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The genetic diversity and population structure of *Genipa Americana* (Rubiaceae) in Northern Mato Grosso, Brazil

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ABSTRACT. *Genipa americana* (Rubiaceae) is an endemic Amazon-region species that may be subject to inbreeding, since it grows in fragmented environments. To examine this possibility, we examined the genetic diversity and population structure of three *G. americana* populations naturally grown in Northern Mato Grosso State using SSR markers. Sixty-four individuals were sampled from the three populations: 20 in AFL, 20 in MTP, and 24 in NBD. DNA extraction was performed according to the CTAB method, with modifications. Six SSR primers developed for the species were used. The allele frequency, the observed and expected heterozygosity, polymorphism information content), and the fixation Index were estimated. Molecular variance and principal coordinates analyses were conducted, and the most likely number of groups was inferred using the Structure software to help understand the genetic structure of the populations. The six microsatellite loci used showed 17 alleles,

in total, ranging from 2 to 4 alleles per locus, with a mean of 2.83. The expected heterozygosity ranged from 0.35 to 0.67 and remained higher than the observed heterozygosity for all loci. The three populations showed genetic diversity, shared genetic material and presented high inbreeding indexes. Cluster analysis results showed genetic material sharing between populations, as well as a lack of genetic structuring among individuals according to their geographical origins. The molecular characterization revealed that the genetic diversity is higher within than between populations. The three populations had shared genetic material and a high inbreeding index due to low observed heterozygosity. This could be a consequence of the fragmented environment where these populations currently live in, since it reduces the number of *G. americana* individuals and can increase inbreeding.

Key words: Genip tree; SSR; Genetic variability; Amazon species

INTRODUCTION

The genip tree (*Genipa americana*) is a species belonging to the family Rubiaceae; it is natural to the Amazon region and is distributed from Northern Argentina to Mexico (Unctad, 2005). The species is known for its use for various purposes, such as construction, cooking (candy and liquor manufacture) and home-made medicine (Lorenzi, 2002; Souza, 2008). In addition, it contains several chemical compounds that make it important to the pharmacological and food industries (Bentes, 2005; Mendes, 2011).

Intensive use of economically important species such as the genip tree, as well as the fragmentation of forest areas, may lead to genetic diversity loss due to a decreased number of individuals within a given population. This can cause genetic variability losses, which lead to genetic drift in the short-term, as well as to increased inbreeding rates in the long-term, as discussed by Kageyama et al. (1998) and Viegas et al. (2011).

According to Ramalho et al. (2016), the genetic structure of populations refers to heterogenous genotype distribution and genetic variability within and between populations. Molecular characterization allows assessing genetic diversity regardless of environmental interferences, as well as inferring the diversity level between individuals and between populations (Costa, 2011).

Molecular markers are a quick and effective instrument in genomic studies, since they directly detect polymorphism at the DNA level, without environmental influence (Souza, 2001). Polymorphism in these genes allows us to make inferences about the relation between an individuals' genotype and phenotype; this type of information can help increase the efficiency of breeding programs and species conservation strategies. These markers have been widely used to estimate the genetic diversity of native Amazon species such as *Bertholletia excelsa* (Lecythidaceae) (Ramalho et al., 2016), *Theobroma grandiflorum* (Malvaceae) (Silva, 2016), *Theobroma Speciosum* (Malvaceae) (Varella, 2016) and *Spondias mombin* (Anacardiaceae) (Silva, 2017). According to Costa et al. (2011), genetic diversity studies are fundamental, especially for native species whose diversity is yet to be fully understood.

Microsatellites, also known as SSR (Simple Sequence Repeat), have been indicated, among different molecular marker classes, for genetic variability studies, allelic frequency calculations, deviations from Hardy-Weinberg equilibrium, genome genetic and physical mapping, genotype identification and discrimination, paternity tests, as well as for population-genetics studies, since they are of a codominant nature and because they are one of the most polymorphic molecular marker classes currently available (Ferreira and Grattapaglia, 1998).

To increment our knowledge of the genip tree, we assessed the genetic diversity and population structure of three *G. Americana* populations naturally grown in Northern Mato Grosso State using SSR markers.

MATERIAL AND METHODS

Study site and material collection

The collections were carried out in Alta Floresta (AFL), Matupá (MTP), and Nova Bandeirantes (NBD) counties, in northern Mato Grosso State, Brazil (Figure 1). The climate in this region is hot and humid, the mean annual temperature is above 24°C, the mean annual rainfall is above 2,400 mm, and the region presents a well-defined dry season (3-5 months). The vegetation types in the region are transitional between Ombrophylous Forest, Seasonal Forest and Savanna (IBGE, 2017; Zappi, 2011).

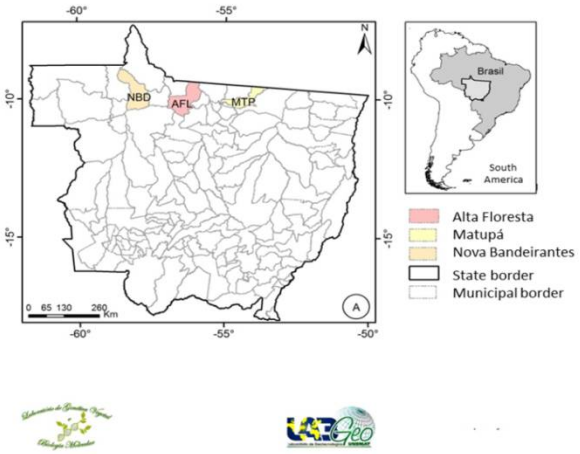

Figure 1. Geographic location of the three *Genipa americana* populations studied in Alta Floresta (AFL), Matupá (MTP) and Nova Bandeirantes (NBD) counties, Mato Grosso State, Brazil.

Sixty-four (64) individuals were sampled from the three populations: 20 in AFL, 20 in MTP, and 24 in NBD. All individuals were found at the edges of the forest fragments where these trees are more common; the minimum distance between them was 50 m. Each individual was tagged and mapped by GPS. The collected material was conserved in silica gel during its transportation to the laboratory. It was later stored in a freezer (-20°C) until DNA extraction.

DNA extraction and quantification

The total genomic DNA was extracted from approximately 100 mg of leaf material, according to the CTAB (cetyltrimethyl ammonium bromide) method described by Doyle and Doyle (1987), with modifications, namely: increased concentration of polyvinylpyrrolidone (PVP) from 1 to 2%; CTAB, from 2 to 5%; and βmercaptoethanol, from 0.2 to 2% in the extraction buffer; and incubation time reduction from 60 to 30 minutes (at 65°C).The DNA quality and quantification was evaluated by electrophoresis in a1% agarose gel stained with ethidium bromide (0.2 mg / mL) and by nanodrop. The quantified DNA was diluted to generate working solutions at 7.5 ng/μL (Manoel et al., 2014).

Amplification and genotyping of SSR loci

Seventeen SSR primers developed for *G. americana* were tested (Manoel et al., 2014)and six of them were selected for analysis (Table 1).

The PCR amplification reactions were performed in a Biocycler thermocycler, with a final volume 12 μL, including: 2 μL DNA (7.5 ng/μL), 2.4 μl 5x buffer (colorless GoTaq), 0.6 μl MgCl2 (50 mM), 2 μL of each primer (2 μM), 2 μl dNTP (1 mM), 0.15 μlTaq polymerase (5 U/μl), and 0.85 μl autoclaved distilled H_2O. The amplification program used was the one proposed by Manoel et al. (2014), with one initial denaturation cycle at 95°C for 5 minutes, followed by 30 cycles of 45 seconds at 95°C, 1 minute at 53-62°C (depending on the primer), 1 minute at 72°C; and a final extension at 72°C for 7 minutes.

The amplification products were separated through horizontal electrophoresis using 3% agarose gel in TBE 1X buffer at constant voltage 80V. The gels were stained with ethidium bromide (0.6 ng / μL) for 20 minutes after electrophoresis; then they were visualized in a UV transilluminator and *photodocumented*. The sizes of the amplified fragments were estimated throughtheir comparison with a 100 bp DNA molecular marker ladder (Kasvi).

Table 1. Primers used in the genetic diversity analysis of *Genipaamericana*and expected size of the amplified fragments.

Loci	Class	Sequence	Expected size
Gam 01	F	CATTCCACATTTGCCCTTG	175
	R	GCTTTCCTGTTCCCTAAATCC	
Gam 02	F	GCACCAGAGTCTAAAGCCAGA	186
	R	TGCACGAGTTCATTGAGATTG	
Gam 03	F	TGAAATTGCCTCTTACACACAC	245
	R	AACCACTTCCTTGAACACTGC	
Gam 06	F	CTCTGCGTGTGTGTGTGT	150
	R	CAAAGACTGTGCGGCATCT	
Gam 11	F	AGCCACTACCACCAGTCCAT	215
	R	GGAGACCGAGTGTTACATTTCA	
Gam 36	F	TGACTTGGTGCTGTGAGACGAG	214
	R	TCAAAATCCTCCCCGCCTT	

Data analysis

The SSR fragments were analyzed with the GelQuantPro® software, DNR, 2006 in order to help build a matrix based on the size of the amplified fragments. The Power Marker V.3.25 software (Liu and Muse, 2005) was used to determine the allele frequency, the observed and expected heterozygosity, PIC (Polymorphism information content) and the fixation index (F). The matrix was analyzed with the GenAlEx 6.5® software (Peakall and Smouse, 2006) in order to enable the principal coordinate (PCA) and molecular variance analyses (AMOVA). The matrix of genetic distance values by Nei et al. (1983) was generated with Power Marker V.3.25 software and imported to MEGA 6.5 software (Kumar et al., 2004) to build the dendrogram, according to the UPGMA method. The Structure software (Pritchard at al., 2000), which is based on Bayesian statistics, was used to infer the number of groups (k). Twenty (20) runs were performed for each K value, as well as 200,000 burn-ins and 500,000 Markov Chain Monte Carlo (MCMC) simulations. The criteria of Pritchard and Wen (2004) and of Evano et al. (2005) were used to define the most likely k in comparison with the proposed values, and the results were recorded in the Structure Harvester website.

RESULTS

The six microsatellite loci used in the genotyping of 64 *G. americana* individuals showed 17 alleles, in total, ranging from 2 to 4 alleles per locus, with a mean of 2.83 alleles (Table 2). The largest number of alleles was found in the locus 'Gam 02' (4 alleles). The polymorphic information content (PIC) ranged from 0.29 to 0.60 (mean 0.45). The observed heterozygosity had a mean value 0.17 and a maximum value of 0.53 in Gam 02. The expected heterozygosity ranged from 0.35 to 0.67and remained higher than the observed heterozygosity for all loci. This relation between observed and expected heterozygosity resulted in positive inbreeding coefficients (f) for all loci, with overall mean f = 0.40. It indicates that the occurrence of homozygotes in the populations was higher than the expected values, according to the Hardy-Weinberg equilibrium model.

Table 2. Number of alleles (N*a*), gene diversity (Dg), expected heterozygosity (*He*), observed heterozygosity (*Ho*) and Polymorphic Information Content (PIC) of six SSR primers, based on the amplification of 64 *Genipa americana* individuals belonging to three populations sampled in Northern Mato Grosso State, Brazil.

Primer	Na	*Dg*	*He*	*Ho*	PIC
Gam01	3	0.87	0.61	0.27	0.54
Gam02	4	0.92	0.67	0.53	0.60
Gam03	2	0.84	0.35	0	0.29
Gam06	2	0.80	0.48	0	0.36
Gam11	3	0.70	0.57	0.13	0.49
Gam36	3	0.64	0.50	0.10	0.44
Total	17	-	-	-	-
Mean	2.83	0.80	0.53	0.17	0.45

The three populations showed genetic diversity; the MTP population showed the highest observed heterozygosity and the lowest fixation index when compared to the other two populations (Table 3). However, all populations showed higher *He* than *Ho*.

Table 3. Genetic diversity among the three *Genipa americana* populations according to the gene diversity index by Nei (H). Genetic diversity (Dg), expected heterozygosity (*He*), observed heterozygosity (*Ho*), fixation index (f) and Polymorphic Information Content (PIC).

Populations	Dg	*He*	*Ho*	F	PIC
AFL	0.87	0.48	0.14	0.72	0.40
MTP	0.83	0.51	0.21	0.60	0.42
NBD	0.87	0.57	0.13	0.78	0.50

The molecular variance analysis (AMOVA) indicated 13% of the total variance as the difference between populations, and 87% of it as the difference within populations. It showed that the greatest genetic differences are in the intrapopulation component rather than in the interpopulation comparisons, (Table 4).

Table 4. Molecular variance analysis (AMOVA) of three natural *Genipa americana* populations found in Northern Mato Grosso State based on six SSR markers.

Variation source	DF*	SQ*	VC*	TV (%)*	F_{ST}	P value
Between populations	2	26.81	0.273	13	0.13	<0.001
Within populations	63	223.10	1.785	87		
Total	**64**	**249.91**	**2.058**			

* Degrees of freedom (DF), Sum of Squares (SQ), Variance Component (VC), Total Variance (TV).P indicates the odds of having a variance component higher than the values observed at random. The odds were calculated through 1023 permutations at random.

The population structure based on the Bayesian analysis performed in the Structure software, according to the ΔK method described by Evanno et al. (2005) allowed us to identify five divergent groups, which contributed to the genetic composition of the three *G. americana* populations. The ΔK was estimated for K and it ranged from 1 to 6; the highest ΔK value was K = 5 (Figure 2).

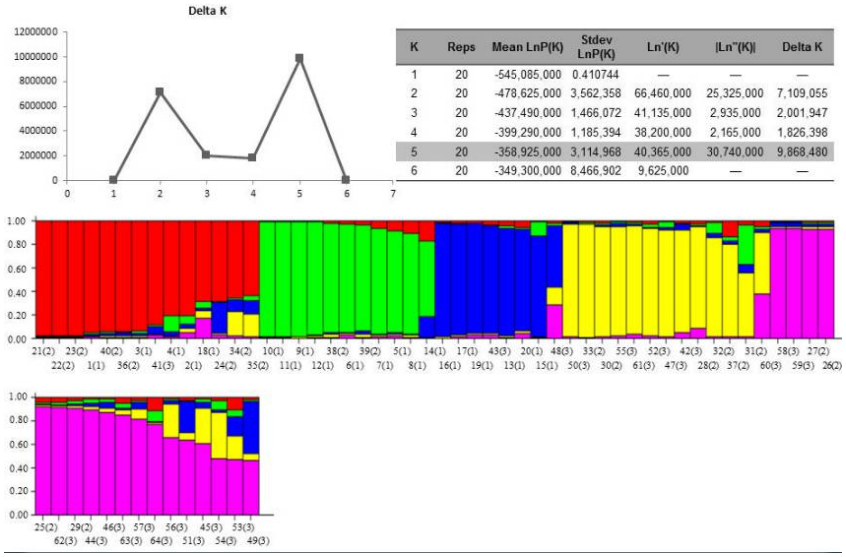

Figure 2. Distribution of 64 *Genipa americana* individuals in groups, according to molecular data of six SSR loci in the Structure software. The individuals are represented by vertical bars; the colors were attributed according to the group formed in the Structure software (five groups, K = 5). 1 (1-20): AFL; 2(21-40): MTP; 3(41-64):NBD.

The three *G. americana* populations share individuals belonging to different genetic groups formed in the analysis conducted in the Structure software (Figure 2). This demonstrates the relation between the geographical origin of the individuals belonging to the original populations and the genetic groups identified through the Structure software. The graphical visualization of the population structure (Figure 2) allowed us to identify several individuals that carry a mixture of more than two genetic groups.

Analysis of the graph resulting from the PCoA (Figure 3) corroborated the result of the Structure software. It showed that the three populations share genetic material and consequently the individuals are not grouped according to geographical origin.

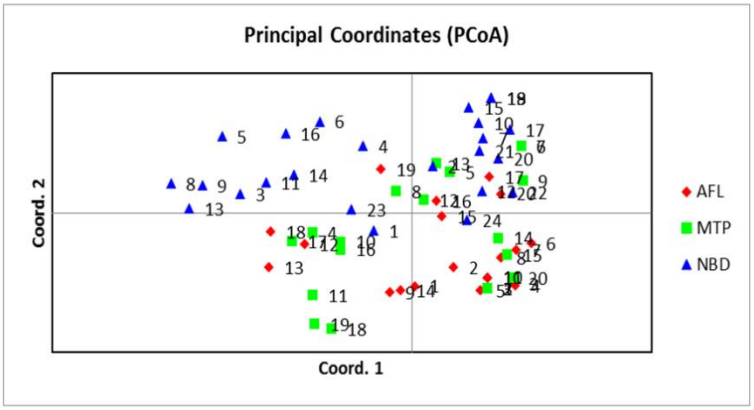

Figure 3. Graphical dispersion based on the principal coordinates analysis conducted on 64 *Genipa americana* individuals from three AFL, MTP and NBD populations sampled in Northern Mato Grosso State.

DISCUSSION

The loci included 17 alleles in 64 individuals (mean 2.83 alleles). This number of alleles is lower than that found by Manoel et al. (2014), who analyzed two genip tree populations in Mato Grosso do Sul (19 individuals) and São Paulo (21 individuals) states and found 33 alleles (mean 5.50) in the first population and 27 (mean 4.50) in the second.

The PIC of the SSR markers was 0.45, on average. This value was higher than that found by Silva et al. (2014) in the same species (0.21) in an ISSR molecular marker analysis. According to Botstein et al. (1980), markers with PIC values between 0.25 and 0.50 are fairly informative for the species under analysis, as was observed in the SSRs applied to *G. americana* in our study.

The observed heterozygosity in the three *G. americana* populations was lower than the expected heterozygosity. Similar results have been found in several studies conducted with tropical species (Silvaet al., 2017; Dardengoet al., 2016; Varella et al. 2016). According to Alves et al. (2007), the number of alleles is correlated with the expected heterozygosity value. The more alleles in the populations, the larger the number of heterozygotes among individuals, according to the ratios provided by the Hard-Weinberg Law (Griffiths et al., 2013). The values found in this study showed that the *G. americana* populations are not in Hardy-Weinberg equilibrium, as was indicated by the mean fixation index.

The mean fixation index of these populations was 0.40; it was positive and high in genip tree individuals, demonstrating in breeding within populations. However, the high fixation index is not always the same in *G. americana* according to Sebben et al. (1998) (mean -0.07) and Manoel et al. (2014) (means -0.04 and -0.09). According to Kageyama et al. (2003), the fixation index (f) is one of the most important population-genetics parameters used to measure the equilibrium between homozygotes and heterozygotes; species showing more homozygosis than heterozygosis may result from a founder effect.

Based on the AMOVA analysis, most of the genetic diversity lies within *G. americana* populations. The group generated through the PCoA method, as well as the distribution in five groups generated in the Structure software, did not correspond to the geographical distribution. It showed that the three original populations share genetic material and that the genetic diversity is not geographically structured in the individuals belonging to these populations. Studies about Amazon species such as *Maurita flexuosa*, *Theobroma speciosum* and *Spondias mombin* showed that the genetic diversity was geographically structured (Silva et al., 2017; Varella et al., 2016; Rossi et al., 2014), unlike what we observed.

The *G. americana* individuals were found in fragmented areas, and this may have influenced the genetic diversity patterns and the fixation indices of the populations. Disturbed landscapes, besides influencing the demography of plant species, also influence the density of pollinators and seed dispersers. Fragmented populations may show increased inbreeding rates due to the higher probability of self-fertilization, as well as of breeding between related individuals, as a consequence of the reduced number of individuals (Carvalho et al., 2010). According to Gaino et al. (2010), isolated populations tend towards reductions in their diversity and size, a fact that reinforces the need to preserve plant species.

CONCLUSION

The three populations the *G. americana* had shared genetic material and a high inbreeding index due to low observed heterozygosity. This could be a consequence of the fragmented environment where these populations currently live in, since fragmentation reduces the habitat and the number of individuals and increases inbreeding. Since the molecular characterization revealed that the genetic diversity is higher within than between populations, we recommend the preservation of several individuals in both populations to maintain the existing intrapopulation genetic diversity.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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