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Genetic diversity between and within half-sib families of Brazil nut tree (Bertholletia excelsa Bonpl.) originating from native forest of the Brazilian Amazon

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ABSTRACT. Brazil nut tree is a species of economic importance for the Amazon region, known for the commercialization of its almonds. The objective of this work was to study the genetic diversity among half-sib progenies from different Brazil nut trees present in native forest in the municipality of Itaúba, MT, belonging to the Brazilian Amazon. In a native forest area of nine hectares, fruits of nine parent trees, randomly selected in the plot, were collected. The seeds were planted at

greenhouse and they were named, according to their origin, identifying seed tree and fruit. After the seed germination and initial development of the seedlings, leaves were collected for DNA extraction and analyzed with microsatellite molecular markers. It was performed analysis of molecular variance and cluster analysis of progenies and seed trees. There is greater genetic diversity between families than among progenies from the same family. The clustering of progenies from different families in the same group can be explained by the low dissimilarity between the seed trees. Among the loci analyzed in this study, eight were informative for evaluations of genetic diversity in Brazil nut, except BET12 and BET16 loci.

KEY WORDS: Genetic Divergence; Microsatellite Markers; Genetic Dissimilarity; Seedlings; Ward Method.

INTRODUCTION

Brazil nut tree (*Bertholletia excelsa* Bonpl.) is a species of economic importance for the Amazon region, known for the commercialization of its almonds (Salomão, 2009). It presents good development in hot and humid climate (Lorenzi, 2000; Santos et al., 2006) and occurs in countries such as Venezuela, Colombia, Peru, Bolivia, Guianas and Brazil (Lorenzi, 2000).

The species reproduces by cross fertilization (allogamy), and its pollination occurs by an exclusive group of bees that can access the flowers reproductive organs (Müller, 1995; Maués, 2002). The fruits are collected in native forests, after maturation, when they fall on the soil, which usually occurs in the rainy season (Borges et al., 2016). They can store approximately 15 to 25 seeds, which have lengths ranging from 4 to 7 cm. The seeds are lined with a ligniform shell, with a single almond inside (Moritz, 1984) and has a recalcitrant behavior (Cunha et al., 1996).

Brazil nut germination process is slow and uneven, and may take between six and eighteen months to occur when there is no treatment, such as breaking the tegument (Müller et al., 1980; Camargo, 1997). The regeneration of the species in native forest is not affected by extractivism (Wadt et al., 2008; Scoles and Gribel, 2011; Ribeiro et al., 2014; Scoles and Gribel, 2015), but deforestation and fires are considered as obstacles to the maintenance of natural populations, genetic diversity and conservation of the species (Maués and Oliveira, 2010).

The maintenance of genetic diversity is the basis for conservation strategies and obtaining improved populations (Yeeh et al., 1996). The distribution of genetic variability in natural populations is influenced by the mode of reproduction, population size, geographic distribution and gene flow (Hamrick, 1982). Tropical forest tree species generally have a high proportion of polymorphic loci, and most genetic variation is maintained within populations rather than between them (Hamrick ,1994; Baldoni et al., 2017).

Knowledge of genetic variability, both within and between populations, can guide genetic breeding programs aiming at improving a specific trait or group of traits by selecting, crossing and / or recombining individuals with high frequency of favorable alleles (Erickson et al., 2004). In addition, studies on genetic diversity can help in the maintenance and conservation of germplasm banks by eliminating redundant individuals (replicates).

In Brazil nut tree, several studies have shown different levels of genetic diversity (Buckley et al., 1988; Kanashiro et al., 1997; O'Malley, 1998; Serra et al., 2006; Sujii, 2011; Silva et al., 2012; Vieira, 2014; Wadt et al., 2015; Baldoni et al., 2017; Cabral et al., 2017), being, in the majority of them, considered high. An important technique available for detecting genetic diversity at the DNA level is the microsatellite markers. Also known as SSR, these are one of the most polymorphic classes of markers currently available, are codominant and easily reproducible, besides have frequent and random distribution, allowing wide genome coverage (Caixeta, 2016).

Studying genetic diversity through molecular markers is important for breeding programs because of the absence of environmental factors. Excoffier et al., (1992) proposed an analysis of molecular variance (AMOVA) to analyze the distribution of genetic variability between and within populations. The method uses a hierarchical analysis scheme to analyze the distance among all pairs of genotypes.

The objective of this work was to study the genetic diversity among half-sib progenies from different seed trees of Brazil nut present in native forest in the municipality of Itaúba, MT, belonging to the Brazilian Amazon.

MATERIAL AND METHODS

In a native forest area of nine hectares, which constitutes a permanent plot, Santo Ângelo Farm, located approximately 30 km from the municipality of Itaúba, MT, Brazil, fruits of nine seed trees, randomly selected in the plot, were collected. The name of each seed tree and its location are represented in Figure 1. The trunk vascular cambium of these trees was collected for DNA extraction.

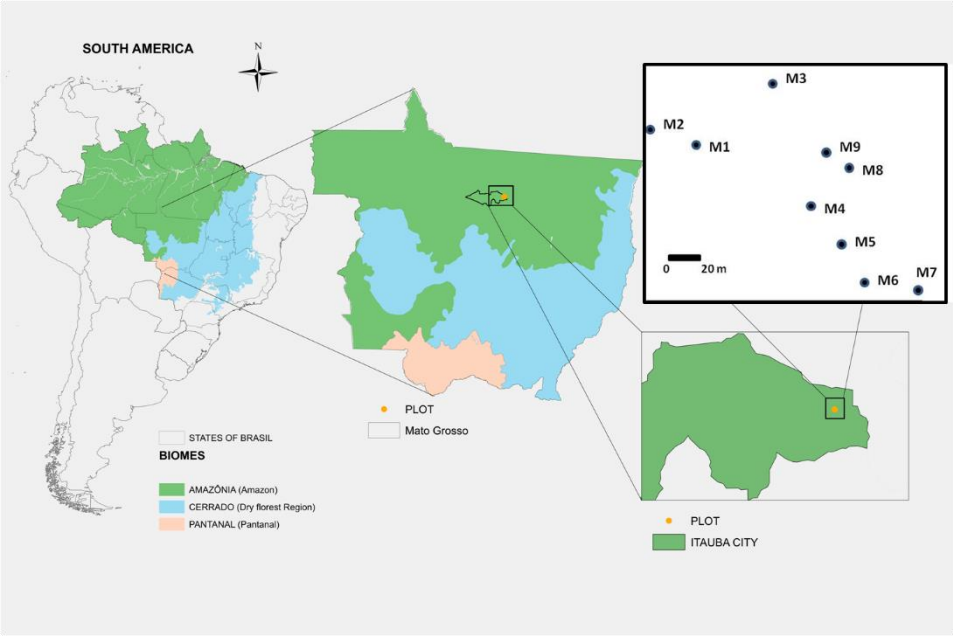

Figure 1. Geographic location of the nine *Bertholletia excelsa* trees used for collecting seeds.

A total of 98 fruits were collected in December 2014 and taken to Embrapa Agrossilvipastoril, Sinop, MT, Brazil. Before planting the seeds, the seeds teguments were removed to accelerate the germination, taking care not to damage its endosperm, mainly in the regions where the apical meristems are located. After removal of the tegument, the almonds were treated with a fungicide solution according to Müller (1982) and seeded in a sandbox in the greenhouse with 50% shading, in January 2015.

Table 1 shows the name of seed tree, diameter at breast height (DBH), number of fruits of each tree, the number of seeds planted and germinated.

Table 1. Name of seed tree, diameter at breast height (DBH), number of fruits collected per tree, number of seeds planted and germinated of *B. excelsa*

Name of seed tree	DBH (cm)	Number of fruits per tree	Number of seeds planted	Number of seeds germinated
M1	58.80	20	253	78
M2	73.10	08	109	10
M3	46.70	10	138	31
M4	101.22	10	146	37
M5	62.40	10	142	22
M6	55.50	10	145	31
M7	147.05	10	115	35
M8	93.90	10	162	41
M9	75.30	10	138	15
Total		98	1348	300

The seeds were planted in rows, where each row was composed of seeds from the same fruit. These seeds were named according to their origin, identifying seed tree and fruit. After the seed germination and initial development of the seedlings, leaves were collected for DNA extraction.

Table 2. Relationship of microsatellite primers used, with sequence, size found in base pairs (SF), and annealing temperature (AT).

Locus	Sequence	SF (bp)	AT °C
BET12	F: ATAAGGACCGCCCATCATC R: ATAGCGAGAGCAACCTTTGAAC	112-118	56
BET14	F: GTGTACTTCTCTGGTTGGGGC R: CCCGAGTTCATTACCCAAACT	106-120	56
BET15	F: ACTGCCATCACCAGCATGTAG R: GTCCCTTGTGGTCTCTCACAAT	184-202	56
BET16	F: TCTTCAAACACTCAAAGGGACA R: TGTCTATAAATAGGGGCCTCCC	128-130	56
BEX02	F: GCCATGTTCTCTACAGTCTC R: AGTCGGACATCCTTCGTGCT	108-130	56
BEX09	F: TATTCCATGGTCCTCCGT R: AGTCAATCATCTTCAAGAGT	108-138	56
BEX22	F: GCATTCTCTCATTTTCGCTTG R: CCCTAGCAATCGTCGTCTTC	124-150	56
BEX27	F: ACTGTTCTGATCCGCCATGT R: TTTCGACCGTTCAAATACGC	128-136	56
BEX33	F: CAAGTCTCTGACTCATCGCCTA R: ACCAGGTTCAGCAGACGTTC	195-249	56
BEX37	F: TGCATGCTATGTTTCATTGCT R: CACGCAACCTCACAGTCTTG	184-212	56

The collection of trunk vascular cambium from the seeds tree and progeny leaves were performed according to Cabral et al., (2017). Genomic DNA extraction followed the protocol proposed by Doyle and Doyle (1987), with modifications (CTAB 4%). Ten microsatellite primers already developed for Brazil nut were used, namely BET12, BET14, BET15, BET16, BEX02, BEX09, BEX22, BEX27, BEX33 and BEX37 (Reis et al., 2009; Sujii et al., 2013), as shown in Table 2. Amplification and genotyping were performed according to Cabral et al., (2017).
Considering that progenies from the same seed tree constitute a half-sib family (HSF), we used nine HSF. The present study considered each of these families as a different population, and the individuals that composed them were used to study the divergence between and within families. The results from the microsatellite data were used to perform the analysis of molecular variance (AMOVA) for each locus (primer), according to the statistical model, as shown in Equation 1:

$$X_{ijk} = \mu + P_i + I/P_{ij} + G_{ijk} \quad (1)$$

Where: X_{ijk} is the variable that identifies the presence of a given A_k allele in the genotype of the j-th progeny of the i-th family; μ is the average frequency of the allele A_k in the studied families; P_i is the effect of the i-th family (i = 1, 2, ..., 9) with $P_i \sim (0, \sigma_p^2)$; I/P_{ij} is the effect of the j-th progeny within the i-th family (j = 1, 2, ..., 300) with $I/P_{ij} \sim (0, \sigma_i^2)$; G_{ijk} is the effect of the presence or absence of the k-th allele (k = 1, 2) with $G_{ijk} \sim (0, \sigma_g^2)$.

Wrigth (1951) statistics were estimated according to Equations 2, 3 and 4, respectively:

$$F = \frac{\sigma_p^2 + \sigma_i^2}{\sigma_p^2 + \sigma_i^2 + \sigma_g^2} \quad (2)$$

$$\emptyset = \frac{\sigma_p^2}{\sigma_p^2 + \sigma_i^2 + \sigma_g^2} \quad (3)$$

$$f = \frac{F - \emptyset}{1 - \emptyset} \quad (4)$$

Subsequently, the distances between the progenies were estimated according to the complement of the unweighted index ($C_{ii'}$), as shown in Equation 5:

$$C_{ii'} = 1 - \frac{1}{2L}\sum_{j=1}^{L} c_j \quad (5)$$

where: L is the total number of loci studied; c_j: number of common alleles between the progeny pairs i and i '. All analyses were performed with Genes software (Cruz, 2013).

With the distance matrix between genotypes, the HSF together with their seed trees were clustered by the modified Ward method, following a proposal by Tardin et al., (2007). Ward's minimum variance method, for initial group formation, considers the individuals that provide the smallest sum of squared deviations. It is assumed that at any stage there is loss of information due to the clustering formed, which can be quantified by the ratio of the sum of squared deviations within the forming group and the total sum of squared deviations. While calculating the sum

of squared deviations within the group, considering only the genotypes within the forming group, the total sum of squared deviations considers all individuals available for cluster analysis (Cruz and Carneiro, 2003). The clustering is carried out from the sum of squared deviations between genotypes or, alternatively, from the squared distance between genotypes. In the case of this work, the Ward's method was modified, because instead of using the Euclidean distance, the squared distance between genotypes obtained by the arithmetic complement of the unweighted index was used.

RESULTS

Table 3 contains the summary of the analysis of variance, proposed by Excoffier et al. (1992) from the ten microsatellite loci evaluated between and within nine half-sib families (HSF) of Brazil nut. Significant differences were observed between the families for the majority of the loci evaluated, that is, from the ten loci used, eight allowed differentiating the nine families, being important for the genetic diversity analysis. It was found that there was no significant difference between the HSF only for the BET12 and BET16 loci. These results show that all the HSF evaluated in this study presented similar polymorphism for these loci, indicating that they did not contribute to the study on genetic diversity in the present study.

There was no significance of the effect of progenies within the family for any of the loci evaluated in this study (Table 3), demonstrating a progenies trend from the same seed tree (family) to present the same alleles.

Table 3. Summary of molecular analysis of variance (AMOVA) with their sources of variation and respective degrees of freedom (DF) and mean squares (MS) for the ten loci quantified in nine families and 300 progenies of Brazil nut, by microsatellite markers.

Sources of variation	DF	MS				
		BET 12	BET 14	BET 15	BET 16	BEX 02
Families	8	1.04^{ns}	4.10*	6.05*	0.25^{ns}	2.41*
Progenies/Families	300	0.16^{ns}	0.42^{ns}	0.50^{ns}	0.13^{ns}	0.40^{ns}
Allele/Progenies	309	0.83	0.45	0.61	0.86	0.44
		BEX 09	BEX 22	BEX 27	BEX 33	BEX 37
Families	8	4.23*	4.90*	3.30*	3.86*	9.24*
Progenies/Families	300	0.34^{ns}	0.37^{ns}	0.13^{ns}	0.51^{ns}	0.50^{ns}
Allele/Progenies	309	0.34	0.50	0.17	0.57	0.60

^{ns} and *: not significant and significant at 5% probability by the F test, respectively.

Figure 2 contains the clustering of the seed trees and their progenies based on the complement of the unweighted index. It can to check the distinction of five well-defined groups. Group I allocated the majority of the progenies originated from M1, besides M1 itself, M3 and M7. Group II allocated M6 and its progenies. The other groups presented different constitution allocating progenies from different seed trees. The greatest distance was observed among the genotypes M1-F1-2 and M6-F5-1 (0.85).

Figure 2. Clustering of nine seed trees and 300 progenies of Brazil nut by the Ward method, based on the complement of the unweighted index.

Variability within families can be observed by the occurrence of several subgroups within the five large groups formed in the dendrogram (Figure 2), indicating the possibility of selecting genotypes with genotypic variance for use in breeding programs. The clustering by Ward's hierarchical method also showed a coincidence among 29 pairs of genotypes, which presented null value of genetic dissimilarity. In the present study, replicate cases were observed, highlighting the progenies from the family 4, in which five genotypes had null genetic distance between them (M4-F6-6, M4-F6-4, M4-F3-3, M4-F3-1 and M4-F3-2). It was also observed that many replicates occurred between plants from the same tree and same fruit (M1-F8-1 and M1-F8-2; M1-F9-8 and M1-F9-10; M4-F3-1, M4-F3-2 and M4-F3-3; M4-F6-4 and M4-F6-6).
Clustering of nine families into five large groups (Figure 2), by Ward's hierarchical method, demonstrated that some of the progenitors are genetically close to each other. This was observed by the calculated values of genetic dissimilarity between the nine seed trees, according to Table 4.

Table 4. Genetic dissimilarity values among the nine Brazil nut trees.

Seed tree	M2	M3	M4	M5	M6	M7	M8	M9
M1	0.2	0.2	0.2	0.2	0.25	0.15	0.1	0.2
M2		0.2	0.35	0.3	0.3	0.2	0.2	0.25
M3			0.25	0.15	0.3	0.3	0.3	0.3
M4				0.1	0.35	0.15	0.2	0.15
M5					0.3	0.25	0.2	0.25
M6						0.4	0.25	0.35
M7							0.15	0.1
M8								0.1

Among the seed trees evaluated, M6 and M7 presented the highest dissimilarity values (0.4). In M6, the highest dissimilarity values were also observed in relation to the other trees (Table 4), as well as in the clustering (Figure 2), in which most progenies from this family were allocated into the same group (Group II). The smallest distances were observed between the trees M1 and M8, M4 and M5, M7 and M9, M8 and M9 (0.1), e consequently, the progenies from these trees were clustered together.

DISCUSSION

The molecular analysis of variance (AMOVA) from the ten microsatellite loci evaluated between and within nine half-sib families (HSF) of Brazil nut showed that progenies trend from the same seed tree (family) to present the same alleles (Table 3). These results are important because they show that even Brazil nut being an allogamous species (Baldoni et al., 2017), the gene flow is low, that is, with small diversity within the family.

In the literature, the genetic diversity evaluated at the Brazil nut population level showed greater diversity within than between populations (Sujii et al., 2015; Wadt et al., 2015; Baldoni et al., 2017). In the present study, the evaluation was performed at the individual level, that is, the genetic diversity of the progenies from the same seed tree (family) was evaluated. In this case, a lower diversity was observed among the progenies from the same family when compared to the diversity among the families. It should also be considered that the progenies from each family evaluated are half-sib, that is, they initially have 50% of the genetic constitution in common, which explains the results observed here.

The variability within families can be observed by the occurrence of several subgroups within the five large groups formed by the clustering dendrogram of the Ward method (Figure 2). These results indicate the possibility of selection genotypes between and within half-sib families with genotypic variance for use in breeding programs.

Twenty-nine pairs of genotypes with null value of genetic dissimilarity were observed, demonstrating the efficiency in using the markers in diversity studies, to identify replicates in germplasm banks, for example, which would allow eliminating replicates and hence reducing maintenance costs of banks. It was also observed that many replicates occurred between plants from the same tree and same fruit, which can be explained by the action of pollinating agents, bees belonging the genus *Bombus*, *Xylocopa* and *Centris* (Müller, 1995; Maués, 2002), which can carry the pollen to different flowers of the same tree (Baldoni et al. 2017). As Brazil nut is reproduced by cross fertilization, another hypothesis would be the existence of apomixis, as already reported for other forest species (Kaur et al., 1978; Goldenberg and Shepherd, 1998; Chaves et al., 2017).

Studies on genetic dissimilarity, through the use of molecular markers, such as microsatellites, contribute to breeding programs (Caixeta et al., 2013), since it is possible to identify replicates, information on levels of heterozygosity, and to identify genotypic differences of the seed trees. Identification of replicates is important in the maintenance and conservation of genetic resources in germplasm banks. The molecular markers used in this study were able to identify the replicates, in addition to being significant in the genetic diversity analysis of the families evaluated.

CONCLUSION

There is greater genetic diversity between families than among progenies from the same family. The clustering of progenies from different families in the same group can be explained by the low dissimilarity between the seed trees. Among the loci analyzed in this study, eight were informative for evaluations of genetic diversity in Brazil nut, except BET12 and BET16 loci.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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