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Characterization of trees, fruits and genetic diversity in natural populations of mangaba

Caracterização de árvores, frutos e diversidade genética em populações naturais de mangaba

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ABSTRACT

The state of Sergipe is the largest mangaba producer, which is a fruit native to Brazil, and has cultural, social and economic importance in its area of occurrence. It is an endangered species due to human actions, and despite its economic potential, there are still no commercial plantations. The study was carried out in order to characterize trees, fruits and the genetic diversity of natural populations of mangaba in Sergipe, Brazil. Fruits from Abaís Beach/Estância (AB) presented, on average, twice the vitamin C content (414.81 mg of vit. C/100g), when compared with the others. The use of ISSR primers was efficient in estimating the genetic similarity of populations. The primers clustered the populations of mangaba according to their origin, which indicates the genetic diversity of mangaba and their isolation. The results can be used to guide the selection of individuals *in situ* and *ex situ* conservation actions of these genetic resources.

Index terms: *Hancornia speciosa* Gomes; postharvest; molecular markers; variability.

RESUMO

O Estado de Sergipe é o maior produtor de mangaba, fruta nativa do Brasil e de importância cultural, social e econômica nas áreas de ocorrência. Encontra-se em risco de extinção devido a ações antrópicas, e apesar de seu potencial econômico, ainda não existem plantios comerciais da espécie. O trabalho foi desenvolvido com o objetivo de caracterizar árvores, frutos e a diversidade genética de populações naturais de mangabeira em Sergipe, Brasil. Frutos oriundos de Abaís/Estância (AB) apresentam em média o dobro do conteúdo de vitamina C (414.81 mg de vit. C/100g) dos demais. O uso de primers ISSR foi eficiente para estimar a similaridade genética das populações, sendo agrupados de acordo com sua origem, o que indica a diversidade genética destas mangabeiras, e seu isolamento. Os resultados poderão ser usados para direcionar a seleção de indivíduos em ações de conservação desses recursos genéticos, *in situ* e *ex situ.*

Termos para indexação: *Hancornia speciosa* Gomes; pós-colheita; marcadores moleculares; variabilidade.

INTRODUCTION

Mangaba (*Hancornia speciosa* Gomes) is a fruit tree native to Brazil. It occurs in the tableland and coastal plains of the Northeast, in the Cerrado of the Central-West, in the North and Southeast regions of Brazil. The state of Sergipe is the largest mangaba producer in the country, especially in the municipalities of Barra dos Coqueiros, Itaporanga D'Ajuda, and Estância (Soares et al., 2016). The deforestation of large areas of natural occurrence of the species provides the genetic erosion of mangaba, and associated with this, the unsustainable use of remaining individuals result in irreparable environmental damage (Santos et al., 2010).

Because of the that, Embrapa Coastal Tablelands, a Unit of Brazilian Agricultural Research Coporation (Embrapa), located in the State of Sergipe, has been employing efforts to promote *ex situ* conservation of the species genetic resources. The Mangaba Active Genebank (BGMangaba) constitute the repositorie of the species and encompasses 271 accesses nominated according to the area of sample collection. New entries have been continuously made to the Bank in order to ensure its enrichment (Silva et al., 2015).

The conservation of the species both *ex situ* as *in situ* requires the knowledge on the structure and on the genetic variability; thus, the understanding of this nature in the study of remaining populations of mangaba

is essential to combat the increasing reduction of the areas of natural occurrence (Amorim et al., 2015), since the genetics of a species is an important factor for the survival of populations in variable environments, and is recognized as a key biodiversity component (Mace et al., 1996). Therefore, the knowledge of how genetic variation is divided between the populations may have important implications, not only in evolutionary biology and ecology, but also in conservation biology.

One way to assess the genetic diversity of populations is the molecular characterization, which allows inferring the polymorphism degree between individuals and populations. These techniques are not influenced by environmental conditions and do not present pleiotropic effects (Amorim et al., 2015). Among the molecular markers used, dominant ISSR markers (Inter Simple Sequence Repeats) present to be useful tools in studies on diversity and genetic structure (Zietkiewicz; Rafalski; Labuda, 1994). The use of this marker has been successfully reported in mangaba (Costa et. al., 2015).

Thus, in order to contribute to the increase of the knowledge of the species for further conservation works, this study was carried out to analyze plants, fruits and the genetic diversity in mangaba populations in the state of Sergipe.

MATERIAL AND METHODS

Mangaba trees were randomly sampled and georeferenced in three areas of occurrence in the State of Sergipe (Figure 1), in the municipalities of Barra dos Coqueiros (BC, 20), Itaporanga D'Ajuda (Reserva Particular do Patrimônio Natural do Caju - RC, 19) and Estância (Abaís Beach - AB, 20), totaling 59 individuals.

Figure 1: Municipalities of Sergipe where the studied mangaba were collected.

For morphoagronomic characterization, all matrices were evaluated for plant height (PH), height of the first bifurcation (HFB), stem diameter (SD), and canopy radius (RC). Of each matrix, 12 fruits were collected, and it was evaluated: a) fruit weight (FW) and fruit diameter (FD), using a scale and a caliper; b) soluble solids (SS) (AOAC, 1992); c) titratable acidity (TTA), determined by titration with NaOH 0.1N solution and 1% phenolphthalein as indicator, and the values were expressed as percentage of citric acid; d) pH using five grams of pulp diluted in 50 mL distilled water and measured in electronic potentiometer; e) vitamin C content (Silva et al., 2012a), and results were expressed in mg of vit.C/100g; and f) peel color, determined by the colorimeter MINOLTA model CR-10. The parameters obtained were: 'L', which indicates luminosity (light/dark, and ranges from 0 to 100); 'a', which indicates the chromaticity from green (-) to red (+); and 'b' indicates the chromaticity from blue (-) to yellow (+).

For the analysis of diversity and population genetic structure, young leaves were collected, identified, stored in plastic bags, and transported under low temperature to the Molecular Biology Laboratory. DNA was extracted based on the standard CTAB protocol (Doyle; Doyle, 1990). Quantification was carried out using the spectrophotometer NanoDrop 2000C Termo Scientific®, and DNA integrity was visualized in 2% agarose gel.

To obtain reliable, reproducible and polymorphic markers, 28 ISSR primers were initially tested (Invitrogen®) (Table 1).

The total volume of reaction for the selection and optimization of primers was 20 µL, which contained: 2 µL genomic DNA solution (X) 1 µL of each primer (Y), together with a mix composed of: 14.4 µL sterile water MilQ; 2 µL 10X buffer ($MgCl_2$ (Z), 100 mM $MgSO_4$, 100 mM KCl, 80 mM $(NH4)_2SO_4$, 100 mM Tris-HCl) (NeoTaq); 0.4 µL dNTP (10mM); 0.2 µL Taq polymerase (5 units/L).

After the testing and selection of optimal variables, 10 primers (844a, 844b, 17899b, HB10, HB12, HB13, HB15, 810, 826, 841) were chosen and applied to the 59 individuals.

In each tube containing 20 µL amplified DNA, it was added 3 µL sample buffer (0.01% bromophenol blue, 40% glycerol). Of this mixture, 10 µL were disposed in 2% agarose gel dissolved in TBE 1X (TRIS 89 mM, boric acid 89 mM, EDTA 2.5 mM, pH 8.3) and subjected to horizontal electrophoresis at 100 V for approximately two hours and 30 minutes.

Table 1: ISSR Primers tested with their respective sequences of nucleotides.

Primer	Sequencia 5′ - 3′	Primer	Sequencia 5′ - 3′
807	AGAGAGAGAGAGAGAGT	855	ACACACACACACACACYT
810	GAGAGAGAGAGAGAGAT	856	ACACACACACACACACYA
814	CTCTCTCTCTCTCTCTTG	17898 A	CACACACACACAAC
823	TCTCTCTCTCTCTCTCC	17898 B	CACACACACACAGT
826	ACACACACACACACACC	17899 A	CACACACACACAAG
828	TGTGTGTGTGTGTGTGA	17899 B	CACACACACACAGG
834	AGAGAGAGAGAGAGAGYP	HB 8	GAGAGAGAGAGAGG
835	AGAGAGAGAGAGAGAGYC	HB 9	GTGTGTGTGTGTGG
841	GAGAGAGAGAGAGAGATC	HB 10	GAGAGAGAGAGACC
843	CTCTCTCTCTCTCTCTRA	HB 11	GTGTGTGTGTGTCC
844 A	CTCTCTCTCTCTCTCTAC	HB 12	CACCACCACGC
844 B	CTCTCTCTCTCTCTCTGC	HB 13	GAGGAGGAGGC
845	CTCTCTCTCTCTCTCTRG	HB 14	CTCCTCCTCGC
848	CACACACACACACACARG	HB 15	GTGGTGGTGGC

Gels were placed in a solution containing ethidium bromide (0.02 µL /mL water) for approximately 60 minutes for visualization under ultraviolet light. For the measurement of the banding pattern, it was used a 1kb molecular weight marker (Promega). The visualization of the results was carried out in a photodocumentation device Gel doc L-pix (Loccus Biotecnologia, Brasil), and recorded for later analysis.

The data obtained were identified by means of polymorphic bands, and each band was designated as a variable, and the presence was represented by (1), and the absence was represented by (0). The binary matrix was used to estimate the number of polymorphic bands and for the study of the genetic diversity.

It was calculated the polymorphic information content (PIC) for the dominant marker (Ghislain et al., 1999). The marker index (MI) was determined as described by Zhao et al. (2007). The Shannon index (I) and the expected heterogozity (He) were calculated as described by Lynch and Milligan (1994) and Maguire, Peakall and Saeger (2002), using the Genalex v.6.3.

The coefficients of similarity were calculated using the genetic similarity of Jaccard (SJ). The dendrogram was constructed using the clustering method UPGMA (Unweighted Pair Group Method with Arithmetic Mean). To determine the robustness of the dendrogram, it was carried out bootstrap with 10,000 replications using the FreeTree software and visualization was carried out using the TreeView software. The Principal Coordinates Analysis was carried out using the Genalex v.6.3.

In addition, it was used five replications for each K value estimated, each one consisting of burning period length of 15.000 steps, followed by 100.000 replicas of Markov chain Monte Carlo. The Structure software estimates the most probable number of clusters (K) by calculating the probability of data log for each value of K. According to Evanno, Regnault and Goudet (2005), it was calculated the change of the second order of the likelihood function, divided by the standard deviation of the probability (ΔK), in order to evaluate the best K value supported by the data (Santos et al., 2011).

RESULTS AND DISCUSSION

The analysis of variance of the morphological characteristics of mangaba trees and fruits revealed the existence of a significant variation for almost all the characters (Table 2). The mean plant height ranged from 3.60 m (AB) to 4.33 m (BC). The height of the first bifurcation showed high coefficient of variation, with no significant difference, although it ranged from 0.39 m (AB) to 0.70 m (RC). The highest mean for stem diameter was 0.65 m in the plants of the BC population. The diameter was measured at 10 cm from the ground, and not at breast height, as usual, since mangaba is quite branched, and do not present straight and uniform bole. The mean for the canopy diameter ranged from 4.90 m (AB) to 6.01 m (BC).

Phenotypic variation may be influenced by not controlled environmental components, such as human disturbance, soil, plant age, and genetic differentiation between individuals. This wide variation was also observed by Ganga, Chaves and Naves (2009) in Cerrado, with high levels of genetic variation for plant height and stem diameter.

Physical characterization of fruits presented higher fruit weight in the population of Barra dos Coqueiros (21.1 g). In relation to the longitudinal and transverse diameters, higher values were observed in the fruits of Estância (44.91 and 476.54 g, respectively). Although it was sought to collect fruits as more homogeneous as possible, the peel color was different for the different origins, in relation to 'a' (red/green component), 'H' (color angle), and 'L' (luminosity). The fruits of Barra dos Coqueiros (BC) were less red and presented more luminosity due to the larger yellow area in the peel (Table 2).

For the physico-chemical attributes, there was significant difference in pH, TTA, and vitamin C content. SS values were similar and ranged from 17.05 to 19.20 ºBrix. The total titratable acidity (TTA) ranged from 0.97 to 1.38% citric acid, being consistent with the results of Silva et al. (2012a). The vitamin C content was quite variable. The fruits of Estância (AB) are rich vitamin C sources, with mean of 414.81 mg vit.C/100 g, and are the only ones which had values similar to those found by Silva et al. (2012a). The fruits of the other individuals presented 158.68 (BC) and 206.26 (RC) mg vit. C/100 g (Table 2). Since the loss of ascorbic acid is correlated with the maturation and ripening, changes in this characteristic after collection can be considered as an indicator of product quality loss (Klein, 1987).

The variation observed in this study is expected since it this is a native population which has not undergone any selection process, and it should be mentioned that there was genetic differentiation between individuals.

For the genetic diversity analysis, it was tested 28 ISSR primers, of which 10 were selected (844a, 844b, 17899b, HB10, HB12, HB13, HB15, 810, 826 and 841) for presenting reproducibility. According to the classification

Table 2: Morphological characteristics of trees [total height (m), height of the first bifurcation (m), stem diameter, and canopy diameter (m)] and morphological characteristics of the fruit (weight, longitudinal and transverse diameters, peel color, soluble solids, titratable acidity, pH, and vitamin C) of natural populations of mangaba located in Sergipe, Brazil.

		Characteristics	Natural populations			DMS	CV%
			Itaporanga D'Ajuda (RC)	Estância (AB)	Barra dos Coqueiros (BC)		
	Tree	Total height (m)	4.15ab	3.60b	4.33a	0.55	21.93
		Height of the 1st bifurcation (cm)	0.70a	0.39a	0.57a	0.31	74.62
		Stem diameter (mm)	52.30b	55.05b	65.65a	10.41	23.72
		Canopy diameter L-O/N-S (m)	5.54ab	4.90b	6.01a	0.92	22.03
Fruit	Physical	Total weight (g) (g)	15.95b	15.46b	21.10a	3.58	12.15
		Longitudinal diameter (mm)	26.56b	44.91a	32.62b	8.89	15.21
		Transverse diameter (mm)	33.73b	47.54a	36.47b	8.98	13.57
	Peel color	a (red/green +/-)	15.11ab	20.25a	9.75b	9.56	37.7
		b (yellow/blue +/-)	45.87a	49.42a	45.84a	4.44	5.6
		L (luminosity)	44,42b	59.54a	60.88a	5.67	6.12
	Physico-chemical	Soluble solids (ºBrix)	17.80a	17.05a	19.20a	2.52	8.31
		pH	3.30a	3.42a	2.90b	0.2	3.84
		Total titratable acidity (% citric acid) - TTA	0.97b	1.38a	1.27ab	0.33	16.15
		Vitamin C (mg de vit. C/100g)	206.26b	414.81a	158.68b	77.43	17.67

Different lowercase letters in the lines show statistical difference (P <0.05).
LSD - least significant difference; CV% - Coefficient of variation.

of Xie et al. (2010), PIC values in the present study were moderately informative (> 0.25) (Table 3), and had mean lower than that reported by Silva et al. (2012b) (0.32), using RAPD in mangaba.

Fifty-five fragments were amplified, and 67% were polymorphic locus. The value found was lower than the the native mangaba fruits using ISSR markers in the state of Pernambuco (89%) (Jimenez et al., 2015), and higher (48%) than those found in the state of Rio Grande do Norte (Costa et al., 2015). Both values reported in mangaba are considered high when compared with other species, such as cupuaçu (34.9%) (Silva et al., 2016).

The high polymorphism degree detected by molecular characterization suggests that the genetic variability of the remaining populations of mangaba in Sergipe can provide genetic material to be used in the conservation of this genetic resource, such as being inserted in germplasm banks and collections.

PIC ranged from 0.21 to 0.29, and was considered little to moderately informative (Xie et al., 2010). The marker index (MI) takes into account the fraction of polymorphic markers, and estimates the overall usefulness of each molecular marker system that ranged from 0 to 3.29, with mean of 0.98 (AB); 1.35 (RC); and 1.53 (BC).

The Shannon index (I) presented means which suggest intermediate diversity: 0.31 (AB); 0.42 (RC and BC), which is lower than those described by Silva et al. (2012b) and higher than those described by Costa et al. (2011). The closer to zero, the smaller is the diversity. This index is considered a good tool for the analysis of populations when using dominant markers (Dawson et al., 1995).

The genetic diversity index (He) was 0.21 (AB) and 0.29 (RC and BC), with mean of 0.26, suggesting an excess of homozygotes or heterozygotes among the evaluated individuals. This may occur for they are natural populations, and are likely to incorporate or lose alleles by genetic drift (Silva et al., 2012b). The values found in this study are higher than those found by Costa et al. (2011), who evaluated mangaba germplasm with RAPD and found mean values for He of 0.17. However, they are lower than those found by Amorim et al. (2015), who used microsatellites, and lower than that observed by Martins et al. (2012) (0.36), who evaluated mangaba fruits in the states of Pernambuco and Alagoas using isoenzymes.

The dendrogram analysis revealed that the formation of clusters corresponds to the region of collection, except for the individuals BC1 and RC1 (0.42 SJ), which were isolated and identified as the most divergent (Figure 2).

The principal coordinates analysis - PCoA (Figure 3) showed 72% of this variability, and indicates that individuals are clustered by collection areas, following the same clustering presented in the dendrogram, and BC1 genotypes and RC1 were isolated again.

The Structure software, based on the Bayesian analysis, was used to infer the number of clusters (K). The best K found was K = 2, i.e., two reconstructed populations. However, by the analysis of Evanno, Regnault and Goudet (2005), the number of reconstructed populations that represents the set of genotypes is K = 3 (Figure 4). Comparing the data of the Bayesian analysis determined by the Structure software, with the clustering of the principal coordinates analysis and the dendrogram, results were similar, which increased the reliability of the clusterings.

Although the area of the state of Sergipe is small, and by consequence the areas of collection are close to each other, based on the genetic clusters formed, it is noted that the mangaba trees are isolated, since the clusters formed were identical to the areas of collections. Thus, the best way to conserve the remaining genetic diversity is keeping them in their habitat. Mangaba is an allogamous species, and thus it is expected high genetic diversity among individuals. However, the fragmentation of the remaining populations results in the low genetic variability and in population isolation (Costa et al., 2015).

Table 3: Percentage of polymorphic loci (%P). Polymorphic Information Content (PIC), Marker Index (MI), Shannon Index (I), Expected Heterozygosity (He) for 60 mangaba genotypes using ISSR markers, collected in three natural populations in the State of Sergipe, Brazil: Abaís Beach/Estância (AB); Reserva do Caju/Itaporanga D'Ajuda (RC) and Barra dos Coqueiros (BC).

Population	%P	PIC	MI	I	He
Estância (AB)	55	0.21	0.98	0.29	0.20
Itaporanga D'Ajuda (RC)	71	0.29	1.35	0.40	0.27
Barra dos Coqueiros (BC)	75	0.29	1.53	0.45	0.31
Average	67	0.26	1.28	0.38	0.26

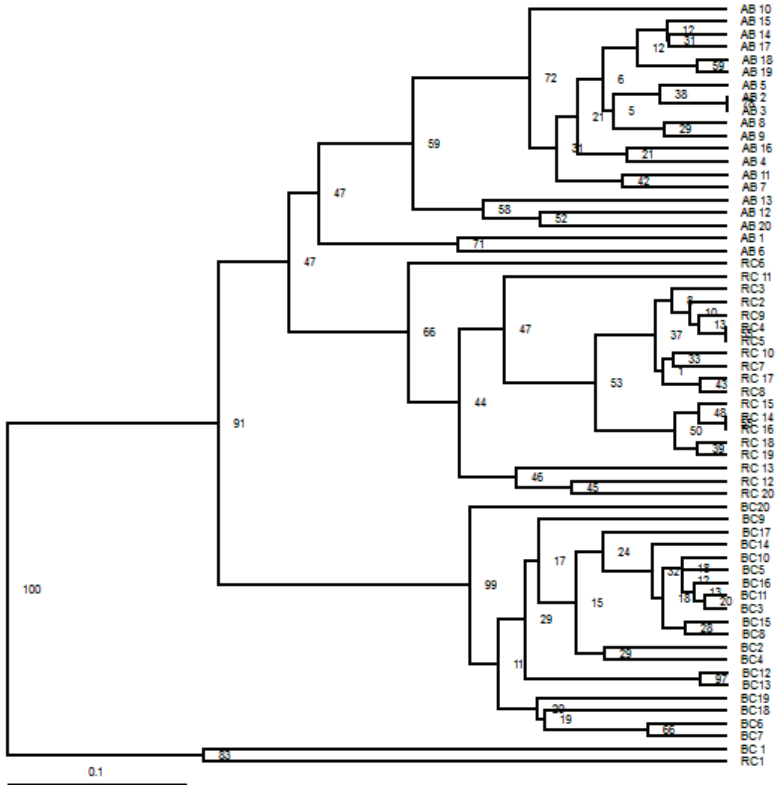

Figure 2: Similarity dendrogram by the Jaccard coefficient, UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and 10.000x bootstraps for 59 mangaba genotypes using ISSR markers, collected in three natural populations in the state of Sergipe, Brazil: Abaís Beach/Estância (AB); Reserva do Caju/Itaporanga D'Ajuda (RC) and Barra dos Coqueiros (BC).

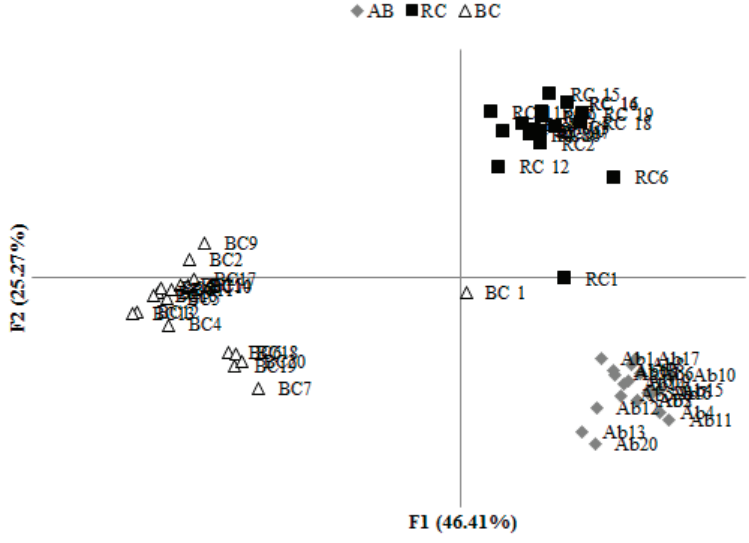

Figure 3: Main Coordinate Analysis for 60 genotypes mangaba using ISSR markers, collected in three natural populations in the State of Sergipe, Brazil: Abaís Beach/Estância (AB); Reserva do Caju/Itaporanga D'Ajuda (RC) and Barra dos Coqueiros (BC).

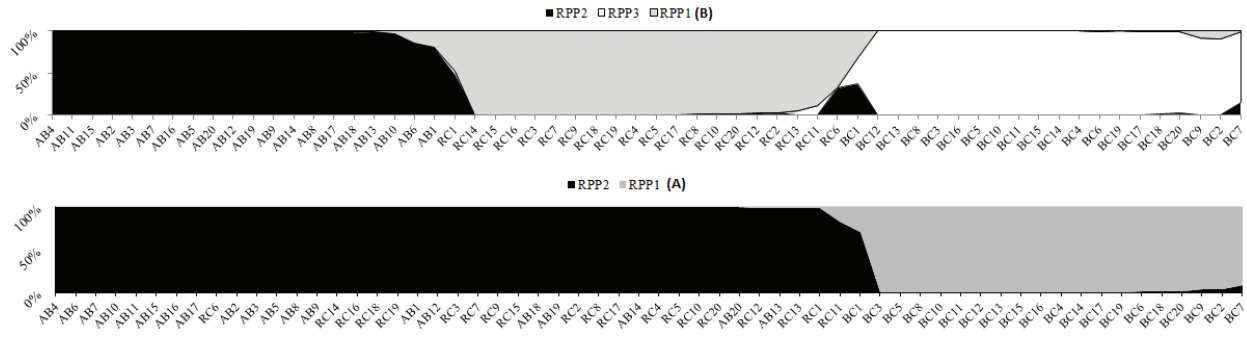

Figure 4: Reconstructed Populations (RPPs) defined by the Structure software (PRITCHARD et al., 2000) for 60 mangaba genotypes using ISSR markers, collected in three natural populations in the state of Sergipe, Brazil: Abaís Beach/Estância (AB); Reserva do Caju/Itaporanga D'Ajuda (RC) and Barra dos Coqueiros (BC). (A) – 2 RPPs; (B) – 3 RPPs.

Due to human activity, there is economic pressure on natural areas and impairment of *in situ* conservation of the species. Thus, measures for the maintenance of the remaining natural populations should be taken to promote the genetic variability of mangabas of the state of Sergipe, and the increase of this variability. Therefore, it is necessary the creation of ecological corridors that connect these areas, in order to promote gene flow in these regions (Jimenez et al., 2015).

CONCLUSIONS

ISSR markers allowed the discrimination of genetically different individuals, with the clustering of individuals according to their geographical location. The fruits from Estância (AB) are superior in size and vitamin C content. Results may be used to assist the selection of individuals for *in situ* and *ex situ* conservation actions of these genetic resources.

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