CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Genetic diversity in populations of Brazil nut

Lucyanna Moura Coelho[1], Maria Teresa Gomes Lopes[1*], Edvan Alves Chagas[2], Rafael Pio[3], Moacir Pasqual[3], Pedro de Queiroz Costa Neto[1] and Magna Aragão Magalhães[1]

Crop Breeding and Applied Biotechnology
17: 382-389, 2017
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332017v17n4a57

Abstract: *The objective of this study was to analyze the genetic diversity among and within populations of Brazil nut by Amplified Fragment Length Polymorphism markers. Five populations with 30 plants each of the state of Amazonas, Brazil, two populations from the Farm Aruanã (Aruanã Carolina and Aruanã Brastor), in Itacoatiara, one from the municipality of Parintins and two from the municipality of Manaus (Instituto Federal do Amazonas - IFAM and Manaus Airport) were evaluated. Four primer combinations were used and 306 polymorphic bands (93.3%) were obtained. Genetic differentiation within and among populations was tested by analysis of molecular variance. The presence of genetic divergence was greater within populations (51.88%) than among populations (48.11%) and the F_{st} value was 0.48. Two groups were formed by interpopulation grouping: the first consisted of populations from plantations in Aruanã Carolina, Aruanã Brastor and Manaus IFAM, and the second of natural populations from Parintins and Manaus Airport.*

Key words*: Bertholletia excelsa H. B. K., lecythidacea, genetic variation, molecular marker.*

INTRODUCTION

Brazil nut (*Bertholletia excelsa* HBK), a tree species of the family Lecythidaceae, is one of the symbol trees of the Amazon, with great social, ecological and economic value for the region, since its fruits and its wood are widely sold (Borém et al. 2009).

In the northern region of Brazil, the nut of this tree is one of the most important non-timber products of the forest economy of the Amazon in commercial terms, second only to açaí (*Euterpe* sp.). In 2015, the nut production in the northern Brazilian region was 40.6 thousand tons (IBGE 2015).

The extraction from natural populations of Brazil nut in the last century was so intense that the replacement of old by younger trees now fails to occur at the desired pace, hampering the succession of generations of Brazil nut trees. This phenomenon is known as demographic collapse, i.e., when the majority of individuals of a population (plants or animals) grows old and cannot generate descendants any more. If the number of young individuals decreases steadily, the species tends to disappear gradually from the region of occurrence (Perez et al. 2003).

RAPD (Random Amplified Polymorphic DNA) molecular markers showed the existence of genetic divergence between and within natural populations of Brazil

***Corresponding author:**
E-mail: mtglopes@hotmail.com

Received: 14 September 2016
Accepted: 31 May 2017

[1] Universidade Federal do Amazonas, Faculdade de Ciências Agrárias, Av. Rodrigo Otávio, 3000, 69.060-000, Manaus, AM, Brazil
[2] Embrapa, Embrapa Roraima, Rod. BR 174, km 08, CP 133, Distrito Industrial, 69.301-970, Roraima, Boa Vista, Brazil
[3] Universidade Federal de Lavras, Departamento de Agricultura, Campus da UFLA, 37.200-000, Lavras, Minas Gerais, Brazil

nut (Serra et al. 2006) and ISSR (Inter Simple Sequence Repeat) markers indicated a significant genetic differentiation between the evaluated genotypes (Ramalho et al. 2016). In Brazil, Embrapa has been developing pre-breeding actions in plantations and in native forests in the States of Roraima, Amazonas, Acre, Amapá, and Pará, to provide information for future breeding programs of the species.

The objective of this study was to obtain knowledge about the distribution of genetic diversity within and among Brazil nut populations based on AFLP (Amplified Fragment Length Polymorphism) markers to support the implementation of exploitation and conservation planning of genetic resources of the species.

MATERIAL AND METHODS

One hundred and fifty Brazil nut trees from five populations were used: two from Manaus, two from the Fazenda Aruanã in Itacoatiara and one from Parintins, with 30 trees per population, all of the State of Amazonas, Brazil. The populations were properly identified and their geographical locations recorded: Aruanã Carolina (lat 2° 59' 43.81" S, long 58° 49' 38.26" W, alt 22 m asl), Aruana Brastor (lat 3° 00' 7.45" S, long 58° 49' 9.68" W, alt 22 m asl), Parintins (lat 2° 40' 12.10" S, long 56° 44' 8.96" W, alt 15 m asl), Manaus IFAM (lat 3° 4' 43" S, long 59° 55' 57" W, alt 45 m asl) and Manaus Airpot (lat 3° 2' 32.92" S, long 60° 1' 43.70" W, alt 70 m asl). Intermediate leaves without stains were collected, wrapped in paper bags with silica gel, stored at -20 °C and then DNA extraction was performed.

Genomic DNA was extracted by the CTAB (cetyltrimethylammonium bromide) method with modifications (Doyle and Doyle 1987) and determined by the comparative method on 1% agarose gel, using markers with known molecular weight (20, 50 and 100 ng) (GIBCO-BRL). The agarose gel was ethidium bromide-stained and visualized by a UV transilluminator.

The AFLP protocol was applied as described by Vos et al. (1995). Genomic DNA was double-digested by combining a rare with a frequent cutting enzyme, both with cohesive cutting. To this end, 6.0 µL DNA (300 ng) were digested using 0.5 µL EcoRI (5 U) (rare cutting) (Promega®) and 0.6 µL *Mse*I (6U) (frequent cutting) (New England Biolabs®) in solution containing 5.0 µL (10X) of the universal One Phor All buffer (Amersham®), 0.5 µL (100X) BSA (Bovine Serum Albumin) (New England Biolabs®) and ultratrapure sterilized water in a final volume of 50 µL reaction solution. The material was incubated in an Esco® PCR Thermal Cycler for 3 h at 37 °C for restriction and then for 15 min at 70 °C to inactivate the restriction endonucleases.

Preamplification was performed in a final volume of 20 µL with 1.2 µL $MgCl_2$ (25 mM), 0.8 µL dNTPs (10 mM), 1 µL of each oligonucleotide containing a selective nucleotide added to end 3': oligo *Eco*RI (25 ng μL^{-1}) + A and oligo *Mse*I (25 ng μL^{-1}) + C (Table 1), 2 µL of Taq DNA polymerase enzyme buffer (10X) (Fermentas®), 0.6 Taq polymerase (5 U μL^{-1}) (Fermentas®), 2 µL digested and ligated DNA plus ultrapure water to complete the volume. The PCR conditions were: 94 °C for 2 min followed by 26 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. The pre-amplified products were diluted in 40 µL of sterile ultrapure water and stored at -20 °C.

For selective amplification, we used 10 combinations of oligonucleotides in a random sample of five plants from the collection: *EcoRI-AAC/ MseI-CAC*, *EcoRI-AAC/ MseI-CTC*, *EcoRI-AAC/ MseI-CAT, EcoRI-ATC/ MseI-CCA*, *EcoRI-AGC/ MseI-CAT*, *EcoRI-AGC/ MseI-CCA*, *EcoRI-ACA/ MseI-CAC*, *EcoRI-ACA/ MseI-CAT*, *EcoRI-ACA/ MseI-CGC,* and *EcoRI-ACA/ MseI-CTC*. The oligonucleotides with the highest number of polymorphic loci were amplified in all plants of the collection (*EcoRI-ATC/ MseI-CCA, EcoRI-AGC/ MseI-CAT*, *EcoRI-AGC/ MseI-CCA and EcoRI-ACA/ MseI-CAC*). The selective PCR amplification reactions were carried out in a solution containing 1.2 µL $MgCl_2$ (25 mM), 0.4 µL dNTPs (10 mM), 1 µL oligo *Eco*RI (25 ng μL^{-1}) + ANN 1.2 µL oligo *Mse*I (25 ng μL^{-1}) + CNN (Table 1), 2 µL *Taq* DNA polymerase enzyme buffer (10X) (Fermentas®), 0.2 Taq DNA polymerase (5 U μL^{-1}) (Fermentas®), 2.5 µL pre-amplified DNA products diluted in ultrapure water to a volume of 20 µL. Polymerase chain reaction was performed under the following conditions: denaturation at 94 °C for 2 min followed by 12 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min in 23 cycles under the same conditions, changing only the annealing temperature to 56 °C and adding a final extension of 72 °C for 2 min.

The silver nitrate (Creste et al. 2001) staining method was used for gel revelation. The loci were analyzed for the presence/absence of amplified fragments. The AFLP markers were identified by codes composed of two initial characters corresponding to the digestion enzymes (*Eco*RI = E and *Mse*I = M), while the third and fourth characters were numbers that identify the combinations according to the variation in the primer extension. After these four characters that

Table 1. Analysis of the results of molecular variance (AMOVA) for the five studied populations of Brazil nut, for the cultivated populations (Aruanã Carolina, Aruanã Brastor and Manaus IFAM) and for the natural populations (Parintins and Manaus Airport)

Five studied populations						
Source of variation	**df**	**Sum of squares**	**Mean squares**	**Components of variation**	**% of variation**	**P**
Among populations	4	3437.573	859.3933	27.6526	48.12	<0.0001
Within populations	145	4323.367	29.8163	29.8163	51.88	<0.0001
Total	149	7760.94	52.0868	57.4689	100.0	
F_{st} = 0.48						
Cultivated populations (Aruanã Carolina, Aruanã Brastor and Manaus IFAM)						
Source of variation	**df**	**Sum of squares**	**Mean squares**	**Components of variation**	**% of variation**	**P**
Among populations	2	9.2794	4.6397	0.1482	43.25	<0.0001
Within populations	87	16.9168	0.1944	0.1944	56.75	<0.0001
Total	89	26.1963	0.2943	0.3426	100.0	
F_{st} = 0.43						
Natural populations (Parintins and Manaus Airport)						
Source of variation	**df**	**Sum of squares**	**Mean squares**	**Components of variation**	**% of variation**	***P***
Among populations	1	4.3077	4.3077	0.1367	39.90	<0.0001
Within populations	58	11.9446	0.2059	0.2059	60.10	<0.0001
Total	59	16.2524	0.2755	0.3427	100.0	
F_{st} = 0.39						

identify the enzymes and primer combination, the amplified fragment size of the locus was added in base numbers. Based on the obtained fragments, a zero-one matrix was constructed, where one indicates the presence and zero the absence of fragments.

The distribution of genetic variation among and within populations was quantified by analysis of molecular variance (AMOVA), using software Genes (Cruz 2013). The genetic distances between populations were calculated based on the Jaccard method (Anderberg 1973). These genetic distances were used to construct a dendrogram by UPGMA (Unweighted Pair Group Method using Arithmetical Averages) cluster analysis.

RESULTS AND DISCUSSION

It was possible to extract the total genomic DNA from the samples with sufficient quantity and quality. Intact DNA samples were obtained because no vertical drag of the DNAs on the gel was observed. The DNA integrity is essential for the clarity and reproducibility of the PCR amplification products. A low polysaccharide concentration in the samples was also observed because the bands were not cone-shaped towards the positive pole. The extraction of high-quality pure DNA is a prerequisite for any molecular analysis. There are several methods available for genomic DNA isolation from plants, but in practice, these procedures are empirical due to the variability in the composition of the plant tissue used. The simple conventional DNA extraction methods are not necessarily replicable for all species, requiring adjustments and modifications (Aras et al. 2003).

Among the 10 tested primer combinations, four were selected for producing fragments with good quality, high intensity and a high number of amplified loci as well as a uniform pattern in the amplification profile for binary readings. The pre-selection of primers to be used for genetic diversity analysis of a population is an important step. Since the nucleotide sequence of the AFLP primers recognizes random primers in the test and selection step, it is essential to measure all samples of a population before using them, to prevent the use of primers that amplify no or generate few fragments and low polymorphism, hampering the study of genetic diversity (Vos et al. 1995).

In all four studied combinations of restriction enzymes (selective primers), a total of 328 loci were amplified, of which 306 (93.3%) were polymorphic and 22 (6.7%) monomorphic. Within a single Brazil nut population, there is a high level

of genetic variation due to the high rate of crosses between individuals (O'Malley et al. 1988). Analyzing the genetic diversity in *B. excelsa* with ISSR (Inter Simple Sequence Repeat) markers revealed six well-defined groups, confirming a significant genetic divergence between genotypes (Ramalho et al. 2016).

Among the studied combinations, *EcoRI-AGC/MseI-CAT* (100) contained the highest number of informative loci and *EcoRI*-ACA/*MseI*-CAC (69) was the least informative and indicated a difference in the amplification pattern between the different combinations. The average polymorphic loci detected in the populations ranged from 44.5 for Aruanã Brastor to 57.5 for Aruanã Carolina. The populations of Parintins, Manaus IFAM and Manaus Airport had intermediate polymorphism levels (55, 50 and 52, respectively).

The polymorphism values detected by AFLP markers were higher than those identified with other dominant molecular markers, e.g., by RAPD and by codominant molecular markers, such as isoenzymes. The genetic diversity in 17 Brazil nut trees from the states of Pará and Mato Grosso observed through 51 RAPD reaction initiators (primers) generated a total of 144 polymorphic loci (Serra et al. 2006). A comparison of the methods used to find polymorphism shows remarkable differences in results. One evidence indicating that the analyses performed here were adequate to detect polymorphism between trees of the analyzed Brazil nut populations is that the amount of polymorphic fragments generated by the AFLP technique was sufficient to differentiate the studied populations.

Based on the similarity coefficient of Jaccard calculated for the studied 150 Brazil nut trees, two groups were identified in the dendrogram, of which the first consisted of cultivated populations (Aruanã Carolina, Aruanã Brastor and Manaus IFAM) and the second of natural populations (Manaus Airport and Parintins) (Figure 1). The dendrograms of genetic distances per population showed high variability within populations (Figures 2, 3, 4, 5, and 6).

For the most closely related populations (AB and AC), the AFLP data ranged from 0.28 to 0.49 for the most distant populations (MA and AB). The dendrogram shows some degree of genetic similarity among the natural and the

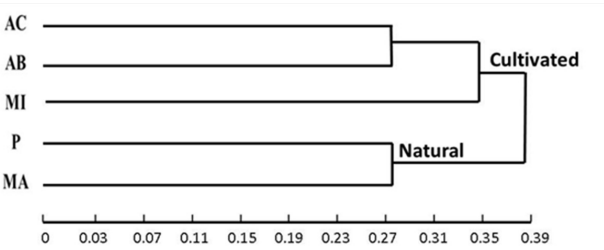

Figure 1. Interpopulational UPGMA dendrogram of Brazil nut, where: AC = Aruanã Carolina; AB = Aruanã Brastor; MI = Manaus IFAM; MA = Manaus Airport. Based on the similarity coefficient of Jaccard. The dashed line represents the division of the groups.

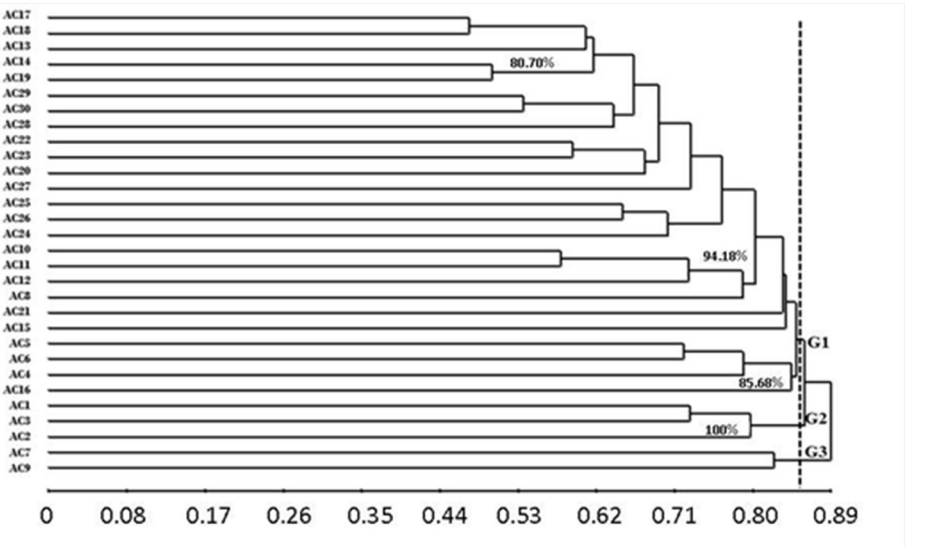

Figure 2. Binary data dendrogram of AFLP based on the similarity coefficient of Jaccard (x-axis) for the Brazil nut population of Aruanã Carolina (accessions AC 1-30) by the UPGMA method; the values in clusters are the highest bootstrap results (resampling 5.000); G1, G2, G3, and G4 are the clusters formed according to the cutting point. The dashed line represents the division of the groups.

cultivated populations, regardless of the origin. Another study reported partially different results, of two groups according to their origins (Pará and Mato Grosso) and one group with genetic similarity between both states, where the plants of Pará were from a genebank and those from Mato Grosso were plants from a reforested area in the municipality of Cláudia (Serra et al. 2006). It is noteworthy that the trees surveyed here were from the same state and the separation of the groups of natural and cultivated plants was evident. Similar results were found in a study of the genetic diversity among 100 Brazil nut plants from five regions of the Brazilian Amazon (Alenquer-PA, Altamira-PA, Marabá-PA, Santarém-PA, and Rio Branco-AC), where plants of a population from Santarem were most similar to plants from Rio Branco, which is geographically more isolated and farther away than other populations (Kanashiro et al. 1997).

One of the factors that probably contributed to this diversity was the high population density of this species. A

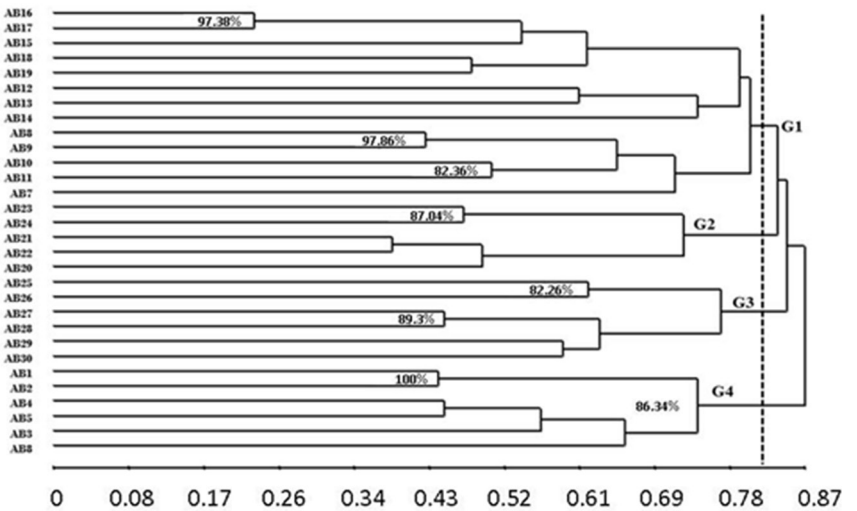

Figure 3. Binary data dendrogram of AFLP based on the similarity coefficient of Jaccard (x-axis) for the Brazil nut population of Aruanã Brastor (accessions AB 1-30) by the UPGMA method; the values in clusters are the highest bootstrap results (resampling 5.000); G1, G2, G3, and G4 are the clusters formed according to the cutting point. The dashed line represents the division of the groups.

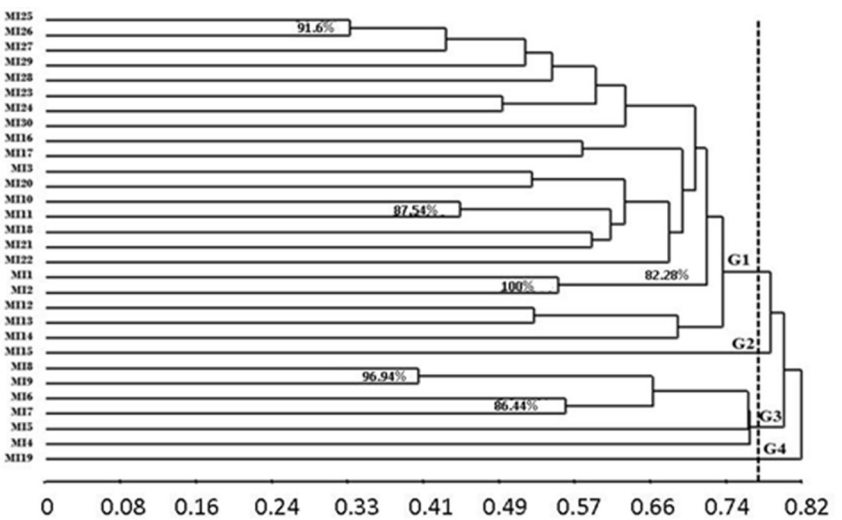

Figure 4. Binary data dendrogram of AFLP based on the similarity coefficient of Jaccard (x-axis) for the Brazil nut population of Manaus (accessions MI 1-30) by the UPGMA method; the values in clusters are the highest bootstrap results (resampling 5.000); G1, G2, G3, and G4 are the clusters formed according to the cutting point. The dashed line represents the division of the groups.

comparison of the genetic diversity in common and rare tropical tree species showed that common species tended to higher diversity levels than rare species (Hamric and Godt 1991). The higher plant density may allow more recombinations and multiple paternity in the progenies, favoring the increase in genetic diversity.

Another factor making the intrapopulation greater than the interpopulation diversity is the restricted dispersion range of these species. The distribution of Brazil nut trees is directly attributed to human intervention, since the agouti, the main dispersers of this species, do not cover long distances (Peres and Baider 1997).

The results of AMOVA, based on 328 informative loci, showed that 48.12% of the genetic diversity is interpopulational and 51.88% intrapopulational (Table 1), suggesting that the maintenance of subpopulations of this species is an important strategy to preserve the natural genetic variability. These indices show that the populations can be isolated. The genetic

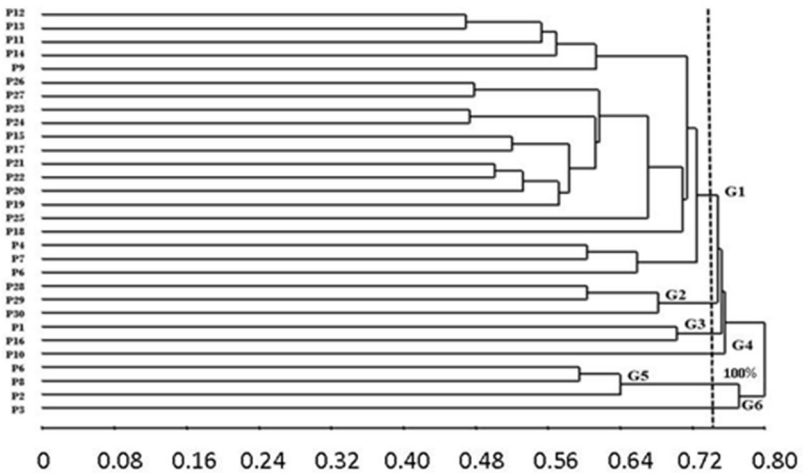

Figure 5. Binary data dendrogram of AFLP based on the similarity coefficient of Jaccard (x-axis) for the Brazil nut population of Parintins (accessions P 1-30) by the UPGMA method; the values in clusters are the highest bootstrap results (resampling 5.000); G1, G2, G3, G4, G5 and G6 are the clusters formed according to the cutting point. The dashed line represents the division of the groups.

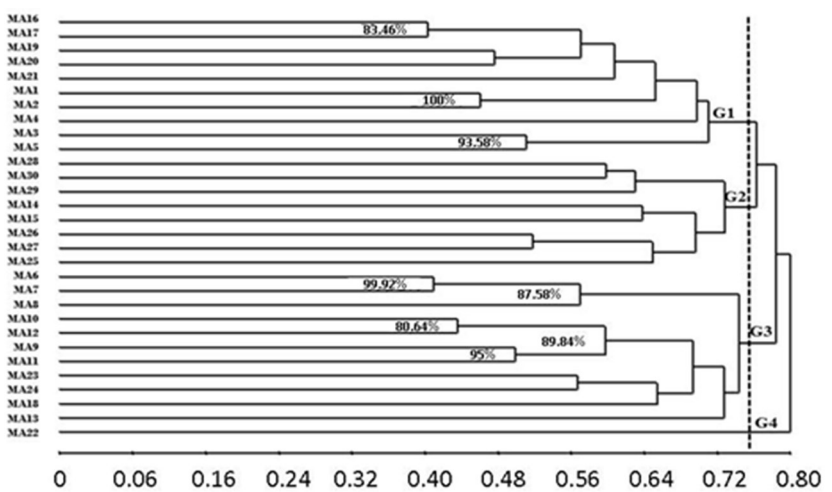

Figure 6. Binary data dendrogram of AFLP based on the similarity coefficient of Jaccard (x-axis) for the Brazil nut population from the airport of Manaus (accessions MA 1-30) by UPGMA; values in clusters are the highest bootstrap results (resampling 5.000); G1, G2, G3, and G4 are the clusters formed according to the cutting point. The dashed line represents the division of the groups.

diversity preserved within and among populations may be a result of historical events and recent evolutionary processes (Lee et al. 2002).

Due to their characteristics of long life, high population density and high cross levels, climactic species probably have gene flow over long distances with low genetic divergence among populations (Kageyama et al. 2003). The reproductive system and genetic structure of biribá (*Eschweilera ovata* [Cambess.] Miers) was analyzed by isoenzyme electrophoresis in a study, addressing three areas of naturally occurring populations of the species under different levels of anthropogenic disturbance, one exploited and two others without exploitation, near the city of Salvador. The genetic divergence among the adult population was only 2.5%, indicating that most of the genetic diversity is distributed within populations (97.5%).

An analysis of molecular variance was performed for the cultivated and another for the natural populations, to detect where variance is highest (Table 1). The results of all analyses of variance were higher within than among populations. The genetic variance within natural populations was higher (60.10%) than in the cultivated populations (56.75%), although the values were similar, as expected for the cultivated populations, since the seeds for these populations had been selected.

These data agree with a multidisciplinary survey that left little doubts (Scoles and Gribel 2011, Shepard and Ramirez 2011). Data were collected ranging from biological properties, based on genetic analyses of Brazil nut populations, to linguistic information, by correlating several indigenous languages, to decipher the historical significance of the species for the regional native populations. One of the most important discoveries by DNA analysis was the result of a comparison of tree populations from all parts of the Amazon region, indicating great uniformity, in other words, little genetic differences between locations. This indicates that the geographical dispersal of Brazil nut occurred quickly and in a relatively recent period, making a natural dispersal mechanism less likely.

In the analysis, F_{st} values of 0.48 were found for all five populations, 0.39 for the natural and 0.43 for the cultivated populations (Table 1), i.e., the level of genetic differentiation of the populations is very high. The F_{st} is a measure of the inbreeding degree within a subpopulation of the total population and F_{st} values between 0 to 0.05 indicate low genetic differentiation; values between 0.05 to 0.15 moderate differentiation and between 0.15 and 0.25 high differentiation (Wright 1978). In natural populations, the inbreeding rate among plants is probably high, since the F_{st} value is lower than in cultivated populations, because inbreeding reduces the frequency of heterozygotes by proportionally increasing the frequency of homozygotes and consequently reducing genetic variation.

The main conclusions of the study were: the content of genetic information detected in Brazil nut by AFLP markers was high, and can be used for genetic analysis to obtain information about genetic sustainability and forest management of the species; the distribution of genetic diversity in natural Brazil nut populations is similar to that observed in most natural populations of tropical tree species, with a higher genetic diversity within than among populations; and the same distribution pattern of genetic variation was observed in the cultivated as in the natural Brazil nut study populations, and the changes in the diversity values were results of selection for new plantations.

REFERENCES

Anderberg MR (1973) **Cluster analysis for applications**. Academic press, New York, 359p.

Aras S, Duran A and Yenilmez G (2003) Isolation of DNA for RAPD analysis from dry leaf material of some *Hesperis L*. specimens. **Plant Molecular Biology Reporter 21**: 461a-f.

Borém A, Lopes MTG, Clement CR and Noda H (2012) **Domestication and breeding: Amazonian species**. Suprema Editora LTDA, Visconde do Rio Branco, 479p.

CONAB - Companhia Nacional de Abastecimento. Ministério da Agricultura, Pecuária e Abastecimento (2012) Conjuntura mensal. Abril, 2012. Available on-line at <http://www.conab.gov.br/OlalaCMS/uploads/arquivos/12_05_17_15_27_18_conjunturacastnhadobrasilabril2012.pdf>. Accessed in November, 2015.

Creste S, Tulmann Neto A and Figueira AVO (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. **Plant Molecular Biology Reporter 19**: 299-306.

Cruz CD (2013) **Programa Genes: análise multivariada e simulação**. Aplicativo computacional na área de genética e estatística experimental. UFV, Viçosa, 278p.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. **Phytochemical Bulletin 19**: 11-15.

Hamric JL and Godt MJ (1991) Allozyme diversity in plant species. In Brown AHD, Clegg MT, Kahler AL and Weir BS (eds) **Plant population genetics, breeding and germplasm resources**. Sinauer Associates, Sunderland, p. 43-63.

IBGE - Instituro Brasileiro de Geografia e Estatística (2015) Produção da extração vegetal e da silvicultura. **Senso Agropecuário**. IBGE, Rio

de Janeiro, 46p.

Kageyama PY, Sebbenn AM, Ribas LA, Gandara FB, Castellen M, Perecim MB and Vencovsky R (2003) Diversidade genética em espécies arbóreas tropicais de diferentes estágios sucessionais por marcadores genéticos. **Scientia Forestalis 64**: 93-107.

Kanashiro M, Harris SA and Simons A (1997) RAPD diversity in Brazil nut (*Bertholletia excelsa* Humb. and Bonpl., Lecythidaceae). **Silvae Genetica 46**: 219-226.

Lee SL, NG KKS, Norwati A, Salwana MHS, Lee CT and Norwati M (2002) Population genetics of *Intsia palembanica* (Leguminosae) and genetic conservation of Virgin Jungle reserves in Peninsular Malaysia. **American Journal of Botany 89**: 447-459.

O'Malley DM, Buckley DP, Prance GT and Bawa KS (1988) Genetics of Brazil nut (*Bertholletia excelsa* Humb. and Bonpl. – Lecythidaceae) 2. Mating system. **Theoretical and Applied Genetics 76**: 929-936.

Peres CA and Baider C (1997) Seed dispersal, spatial distribution and population structure of Brazil nut trees (*Bertholletia excelsa*) in southeastern Amazonia. **Journal of Tropical Ecology 13**: 595-616.

Perez CA, Baider C, Zuidema PA, Wadt LHO, Kainer KA, Gomes-Silva DAP, Salomão RP, Simões LL, Franciosi ERN, Valverde FC, Gribel R, Shepard Jr GH, Kanashiro M, Coventry P, Yu DW, Watkinson AR and Freckleton RP (2003) Demographic threats to the sustainability of Brazil Nut exploitation. **Science 302**: 2112-2114.

Ramalho AB, Rossi AA, Dardengo JFE, Zortéa KÉM, Tiago AV and Martins KC (2016) Diversidade genética entre genótipos de *Bertholletia excelsa* por meio de marcadores moleculares ISSR. **Floresta 46**: 207-214.

Scoles R and Gribel R (2011) Population structure of Brazil Nut (*Bertholletia excelsa*, Lecythidaceae) stands in two areas with different occupation histories in the Brazilian Amazon. **Human Ecology 39**: 455-464.

Serra AGP, Paiva R, Paiva E, Nogueira RC, Soares FP and Paiva PDO (2006) Estudo da divergência genética em castanha-do-brasil (*Bertholletia excelsa* H. B. K.) utilizando marcadores moleculares RAPD (Random Amplied Polymorphic DNA). **Magistra 18**: 42-47.

Shepard Jr GH and Ramirez H (2011) "Made in Brazil": human dispersal of the Brazil nut (*Bertholletia excelsa*, Lecythidaceae) in ancient amazonia. **Economic Botany 65**: 44-65.

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. **Nucleic Acids Research 23**: 4407-4414.

Wright S (1978) **Evolution and genetic population**: Variability within and among natural populations. v.4, University of Chicago Press, Chicago, 590p.