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Genetic diversity and population structure of mangabeira (*Hancornia speciosa*) estimated using ISSR markers

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ABSTRACT. The mangabeira is a native fruit tree from Brazil with fruits that present significant potential for exploitation. This species is experiencing genetic erosion, which increases the importance of elucidating the genetic diversity that exists in mangabeira populations to support conservation programs. Thus, this study aimed to evaluate the diversity and genetic structure of mangabeira populations from the Germplasm Bank of Embrapa Meio-Norte using inter simple sequence repeat (ISSR) markers. A total of 29 accessions from Brazil were characterized, including one from Sergipe, one from Bahia, three from the Distrito Federal, 11 from Piauí and 13 from Paraíba. The 11 ISSR primers provided 166 loci, among which 120 were polymorphic. The analysis of molecular variance (AMOVA) indicated that 69.66% of the observed genetic variability occurred within populations and that the populations showed high genetic differentiation. The results obtained from the STRUCTURE analysis indicated the existence of two genetic groups. The Nei and Shannon indices of genetic diversity varied from 0.15 to 0.24 and from 0.22 to 0.34, respectively. The coefficient of similarity ranged from 0.57 to 0.94, with a mean of 0.76. The mean was used as the cut-off point in the dendrogram, and seven groups were identified. In conclusion, this study demonstrates the presence of low or moderate genetic diversity within the studied mangabeira populations and high genetic differentiation between the populations. The results indicate a need to increase the number of mangabeira population samples from different collection sites as a strategy to achieve more significant results for the conservation and genetic improvement of this species.

Keywords: Apocynaceae; genetic structure; genetic variability; ISSR markers.

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Introduction

Fruticulture is an agribusiness sector that plays an important socioeconomic role in Brazil. The country is one of the main centers of genetic diversity of native fruit trees (Ferreira, Araújo, Alves, Costa, & Silva, 2013) and the third largest producer of fruit in the world (Santos et al., 2015). However, many native species with unique organoleptic characteristics, such as high nutritional value and potential for agroindustry use, including the mangabeira (*Hancornia speciosa* Gomes), are not well studied (Cardoso, Martino, Moreira, Ribeiro, & Sant'ana, 2011; Silva et al., 2014).

The mangabeira is a tree plant of the family Apocynaceae that is found mainly in areas of open vegetation, such as savannas, coastal tablelands and coastal lowlands (Silva, Rabbani, Sena-Filho, Almeida, & Feitosa, 2012; Costa et al., 2017). This species is very promising due to the potential of its fruits, which exhibit excellent taste and high nutritional value (Martins, Martins, Veasey, Lederman, & Silva, 2012; Maia, Ávila, Mezzomo, & Lanza, 2018). The mangabeira plays an important role in the local economy of the midwestern and northeastern regions of Brazil, where its fruit is consumed in its natural form or processed to produce juices, ice cream and sweets, among other products (Collevatti, Olivatti, Telles, & Chaves, 2016). In addition, the fruit (mangaba) is an excellent source of folate (Cardoso, Reis, Oliveira, & Sant'ana, 2014) and ascorbic acid, and it exhibits antioxidant activity (Lima et al., 2015).

The mangabeira also has pharmacological potential. In folk medicine, its derivates are used in the treatment of gastritis, inflammation, and diabetes, among other ailments (Almeida et al., 2014). In addition, studies have demonstrated the anti-inflammatory (Marinho, Alviano, Matheus, Alviano, & Fernandes, 2011), osteogenic (Neves et al., 2016), and angiogenic (Almeida et al., 2014) activities of the mangabeira latex. Furthermore, the hypotensive (Silva et al., 2011) and anti-diabetic (Pereira et al., 2015) effects of the leaf ethanol extract from mangabeira have been reported. The anti-inflammatory activity (Torres-Rêgo et al., 2016) and antimutagenic and protective effects of mangabeira fruit extract were also demonstrated, thus emphasizing its potential as a functional food (Lima, Azevedo, Souza, Nunes, & Boas, 2015).

Despite its economic potential, the mangabeira has mainly been exploited in an extractive manner (Soares, Melo, Vitória, & Silva, 2015). This species is undergoing the process of domestication and experiencing genetic erosion (Sá, Lédo, & Lédo, 2011), which can lead to a loss of alleles as well as to genetic combinations that help the species adapt in response to environmental variations (Rogers & McGuire, 2015). The observed loss of genetic variability results from many factors, such as the intensification of agricultural activity, forest fragmentation and real estate expansion in areas of natural occurrence of the mangabeira (Silva, Amorim, Melo, Ledo, & Rabbani, 2017). It is also worth noting that mangabeira is on the list of endangered species, which reinforces the importance of studies aimed at developing conservation strategies for this species (Silva et al., 2017).

A key step in the development of strategies for the conservation, breeding and domestication of a species is obtaining knowledge of its genetic diversity (Costa, Silva, Lédo, Santos, & Silva Júnior, 2011). Such information may reveal the best ways to use genetic resources or obtain promising breeds for crosses and hybrids with superior characteristics and lead to the identification of duplicates in germplasm banks (Oliveira, Oliveira, Pereira, Lima, & Aloufa, 2014; Santos et al., 2017).

One way to determine the genetic diversity of a species is the use of molecular markers that indicate differences between plants and that are not influenced by environmental factors. Among the markers that are available, the application of inter simple sequence repeats (ISSRs) is a simple technique that employs a single repeat sequence primer that is complementary to microsatellite regions. Thus, this type of marker provides a fast and reproducible technique through which DNA fragments shared between identical microsatellite regions are amplified. In addition, the use of ISSRs allows broad coverage of the genome and is applicable in the study of genetic diversity in numerous species since it does not require previous knowledge of the target DNA sequences (Ng & Tan, 2015).

Therefore, this study aimed to evaluate the diversity and genetic structure of mangabeira populations from the Germplasm Bank of Embrapa Meio-Norte using ISSR markers.

Material and methods

Plant samples

Twenty-nine mangabeira accessions from the Germplasm Bank of Embrapa Meio-Norte, located in Teresina-PI at a latitude of 5°02'05.9" S and longitude of 42°48'15.3" W, were used in this study. All of the accessions belong to the species *H. speciosa* var. *speciosa* except for three accessions of the species *H. speciosa* var. *pubescens* (Table 1).

DNA isolation and quantification

Young leaves collected from the 29 accessions of mangabeira were kept in hermetically sealed plastic bags containing silica for 16 hours. Genomic DNA was isolated from 0.02 g of dehydrated leaves using the DNeasy plant mini kit (QIAGEN, USA) following the protocol suggested by the manufacturer. The obtained DNA was analyzed via 0.8% agarose gel electrophoresis with 0.5X TBE, stained with GelRed ™ (Biotium) and photographed under ultraviolet light. Quantification was performed by comparing the DNA samples with the DNA λ value determined for 100 ng and using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), which was also employed to verify the purity of the samples.

Table 1. Origin of the 29 accessions of mangabeira from the Germplasm Bank at Embrapa Meio-Norte used for molecular characterization.

Accession	Species	Country	State
M1	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M2	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M3	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M4	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M5	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M6	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M7	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M8	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M9	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M10	*H. speciosa* var. *speciosa*	Brazil	Paraíba
[a]M10[2]	*H. speciosa* var. *speciosa*	Brazil	Bahia
[a]M12[1]	*H. speciosa* var. *speciosa*	Brazil	Sergipe
M12	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M13	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M14	*H. speciosa* var. *speciosa*	Brazil	Paraíba
M15	*H. speciosa* var. *pubescens*	Brazil	Distrito Federal
M16	*H. speciosa* var. *pubescens*	Brazil	Distrito Federal
M17	*H. speciosa* var. *pubescens*	Brazil	Distrito Federal
M18	*H. speciosa* var. *speciosa*	Brazil	Piauí
M19	*H. speciosa* var. *speciosa*	Brazil	Piauí
M20	*H. speciosa* var. *speciosa*	Brazil	Piauí
M21	*H. speciosa* var. *speciosa*	Brazil	Piauí
M22	*H. speciosa* var. *speciosa*	Brazil	Piauí
M23	*H. speciosa* var. *speciosa*	Brazil	Piauí
M24	*H. speciosa* var. *speciosa*	Brazil	Piauí
M25	*H. speciosa* var. *speciosa*	Brazil	Piauí
M27	*H. speciosa* var. *speciosa*	Brazil	Piauí
M29	*H. speciosa* var. *speciosa*	Brazil	Piauí
M30	*H. speciosa* var. *speciosa*	Brazil	Piauí

[a]Accessions not included in the analysis of the genetic structure of the populations.

DNA amplification reactions

DNA amplification reactions were performed using the following reagent concentrations: 1X buffer [20 mM Tris-HCl pH 8.4; (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 1 mM dNTPs, 0.4 µM primers, and 0.5 U Taq DNA polymerase (Invitrogen), with 1 µL of DNA (7 ng μL^{-1}) and ultrapure water to a final volume of 10 µL. The reactions were conducted in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA), and the reaction program was as follows: 1 initial denaturation cycle of 1.5 minutes at 94°C, followed by 40 cycles of 40 seconds at 94°C, 45 seconds at the annealing temperature for each primer, and 2 minutes at 72°C, and a final extension of 7 minutes at 72°C. Among the 30 ISSR primers that were tested, which were developed by the University of British Columbia, 11 primers were selected based on their polymorphism and the number and resolution of the bands observed in virtual gels.

The obtained amplification fragments were separated through capillary electrophoresis using the DNF-915 (35-5000 bp) Kit in a Fragment Analyzer™ (Advanced Analytical Technologies, USA) at 9 KV for 90 or 120 minutes. The time was determined based on the number of bands obtained with the primers. For the analysis, the samples were diluted to a final volume of 24 µL at a ratio of 4 to 6 µL of amplified DNA from each accession to 18 to 20 µL of elution buffer, where the volume was determined based on the fragments amplified by the primers.

Statistical analysis

A matrix was constructed for the amplified fragments encoded in the binary system, with the fragments being assigned a value of (1) for the presence and (0) for the absence of bands in the virtual gels. Based on the matrix, the genetic similarities among the mangabeira accessions were determined using the Jaccard coefficient, and a clustering analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). The bootstrap reliability index was also estimated based on 1000 repetitions and the cophenetic correlation coefficient (r). The data analyses were carried out using Past v. 3.11 software (Hammer, Harper, & Ryan, 2001).

To estimate the structure of the genetic variability of the populations and genetic differentiation (Φ_{ST}), an analysis of molecular variance (AMOVA) was performed using ARLEQUIN v. 3.5.1.2 software (Excoffier & Lischer, 2010). In the analysis of genetic diversity, the parameters estimated in POPGENE v. 1.32 software (Yeh, Yang, & Boyle, 1999) were as follows: percentage of polymorphic loci (P), Shannon index (I) (Lewontin, 1972), Nei's genetic diversity (h) (Nei, 1973), genetic differentiation coefficient (G_{ST}) (Nei, 1987) and gene flow based on the estimated G_{ST} (McDermott & McDonald, 1993).

The population structure was inferred via Bayesian analysis in STRUCTURE v. 2.3.4 software (Pritchard, Stephens, & Donnelly, 2000). The analyses were carried out with a burn-in of 100,000 using the Markov Chain Monte Carlo (MCMC) algorithm with 1,000,000 simulations. Ten independent runs were performed for each value of K, which ranged from 1 to 8. An admixture ancestry model correlated with the frequency of alleles was adopted in the analysis. The most likely K value was determined based on the ΔK values according to Evanno, Regnaut, and Goudet (2005), which were obtained using STRUCTURE HARVESTER v. 0.6.9 software (Earl & VonHoldt, 2012).

Results

Polymorphism of ISSR markers and genetic diversity

The 11 ISSR primers amplified 166 loci, of which 120 (72.29%) were polymorphic in the studied species. The size of the amplified fragments ranged from 271 to 3973 bp. The number of amplified loci ranged from 4 (UBC 813) to 25 (UBC 826), with a mean of 15.09 (Table 2). A specific locus for *H. speciosa* var. *speciosa* was found using the primer UBC 822. The pattern of the bands obtained with the UBC 857 primer is shown in Figure 1.

Table 2. Details on the DNA amplification for the 29 mangabeira accessions from the Embrapa Meio-Norte Germplasm Bank using the 11 ISSR primers.

ISSR Primers	Sequence 5'-3'	Ta (ºC)	Amplified fragments		Percentage of Polymorphisms	Band size (bp)
			Total	Polymorphic		
UBC 808	$(AG)_8C$	54	11	7	63.64	292-1388
UBC 813	$(CT)_8T$	49	4	2	50.00	900-1930
UBC 822	$(TC)_8A$	50	8	8	100.00	494-1830
UBC 826	$(AC)_8C$	55	25	20	80.00	271-3973
UBC 827	$(AC)_8G$	52	14	10	71.43	500-2500
UBC 834	$(AG)_8YT$	46	21	14	66.67	534-2500
UBC 836	$(AG)_8YA$	50	18	9	50.00	297-1950
UBC 844	$(CT)_8RC$	52	12	9	75.00	283-3400
UBC 855	$(AC)_8YA$	57	11	7	63.64	475-887
UBC 857	$(AC)_8YG$	55	23	20	86.96	222-2263
UBC 886	$VDV(CT)_7$	46	19	14	73.68	283-2260
Total			166	120	72.29	271-3973

Ta = annealing temperature.

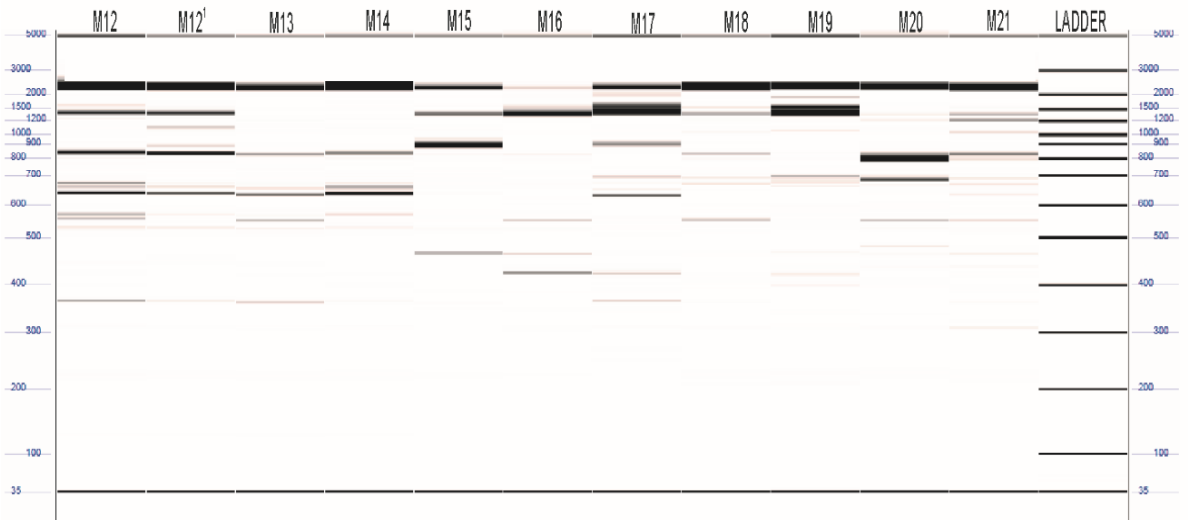

Figure 1. Virtual gel obtained via capillary electrophoresis, showing the amplified DNA fragments of the 11 mangabeira accessions from the Germplasm Bank of Embrapa Meio-Norte obtained using the ISSR marker UBC 857.

The number of polymorphic loci ranged from 64 (Distrito Federal population) to 95 (Piauí population). Nei's genetic diversity (h) was 0.26 when considering all accessions, and the values for each population ranged from 0.15 to 0.24. The Shannon index (I) ranged from 0.22 to 0.34 in the populations and 0.39 when considering all accessions. Therefore, the studied populations of mangabeira presented low or moderate levels of genetic diversity. Among the populations, the Piauí population showed the highest level of genetic diversity (Table 3).

Table 3. Genetic diversity parameters obtained for three mangabeira populations from the Embrapa Meio-Norte Germplasm Bank.

Population	L	%P	h	I
Paraíba	65	39.16	0.15	0.22
Distrito Federal	64	38.55	0.16	0.23
Piauí	95	57.23	0.24	0.34
All Samples			0.26	0.39

L = polymorphic loci, %P = percent of polymorphic loci, h = Nei's Genetic diversity, and I = Shannon index.

Population structure and genetic differentiation

The AMOVA showed that most of the genetic variability existed within the populations (69.66%). In addition, there was high genetic differentiation between the populations, with a Φ_{ST} value of 0.30 (Table 4), and the G_{ST} indicated that 32.46% of the variation existed between the populations, with gene flow (Nm = 1.04) occurring between them.

Table 4. Analysis of molecular variance showing the genetic variability between and within the mangabeira populations from the Embrapa Meio-Norte Germplasm Bank.

Sources of variation	Sum of squares	Components of variance	Percentage of variance	Fixing index (Φ_{ST})	P-value
Between the populations	123.88	6.04	30.34	0.30	0.00
Within the populations	322.71	13.86	69.66		
Total	456.59	19.90			

P-value = probability of significance.

The ΔK statistic of Evanno et al. (2005) indicated K = 2 as the probable number of genetic groups for the three mangabeira populations. Thus, the population from Paraíba was composed of one genetic group (green), and the populations from the Distrito Federal and Piauí composed another group (red). In addition, allelic sharing was observed between these two genetic groups, and accession M23 from Piauí showed a greater chance of belonging to the Paraíba genetic group, which aligned with the UPGMA grouping in which this accession was grouped within the Paraíba accessions (Figure 2 and 3).

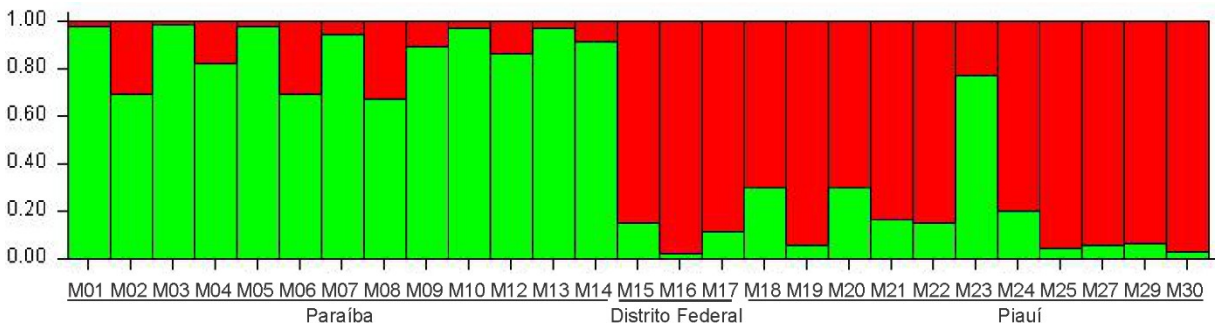

Figure 2. Bar plot with K = 2 for the mangabeira populations (Paraíba, Distrito Federal and Piauí) from the Germplasm Bank of Embrapa Meio-Norte. Plot was obtained via a Bayesian analysis using an admixture ancestry model correlated with the frequency of alleles in STRUCTURE software, with 120 polymorphic ISSR loci.

Genetic relationships between accessions

The value of the Jaccard similarity coefficient among the accessions ranged from 0.57 (from M16 to M23) to 0.94 (from M1 to M10). The value of the cophenetic correlation (r) was equal to 0.90, indicating the reliability of the grouping obtained with the UPGMA method, which generated a dendrogram representative of the original data matrix. The average value of the Jaccard coefficient considering all loci was 0.76. This value was used as the dendrogram cut-off point. Thus, the accessions were separated into seven groups. Group I was composed of six accessions from Piauí, identified as M18, M21, M22, M24, M25 and M29. Group II was composed of two accessions from Piauí, identified as M19 and M30. Groups III, VI and VII were

composed of single accessions, identified as M20, M27 and M16, respectively, with accessions M20 and M27 from Piauí and M16 is from the Distrito Federal. Group IV included accessions from Paraíba except for accessions M10[2], M12[1] and M23, which were from Bahia, Sergipe and Piauí, respectively. Group V included two accessions from the Distrito Federal, identified as M15 and M17 (Figure 3).

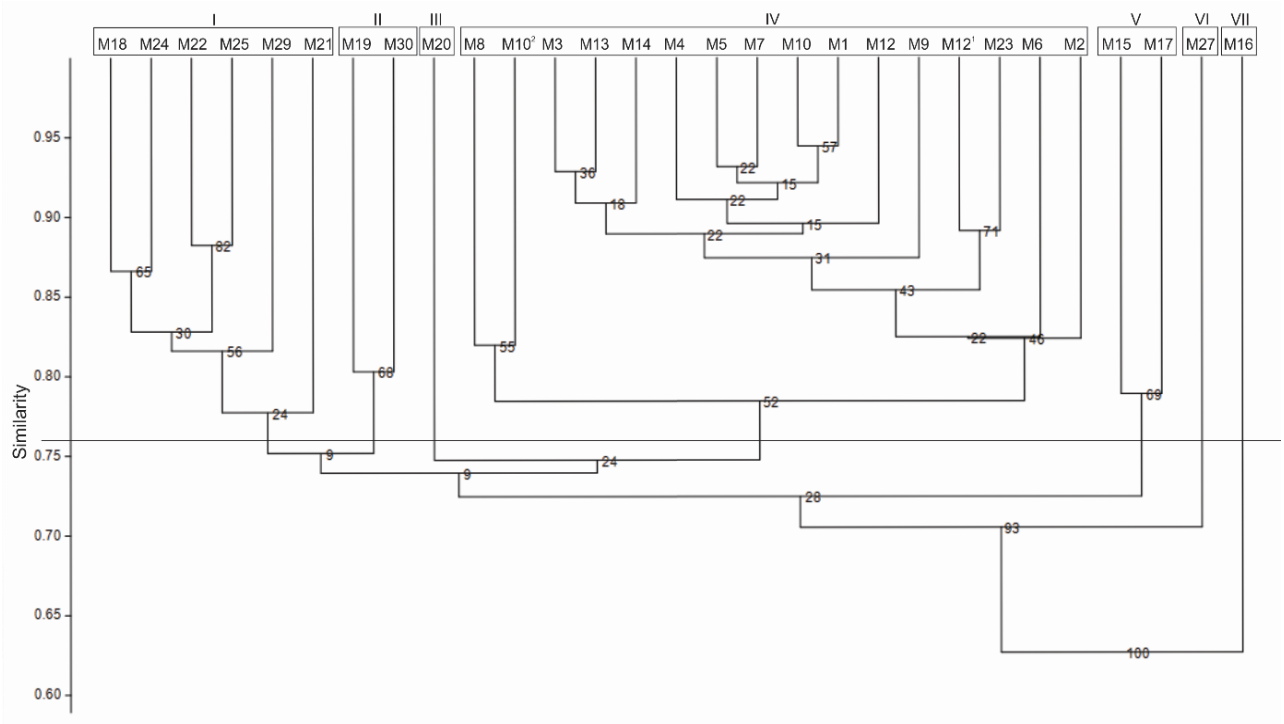

Figure 3. Dendrogram obtained with the UPGMA method based on Jaccard's similarity, showing the genetic relationships between the 29 mangabeira accessions from the Germplasm Bank of Embrapa Meio-Norte determined using 11 ISSR primers, which generated 166 loci, of which 120 were polymorphic.

Discussion

The analysis of the diversity and genetic structure of the mangabeira populations using molecular markers supports the development of conservation strategies and the identification of promising genotypes that can be used in breeding programs (Singh et al., 2016). In this study, the applied ISSR markers revealed a polymorphism rate of 72.29% and were therefore considered useful for obtaining information on the genetic diversity of mangabeira. Previous studies have identified a polymorphism rate between 47.62 and 100% for mangabeira using ISSR markers, which is consistent with the results obtained in this study (Costa, Vieira, Fajardo, & Chagas, 2015; Jimenez et al., 2015; Soares et al., 2016).

The genetic structure of the populations is a result of evolutionary forces acting over generations, such as genetic drift and natural selection (Ellstrand, 2014), as well as ecological factors that affect species dispersal and reproduction (Loveless & Hamrick, 1984; Riguete, Rangel, & Silva, 2012). The observed genetic variability was highest within the populations because of the reproductive system of the investigated species: genetic variability is expected to be larger within populations of perennial and allogamous plants, such as mangabeira, while genetic variability is higher between populations of annual and autogamous plants (Hu et al., 2010; Chhajer, Jukanti, Bhatt, & Kalia, 2018).

The high genetic differentiation of the mangabeira populations identified in this study was consistent with the Φ_{ST} value of 0.30 reported by Jimenez et al. (2015) based on an ISSR marker analysis of mangabeira from the coastal region of Pernambuco. Such high genetic differentiation may be related to random events during the transfer of alleles from one generation to another or to differences in the allelic frequencies of the individuals of these populations (Hartl & Clarck, 2010). Studies of genetic diversity in mangabeira using SSR markers have also highlighted a positive intrapopulation fixation index (f) and suggested that inbreeding plays an important role in the genetic differentiation of populations (Amorim, Mata, Lédo, Azevedo, & Silva, 2015; Collevatti et al., 2018; Costa et al., 2017). Thus, the crossing of related individuals may favor the high degree of genetic structuring observed in the mangabeira populations sampled in this study.

The gene flow value equal to 1.04 obtained among the mangabeira populations is considered sufficiently high to prevent differentiation between these populations due to genetic drift (Sheidai, Mosaferi, Keshavarzi, Noormohammadi, & Baraki, 2016; Vianna, 2015). Gene flow can significantly affect the distribution of genetic variation since it tends to introduce new allelic combinations within populations and reduce differentiation between them (Hamrick, 2012). However, the gene flow estimated from the index of genetic differentiation among populations refers to allelic exchanges that occurred in the past (Ellstrand, 2014). The gene flow between the two genetic groups obtained after the Bayesian analysis using STRUCTURE software probably resulted from seed dispersal in an anthropogenic manner and not from insect pollinators, such as bees and moths (Darrault & Schlindwein, 2005), which generally do not travel great distances in search of food.

Genetic diversity is one of the key factors in maintaining the natural ability of species to respond to environmental variations (Rogers & McGuire, 2015). In addition, genetic diversity is essential for breeding programs that can meet the needs of farmers and the market by generating more productive and better adapted genotypes through crosses between genetically distant individuals and gene manipulation (Nass, Sigrist, Ribeiro, & Reifschneider, 2012). Although mangabeira is a cross-pollinating species (Pinto, Oliveira, & Schlindwein, 2008) in which the recombination of genes from different plants favors the appearance of new genetic combinations that did not exist in the progenitors, this study demonstrated that low or moderate levels of intrapopulation genetic variation occur in the mangabeira populations.

The excess of homozygotes found in the populations is at least partly related to inbreeding and genetic drift, which reduce heterozygosity within populations (Collevatti et al., 2018; Wu, Shen, Zhang, Wang, & Sun, 2015). Additionally, the genetic diversity of the population from the Distrito Federal may be underestimated due to the small sample size. This result is in accordance with previous studies that were carried out using ISSR markers and reported low genetic diversity in mangabeira populations from Rio Grande do Norte, Bahia and Sergipe (Costa et al., 2015; Santos et al., 2017). In addition, Sá et al. (2011) noted that the genetic variability of mangabeira populations in northeast Brazil is decreasing due to anthropic pressure in environments where this species occurs naturally.

In this study, a specific locus of *H. speciosa* var. *speciosa* was identified using the primer UBC 822 and accessions $M10^{2}$, $M12^{1}$ and M23 were grouped in the dendrogram, independent of their collection site. Nogueira et al. (2015) evaluated the genetic diversity of mangabeira accessions from nine Brazilian states using ISSR and random amplified polymorphic DNA (RAPD) markers and found that accessions from distant regions belonged to the same group and no specific loci were observed for the six studied botanical varieties of mangabeira.

Conclusion

This study demonstrated a low-to-moderate level of genetic variation within mangabeira populations and high genetic differentiation between them. These findings are consistent with the occurrence of factors that reduce heterozygosity and increase the differences between populations, such as genetic drift and endogamy. Therefore, we propose that the largest possible number of mangabeira populations should be sampled from different collection sites to enhance the efficiency of conservation programs and the genetic breeding of mangabeira.

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