Genetic diversity in a jaborandi (*Pilocarpus microphyllus* Stapf.) germplasm bank assessed by RAPD markers

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ABSTRACT: The aim of this work is to assess the occurrence of genetic variability in a *P. microphyllus* germplasm bank from Embrapa Amazonia Oriental in Belém, PA, using RAPD markers. DNA was extracted from 93 plants, belonging to 12 sample areas owned by the *P. microphyllus* germplasm bank. Dendrograms were constructed using Nei & Li's genetic similarity coefficient among plants and among sample areas using NTSYS-pc program. To verify variability distribution, an analysis of molecular variance (AMOVA) was obtained, generating among and within sample areas variance. The Shannon index was used to verify the genetic diversity of each area. It was obtained a fine amplitude of similarities among individuals, but the dendrogram did not show a complete clustering of the individuals according to their origin. AMOVA obtained 24.16% among areas variation and 75.84% within areas.

Key words: molecular markers, medicinal plants, AMOVA, genetic markers

RESUMO: Diversidade genética em banco de germoplasma de jaborandi (*Pilocarpus microphyllus* Stapf.) por meio de marcadores RAPD. O objetivo deste trabalho é avaliar a presença de variabilidade genética no banco de germoplasma de *P. microphyllus* da Embrapa Amazônia Oriental por meio de marcadores RAPD. Foi extraído o DNA de 93 indivíduos, pertencentes a 12 áreas de coleta presentes no banco de germoplasma. Foram construídos dendrogramas entre indivíduos e entre áreas de coleta, usando os coeficientes de similaridade de Nei & Li por meio do programa NTSYS-pc. Para verificar a distribuição da variabilidade, foi realizada a análise de variância molecular (AMOVA), sendo obtidas as variâncias entre e dentro de áreas de coleta. Foi usado o índice de diversidade de Shannon, para medir a diversidade de cada área. Foi verificada uma boa amplitude de similaridades entre os indivíduos, sendo que o dendrograma não agrupou por completo os indivíduos de acordo com sua origem. A AMOVA obteve 24,16% de variação entre áreas e 75,84% dentro de áreas.

Palavras-Chave: marcadores moleculares, plantas medicinais, genética populacional , AMOVA Marcadores genéticos

INTRODUCTION

Jaborandi (*Pilocarpus microphyllus* Stapf.) is a shrubby tree which belongs to the Rutaceae family. Its leaves contain the imidazolic alkaloid named pilocarpine, used mainly in the first stages of glaucoma treatment. Pilocarpine also stimulates lacrimal and salivar glands (Merck, 1989), and some believe that it prevents hair loss and, as a result, it has been used in cosmetics. It occurs in semi-shady areas and, according to Skorupa (2000), it is found in parts of the Amazon region, mainly at East of the state of Pará, West and North of the state of Maranhão and North of the state of Piauí. The search for *P. microphyllus* leaves, stimulated by the pharmaceutical industry, lead to a reduction of many natural populations, especially in Maranhão (Pinheiro, 2002). Even though the specie has been cultivated in some areas, irrational exploration still occurs (Pinheiro, 2002). To minimize the losses caused by irrational exploration, Embrapa Amazonia Oriental at Belém, PA, Brazil, created, in 1992, a germplasm bank, aiming *P. microphyllus* domestication and conservation.

Analyses of accessions genetic diversity sampled in germplasm banks is important to establish conservation strategies, set the guidelines for sample

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collections and help in genetic breeding programs. According to Brown (1992), the first collections must be based on the assumption that great diversity occurs among regions, with efforts to collect in as many different places as possible, and then study the genetic distribution within and among regions. Based on this structuring, a more efficient plan of sample collections can be established.

Molecular markers have been extensively used to assay the genetic diversity in natural populations (Pavek *et al*., 2003; Kaundun & Park, 2002; Oiki *et al*., 2001; Sun & Wong, 2001) and in germplasm banks (Wadt *et al*., 2004; Reif *et al*., 2003; Rouf Mian *et al*., 2002; Nienhuis *et al*., 1995). SSR markers can supply more detailed studies of population genetics, since they can measure heterozigote rates, but primers construction for each species is a laborious and expensive work (Ferreira & Grattapaglia, 1998). RAPD markers, as described by Williams *et al*. (1990), are favored because of their fine accessibility and low costs, reason why they have often been used in several studies assessing natural population genetic variation (Schmidt & Jensen, 2000; Allnut *et al*., 1999; Gillies *et al*., 1999; Yeh *et al*., 1995) and in germplasm characterization (Reis & Grattapaglia, 2004; Wadt *et al*., 2004; Lima, 2002). The dominance characteristic of RAPD markers represents its main disadvantage in the use for population genetic studies, since parameters like heterozigote rates, endogamy rates, gene flow, and others, cannot be directly estimated. However, several methodologies have been created to afford its use in genetic populations study. The AMOVA program developed by Excoffier *et al*. (1992) uses the molecular variance among haplotipes to evaluate the genetic variation distribution in a hierarchic structure of regions and populations and was used with dominant markers for the first time by Huff *et al*. (1993) with *Buchloë dactyloides* populations. Reis & Grattapaglia (2004), Wadt & Kageyama (2004), Kaundun & Park (2002) and Gillies *et al*. (1999) also used the AMOVA program to estimate the genetic variation among populations of plant species and could even come to some conclusions about the population's gene flow and endogamy rates. The Shannon's diversity index is not based on the heterosigosity estimative, neither on Hardy-Weinberg's equilibrium, so it has also been used in studies using RAPD markers (Oiki *et al*., 2001; Sun & Wong, 2001; Lanteri *et al*., 2003). This index is based on the frequency of individuals having a band in a group of polymorphic loci.

The aim of this work is to evaluate the presence of genetic variation in the *P. microphyllus* germplasm bank of Embrapa Amazônia Oriental and, based on the data obtained, conclude about the genetic structure of the sampled populations and suggest new sample strategies.

MATERIAL AND METHOD

Plant material

Leaves were sampled from 93 plants, which belong to 12 sample areas present at the *P. microphyllus* germplasm bank of Embrapa Amazônia Oriental in Belém, PA, Brazil. The sample areas with respective number of plants collected are indicated on Table 1.

DNA extraction

For DNA extraction, 2.0 g of fresh leaves were used. The leaves were macerated in 10 ml of extraction buffer with CTAB at 65°C, together with 200 ml de b-mercaptoethanol. The extraction buffer contained 2% CTAB, 100 mM Tris (pH = 8.0), 20 mM EDTA (pH = 8.0), 1.4 M de NaCl and 1% PVP (polivinilpirrolidone). The macerate was kept in double boiler for 10 minutes, and shacked every 10 minutes. Then, it was added 10 mL chloroform: isoamilic alcohol solution (24:1), followed by homogenization and centrifugation for 10 minutes at 5000 rpm. The supernatant was mixed with 30 mL ethylic alcohol 95%: ammonium acetate 7.5M solution (6:1) and kept in a freezer at –20°C for an hour. To the collected DNA it was added 300 ml TE (Tris 1 mM e EDTA 0.1 mM, pH 7.7). The dissolved DNA went through a new extraction with chloroform: isoamilic alcohol, and the supernatant was collected and mixed three times its volume with ethylic alcohol at 95%: sodium acetate 3M (20:1) and kept in a freezer at –20°C for another hour. After that, purified DNA was dissolved in 50-100µL TE. Samples were diluted in TE to a concentration of 10 ng/µL for RAPD reactions.

TABLE 1. Sample areas and corresponding number of plants collected.

Sample area	Number of sampled plants
Dom Eliseu,PA (DE)	5
São Félix do Xingu, PA (SF)	6
Serra dos Carajás, PA (SC)	18
Breu Branco, PA (BB)	13
Merck-MA (ME)	10
Moju, PA (1) (MO1)	10
Moju, PA (2) (MO2)	14
Açailândia, MA (AÇ)	6
Nina Rodrigues, MA (NR)	3
Mata Roma, MA (MR)	2
Brejo, MA (BR)	4
Santa Quitéria, MA (SQ)	2

RAPD reactions

Each reaction contained 30 ng of DNA; 100 mM of each dNTPs; 0.4 mM primer; 0.6 U Taq DNA polymerase; 2.5 mM $MgCl_2$; 20mM KCl; 50 mM tris pH 8.3; 250 mg/mL bovine serum albumin; 1% Ficoll 400; 1mM tartrazine and pure water up to final volume

of 16 ml (Hagiwara *et al*. 2001). The amplification reaction was conducted in a Gradient 5331 Eppendorf Master Cycler thermocycler, programmed for 39 cycles. In the first two cycles, the denaturation was conducted at 94°C for two minutes, the annealing at 37°C for 15 seconds, and the elongation at 72°C for 60 seconds, followed by additional 37 cycles, which differed from denaturation by 15 seconds. A final extension was done for two minutes at 72° C. The DNA fragments were analyzed in 1% agarose gel electrophoresis (75V 4-5 h), with 0.5% bromide ethidium and photographed under UV light.

Statistical analyses

Presence of band was computed as 1 and absence of band as 0, to build a matrix. The genetic similarity analyses were made using NTSYS-pc 2.0 program (Rholf, 1992), using Nei & Li's coefficient. The errors associated to each similarity and the maximum similarity value between two accessions were estimated using methodology mentioned by Hagiwara *et al*. (2001). The genetic similarities among sample areas were the media of two groups of individuals genetic similarities, each belonging to different sample areas. The similarity clustering analyses was made with UPGMA, and NTSYS-pc 2.0 program generated the dendrogram.

The Pearson's correlation calculus was made between geographic distances and genetic similarities and significance *t* test to verify if $r = 0$.

To obtain the genetic variation partition among and within sample areas, analyses of molecular variance was made, using WINAMOVA 1.55 program (Excoffier *et al*., 1992). To achieve this result, genetic distances were obtained from the Nei & Li's genetic similarities previously obtained.

To study genetic diversity, it was used Shannon's index, which is not influenced by RAPD dominance, and is given by $H = \acute{O}\text{-}p_i (\log_2 p_i)$, where p_i is the proportion of individuals having the band in a locus. Ten individuals randomly sampled were used from populations 1 and 2 from Moju, PA, Serra dos Carajás, PA, Breu Branco, PA and Merck population.

RESULT AND DISCUSSION

Operon Technologies primer kits OPAH, OPAM, OPAN, OPAO and OPAP, totalizing 100 primers, were screened for polymorphism and best resolution. Among these, 17% were viable to be used. These results are similar to those showed by Reis & Grattapaglia (2004), who screened 165 primers with *Myracrodruon urundeuva* tree, 27 being viable, obtaining 16.36% results. Wadt *et al*. (2004) obtained 10% of primer results screened with three *Piper* species, indicating that this low result is typical in native plants.

The 17 chosen primers amplified 143 bands, of which 85 were polymorphic, generating 58.04% polymorphism. Theses values are above the ones obtained with *Piper hispidinervium* accessions from a germplasm bank, which showed 79,4% polymorphism (Wadt *et al*., 2004). The polymorphic primers were: OPAH-10 (5 polymorphic bands), OPAM-04 (4), OPAM-7 (5), OPAM-10 (5), OPAM-14 (4), OPAM-20 (4), OPAN-7 (3), OPAN-10 (6), OPAN-17 (4), OPAO-5 (4), OPAO-6 (5), OPAO-8 (7), OPAO-17 (4), OPAP-1 (7), OPAP-6 (6), OPAP-7 (6) e OPAP-14 (8), averaging 5,0 polymorphic bands/primer.

Nei & Li's genetic similarities among accessions ranged from 0.55 to 0.91, averaging 0.67.The genetic analyses in germplasm banks of different Brazilian plants were also very variable. Lima

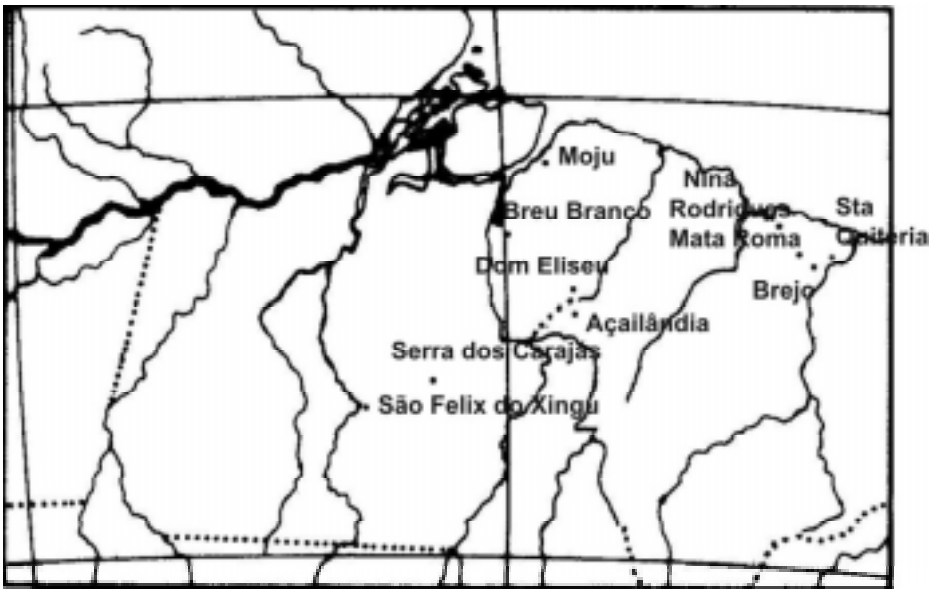

FIGURE 1. Pará and Maranhão states's map showing sampled areas.

(2002) obtained Nei & Li's similarities from 0.24 to 0.99 for *Psycothria ipecacuanha* accessions, while Reis & Grattapaglia (2004) found genetic similarities from 0.54 to 0.86 for *Myracrodruon urundeuva*. It means that the genetic similarities obtained between *P. microphyllus* accessions are comparable to other native species, indicating that its germplasm bank has a considerable genetic variation.

The maximum similarity value (at 1% significance) was 0.86 and 11 duplicate pairs were observed (Figure 2). The duplicate pairs are on the right to the traced line on the dendrogram. Excepting two cases, one where an individual from São Félix do Xingu, PA and one from Serra dos Carajás region, PA, grouped together, and a second one where an individual from Santa Quitéria, MA and another one from Açailândia, MA were also considered genetically identical, all other duplicate pairs were from the same sample area. The population 2 from Moju, PA had the highest duplicate pair numbers (3). The high genetic similarity among individuals from regions far from one another, as those between Santa Quitéria and Açailândia, may be due to an efficient *P. microphyllus* gene flow system or to human action. The decision to discard one of the duplicate pairs individuals may also be complemented with phenotypic data, since those are more relevant to genetic breeding programs and, are usually explained by a narrow genome portion, which is not commonly sampled with standardized molecular markers, such as RAPD.

P. microphyllus was considered an alogamous species by Marques & Costa (1994). However, genetic similarities obtained among sample areas varied from 0.61 to 0.73, which are relatively low for an alogamous species. In contrast, Castellen (2000) obtained similarities among populations of *Esenbeckia leiocarpa*, an alogamous tree, which ranged from 0.81 to 0.98. These low similarity values are corroborated by the analysis of molecular variance (AMOVA), which verified that genetic differentiation among sample areas was highly significant ($\hat{O}_{ST}$ = 0,242, p<0,001), with 24.16% genetic variation among sample areas and 75.84% within sample areas (Table 2). These results agree with the ones obtained from other alogamous and shrubby species. It was found 28.1% genetic variation among *Piper hispidinervium* populations (Wadt & Kageyama, 2004), 28.58% with *Digitalis minor* (Sales *et al*., 2001) and 16.2% with *Camellia sinensis* (Kaundun & Park, 2002). Results obtained for tree species also showed significant differentiation among populations, but the values were much lower, as those found in *Swietenia macrophylla* with 12.58% (Gillies *et al*., 1999), 10.06% in *Esenbeckia leiocarpa* (Castellen, 2000), 8% with *Myracrodruon urudeuva* (Reis & Grattapaglia, 2004) and 2.6% with *Populus tremuloides* (Yeh *et al*. 1995). Thus, the variation percentage among *P. microphyllus* sample areas may be considered typical, and little affected by possible reductions in the population, since it is comparable to other percentages of similar species, without a history of population reduction. Landergott *et al*. (2001) obtained high genetic differentiation among *Dryopteris cristata* populations, showing 49% genetic variation and attributed it to bottleneck effects, since, according to them, this species has a very efficient spores dispersion system.

TABLE 2. Analysis of molecular variance among and within *P. microphyllus* sample areas.

	Variance	% Total	P	Φ_{ST}
Among sample areas	0,0437	24,16	0,001	0,232
Within sample areas	0,1276	75,84		

The affirmative that *P. microphyllus* is an alogamous species may be correct, but many papers have been demonstrating that the reproductive system of many tropical species of trees or shrubby trees is mainly alogamous, in a mixed reproductive system (Kageyama *et al*., 2003; Souza *et al*., 2003; Guidice-Neto & Kageyama, 2000). The occurrence of self-pollination rates in *P. microphyllus* could explain the degree of differentiation verified among sample areas. Also, according to Kageyama *et al*. (2003), tree species that have short life cycle (~20 years), high population density, joined occurrence and low distances seed dispersion have higher rates of endogamy, as they are more likely to have parental crosses, and this also explains genetic differentiation among areas.

The dendrogram (Figure 3) obtained with the Nei and Li's genetic similarities among sample areas did not show separation among natural populations and the population from Merck, which had already gone through higher dry matter and leaf area artificial selection (data not show), indicating that probably few genes were involved, which means, a small genome portion, not sufficient to molecularly separate this population from the others.

Still on Figure 3, it can be observed a clustering among the populations from Serra dos Carajás, PA and São Félix do Xingu, PA, which are close geographically (145 km), between Nina Rodrigues, MA and Brejo, MA populations (130 km) and between Breu Branco, PA and population 2 from Moju, PA (193 km). The correlation value between the geographical distances and the genetic similarities was low, but significant, r = -0,41**, according to Pearson´s index. There is possibly a degree of genetic structuring according to the geographic distance up to a certain distance, but this might be verified later. According to Sousa & Souza (2001), the geographic distance is not always correlated with the genetic distance for Amazonian species meaning that it should be verified for each case. In this case, the pollen and seeds dispersion by different agents, as well as the

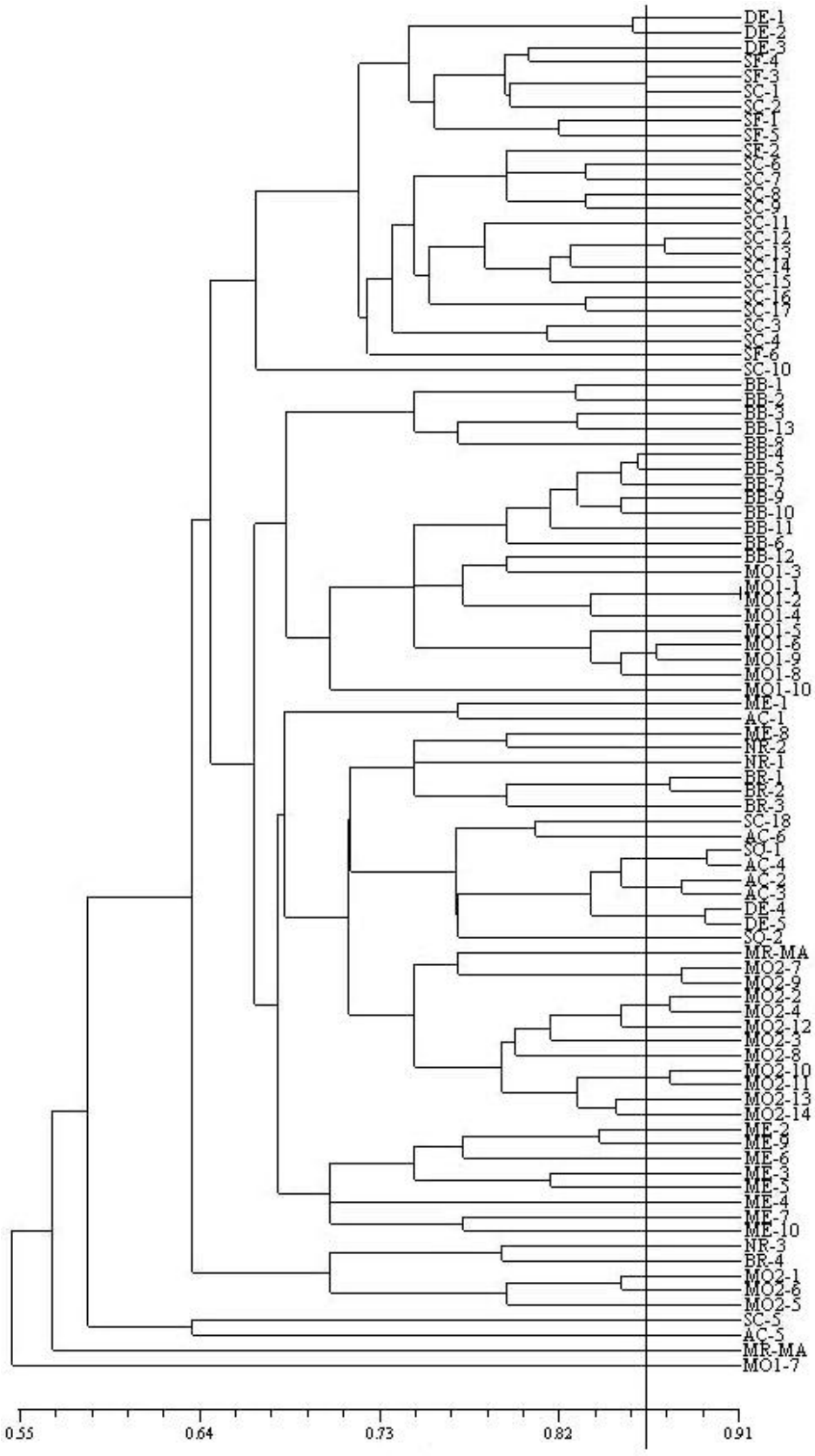

FIGURE 2. Dendrogram obtained from genetic similarities among 93 *P. microphyllus* acessions. DE = Dom Eliseu, PA; SF = São Felix do Xingu, PA; SC = Serra dos Carajás, PA; MO1= Moju,PA (1); BB = Breu Branco, PA; MO2 = Moju, PA (2); ME = fazenda Merck, MA; AÇ = Açailândia, MA; NR = Nina Rodrigues, MA; MR = Mata Roma, MA; BR = Brejo, MA; SQ = Santa Quitéria, MA. Duplicate pairs are on the right of the traced line.

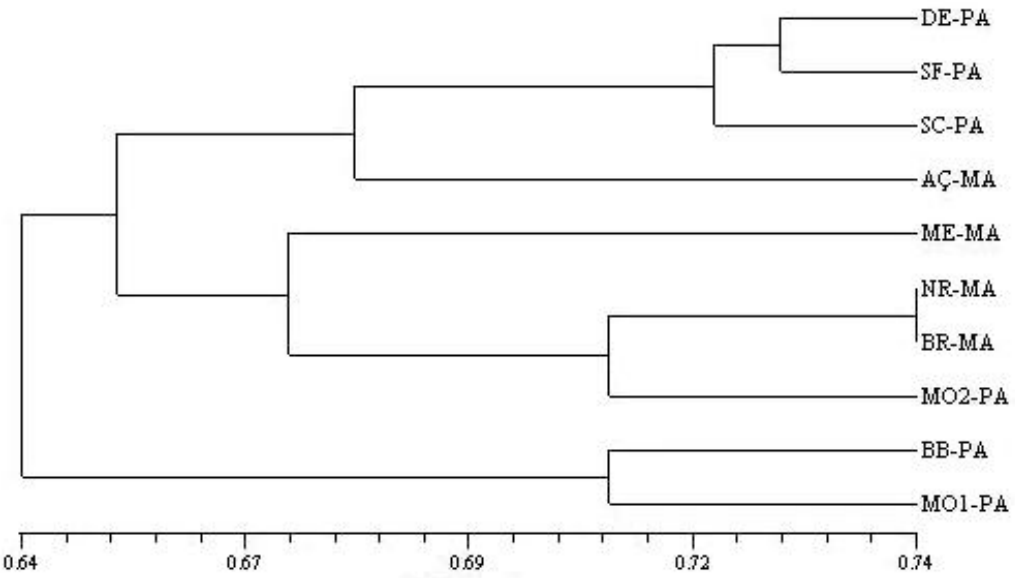

FIGURE 3. Dendrogram of genetic similarities among *P. microphyllus* sample areas. DE = Dom Eliseu, PA; SF = São Felix do Xingu, PA; SC = Serra dos Carajás, PA; BB = Breu Branco, PA; ME = Fazenda Merck, MA; MO1 = Moju, PA (1); AÇ = Açailândia, MA; NR = Nina Rodrigues, MA; BR = Brejo, MA; MO2 = Moju, PA (2).

material introduction by men, can have contributed for the intercross between different genotypes, resulting in the low obtained correlation .

The low genetic similarity found between two populations from Moju, PA (0.67) may be the result of local human population influence on the genetic composition of these populations, since *P.microphyllus* is a medicinal plant, and rural populations use it empirically. Besides, there may have been sample efforts in different seasons, and this low similarity may be the result of generation overlaps. This may also be a sample effect, since 10 to 13 plants were sampled per populations.

The Shannon diversity indexes ranged from 0.2855 to 0.3147 (Table 2). The Shannon index ranges from 0 to 1, and the closer it is to 0, the lower are the genetic diversities. These values were below the ones obtained for the tree species *Populus tremuloides*, from 0.58 to 0.69 (Yeh *et al.*, 1995), *Fitzroya cupressoides*, with 0.42 to 0.56 (Allnut *et al.*, 1999) and *Swietenia macrophylla* from 0.27 to 0.41 (Gillies *et al.*, 1999). This low diversity may be the result of population reductions suffered by *P. microphyllus* due to intense extrativism (Pinheiro, 2002). Besides, the natural populations of Pará genetic diversity is comparable to Merck´s genetic diversity, a population which had already gone through an artificial selection, expected to have lower diversity. According to Hamrick & Lovelles (1989), highly distributed tree species tend to have higher genetic diversity compared to lower distributed endemic shrubby species. *P. microphyllus* geographical distribution is limited to the East of Pará, West and North of Maranhão and North of Piauí (Skorupa, 2000). For an herbaceous species of reduced geographical distribution, *Campanula microdonta*, the Shannon diversity indexes ranged from 0.076 to 0.122 (Oiki *et al.*, 2001). The Shannon indexes for *Zeunine gracilis*, an orchid species, ranged from 0.018 to 0.224, and the authors attributed this to a reduced population size, a result of human impact on forests (Sun & Wong, 2001). Low diversities were also found for orchid *Goodyera procera*, with 0.16 to 0.37 (Wong & Sun, 1999), and the authors mentioned founder's effect and genetic drift as factors that contributed to low diversity values. Then, low genetic diversity values for *P. microphyllus* may be the result of many factors, such as sample effects, geographical distribution, type of reproduction and historical population reductions.

CONCLUSION

The results obtained for *P. microphyllus* germplasm bank from Embrapa at Belém,PA, show that there is considerable genetic variation sampled, when compared to other native plant species. This guarantees the creation of genetic breeding programs, since there is genetic divergence among accessions.

The high variation among *P. microphyllus* sample areas must be investigated, since it may show clearly the occurrence of low endogamy rates, as a result of population reductions or from its own reproduction system.

The low diversity observed, estimated by Shannon's index, may be explained by many factors, as it has already been mentioned. The number of samples must be emphasized, since the number of accessions by area is low at *P. microphyllus* in the germplasm bank of Embrapa in Belém,PA. So, new

sample efforts must be carried out to obtain a better representation of *P. microphyllus* from the areas of occurrence. Considering that the highest portion of genetic variation was inside sample areas and that genetic similarities of nearby populations were high, sample efforts must be conducted in areas far from one another, collecting a high number of plants or seeds per area.

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