Diversity and genetic structure of mangaba remnants in states of northeastern Brazil

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ABSTRACT. In this study, we analyzed the genetic diversity and structure of remnants of mangaba populations in states of northeastern Brazil by applying 9 microsatellite markers previously developed to establish conservation strategies for germplasm and species preservation. Six to 20 individuals per population were analyzed, with a total of 94 individuals and 6 populations from the states of Ceará, Pernambuco, and Sergipe, Brazil. The intra-population positive fixation index (f) in all populations indicated inbreeding resulting from the lack of random mating. The mean genetic diversity index values G_{ST}, F_{ST}, and R_{ST} estimated for divergence among the 6 populations were 0.14 (P < 0.05), revealing moderate genetic differentiation. The smallest F_{ST} value (P ≥ 0.05) was observed between the Jacarecoara and Tapera populations (0.005) and the highest between the Barra dos Coqueiros and Jacarecoara populations (0.287). The Jacarecoara population was the most divergent among the populations analyzed. According to analysis of molecular variance results, the largest variation percentage resulted from variability within populations (83.18%). Bayesian

clustering analysis showed the formation of 2 sets (K = 2). Our results are important for developing strategies for *in situ* conservation of the species, seed collection, and *ex situ* conservation. For both methods, conservation of the greatest possible genetic variability of the species is essential.

Key words: Genetic variability; *Hancornia speciosa*; Microsatellite markers; Species preservation

INTRODUCTION

Genetic diversity is important for sustaining populations of plant species (Wang et al., 2007). One method of evaluating genetic diversity is through molecular characterization, which allows inference of the degree of polymorphism among individuals and populations (Costa et al., 2011) using molecular markers or DNA techniques that are not influenced by environmental conditions and do not show pleiotropic effects (Young et al., 1989).

The genetic structure of plant populations reflect the interactions of many different processes such as the long-term evolutionary history of species (such as changes in distribution, habitat fragmentation, and population isolation), mutation, reproductive, geographical, and ecological isolation, genetic drift, cross system, gene flow, and selection (Thendral Hepsibha et al., 2010; Silva et al., 2012).

Mangaba (*Hancornia speciosa* Gomes) is a native fruit tree widely distributed in Brazil. It occurs naturally in the areas of coastal tablelands and coastal plains in northeastern Brazil and the Cerrado region of mid-western, northern, and southern Brazil (Moura et al., 2005; Soares et al., 2006).

Because of its high soluble solid content associated with high acidity, giving the fruit good flavor (Soares et al., 2006), mangaba pulp is widely consumed fresh and used as raw material in the preparation of various products such as jelly, jam, syrup, wine, vinegar, and mainly juice and ice cream (Silva et al., 2011). The fruit has large economic potential, which has attracted increasing interest from sectors linked to industry and trade (Ganga et al., 2010).

The state of Sergipe is the largest mangaba producer, particularly the municipalities of Itaporanga D'Ajuda, Pirambu, Estância, Indiaroba, and Barra dos Coqueiros (IBGE, 2012). The collection of fruits from natural populations is an extractive activity performed mainly by the so-called "mangaba pickers" with consequent socio-economic and cultural importance to the state.

Several factors, such as forest fragmentation, real estate expansion, tourism, and increase in areas cultivated with coconut, pasture, and sugarcane (Moura et al., 2005; Silva Junior et al., 2007), have contributed to the significant reduction of naturally occurring growing areas and have exhibited strong pressure on native mangaba tree populations. Therefore, studies examining the genetic diversity and structure of remnant mangaba populations are fundamental for establishing conservation strategies for germplasm and species preservation.

Among molecular markers, microsatellites or simple sequence repeats are the most suitable for polymorphism studies of DNA sequences to examine population genetics because they are codominant, abundant, apparently distributed throughout the genome, and multi-allelic; further, the procedure requires the use of only a small amount of DNA, is based on polymerase chain reaction (Buso et al., 2003), and has high reproducibility.

Few studies have used microsatellite molecular markers to examine the variability in natural mangaba populations (Rodrigues, 2009; Souza et al., 2010; Costa et al., 2012).

In this study, we analyzed the genetic diversity and structure of remnant mangaba populations in 3 states in northeastern Brazil (Ceará, Pernambuco, and Sergipe) using microsatellite markers to assist in the establishment of *in situ* and *ex situ* conservation strategies.

MATERIAL AND METHODS

Sampling, collection, and DNA extraction

Approximately 6-20 individuals per native *H. speciosa* population were sampled in a total of 6 populations and 94 individuals, 59 from the state of Sergipe (SE), 20 from Ceará (CE), and 15 from Pernambuco (PE), Brazil (Figure 1, Table 1). Because these are remnant populations, the total number of individuals in some cases was exactly the size of populations, making the collection of larger sample sizes impossible. Young leaves without mechanical damage and signs of disease were collected, identified, placed in plastic bags, and transported to the Laboratory of Molecular Biology at Embrapa Coastal Tablelands, in which genomic DNA was extracted based on the CTAB standard protocol (Doyle and Doyle, 1990), modified by Costa et al. (2011). DNA was quantified by comparison with known standard molecular weight using bacteriophage λ DNA on a 2% agarose gel stained with ethidium bromide. Samples were diluted to a concentration of 3.0 ng/μL.

Figure 1. Locations of natural *Hancornia speciosa* populations in the states of Sergipe, Ceará, and Pernambuco, Brazil, where samples were collected to examine genetic diversity and structure.

Table 1. Identification, geographical location, and sample size of the 6 *Hancornia speciosa* populations.

Populations	Geographical coordinates	No. Individuals sampled
Reserva do Caju (SE)	11°11'6"S and 37°11'18"W	19
Abaís (SE)	11°18'18"S and 37°17'18"W	20
Barrados Coqueiros (SE)	10°49'10"S and 36°56'52"W	20
Jacarecoara (CE)	4°07'10"S and 38°10'34"W	14
Tapera (CE)	3°56'20"S and 38°20'18"W	6
Tamandaré (PE)	8°43'50"S and 35°6'10"W	15
Total		94

Genotyping of microsatellites

Individuals were genotyped at the Laboratory of Plant Genetics, Embrapa Genetic Resources and Biotechnology (Cenargen), Brasília, DF. Overall, 16 pairs of primers developed by Rodrigues (2009) were tested. For each pair of primers, the forward with fluorescence was marked (Table 2). The polymerase chain reaction consisted of the following combination of reagents: 9.0 ng DNA, 0.25 mg/mL bovine serum albumin, 0.2 µM of each primer (forward and reverse), 1X polymerase chain reaction buffer (10 mM Tris-HC, pH 8.3, and 50 mM KCl), 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 U *Taq* DNA polymerase, and sterile ultrapure water to a final reaction volume of 13.0 µL. For the amplification reactions, the thermocycler used with the following program: denaturation at 94°C for 1 min, 35 cycles at 95°C for 1 min, annealing temperature specific for each locus (Table 2) for 1 min, extension at 72°C for 1 min, and 72°C for 10 min.

Multiplex systems consisting of 2-3 loci were assembled using primers marked with different or the same fluorochrome, but with different allele amplitudes. These systems were mixed to 11 µL cocktail containing 10 µL HiDi formamide and 1 µL internal marker developed by Brondani and Grattapaglia (2001). The resulting mixture was denatured for 5 min at 95°C. Next, fragments were separated on an ABI automated DNA analyzer 3730 (Applied Biosystems, Foster City, CA, USA).

Data analysis

The presence of null alleles and their interference in allele frequencies was verified using the Micro-Checker software (Van Oosterhout et al., 2004). A confidence interval of 95% was used and 1000 Monte Carlo simulations were performed. Equation 1 described by Brookfield (1996) was used to estimate the frequency of null alleles. Linkage disequilibrium and Hardy-Weinberg equilibrium were evaluated based on allelic permutations within populations using the FSTAT software (Goudet, 1995).

The GDA software was used for descriptive analysis of intra-population genetic diversity (Lewis and Zaykin, 2002), estimating the following parameters: allelic number per locus (N_A), observed heterozygosity (H_O), expected heterozygosity under Hardy-Weinberg equilibrium (H_E), and the inbreeding coefficient of populations (*f*). The consistency of estimates of the inbreeding coefficient was evaluated using the bootstrap method with resampling of 10,000 and confidence interval of 95% (Weir, 1996). Allelic richness (R) and allele frequencies were calculated using the FSTAT software (Goudet, 1995).

To characterize inter-population genetic variability, Weir and Cockerham (1984) parameters were estimated, including total fixation index (F_{IT}), genetic divergence among popu-

lations (F_{ST}), and fixation index within populations (F_{IS} is equivalent to the f index), using the FSTAT software (Goudet, 1995). Similarly, other indices similar to F_{ST} such as G_{ST} (Nei, 1973) and R_{ST} (Slatkin, 1995) were determined. The consistency of results was assessed using the bootstrap method for all loci, with a confidence interval of 99%.

An array of pairs of F_{ST} (Weir and Hill, 2002) was calculated for the 6 populations and the average gene flow was estimated using the following formula: $Nm = (1/F_{ST} - 1)/4$ (Whitlock and Mccauley, 1999) and the absolute number of migrants among populations (M $= 2Nm$). These analyses were performed using the Arlequin software v. 3.1 (Excoffier et al., 2006). Significance was tested with 1000 permutations. To identify the distribution patterns of variability and genetic differentiation among *H. speciosa* populations, analysis of molecular variance was performed.

Inference of the population structure, number of populations or existing clusters (K), and assignment of sampled individuals to each group was performed using the Structure software v. 2.3.4 (Pritchard et al., 2000). K values ranging from 1-9 were estimated with resampling of 1,000,000 (rejecting the first 250,000) and 20 iterations. To detect the most probable number of groups, we used the ΔK statistics described by Evanno et al. (2005).

RESULTS AND DISCUSSION

Among the 16 microsatellite loci tested, 09 (HS01, HS05, HS06, HS08, HS10, HS11, HS16, HS27, and HS33) allowed amplification of fragments with clear and consistent bands on the agarose gel and showed polymorphisms. Therefore, these loci were further analyzed. The optimal annealing temperature defined for each of the 9 loci ranged from 52°-56°C (Table 2).

Table 2. Annealing temperature (Ta), reasons for repeats, allele amplitude (bp[1]), and fluorescence of microsatellite primers tested for the genetic characterization of *Hancornia speciosa* populations (Rodrigues, 2009).

Primer	Ta (°C)	Reasons for repeats	Allele amplitude (bp)	Fluorescence
HS 01	56	$(GCA)_6(TC)_{20}(GCA)_8$	250-310	HEX
HS 03	56	$(CT)_5(CT)_6$	120-180	6-FAM
HS 05	56	$(GA)_{15}(TGC)_6$	200-300	HEX
HS 06	54	GA_{14}	100-150	HEX
HS 08	52	$(CA)_6(CT)_{17}$	200-250	6-FAM
HS 09	56	$(CT)_7(CT)_{10}(CT)_6$	200-300	6-FAM
HS 10	56	$(CT)_{14}(CT)_8$	100-200	HEX
HS 11	56	$(GA)_{17}$	100-200	6-FAM
HS 16	54	$(GA)_{12}$	100-150	6-FAM
HS 17	56	$(GA)_{16}$	100-200	HEX
HS 18	56	$(AG)_{14}$	300-350	6-FAM
HS 20	56	$(CT)_{11}$	200-250	HEX
HS 27	54	$(GA)_{14}$	100-150	6-FAM
HS 30	56	$(AG)_{10}$	180-200	HEX
HS 32	56	$(CT)_9$	200-300	6-FAM
HS 33	56	$(AG)_{24}$	80-120	6-FAM

[1]bp = base pairs.

No locus showed deviation in the proportions of the Hardy-Weinberg equilibrium and linkage disequilibrium probability tests in all populations showed no associations among loci.

The allelic number per locus (N_A) appeared to be low for all populations evaluated, with mean values ranging from 3.56-5.56, respectively, for the Tamandaré (PE) and Abaís

(SE) populations (Table 3), with a difference of 36% between populations. Therefore, despite the low level, the difference was relevant. This difference may be important for future adaptation of populations with low levels of genetic variability among individuals (Diniz-Filho et al., 2012).

Table 3. Average sample size (N), number of alleles (N_A), allelic richness (R), expected heterozygosity (H_E), observed heterozygosity (H_O), and inbreeding coefficient (*f*) in relation to the 9 loci analyzed for 6 *Hancornia speciosa* populations.

Populations	N	N_A	R	H_E	H_O	*f*	95%CI
Reserva do Caju (SE)	19	5.11	3.42	0.58	0.43	0.26	0.11 to 0.42*
Abaís (SE)	20	5.56	3.31	0.57	0.50	0.12	0.03 to 0.25*
Barrados Coqueiros (SE)	20	4.56	2.99	0.49	0.35	0.30	0.10 to 0.53*
Jacarecoara (CE)	14	5.00	4.03	0.72	0.61	0.16	0.06 to 034*
Tapera (CE)	6	4.33	4.11	0.67	0.41	0.40	0.22 to 0.60*
Tamandaré (PE)	15	3.56	2.93	0.56	0.49	0.12	0.01 to 0.31*
Mean		4.69	3.47	0.60	0.47	0.23	

95%CI = 95% confidence interval. *Significant at 95% probability, based on bootstrap resampling of 10,000.

Despite the variation in sample size among populations, the allelic richness result (R) indicated no direct relationship between sample size and mean alleles per locus, and Tamandaré population (15 individuals) showed the lowest allelic richness (2.93), while the Tapera population (6 individuals) showed the highest allelic richness (4.03) (Table 3). These results suggest that no direct relationship exists between sample size and variability.

The populations with the lowest and the highest genetic diversity among those analyzed were Barra dos Coqueiros and Jacarecoara, respectively, with mean expected heterozygosity (H_E) values equal to 0.49 and 0.72 and observed heterozygosity (H_O) values equal to 0.35 and 0.61. All populations showed H_E values greater than H_O values, and therefore excess homozygotes as expected for a population in Hardy-Weinberg equilibrium (Table 3).

The intra-population positive fixation index (*f*) shown by all populations indicated inbreeding resulting from a lack of random mating. This can be caused by the crossing of related individuals or self-fertilization.

The mean H_O, H_E, and *f* values obtained in this study are within standards reported for other *H. speciosa* populations (Costa et al., 2012). However, compared to the results of Souza et al. (2010), we found that mean H_O values were higher than previously reported values, indicating that the population they studied had a lower proportion of heterozygous individuals, resulting in higher *f* values and lower genetic diversity.

Population genetics studies examining tree species have shown that trees are generally allogamous, but with positive inbreeding coefficients (Azevedo et al., 2007; Santos et al., 2011; Alves et al., 2013).

F_{ST} values less than 0.05 indicate low genetic differentiation among populations; 0.05-0.15, moderate; 0.15-0.25, high; and above 0.25, very high, according to Wright (1969). The genetic differentiation among populations at the state level (among the 3 states) was highly significant (P < 0.01), with G_{ST}, F_{ST}, and R_{ST} values equal to 0.14 (Table 4), revealing moderate genetic differentiation among these populations. Moderate diversity was observed for the average of the 3 populations of Sergipe when the indices were calculated for these populations (G_{ST} = 0.07, F_{ST} = 0.07, and R_{ST} = 0.05). Low genetic differentiation was also verified between the 2 populations from the state of Ceará (G_{ST} = 0.01, F_{ST} = 0.01, and R_{ST} = -0.05).

Table 4. Genetic diversity indices among populations (G_{ST}, F_{ST}, R_{ST}), total fixation index (F_{IT}) obtained for different groups of *Hancornia speciosa* populations.

Group (size)	G_{ST}	R_{ST}	F_{ST}	F_{IT}
States (3)	0.14	0.14	0.14**	0.34**
Sergipe (3)	0.07	0.05	0.07**	0.28**
Ceará (2)	0.01	-0.05	0.01^{ns}	0.26**

**Highly significant (P < 0.01). ns = not significant.

The comparison results of F_{ST} for pairs of populations (Table 5) indicated that the lowest F_{ST} value was observed for Jacarecoara (CE) and Tapera (CE) populations (0.005), which was not significant (P ≥ 0.05). The highest F_{ST} value was found for pairs of Barra dos Coqueiros (SE) x Jacarecoara (CE) populations (0.287). The Jacarecoara population was the most divergent compared to the other populations, except in comparison to the population from the same state, Tapera. Our results will be useful for supporting conservation strategies aimed at preserving genetically diverse populations. For the *ex situ* conservation of the greatest possible genetic diversity of the species, these same populations are essential as a source of germplasm to enrich the collection stored in the Mangaba Germplasm Active Bank (BAG Mangaba) at Embrapa Coastal Tablelands. Additionally, these results may guide the selection of parents for use in breeding programs.

Table 5. Divergence array correlating F_{ST} (genetic divergence index, diagonal below) and M = 2Nm (absolute number of migrants, diagonal above) between *Hancornia speciosa* populations.

	Reserva do Caju	Abaís	Barra dos Coqueiros	Jacarecoara	Tapera	Tamandaré
Reserva do Caju	-	44.196	5.089	1.795	3.079	9.210
Abaís	0.011^{ns}	-	2.988	2.295	4.446	24.824
Barra dos Coqueiros	0.089*	0.143*	-	1.244	1.560	3.053
Jacarecoara	0.218*	0.179*	0.287*	-	102.399	2.958
Tapera	0.140*	0.101*	0.243*	0.005^{ns}	-	7.745
Tamandaré	0.051*	0.020^{ns}	0.141*	0.145*	0.061*	-

ns = not significant. *Significant (P < 0.05).

For the absolute number of migrants (M = 2 Nm), the maximum and minimum values were equal to 102.4 and 1.2, respectively, between the Jacarecoara (CE) and Tapera (CE) populations and the Jacarecoara (CE) and Barra dos Coqueiros (SE) populations (Table 5). These results agree with the maximum and minimum F_{ST} values for pairs of population; the smaller the genetic distance between populations, the higher the gene flow between them (Mangaravite, 2012).

Thus, the Jacarecoara x Tapera pair of populations showed the lowest genetic divergence, while the Barra dos Coqueiros x Jacarecoara pair was the most divergent. This can be explained by the fact that Jacarecoara and Tapera are geographically close populations. However, Jacarecoara and Barra dos Coqueiros populations are in different states and geographically distant. Although these results suggest a positive correlation between geographical and genetic differentiation, the geographically close Reserva do Caju (SE) and Abaís (SE) populations showed intermediate F_{ST} values compared with the Tamandaré (PE) population.

According to the analysis of molecular variance, the largest variation percentage resulted from variability within populations (83.18%), with 10.82% of variation among states

and 6.00% among populations within each state (Table 6). Similar results for variation within *H. speciosa* populations were observed by Costa et al. (2011) and Moura et al. (2011). High genetic diversity within populations is commonly found in tree species.

Table 6. Genetic variability distribution of *Hancornia speciosa* populations based on the analysis of molecular variance of 9 loci.

Source of variation	df	Sum of squares	Variation components	Variance (%)	P value
Among states	2	47.59	0.34	10.82	<0.001
Among populations within states	3	25.16	0.19	6.00	<0.001
Within populations	182	450.12	2.63	83.18	<0.001
Total	187	522.87	3.16		

Based on Bayesian clustering analysis conducted using the Structure software to evaluate the population genetic structure, the highest ΔK value converged for K = 2 (Figure 2), with little variation between values estimated for different repeats, where K represents the number of genetic groups identified (Evanno et al., 2005).
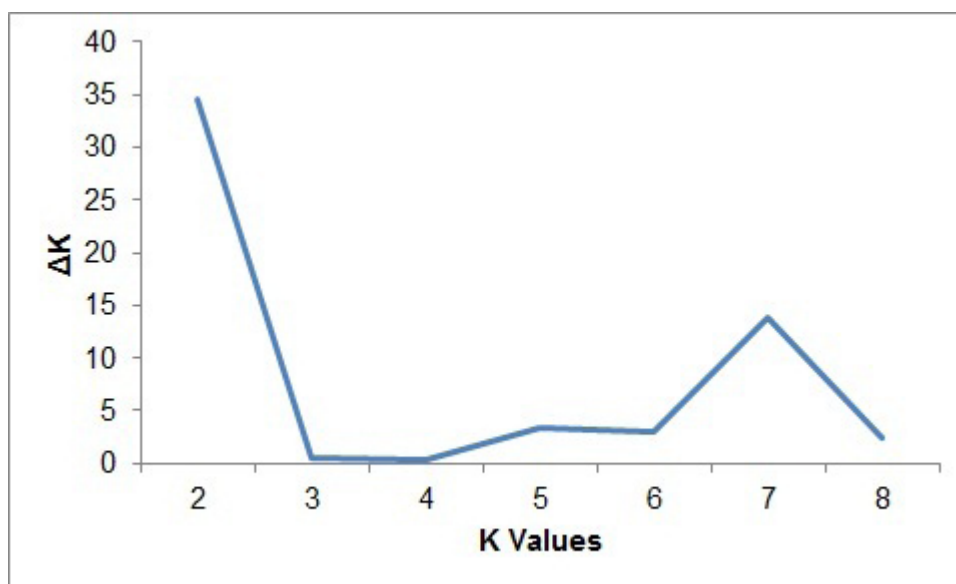

Figure 2. Correlation among K values (number of genetic groups identified) and ΔK for *Hancornia speciosa* populations.

This result indicates the formation of 2 groups. Populations from Ceará (Jacarecoara and Tapera) were more homogeneous with regard to the distribution of individuals (predominance of red bars) and were very similar to each other, representing a cluster. The Barra dos Coqueiros population was also one of the most homogeneous (predominantly green bars compared to red bars), representing another cluster. However, the other 3 populations were heterogeneous, as a mixture of these 2 clusters (Figure 3). This result reinforces the F_{ST} values

found, showing almost no differentiation between populations from Ceará (0.5 %), and high values for Barra dos Coqueiros population compared to these 2 populations (Table 5).
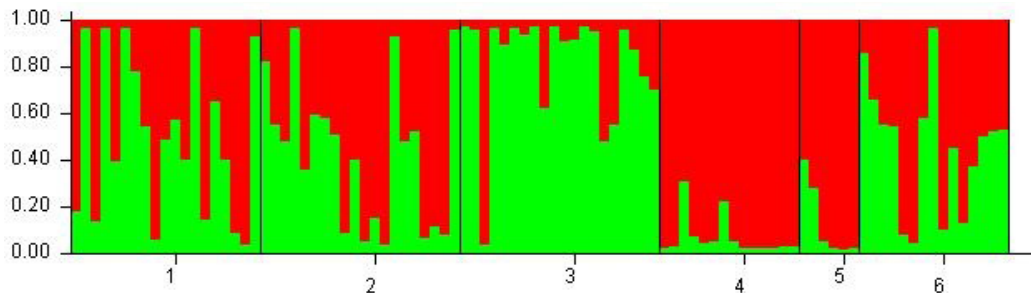
Figure 3. Genetic structure of *Hancornia speciosa* populations according to Bayesian clustering analysis implemented by the Structure software for K = 2, with samples sorted by population: 1 = Reserva do Caju (SE), 2 = Abaís (SE), 3 = Barra dos Coqueiros (SE), 4 = Jacarecoara (CE), 5 = Tapera (CE) and 6 = Tamandaré (PE). Each group is represented by a color and each individual by a vertical bar.

The results obtained for the *H. speciosa* populations revealed moderate inter-population diversity, with the highest estimated variation resulting from divergences within populations. However, significant differentiation was observed when comparing the populations from Ceará with the population from Sergipe, Barra dos Coqueiros, and intermediate differentiation, compared with the Tamadaré (PE), Reserva do Caju (SE), and Abaís (SE) populations. The identification of these 3 groups allows determinating collection strategies in at least 1 population of each of the 3 groups: 1. Jacarecoara or Tapera; 2. Barra dos Coqueiros; 3. Reserva do Caju, Abaís, or Tamandaré.

The *in situ* conservation of these 3 groups is also essential. Because the population fragments are very small, the recovery of areas with mangaba plantations in these regions should also be encouraged.

This study was the first to analyze genetic diversity and the structure of remnant natural mangaba populations in the states of Ceará, Pernambuco, and Sergipe using microsatellite markers. Thus, because the number of studies of *H. speciosa* species using this type of molecular marker is limited, further analysis should be conducted.

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