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Genetic diversity of the mangaba GeneBank using microsatellites

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ABSTRACT. The mangaba, *Hancornia speciosa*, (Apocynaceae) is a fruit tree native to Brazil with predominantly extractivist production. The fruit can be consumed *in natura*; however, it is widely consumed as frozen pulp and ice cream produced by agro-industry companies. We evaluated the genetic diversity of 213 individuals that make up the mangaba GeneBank of Embrapa Tabuleiros Costeiros, using nine microsatellite markers (SSR). A total of 147 alleles were identified, with a mean of 16 alleles per locus; 100% polymorphism was observed among accessions. Reliability of the result was verified based on stress (0.042) and correlation (0.988) values. The alleles presented a high frequency of heterozygosity (He > Ho). The F_{st} (0.22) and *f* (0.07) values indicated moderate population structure, with great diversity within accessions. Bayesian analysis indicated the most adequate grouping with k = 2. The Unweighted Pair Group Method analysis showed three distinct groups according to similarity. The BI accession had the best genetic structure. The PM5/GX2, CN1/CN9, G18/PA1, JA14/JA15, and OI8/OI9 pairs of individuals are the closest genetically. We conclude

that the Mangaba GeneBank has high diversity; this knowledge is relevant to develop strategies for the management of these genetic resources.

Key words: *Hancornia speciosa*; Variability; Conservation; SSR; Brazilian fruit; Germplasm

INTRODUCTION

The mangaba, *Hancornia speciosa*, (Apocynaceae) is a fruit tree native to Brazil and is also found in Peru, Bolivia and Paraguay. Despite its importance and potential for increased production and consumption, there are no commercial varieties, and production is predominantly extractivist. In Brazil, it is naturally found from the coastal tablelands and coastal littoral lowlands in the northeastern region to the cerrado in the midwestern, northern, and southeastern regions of the country (Silva et al., 2017a).

The fruit is the main product, which in addition to being consumed *in natura*, is widely used in the production of frozen pulp, ice creams, and jellies. The pulp has antioxidant activity and a high vitamin C content (Lima et al., 2015).

The species natural occurrence areas have suffered accelerated reduction in the last decade, mainly in the northeastern coastal region, due to real estate activity expansion and deforestation. As a consequence, various collections and germplasm banks have been implemented and maintained by teaching and research institutions in order to conserve these genetic resources. There are nine mangaba germplasm banks distributed among Brazilian Agricultural Research Corporations - Embrapa (4); State Agricultural Research Corporation of Paraíba - Emepa (1); Federal University of Lavras - UFAL (1); Federal University of Goiás - UFG (1), and State University of Goiás - UEG (1). The Mangaba GeneBank (BGMangaba) belongs to Embrapa Coastal Tablelands; it is accredited by the Ministry of the Environment, and it is a reliable custodian of this specie's genetic patrimony (Soares et al., 2018). Knowledge of the genetic diversity preserved in this germplasm assists in conservation strategies and provides key information for breeding programs.

The genetic diversity of this species has been studied in recent years. Some studies were carried out on natural populations (Amorim et al., 2015; Soares et al., 2016; Silva et al., 2017a), germplasms (Costa et al., 2011; Silva et al., 2012; Collevatti et al., 2016), and among botanical varieties (Nogueira et al., 2015). Mangaba microsatellites were developed by Rodrigues et al. (2015) and tested by Amorim et al. (2015).

We evaluated the diversity and genetic structure of accessions in the Mangaba GeneBank of the Embrapa Tabuleiros Costeiros using microsatellite markers (SSR)

MATERIAL AND METHODS

The Mangaba GeneBank is located in Itaporanga d'Ajuda, Sergipe, Brazil (latitude 11°06'40" S; longitude 37°11'15" N and altitude 9 m). The first accessions were implanted in 2006; currently, there are 213 individuals representing 22 accessions originated from seven states (Bahia, Sergipe, Paraíba, Pernambuco, Alagoas, Pará, and Minas Gerais; Table 1).

Table 1. Origin of the accessions in the Mangaba GeneBank of Embrapa Tabuleiros Costeiros.

Origin	Accession	Number of plants	Latitude	Longitude
Jandaíra, BA	Costa Azul - CA	6	11º33'11'S	37°40'52"W
Conde, BA	Barra de Itariri – BI	6	11°48'34"S	37°40'52"W
Mata de São João, BA	Lagoa Grande – LG	6	12º32'52"S	38°18'04"W
Indiaroba, SE	Terra Caída – TC	6	11°31'10"S	37°30'47"W
Indiaroba, SE	Preguiça – PR	6	11°30'43"S	37°27'11"W
Indiaroba, SE	Pontal – PT	6	11°28'24"S	37°24'16"W
Salvaterra, PA	Água Boa – AB	6	00°45'22"S	48°30'55"W
Conde, PB	Ipiranguinha – IP	5	07°15'37"S	34°54'43"W
Alhandra, PB	Mata Redonda – AD	5	07°20'38"S	34°56'07"W
Conde, PB	Guaxinduba – GX	1	07°15'38"S	34°54'30"W
João Pessoa, PB	Paratibe – PA	4	07°12'25"S	34°49'36"W
Barra dos Coqueiros, SE	Capoã – CP	18	10°51'50"S	36°58'51"W
Palmeiras, BA	Casas Velhas – CV	18	12°25'38"S	41°29'26"W
Rio Pardo de Minas, MG	Chapada do Areião - CH	17	15°29'21"S	42°28'10"W
Montes Claros, MG	Tabua – TA	11	16°43'43"S	43°51'29"W
Couto Magalhães, MG	Magalhães de Minas – CM	15	18°04'18"S	43°28'17"W
Sinhaém, PE	Guaiamum – GU	15	08°37'17"S	35°03'22"W
Ipojuca, PE	Oiteiro – OI	17	08°23'53"S	35°03'39"W
Japaratinga, AL	Japaratinga – JA	17	09º05'23"S	35°15'29"W
Maragogi, AL	Ponta do Mangue – PM	14	09°00'37"S	35°13'14"W
Tamandaré, PE	Tamandaré – TM	2	08°45'36"S	35°06'18"W
Tamandaré, PE	Carneiros – CN	12	08º45'35"S	35º06'17"W

Young leaves were collected and stored in a cooler with ice for transportation and later stored at -80ºC until DNA extraction for genotyping. Genomic DNA was extracted from young leaves from 213 specimens of mangaba. Approximately 200 mg of leaves were macerated in liquid nitrogen according to the protocol described in Doyle and Doyle (1990), with adaptations from Alzate-Marin et al. (2009). The extraction solution consisted of 2% CTAB (100 mM Tris HCl pH 8.0, 1.4 M sodium chloride, 20 mM EDTA); 2% PVP (polyvinylpyrrolidone) and 2% β-mercaptoethanol) preheated to 65°C. The DNA concentration was estimated by spectrophotometry (NanoDrop 2000C - Thermo Scientific, Massachusett, USA) and its integrity verified in 1% (w/v) agarose gel electrophoresis stained with ethidium bromide (Sambrook et al., 1989). After standardization (at 3 ng/μL), the samples were used in PCR reactions. Nine pairs of microsatellite markers (Rodrigues et al., 2015) labeled with the fluorochromes 6-FAM or HEX (Table 2) were tested.

Table 2. Relationship between pairs of microsatellite markers used to study the genetic diversity in the Mangaba GeneBank of the Embrapa Tabuleiros Costeiros.

Primer	Ta (°C)	Repeats	Allele amplitude(bp)	Fluorescence
HS01	52	$(GA)_6(TC)_{20}(GCA)_8$	250 - 310	HEX
HS03	56	$(CT)_5(CT)_6$	120 - 180	6-FAM
HS05	56	$(GA)_{15}(TGC)_6$	200 - 300	HEX
HS08	56	$(CA)_6(CT)_{17}$	200 - 250	6-FAM
HS10	56	$(CT)_{14}(CT)_8$	100 - 200	HEX
HS11	56	$(GA)_{17}$	100 - 200	6-FAM
HS16	60	$(GA)_{12}$	100 - 150	6-FAM
HS27	52	$(GA)_{14}$	100 - 150	6-FAM
HS33	56	$(AG)_{24}$	80 - 120	6-FAM

Ta: annealing temperature of each primer; Reason for repetition: Reason for repetition of the microsatellite region; AVA: interval in the allele amplitude variation of each microsatellite locus (bp = base pairs) and fluorescence: fluorochrome used in the primer.

Each PCR reaction was performed in a total volume of 13 μL of a reaction mix containing: 9 ng DNA, 0.25 mg/mL BSA (serum bovine albumin), 0.2 μM of each marker (25 pmol), 1 x PCR buffer (10X), 0.25 mM dNTP mix (2.5 mM), 1 U of Taq DNA polymerase, and sterile ultrapure water.

The PCR reactions were performed on a Veriti 96 Fast thermal cycler (Applied Biosystems, California, USA) under the following conditions: one initial cycle at 94°C for 1 min followed by 35 cycles at 95°C for 1 min, at the ideal temperature for each locus (Table 2) for 1 min, at 72°C for 1 min, and one final extension cycle at 72°C for 20 min followed by indefinite cooling at 12°C.

The PCR products were verified in 2% (w/v) agarose gel electrophoresis stained with ethidium bromide (0.5 $\mu g.ml^{-1}$) (Sambrook et al., 1989) and photo documented under ultraviolet light on a gel documentation machine (Kodac Gel Logic Imaging System, NY, USA). A 1 Kb Ladder DNA marker (Invitrogen) was used as the standard in the gel.

The fragments were analyzed in a solution containing 1 µL of each reaction mixed with 10 µL of HiDi formamide (Applied Biosystems, CA, USA) and 1 µL of internal carboxy-X-rhodamine (ROX) marker developed by Brondani and Grattapaglia (2001). This solution was denatured for 5 min at 95°C and the fragments were separated in an ABI 3730 automatic DNA analyzer (Applied Biosystems, CA, USA).

The band patterns were transformed into data matrices to estimate all analyses of genetic diversity. Detection of fluorescence peaks and genotyping were performed with Genemapper version 4.1 (Applied Biosystems, CA, USA). Allele sizes were adjusted for the allelic classes defined by the AlleloBin program.

The genotyping based on the analysis of fragments allowed inferring about the genetic distance between individuals, reflecting the genetic variability. The determination of the number of amplified fragments required in diversity studies was based on correlation estimates (r) of similarity matrix values and the stress value (E), which express the fit between the original and simulated matrices. The optimal number of fragments was calculated in the GENES program version 2007.0.0 and considered acceptable for the analyses when the stress value was less than 0.05 (Kruskal, 1964) and the correlation was close to 1.

The intra-population genetic variability (within accessions) was determined from estimates of allelic frequencies and the mean number of alleles per locus (A), observed heterozygosity (Ho), expected heterozygosity (He), and the fixation index (f), assuming that the loci were in Hardy-Weinberg equilibrium; these analyses were calculated using the GDA software (Genetic Data Analysis, version 1.0).

Null hypotheses were tested for the accessions and loci in order to evaluate Hardy-Weinberg equilibrium and linkage using the Bonferroni correction at the $P < 0.05$ level of significance in the FSTAT software, version 2.9.3. The Weir and Cockerham (1984) parameters were estimated in order to characterize the genetic variability intra-population (within accessions) and between groups (origins): F_{it}, which estimates the global heterozygote deficiency; F_{st}, which estimates the heterozygous deficiency between populations (accessions), and F_{is}, which estimates the heterozygote deficiency within populations (accessions). We also calculated G_{st} (Nei, 1973) and R_{st} (Goodman, 1997), considering the stepwise mutation model.

The software ARLEQUIN, version 3.1 was used to perform the molecular variance analysis (AMOVA) and to calculate the fixation index (F_{st}) among accessions (Weir and Hill, 2002). The Structure, version 2.3.4 program was used in the analyses of population genetic structure. The number of clusters was inferred using the method proposed by Evanno et al. (2005), and the mean Euclidean distance matrix was determined among

accessions as a measure of dissimilarity using the GENES program. A dendrogram was generated through the mean distance grouping method (UPGMA) using the R software.

RESULTS AND DISCUSSION

The nine SSRs previously tested by Amorim et al. (2015), resulted in 147 alleles with 100% polymorphism. The number of alleles varied from 6 (HS03) to 33 (HS01), with an average of 16, which is close to the mean of 17.1 reported by Collevatti et al. (2016), who evaluated the mangaba germplasm of the Federal University of Goiás.

All analyzed loci indicated a tendency towards Hardy-Weinberg equilibrium. The probability tests of linkage disequilibrium did not show associations between loci, assuming statistical independence. The HS10 accession was the only one that presented a high proportion of heterozygotes. The highest observed f value was 0.704 for the HS03 marker; the remaining values were low (Table 3). The number of alleles being potentially influenced by differences in sample sizes (Kalinowski, 2004) might explain how this result influenced the mean number of alleles per locus.

Table 3. Genetic diversity within accessions in the Mangaba GeneBank of the Embrapa Tabuleiros Costeiros obtained in the analysis of nine microsatellite loci.

Primer	N_a	He	Ho	f
HS01	33	0.872	0.706	0.191
HS03	6	0.246	0.073	0.704
HS05	18	0.772	0.461	0.404
HS06	21	0.765	0.584	0.237
HS08	15	0.803	0.589	0.266
HS10	8	0.564	0.657	-0.165
HS16	22	0.760	0.718	0.055
HS27	11	0.430	0.243	0.435
HS33	13	0.689	0.584	0.152
Mean	16	0.656	0.513	0.253

Proportion of polymorphic loci = 100% for all; N_a = number of alleles; Ho = frequencies of observed heterozygotes; He = frequencies of expected heterozygotes; f = intra-population inbreeding/fixation index.

The results' reliability was verified by observing the stress value (0.042) and cophenetic correlation (0.988), which reached stability with 12 amplified loci (r = 0.9) for the 213 mangaba individuals (Figure 1). The value of E < 0.05 indicates precision in the estimates (Kruskal, 1964).

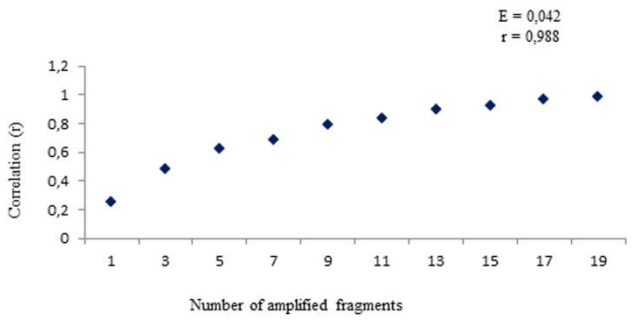

Figure 1. Determination of the correlation coefficient for the number of polymorphic fragments among 213 individuals in the Mangaba GeneBank of Embrapa Tabuleiros Costeiros using microsatellite markers.

The percentage of polymorphism observed was 91% (Table 4), which is higher than that reported by Amorim et al. (2015) and Jimenez et al. (2015) in natural populations of mangaba in northeastern Brazil. The positive and negative f values indicated that some accessions tend to endogamy (CM, CP, TA, CV, CH, GX, AD, PR, TC, LG, BI, CA, and TM), and others to Hardy-Weinberg equilibrium (JA, GU, OI, PM, CN, PA, IP, AB, and PT). The mean value of He was higher than Ho (0.566) and similar to that reported by Rodrigues et al. (2015). However, 11 accessions showed high frequencies of heterozygosity (CM, CP, TA, CV, CH, AD, TC, LG, BI, CA, and TM); 11 presented low frequencies of heterozygotes and may require more individuals to be representative in the future (Table 4).

Table 4. Determination of the genetic diversity within the accessions in the Mangaba GeneBank of Embrapa Tabuleiros Costeiros using nine microsatellite loci.

Accession	n	P (%)	He	Ho	f
JA	15	100	0.494	0.478	-0.036
GU	13	89	0.523	0.521	-0.002
OI	16	89	0.454	0.432	-0.054
CM	12	100	0.568	0.675	0.164
CP	15	100	0.414	0.450	0.082
TA	9	100	0.567	0.609	0.072
CV	15	89	0.523	0.562	0.068
CH	15	100	0.546	0.749	0.278
PM	13	100	0.547	0.531	-0.032
CN	11	88	0.534	0.485	-0.107
PA	4	78	0.472	0.373	-0.328
GX	1	78	0.778	0.778	0.000
AD	4	89	0.539	0.585	0.089
PR	5	78	0.511	0.511	0.001
TC	5	89	0.289	0.497	0.444
LG	5	89	0.420	0.588	0.301
IP	4	89	0.606	0.594	-0.019
AB	5	87	0.646	0.536	-0.232
PT	5	89	0.659	0.608	-0.115
BI	5	100	0.489	0.701	0.320
CA	5	89	0.396	0.570	0.326
TM	2	89	0.611	0.629	0.043
Mean		91	0.566	0.527	0.082

n = number of individuals analyzed per locus; P = proportion of polymorphic loci; Ho = frequency of observed heterozygotes; He = frequency of expected heterozygotes; f = intra-population inbreeding/fixation index.

The low inbreeding coefficient in BGMangaba was confirmed by the mean value of f (0.082), which allows inferring that there is high genetic diversity. Nevertheless, the accessions TC, LG, BI, and CA tend to be inbred because they presented the highest f values. When genetic diversity is analyzed between accessions and/or populations, it is known that a high inbreeding coefficient can indicate deleterious effects for the species due to undesirable genetic combinations (Govindaraj et al., 2015). Loiola et al. (2016) also observed low f values, ranging from 0.12 to 0.37, when analyzing the genetic diversity of the Coconut GeneBank using SSR markers; this result indicates high diversity and conservation in the accessions we examined. Using the same SSR markers, Amorim et al. (2015) also observed f values < 0.5 in other native mangaba populations

The genetic diversity among the 22 evaluated accessions (AB was excluded from this analysis because it was represented by only one individual) was also estimated with the G_{st} (0.147), R_{st} (0.2511), F_{st} (0.166), and low inbreeding coefficient (Fis = 0.025) values. With the exception of F_{is}, the estimated indexes were significant for all accessions ($P < 0.01$) confirming the presence of high diversity (Table 5). Amorim et al. (2015) found similar results with G_{st}, F_{st}, and R_{st} values equal to 0.14 ($P < 0.05$). According to the classification of Wright (1951), the

accessions from Pernambuco and Alagoas presented low genetic diversity, with G_{st} from 0.035 to 0.027; R_{st} from 0.0517 to 0.0179; and F_{st} from 0.045 to 0.053.

Table 5. Genetic diversity indexes among accessions (G_{st}, R_{st}, and F_{st}), total fixation index (F_{it}), and fixation index within accessions (F_{is}) obtained for different accessions of the Mangaba GeneBank of the Embrapa Tabuleiros Costeiros.

Group	G_{st}	R_{st}	F_{st}	F_{it}	F_{is}
Accessions (22)	0.147**	0.251**	0.166**	0.225**	0.071^{ns}
Bahia (4)	0.069**	0.2723**	0.129**	0.291**	0.186^{ns}
Sergipe (4)	0.131**	0.2605**	0.194**	0.277**	0.103^{ns}
Paraíba (4)	0.052**	0.0308**	0.070**	0.027^{ns}	-0.047^{ns}
Minas Gerais (3)	0.107**	0.0650**	0.107**	0.300**	0.216**
Pernambuco (4)	0.035**	0.0517**	0.045**	-0.002^{ns}	-0.050^{ns}
Alagoas (2)	0.027**	0.0179**	0.053**	0.022^{ns}	-0.033^{ns}

The results of the molecular variance analysis (AMOVA) indicated significantly greater genetic diversity within accessions than among them (Table 6). This was also observed by Costa et al. (2011), who evaluated the diversity of the first accessions introduced in the BGMangaba, and by Amorim et al. (2015) in native populations of mangaba, both using RAPDs. Conversely, Soares et al. (2016) found 23% divergence within accessions and 77% between them in natural populations in Sergipe. The pattern observed in our study is what is expected for allogamous arboreal species (Silva et al., 2017b).

Table 6. Molecular variance analysis of the 22 accessions of the Mangaba GeneBank of Embrapa Tabuleiros Costeiros using nine microsatellite markers.

Source of variation	df	Sum of squares	Variation components	Variance (%)	P value
Among accessions	21	204.099	0.44277	17.09	< 0.001
Within accessions	403	788.314	214.806	82.91	< 0.001
Total	424	992.413	259.083		

df = degrees of freedom

The Bayesian approach implemented in the Structure software was used to estimate the BGMangaba structure. In the ΔK analysis, the highest value was K = 2 (Figure 2), where K represents the number of groups identified (Evanno et al., 2005).

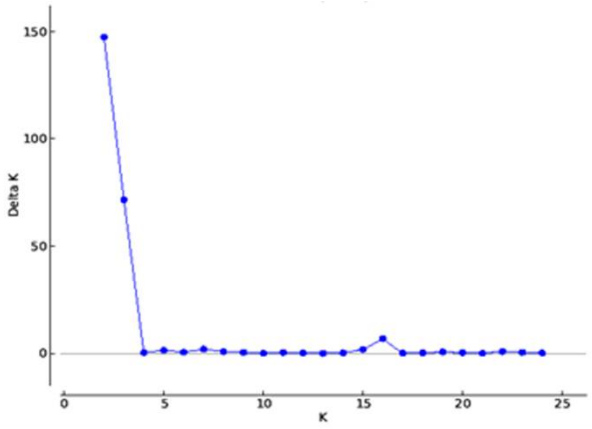

Figure 2. Correlation between the values of K (number of identified genetic groups) and ΔK in 213 individuals in the Mangaba GeneBank.

The Structure software grouped BGMangaba individuals into two groups (Figure 3). The most homogeneous accessions regarding allele distribution (predominance of one color) were JA, GU, OI, CM, CP, TA, CH, PM, CN, AD, TC, AB, PT, and TM. All accessions from Alagoas, Pernambuco, and Minas Gerais, some from Sergipe and Paraiba, and the only individual from Pará were identified in this group. The greatest variation of alleles (with two-color bars distribution), suggesting high diversity, was observed in the CV, PA, GX, PR, LG, IP, BI, and CA accessions (Figure 3). Based on the geographic origin, it can be said that all accessions from Bahia and Paraiba (with the exception of AD) grouped together and showed great divergence.

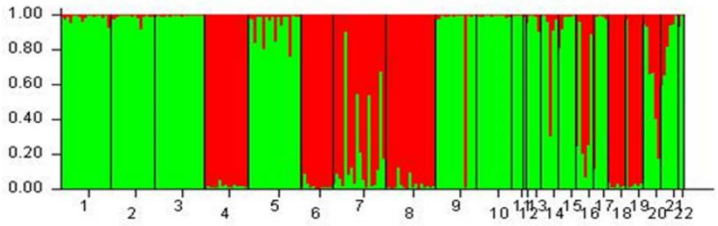

Figure 3. Genetic structure of the Mangaba GeneBank of Embrapa Tabuleiros Costeiros according to the Bayesian clustering analysis implemented in the Structure software for K = 2 with samples ordered by accession: 1.JA; 2.GU; 3.OI; 4.CM; 5.CP; 6.TA; 7.CV; 8.CH; 9. PM; 10.CN; 11.PA; 12.GX; 13.AD; 14. PR; 15.TC; 16. LG; 17. IP; 18. AB; 19. PT; 20. BI; 21. CA; and 22.TM.

The UPGMA grouping method allowed the formation of two large groups (Figure 4) according to similarity. The CA5 individual in the G1 group was the most distant from the others. The G2 group was divided into seven subgroups (SG1 to SG7); in this group, the pairs of individuals PM5/GX2, CN1/CN9, GU8/PA1, JA14/JA15, and OI8/OI9 were the closest genetically. Because they belonged to the same accession and were genetically close, the pairs CN1/CN9; JA14/JA15; and OI8/OI9 (highlighted in Figure 6) may be duplicates.

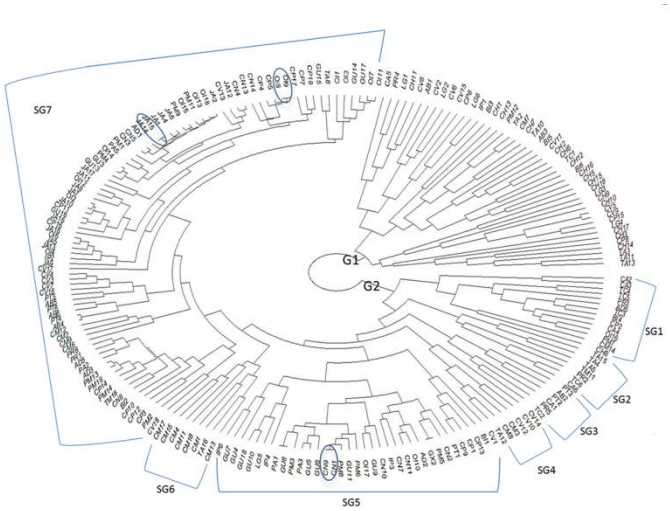

Figure 4. Dendrogram of the 213 individuals that compose the Mangaba GeneBank based on the genetic dissimilarity matrix (weighted average index).

In 2011, when the genetic diversity of the mangaba germplasm was estimated for the first time (Costa et al., 2011), the BGMangaba was composed of 11 accessions with 55 individuals. Significant enrichment is observed after six years as the result of collection expeditions conducted by curators and constant evaluations of these genetic resources. The information obtained in this study will be used in the management of this germplasm, including new introductions involving collection, exchange, selection, and pre-breeding improvement.

CONCLUSIONS

The mangaba accessions present high genetic diversity; however, the greatest variability was observed within accessions. The CA5 individual was the most divergent, and the pairs PM5/ GX2; CN1/CN9; GU8/PA1; JA14/JA15; and OI8/OI9 were the most similar. The accessions belonging to the Mangaba GeneBank presented significant genetic amplitude and diversity. This information will be useful for the development of strategies for selection and use in future programs of genetic improvement, with emphasis on the BI accessions, which presented the best genetic structure.

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