material for the manufacture of products such as jam, ice cream, juice, jelly, sauce, wine, and vinegar (Costa et al., 2011). Consequently, the species has aroused the interest of the industry and trade sectors (Ganga et al., 2010).

In the Brazilian Northeast, the extraction of genetic resources undergoes accelerated genetic erosion due to large real estate pressure on the coastal lowlands (Santos et al., 2010). Therefore, studies on the genetic diversity and structure of the remaining populations of mangaba are extremely important, in order to find alternatives for the species' preservation (Amorim et al., 2015). In addition, the domestication and incorporation of this species into production systems is directly related to knowledge of the range and distribution of genetic variability in natural populations (Costa et al., 2011).

One way of evaluating genetic diversity is by using molecular markers, which are defined as DNA-identifiable sequences specific to the genome, and provide information on genetic variability by eliminating environmental effects. Few studies have used molecular markers to describe this variability in natural populations of mangaba (Silva et al., 2012b). Among the different molecular markers, inter-simple sequence repeats (ISSRs) are widely used to assess genetic diversity in plants (Liu et al., 2011). They are dominant markers, are based on the amplification of DNA regions by polymerase chain reaction (PCR), and combine many of the advantages of amplified fragment length polymorphism markers and microsatellites. They are highly polymorphic, reproducible, and do not require prior knowledge of the genome. Moreover, they are relatively cheap (Souza et al., 2004). ISSR markers are important tools for the analysis of genetic diversity, as well as for the characterization of accessions and cultivars of several species.

Morales et al. (2011) evaluated the genetic divergence of 11 strawberry cultivars, and found that this type of molecular marker is effective in the formation of groups in the study of genetic diversity. Dias et al. (2015) used nine ISSR markers in early and erect cowpea genotypes, and concluded that these markers were effective in estimating genetic variability.

ISSRs used in a study on the genetic diversity of *Elaeis guineensis* were effective in detecting genetic variability in this species (Chagas et al., 2015).

Using microsatellite markers in mangaba, Amorim et al. (2015) estimated the divergence and genetic structure of natural populations in the Brazilian Northeast, and found low genetic diversity in populations in Sergipe State. Therefore, the objective of this study was to estimate genetic variability in 10 natural populations of mangaba in the State of Sergipe using ISSR markers.

MATERIAL AND METHODS

Sampling, collection, and DNA extraction

Young leaves were collected from 155 individuals in 10 natural populations of mangaba in the State of Sergipe (Figure 1 and Table 1).

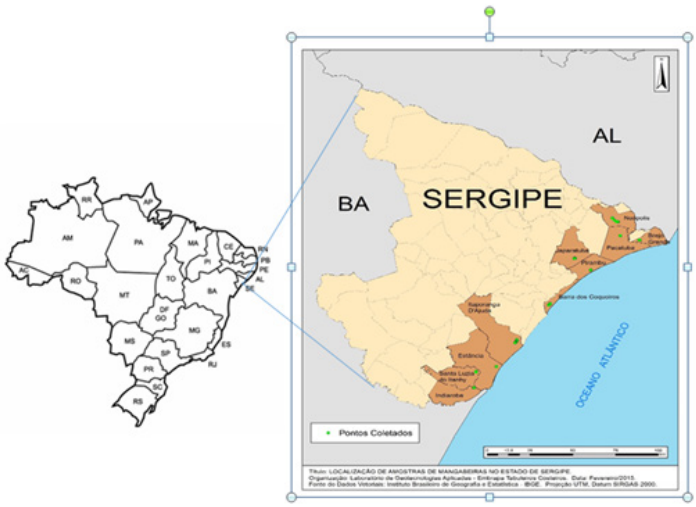

Figure 1. Location of natural populations of *Hancornia speciosa* Gomes in the State of Sergipe, Brazil, where samples were collected for the analysis of structure and genetic diversity.

Table 1. Identification, geographical location, and number of *Hancornia speciosa* Gomes samples collected in Sergipe, Brazil.

Population	Geographical coordinates	No. of individuals	Code
Itaporanga	11°8'30"S and 37°11'18"W	19	Ita
Pacatuba	10°29'35"S and 36°32'42"W	20	Pac
Indiaroba	11°26'25"S and 37°24'30"W	20	Ind
Estância	11°18'20"S and 37°17'42"W	20	Est
Barra dos Coqueiros	10°54'21"S and 37°1'18"W	20	Bc
Pirambu	10°40'15"S and 36°47'27"W	19	Pir
Japaratuba	10°36'52"S and 36°52'10"W	5	Jap
Brejo Grande	10°27'45"S and 36°38'14"W	15	Bg
Neópolis	10°22'19"S and 36°39'22"W	15	Neo
Santa Luzia	11°19'25"S and 37°24'12"W	2	Sant
Total		155	

Leaves of each individual were collected separately, and were ice-packed in order to prevent oxidation. Subsequently, they were transported to the Molecular Biology Laboratory of Embrapa Coastal Tablelands, Aracaju, SE, Brazil. Samples remained in the freezer at -80°C until genomic DNA extraction. DNA extraction was conducted using the method described by Doyle and Doyle (1990) and modified by Alzate-Marin et al. (2009). DNA quantification was performed using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, USA). Samples were stored at -20°C for subsequent use in PCRs.

PCR amplification

Fifteen ISSR primers obtained from the University of British Columbia, Vancouver, Canada, were used to estimate the genetic diversity of the 155 mangaba individuals (Table 2).

For the PCR, the total volume of the reaction was 20 µL, which contained 2 µL genomic DNA solution, 2 µL of each primer with a compound mix of 2 µL 10X PCR buffer, 0.4 µL dNTP (10 mM), 0.6 µL $MgCl_2$ (50 mM), 0.2 µL *Taq* DNA polymerase (5 U/µL; Invitrogen), and 12.8 µL ultrapure water. For reaction amplification, the thermal cycler (ProFlex™, Applied Biosystems, USA) was programmed so that the samples were denatured at 95°C for 5 min followed by 45 amplification cycles. At each cycle, the samples underwent denaturation at 94°C for 1 min, annealing at different temperatures for 45 s, and a final extension at 72°C for 2 min.

The result of the amplification was subjected to horizontal electrophoresis on 2% agarose gels. The gels were then placed in contact in a solution containing ethidium bromide (0.5 µL/mL water) for 1 h, and visualized under ultraviolet light. For the measurement of fragment patterns, we used a 1-kb molecular weight marker (Promega, Madison, SD, USA). Visualization of the results was conducted using a Gel Doc L-Pix photodocumentation device (Loccus Biotecnologia, Cotia, SP, Brazil).

Data analysis

The ISSR fragments were converted into a binary matrix based on the presence (1) or absence (0) of fragments. Data were imported into the TreeView software (Page, 1996), and the distribution of genetic variability in each population was estimated based on the Shannon index (*I*), the expected heterozygosity (H_E), and analysis of molecular variance (AMOVA). The same software was used to estimate genetic distances according to the Jaccard coefficient, and the respective dendrograms were constructed based on the unweighted pair group method with arithmetic mean (UPGMA).

The consistency of the clustering was analyzed by bootstrap resampling (10,000 iterations). Principal coordinates analysis (PCoA) was conducted using the GenALEx 6.3 software (Peakall and Smouse, 2006), which was also used to estimate *I* (Brown and Weir, 1983) and H_E (Lynch and Milligan, 1994).

RESULTS AND DISCUSSION

Of the 15 primers tested, 162 fragments were amplified with 100% polymorphism, showing high genetic variability between the populations. This may have been due to the allogamy of this species that results in self-incompatibility, and thus makes plants derived

from seeds highly divergent from each other in relation to the parent plant (Darrault and Schlindwein, 2006). The number of fragments ranged from five (UBC 810) to 22 (UBC 817) (Table 2).

Table 2. Inter-simple sequence repeat (ISSR) primers used in natural populations of *Hancornia speciosa* Gomes from the State of Sergipe, Brazil, with their respective sequences, annealing temperatures, total fragments, polymorphic fragments, and polymorphism percentages (PP).

ISSR primer	Sequence (5'-3')	Annealing temperature (°C)	Total fragments	Polymorphic fragments	PP (%)
UBC 807	AGA GAG AGA GAG AGA GT	50.4	7	7	100
UBC 809	AGA GAG AGA GAG AGA GG	57.2	12	12	100
UBC 810	GAG AGA GAG AGA GAG AT	50.4	5	5	100
UBC 811	GAG AGA GAG AGA GAG AT	53.0	7	7	100
UBC 812	GAG AGA GAG AGA GAG AA	54.8	13	13	100
UBC 815	CTC TTC TCT CTC TCT CTG	52.8	9	9	100
UBC 816	CAC ACA CAC ACA CAC AT	54.8	6	6	100
UBC 817	CAC ACA CAC ACA CAC AA	50.4	22	22	100
UBC 818	CAC ACA CAC ACA CAC AG	57.2	14	14	100
UBC 823	TCT CTC TCT CTC TCT CC	57.2	6	6	100
UBC 825	ACA CAC ACA CAC ACA CT	54.8	15	15	100
UBC 828	TGT GTG TGT GTG TGT GA	54.8	8	8	100
UBC 834	AGA GAG AGA GAG AGY T	52.8	7	7	100
UBC 848	CAC ACA CAC ACA CAC ARG	53.0	18	18	100
UBC 855	ACA CAC ACA CAC ACA CYT	53.0	13	13	100

Several studies on natural populations use the percentage of polymorphic loci as an important measure of genetic diversity. However, although it is widely used, wide variations in this value are found, e.g., Black-Samuelsson et al. (1997) reported very low polymorphism levels (around 7%) in *Vicia pisiformis*, whereas Brandão et al. (2011) found between 83 and 89% polymorphic loci in *Myrcia splendensm* using ISSR markers. There is significant variation in these percentages, probably due to the ecological characteristics of each species, the different individuals and populations sampled, and the type of marker used.

Coefficient of variation stabilization occurred in 120 fragments, with values lower than 10%, indicating that the results could be used to analyze the genetic diversity of these populations (Figure 2). Markers based on ISSR have been successfully used in estimating genetic variability in wild and cultivated species, both between and within populations (Uysal et al., 2010).

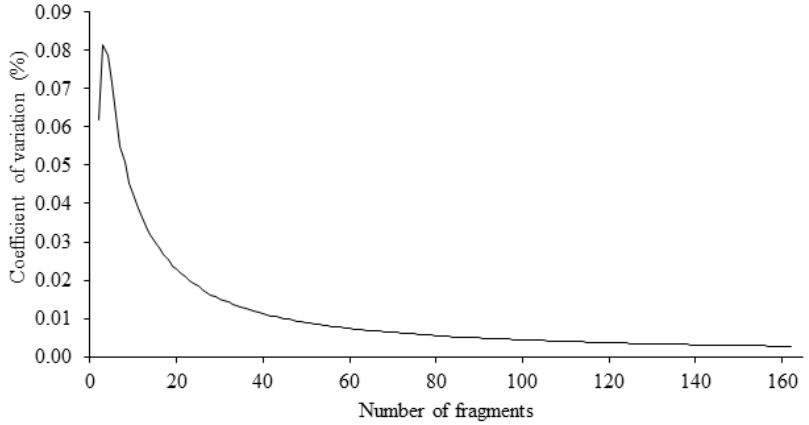

Figure 2. Coefficient of variation for the number of polymorphic fragments using ISSR markers among 10 natural populations of *Hancornia speciosa* Gomes of the State of Sergipe, Brazil.

The 100% polymorphism value that we obtained was higher than that reported in the literature for the use of ISSR molecular markers in *Spondias* sp, in which the polymorphism percentage was reported to be 80% (Santana et al., 2011). Amorim et al. (2013) obtained 51 fragments, 86% being polymorphic, when conducting the molecular characterization of native mangaba using 14 ISSR primers. Several studies that have used dominant markers, such as ISSRs, consider the percentage of polymorphic loci as a genetic diversity measure (Lorenzoni et al., 2014).

The mean genetic diversity value was 0.83 and the mean *I* value was 1.26. Estimated values for *I* within each population ranged from 0.05 (Pacatuba population) to 0.16 (Barra dos Coqueiros and Neópolis populations) (Table 3). The closer to zero is the value of *I*, the lower is the diversity (Souza-Sobreira et al., 2015). Under natural conditions, it is expected that these values are different from zero. Genotypes may incorporate new alleles by crossing, losses in small populations, or in populations fragmented by genetic drift (Barreira et al., 2006). These results agree with those reported by Costa et al. (2011), who obtained low values of *I* and H_E in *H. speciosa* Gomes germplasm.

Table 3. Number of individuals, Shannon index (*I*), and expected heterozygosity (H_E) in natural populations of *Hancornia speciosa* Gomes obtained by inter-simple sequence repeat markers.

Population	No. of individuals	*I*	H_E
Itaporanga	19	0.13	0.09
Pacatuba	20	0.09	0.06
Indiaroba	20	0.12	0.08
Estância	20	0.16	0.11
Barra dos Coqueiros	20	0.16	0.1
Pirambu	19	0.14	0.09
Japaratuba	5	0.10	0.07
Brejo Grande	15	0.15	0.10
Neópolis	15	0.16	0.10
Santa Luzia	2	0.05	0.03
Total	155	1.26	0.83

The genetic diversity indices used in this study (*I* and H_E) revealed low levels of genetic diversity within the populations evaluated. By observing the standard deviations of the H_E estimates, it was noted that the diversity levels were similar in all of the populations. The existence of high genetic diversity among populations can be confirmed when comparing values of *I* with those of other tree species. Similar results were found by Rivas et al. (2013) and Giustina et al. (2014) in natural populations of two allogamous species of the Amazon forest (*Theobroma subincanum* Mart. and *Theobroma speciosum* Willd. Ex Spreng.), who reported that there was great interpopulation genetic variability. However, Amorim et al. (2015), using microsatellite markers in mangaba, observed greater variation within populations (83.18%) than between them (10.82%).

Based on the ISSR markers, the similarity matrix obtained from the 155 individuals resulted in 18,769 values for the dissimilarity index, 21 of which stood out as having the highest and lowest dissimilarities (Table 4). The pairs formed between the individuals 15 x 14 (IN x IN) and 2 x 10 (S x E) had the highest (0.996) and lowest (0.555) similarities, respectively, and most pairs with the highest similarities belonged to the same populations and those with the lowest belonged to different populations. The reproductive system can influence the degree of genetic variability by both homogenizing and increasing divergence between individuals and populations (Zanella et al., 2012).

Table 4. Pairs of genotypes that had extreme values of high and low similarity (s) based on the Jaccard coefficient from 15 inter-simple sequence repeat markers in 155 individuals of *Hancornia speciosa* Gomes.

Order	Highest similarity			Lowest similarity		
	Individuals	Populations	s	Individuals	Populations	s
1	7 x 6	I x I	0.958	5 x 1	BG x I	0.075
2	11 x 12	I x I	0.885	13 x 1	BG x I	0.082
3	12 x 9	I x I	0.935	10 x 7	BG x I	0.067
4	3 x 2	P x P	0.881	2 x 3	J x I	0.093
5	3 x 4	P x P	0.968	2 x 6	J x I	0.086
6	4 x 2	P x P	0.955	1 x 6	NE x I	0.081
7	2 x 5	IN x IN	0.911	14 x 7	NE x I	0.093
8	3 x 1	IN x IN	0.934	2 x 13	S x I	0.095
9	15 x 14	IN x IN	0.996	2 x 17	S x I	0.098
10	1 x 2	E x E	0.943	2 x 2	S x IN	0.091
11	1 x 10	E x IN	0.94	2 x 5	S x IN	0.086
12	4 x 14	E x IN	0.875	2 x 10	S x E	0.055
13	3 x 7	BC x BC	0.839	2 x 12	S x E	0.077
14	5 x 8	BC x BC	0.845	7 x 3	BG x BC	0.088
15	4 x 5	J x J	0.832	13 x 2	BG x BC	0.09
16	3 x 4	J x J	0.821	1 x 2	I x J	0.08
17	3 x 4	BG x J	0.867	6 x 1	I x J	0.092
18	3 x 2	NE x NE	0.87	4 x 13	I x NE	0.099
19	6 x 11	NE x NE	0.928	6 x 1	I x NE	0.085
20	15 x 14	NE x S	0.853	6 x 1	I x BG	0.083
21	14 x 15	BG x BG	0.846	6 x 5	I x BG	0.074

For the species under study, the reproductive system probably caused the high polymorphism percentage, and consequently the high genetic diversity observed among the populations. Contrary results were obtained by Souza-Sobreira et al. (2015) in a study on the genetic diversity of natural populations of *Pitcairnia flammea* (L.) John (Bromeliaceae), who reported that the shortest distance was among individuals from the same population and the greatest distance was among populations.

Genetic relationships between individuals and populations were estimated based on a dendrogram produced by the Jaccard coefficient and the UPGMA, which resulted in five large clusters (I, II, III, IV, and V) and one individual (E10) that was clustered separately, which was the most divergent among the populations (Figure 3). This distance may indicate a significant differentiation process, which is the result of the genetic erosion that these individuals are subjected to in fragmented habitats in Sergipe State.

The high genetic variability found in mangaba may be a consequence of adaptation to habitat change; variation caused by selection pressure can maintain genetic polymorphisms (Sheng et al., 2004). Another factor that may cause high genetic variability in this species is the pollination mechanism it possesses, which reduces the loss of pollen, prevents autogamy, and favors cross-pollination (Darrault and Schlindwein, 2005). Luz et al. (2015) concluded that ISSR markers are effective in detecting genetic variability among *Cratylia argentea* (Desv.) Kuntze accessions. In a study on the genetic diversity of *Cattleya labiata*, ISSR markers effectively estimated the genetic variability of this species (Pinheiro et al., 2012).

The similarity data were in accordance with the PCoA results (Figure 4). Four groups were identified among the different populations, and the first two principal components explained 67.77% of the variation. This result confirms the effectiveness of ISSR markers and the existence of genetic diversity among the populations studied. A joint analysis of the UPGMA clustering and PCoA results revealed that individual E10 was the most divergent

because it was isolated from the other groups. Because of differences in ranking, optimization, and cluster classification, the use of more than one clustering method allows the classification to be complemented by the criteria that each technique uses, and prevents the adoption of erroneous inferences in the allocation of materials within a certain genotype subcluster (Silva et al., 2012a).

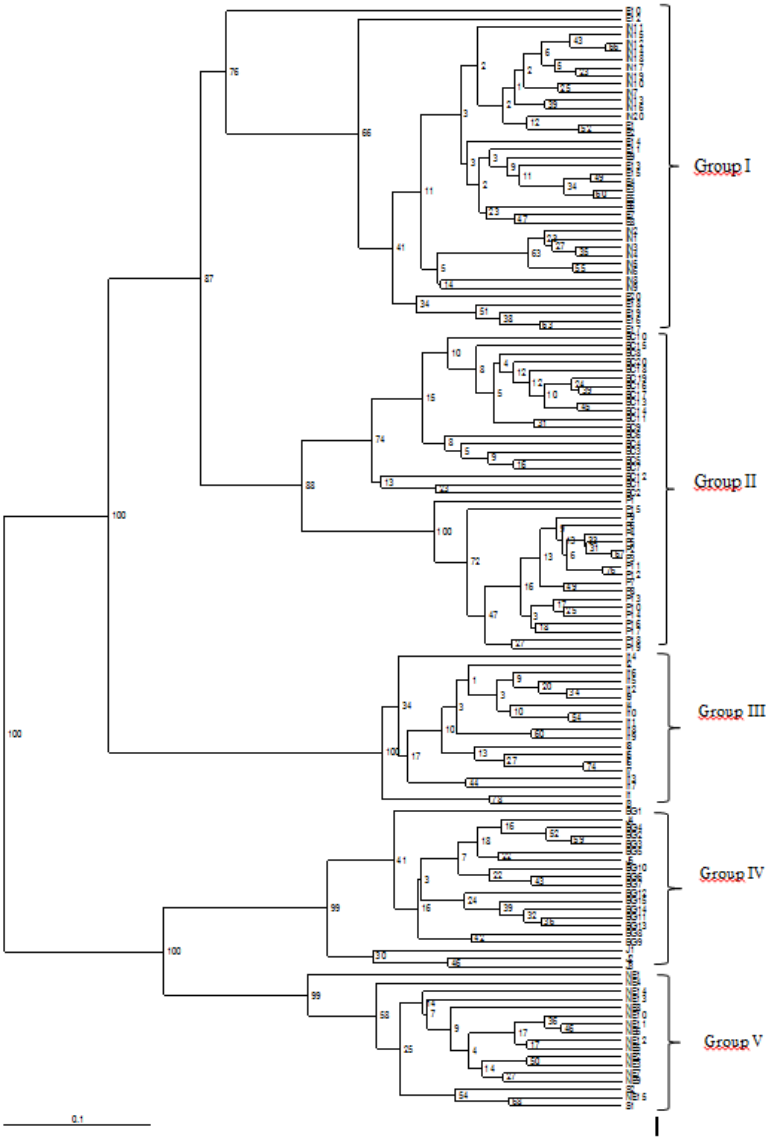

Figure 3. Phylogenetic representation by the UPGMA clustering method, genetic similarity by the Jacard coefficient (1908), and bootstrap analysis (10,000X) for 10 natural populations of *Hancornia speciosa* Gomes of the State of Sergipe, Brazil.

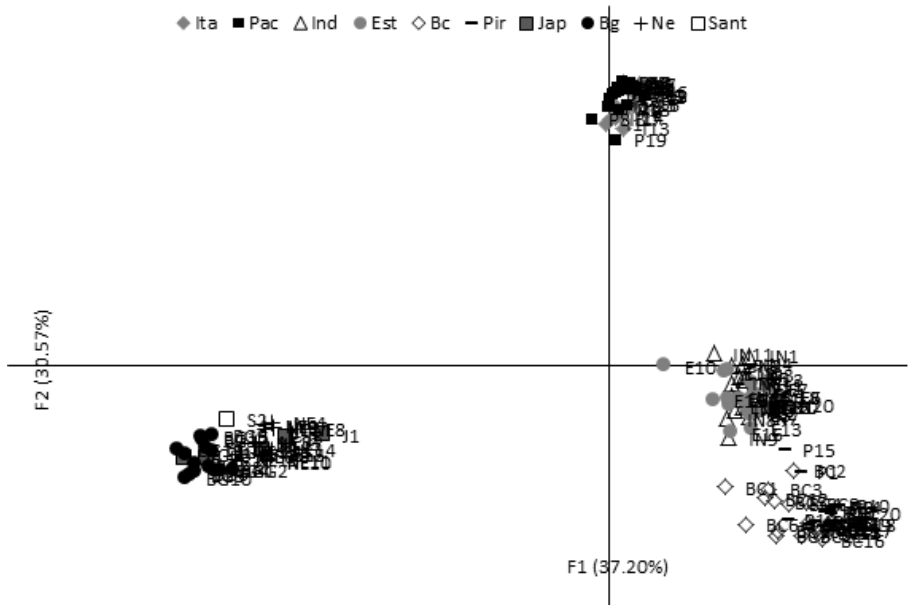

Figure 4. Principal coordinates analysis (PCoA) for natural populations of *Hancornia speciosa* Gomes of the State of Sergipe, Brazil.

Table 5 shows the results obtained by AMOVA, which indicate that most of the genetic diversity (77%) was found between populations while 23% was within populations. Contrary results were found by Jimenez et al. (2015) in a study on natural mangaba populations.

Table 5. Analysis of molecular variance between and within 10 populations of *Hancornia speciosa* Gomes in the State of Sergipe, Brazil.

Source of variation	d.f.	SS	MS	Variance components (CV)	Total (%)
Among populations	9	2751.4	305.7	19.8	77
Within populations	144	847.3	5.9	5.9	23
Total	153	3598.7		25.7	100

d.f. - degrees of freedom; SS - sum of squares; MS - mean square; CV - coefficient of variation.

Large genetic variation exists within populations of tree species with mixed reproductive systems and efficient pollen and seed dispersion (Yun et al., 1998), which result in long-distance gene flow, reduced variation between populations, and increased variation within them (Loveless and Hamrick, 1984). The pattern we observed was not in accordance with those of other tree species, and with what is expected in allogamous species. In the mangaba population evaluated, gene flow could be restricted and consequently the intrapopulation diversity was low, whereas it was high among populations.

The genetic structures of plant populations are dependent upon the interactions of many different processes, such as habitat fragmentation and/or population isolation, range changes, mutation, ecology, reproductive isolation, genetic drift, reproductive mechanism,

gene flow, and selection (Thendral Hepsibha et al., 2010). Genetic diversity greatly contributes to the sustainability of plant populations (Wang et al., 2007), and its study is necessary to understand what occurs in populations (Silva et al., 2012b).

Mangaba is an endangered species in northeastern Brazil, and Sergipe State is subject to high human activity. This compromises the survival of this species, which is part of the social and economic heritage of this state. Habitat fragmentation caused the main limitation of this study, as it was difficult to collect a large number of individuals in some populations. Studies that investigate species distributions and genetic compositions are very important for the conservation and improvement of this species. A good strategy for the effective conservation of the genetic variability of these genetic resources would be the conservation of populations in the geographical range of the species. This has been conducted by the maintenance of a mangaba gene bank and the creation of private natural heritage reserves. The mangaba gene bank of the Embrapa Coastal Tablelands is in Sergipe, and 1232.30 ha of private reserves exist, the largest being "Caju" reserve in Itaporanga d'Ajuda, with 763.37 ha (ICMBIO, 2016).

The ISSR markers used in this study were effective in estimating genetic variability between mangaba populations, and the high degree of polymorphism detected suggests that the genetic diversity of remnant populations in the State of Sergipe can provide important information for the conservation and future improvement of this species.

Conflicts of interest

The authors declare no conflict of interest.

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