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Effect of fragmentation on the natural genetic diversity of *Theobroma speciosum* Willd. ex Spreng. populations

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ABSTRACT. Forest fragmentation reduces the effective size of natural populations, isolates individuals in the landscape, and, consequently, changes species' mating systems by increasing the degree of relatedness between individuals and inbreeding. Investigating the impact of habitat degradation on forest fragments helps to assess the genetic and ecological consequences of these changes, and allows the development of effective and sustainable conservation strategies to manage the genetic resources of species living in degraded landscapes. The aim

of the present study was to assess the genetic diversity of fragmented *Theobroma speciosum* populations using microsatellite markers. Three urban forest fragments were selected in the municipality of Alta Floresta, Mato Grosso State, Brazil, namely C/E park, J park, and Zoo Botanical park. Seventy-five individuals (25 in each fragment) were sampled by collecting their leaves for genomic DNA extraction. Polymerase chain reaction amplifications were performed using nine polymorphic simple sequence repeat primers, which amplified 84 alleles. The mean expected heterozygosity was 0.970, and it was always higher than the observed heterozygosity. Analysis of molecular variance revealed that most variability occurred within populations (64%) rather than between them (36%). The Structure software and an unweighted pair group method with arithmetic mean dendrogram revealed three distinct groups, showing that individuals were allocated to their correct populations. Genotype number 3 from C/E park, number 45 from J park, and number 51 from Zoo Botanical park could be used as stock plants in breeding programs, because they were the most dissimilar within the populations studied. The high genetic diversity levels detected in all three populations studied emphasize the importance of protecting this species in its natural habitat.

Key words: Genetic variability; SSR marker; Amazon

INTRODUCTION

Brazil contains a wide variety of soil and climate types, which favors the cultivation of many tropical fruit trees (Simão, 1998). The most diverse botanical families in the Amazon are the Fabaceae, Bignoniaceae, Lauraceae, Lecythidaceae, Rubiaceae, Anacardiaceae, Malvaceae, Asteraceae, Araceae, Arecaceae, and Poaceae, among others (Neto and Silva, 2011). The *Theobroma* genus (Malvaceae) comprises 22 species that are found in the Americas (Cuatrecasas, 1964). Eight of these species, including the trees cacau (*Theobroma cacao* L.), cupuassu [*Theobroma grandiflorum* (Willd.) Schum.], cacauhy (*Theobroma speciosum* Willd. ex Spreng.), and cupuí (*Theobroma subincanum* Mart.), are found in the Brazilian Amazon. According to Souza and Venturieri (2009), *T. speciosum* is important because it represents a possible source of genetic resistance for other more economically important species, such as *T. cacao* and *T. grandiflorum*. *T. speciosum* is found in the Amazon basin, and is widely distributed in Mato Grosso State. It has an edible fruit, and its flesh can be consumed fresh or as a juice (Neto and Silva, 2011). Its seeds are commonly used to prepare homemade chocolate (Ferrão, 2001).

Wild species, such as *T. speciosum*, provide a source of income for native communities. These species are very profitable for regional development, promote the rational use of native genetic resources, and encourage native communities in the conservation of plants' genetic resources.

However, native *Theobroma* species have been subjected to strong anthropogenic genetic erosion (Alves et al., 2013), which has isolated populations into small fragments, decreased the number of individuals and the population density, and affected genetic processes such as genetic drift, gene flow, selection, and mating systems (Young and Boyle, 2000). According to Costa et al. (2011), knowing the genetic variation of native species enables the

development of strategies that allow the domestication and incorporation of these species in regional productive systems, as well as minimizing the environmental impact and establishing effective conservation plans.

Molecular markers are used in genetic assays because of the relative ease of amplifying particular genome regions in order to characterize the genetic diversity and genetic structures of different populations (Sena et al., 2007). Assessing genetic variability is fundamentally important, particularly for understudied native species the diversity of which is not fully known (Costa et al., 2011).

Therefore, the aim of the current study was to characterize the genetic diversity of *T. speciosum* in three urban forest fragments in the municipality of Alta Floresta, Mato Grosso State, Brazil using microsatellite markers, in order to increase our knowledge of the population dynamics of this species and help develop programs for its conservation and exploitation.

MATERIAL AND METHODS

Study area and sampling

The study was conducted in three municipality parks in Alta Floresta, Mato Grosso State, Brazil: C/E, with an area of 9.19 ha; J, with 7.38 ha; and the Zoo Botanical, with 17.6 ha (Figure 1).

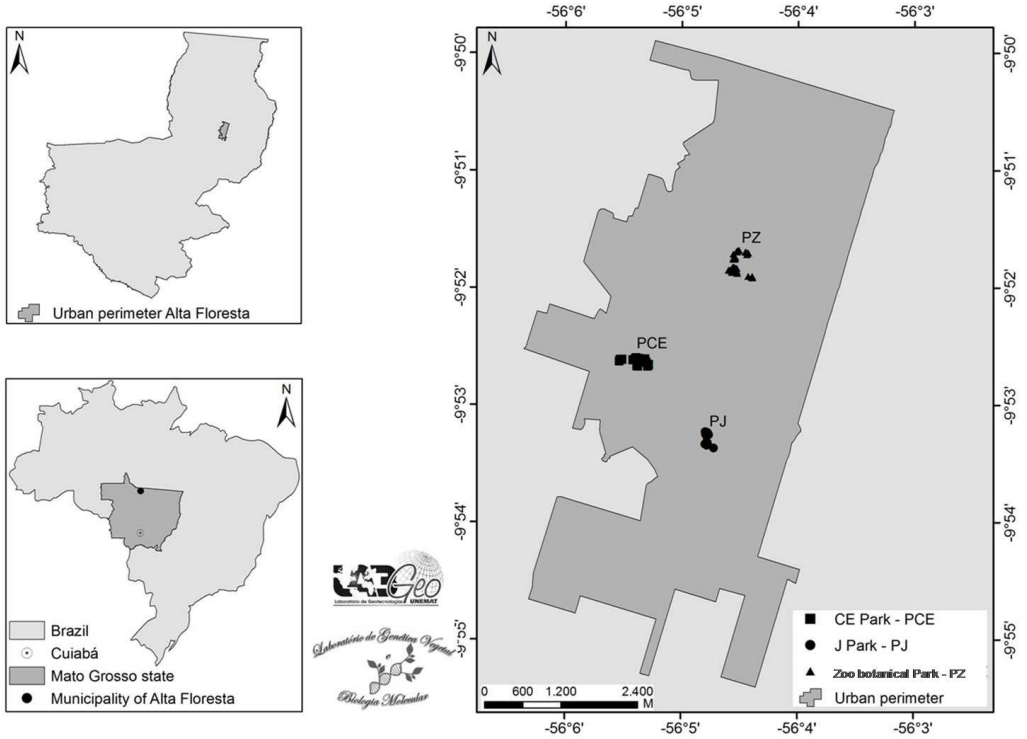

Figure 1. Location of Alta Floresta, Mato Grosso, Brazil and the three urban fragments under study.

To characterize genetic diversity, 75 *T. speciosum* individuals were identified, sampled, and georeferenced in the urban parks (25 in each park). Leaf tissue samples were collected for DNA extraction from all of the trees found within the study parks. The samples were stored with silica gel in the field, and subsequently at -20°C in the Genetics Laboratory of the University of Mato Grosso.

DNA extraction

Total genomic DNA was extracted using the cetyltrimethylammonium bromide method as described by Doyle and Doyle (1990). DNA was applied to an agarose gel (1% w/v) and stained with ethidium bromide for quantification. Bands were compared with a standard DNA (lambda phage) of known concentration. The gels were then examined using an ultraviolet transilluminator (UVB LTB-21x26) and photographed.

Polymerase chain reaction (PCR) amplification

Twenty-three microsatellite loci (simple sequence repeats) that were characterized by Lanaud et al. (1999) were tested in an initial PCR amplification using one *T. speciosum* individual. Of the 23 loci tested, 9 were selected for genetic variability analysis. The amplification protocol followed that described by Lanaud et al. (1999), with some modifications: one initial cycle at 94°C for 4 min, followed by 32 cycles at 94°C for 30 s, 46° or 51°C (depending on the primer used) for 1 min, 72°C for 1 min, and a final cycle at 72°C for 5 min. The amplification products were separated by electrophoresis on a 4% (w/v) agarose gel in 1X TBE running buffer at a constant 85 V for approximately 5 h. The gel was stained with 0.2 mg/mL ethidium bromide and subsequently photographed under ultraviolet light using the transilluminator.

Data analysis

The amplification products were analyzed using the GelQuant Pro program (http://www.dnr-is.com/Product.asp?Par=3.19&id=81), in order to develop a matrix based on the fragment sizes of the bands. Principal coordinate analysis (PCA) and analysis of molecular variance (AMOVA) were performed using the GenAlEx 6.5 program (Peakall and Smouse, 2006). We used the PowerMarker program (Liu and Muse, 2005) to assess allelic frequency, genetic diversity, the observed and expected heterozygosity, and the polymorphism information content (PIC). Nei et al.'s (1983) matrix of genetic distance between *T. speciosum* trees was estimated using the same program. This matrix was imported by MEGA 3.1 (Kumar et al., 2004) to construct a dendrogram of mean distance using the unweighted pair group method with arithmetic mean (UPGMA). The Structure program (Pritchard et al., 2000), which is based on Bayesian statistics, was used to indicate the number of genetic groups (K). We conducted 10 runs for each K value, with 200,000 burn-ins and 500,000 Markov chain Monte Carlo simulations. To determine the most probable value of K, we used the criteria proposed by Pritchard and Wen (2004) and Evanno et al. (2005).

RESULTS

The nine primers used in the analysis were polymorphic and amplified 84 alleles,

with a mean of 9.33 alleles per locus. The highest number of alleles (13) was found at locus mTcCIR19, and the lowest (6) at locus mTcCIR7 (Table 1). The genetic diversity varied between 0.69 (mTcCIR22) and 0.89 (mTcCIR28), with a mean of 0.79 per primer (Table 2).

Table 1. Number of alleles (N_A), genetic diversity (D_G), expected heterozygosity (H_E), observed heterozygosity (H_O), and the polymorphism information content (PIC) of nine simple sequence repeat primers in 75 *Theobroma speciosum* individuals from three populations in Alta Floresta, Mato Grosso State, Brazil.

Locus	N_A	D_G	H_E	H_O	PIC
mTcCIR7	6	0.78	0.94	0.02	0.75
mTcCIR9	8	0.80	0.90	0.02	0.77
mTcCIR10	8	0.75	0.96	0.34	0.71
mTcCIR11	8	0.71	0.97	0.04	0.67
mTcCIR17	11	0.85	0.97	0.10	0.83
mTcCIR19	13	0.85	1.00	0.36	0.84
mTcCIR22	10	0.69	0.97	1.00	0.65
mTcCIR26	8	0.79	1.00	0.00	0.77
mTcCIR28	12	0.89	1.00	0.33	0.88
Total	84	-	-	-	-
Mean	9.33	0.79	0.97	0.25	0.76

Table 2. Genetic diversity of three *Theobroma speciosum* populations in Alta Floresta, Mato Grosso State, Brazil.

Population	D_G	H_E	H_O	F	PIC
C/E	0.62	0.97	0.24	0.61	0.55
J	0.56	0.94	0.23	0.59	0.50
Zoo Botanical	0.54	0.99	0.26	0.52	0.47

D_G, Nei's genetic diversity; H_E, expected heterozygosity; H_O, observed heterozygosity; F, fixation index; PIC, polymorphism information content.

All of the loci had high PIC values that varied between 0.65 and 0.88, with a mean of 0.76 (Table 1), and the loci mTcCIR17, mTcCIR19, and mTcCIR28 had values that were higher than 0.75.

The mean observed heterozygosity was 0.24, and it ranged from 0.02 (mTcCIR7) to 1.00 (mTcCIR22), whereas the locus mTcCIR26 did not exhibit any observed heterozygosity (0.00) (Table 1). The mean expected heterozygosity was 0.97. The observed heterozygosity was lower than the expected heterozygosity, except for locus mTcCIR22 (Table 1). The *T. speciosum* populations had high levels of inbreeding, with a mean of 0.61 for the C/E park population, 0.59 for J park, and 0.52 for the Zoo Botanical park (Table 2).

All of the populations exhibited high gene diversity, with the C/E park population having the highest (0.62). The expected heterozygosity was 0.97 for the C/E park population, 0.94 for the J park population, and 0.99 for the Zoo Botanical park population. The observed heterozygosity was lower than the expected heterozygosity for all of the populations analyzed (Table 2). The PIC was high for all of the fragments (0.55 for C/E park, 0.50 for J park, and 0.47 for Zoo Botanical park).

The PCA explained 41.11% of the total variation, with 18.45% for the first component, 13.96% for the second, and 8.70% for the third. We observed the formation of three distinct groups, which were based on genetic similarity and geographical distribution (Figure 2).

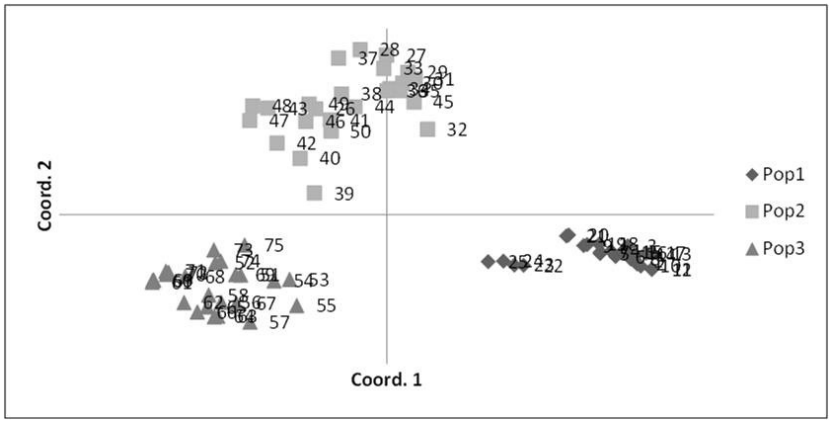

Figure 2. Principal coordinates analysis of 75 *Theobroma speciosum* individuals from urban forest fragments in Alta Floresta, Mato Grosso State, Brazil.

AMOVA revealed that 64% of the total variance occurred within populations and 36% between populations. The dendrogram revealed three principal groups (Figure 3); each group represented a different population, revealing that genetic similarity was high inside each population.

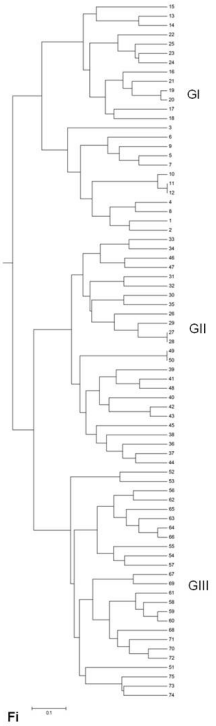

Figure 3. Dendrogram of the genetic similarity among 75 *Theobroma speciosum* trees. GI, C/E park; GII, J park; GIII, Zoo Botanical park.

Group I consisted of the C/E park population (genotypes 1-25), which was subdivided into three subgroups: subgroup I, which contained 13 individuals; subgroup II, which included only one individual (genotype 3) and subgroup III, with 11 individuals (Figure 3). In this group, the most similar individuals were 11 and 12, and the most dissimilar was individual 3.

Group II contained the J park population (genotypes 26-50), which was subdivided into two subgroups: subgroup I, which contained the individuals 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 46, and 47; and subgroup II, which contained the remainder. In this population, the most similar individuals were 27-28, and 49-50, and the most dissimilar was 45 (Figure 3).

Group III consisted of the Zoo Botanical park population (genotypes 51-75), which contained three subgroups: subgroup I, which included only two individuals (52 and 53); subgroup II, which contained nine genotypes; and subgroup III, which contained the remaining 14 *T. speciosum* plants. The most similar individuals were 59, 60, 64, and 66, and the most dissimilar was 51 (Figure 3).

The best K value was found by dividing the samples into three groups (K = 3), which was supported by the clear distributions of individuals in their respective populations (Figure 4).

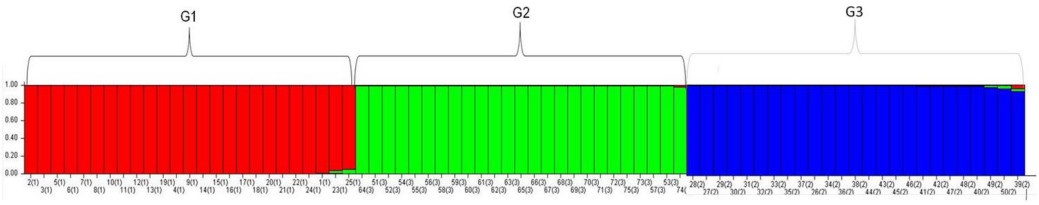

Figure 4. Division of 75 *Theobroma speciosum* trees into three groups based on molecular data from nine simple sequence repeat loci using the Structure program. Individuals are represented by vertical columns and are shaded according to their group (three genetic groups, K = 3). G1, C/E park; G2, J park; G3, Zoo Botanical park.

DISCUSSION

According to Alves et al. (2007), most tropical tree species have a large number of alleles per locus, and, consequently, a high expected heterozygosity. However, Lanaud et al. (1999) and Alves et al. (2013) reported a lower mean number of alleles per locus for *T. cacao* and *T. grandiflorum*, respectively, than that found for *T. speciosum* in the current study, i.e., 4.4 alleles per locus for *T. cacao* and 3.21 alleles per locus for *T. grandiflorum*.

The mean PIC values obtained demonstrate the high quality of the markers used. Markers with PIC values higher than 0.5 are considered highly informative, those with values between 0.25 and 0.5 are considered moderately informative, and those with values below 0.25 are minimally informative (Botstein et al., 1980). According to Boza et al. (2013), highly polymorphic markers are useful in identifying genetic diversity.

The observed heterozygosity was lower than the expected heterozygosity in all of the loci analyzed. Our results support those obtained by Zhang et al. (2012), who found higher expected (0.56) than observed (0.38) heterozygosity values in *T. cacao* populations. Alves et al. (2007) obtained a similar result for *T. grandiflorum* in a study conducted with 21 microsatellite markers, in which the expected (0.41) heterozygosity was higher than the observed (0.35). Motamayor et al. (2002) and Sereno et al. (2006) obtained similar values for *T. cacao* (expected heterozygosity = 0.54 and 0.56, respectively; observed heterozygosity = 0.34 and 0.41, respectively).

According to Carvalho et al. (2010), a low heterozygosity rate is associated with reproductive isolation caused by forest fragmentation, because a continuous reduction in population size leads to genetic variability loss through genetic drift.

The loss of genetic diversity in the forest fragments studied here has resulted in *T. speciosum* exhibiting high inbreeding values. A high inbreeding rate can be caused by the action of monkeys (dispersers), while feeding the fruits of *T. speciosum*, they leave the seeds fall close to the mother tree, where the same germinate and form a new individual. Favoring with this proximity crossings between related individuals and a pattern of aggregate distribution for the species.

Seed dispersal close to the maternal tree favors the formation of a spatial genetic structure (Sobierajski et al., 2006), and increases the possibility of crossings between individuals from the same family, thus generating biparental inbreeding, and, consequently, biparental inbreeding depression. Sebbenn et al. (2011) conducted a study on *Copaifera langsdorffii*, and found that a small urban fragment may influence seed dispersal distance by limiting the area available for the establishment of seedlings.

AMOVA revealed that much of the genetic variation occurs within populations and not between them. *T. speciosum* is a perennial species with outcrossing, and consequently accumulate more genetic variability within populations than between them. According to Hamrick et al. (1991), these species exhibit low population differentiation. *T. speciosum* has a self-incompatible breeding system (Souza and Venturieri, 1998), so it is only effectively pollinated if its pollinators (*Drosophila* spp) visit other individuals in the population (Silva and Martins, 2004), which increases intrapopulation genetic variability.

The C/E park population formed a separate group from the other two populations, probably because it is located in the city center and was the first fragment to be isolated in Alta Floresta county in the 1990s. Because the J park and Zoo Botanical park populations are on the same side of the county and were more recently isolated, we can infer the existence of a corridor that allowed the exchange of genetic material. The most dissimilar individuals in the three populations have the potential to select as stock plants in the formation of germplasm banks, because they were the most genetically distant individuals within the populations. There were similarities between the groupings generated by the Structure program and the dendrogram produced by UPGMA; however, the two methods did not provide the same results, possibly because clusters produced by the Structure program tend to generate greater subgroup differentiation (Romão et al., 2011).

The high genetic diversity detected in the three park populations highlights the importance of maintaining and protecting this species by *in situ* conservation, as well as the importance of collecting germplasm for *ex situ* conservation. Because these are fragmented populations wherein the predatory exploitation of fruits prevents their dispersal, and, consequently, natural seedling establishment, the loss of genetic diversity and the genetic erosion of this species may occur in the next few generations.

To conclude, the genotypes of each population were grouped within their population of origin, and exhibited a geographical structure. Genetic diversity was higher at the intrapopulation level than at the interpopulation level. Genotype number 3 from C/E park, number 45 from J park, and number 51 from Zoo Botanical park could be used as stock plants in breeding programs, because they were the most dissimilar within the populations studied. This study provides important information for the adoption of strategies to conserve, characterize, and study *T. speciosum* in urban forest fragments, by generating indicators that can be used to establish and manage genetic reserves *in situ*.

Conflicts of interest

The authors declare no conflict of interest.

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