



Genetic diversity of *Pilocarpus microphyllus* accessions using leaf traits

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ABSTRACT - *Jaborandi* (*Pilocarpus microphyllus*) is a medicinal species that produces pilocarpine, which is used to treat glaucoma, to stimulate lacrimal and salivary glands and in hair treatments. The objective was to study the genetic variation in a *jaborandi* genebank of Embrapa Eastern Amazon through quantitative traits and multivariate approaches. Leaves of 152 individuals from 13 natural populations from the states of Maranhão and Pará plus a commercial one were collected in two seasons. Six leaf traits were evaluated. The univariate and multivariate analyses showed the existence of genetic differentiation among sample areas. In the dispersion graph the accessions did not group according to their sample area. The first two principal components explained 79.9% of the total variation and the six original traits were highly correlated with them, so no variable was discarded.

Key words: Multivariate analysis, principal components, medicinal plant, *Pilocarpus*.

INTRODUCTION

Among plants with proven medicinal properties, *jaborandi* (*Pilocarpus microphyllus* Stapf.) is ascribed priority in conservation programs (Vieira 1999). It is a Rutaceae shrubby tree that occurs in penumbra areas and, according to Skorupa (2000), is found in parts of the Amazon region, mainly in the East of the state of Pará, West and North of Maranhão and North of Piauí. Its leaves contain the imidazole alkaloid named pilocarpine, used mainly in the first stages of glaucoma treatment.

Pilocarpine also stimulates lacrimal and salivary glands (Merck 1989). It is believed to prevent hair loss and is therefore used in cosmetics. With increasing demands for *P. microphyllus* leaves, mainly during the 70s and 80s, the species was threatened with over-exploitation which caused a significant reduction in its natural populations (Pinheiro 2002).

The species' domestication process consists of several stages, including efforts of germplasm sampling and genetic breeding. Germplasm characterization is one of the methods that contribute to this process by in-depth

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descriptions of the available variability and species traits. Quantitative traits associated to multivariate techniques are very useful in this kind of analyses since they can estimate genetic distribution within and among populations, presume genetic distances between genotypes and help select germplasm descriptors. The morphological characterization of a germplasm bank is the passable way to verify the genetic diversity and compile information for genetic breeding programs. This has been done extensively for a range of crops (Ayana and Bekele 1999, Harrison et al. 1999, Li et al. 2000, Granati et al. 2003, Mathur et al 2003, Yeater et al. 2004). Furthermore, insights into the magnitude of morphological variation present in the species can provide the basis for effective selection.

Several multivariate techniques may be applied in the analyses of germplasm bank accessions. Such techniques consider all measured traits simultaneously to identify differences between populations. The most commonly used are canonical variables, principal components and agglomerative methods (Cruz and Regazzi 2001). These latter also need genetic similarity or dissimilarity estimates.

In 1992, a *P. microphyllus* genebank was established in Embrapa Eastern Amazon in Belém, Northern Brazil, aiming at the species' conservation and use in genetic breeding programs. However, there have been few efforts to characterize the accessions so far.

The aim of our study was to assess the genetic relationships between accessions and the genetic variability present in the bank using quantitative traits of leaves.

MATERIAL AND METHODS

A total of 152 accessions were sampled from 13 sample areas in Oriental Amazonia plus a population that had already gone through selection cycles. These accessions belong to the germplasm bank of Embrapa Eastern Amazon in Belém, PA, Brazil and had been sampled in two Brazilian States: Pará (PA) and Maranhão (MA). The sample areas with the corresponding number of sampled plants were: Dom Eliseu, PA (5); Serra dos Carajás-1, PA (5); Serra dos Carajás-2, PA (20); Moju-1, PA (18); Moju-2, PA (16); Moju-3, PA (11), Moju-4, PA (12); Breu Branco, PA (20); Açailândia, MA (10); Nina Rodrigues, MA (4); Mata Roma, MA (4); Brejo, MA (5); Santa Quitéria,

MA (2) and Fazenda Merck, MA (20) (map in Figure 1). All sample areas corresponded to natural populations, except in the case of the population from Fazenda Merck, MA.

Eight leaves per plant were collected at different times, during the dry and rainy seasons. Six traits were evaluated: the leaf dry matter (DM), number of leaflets/leaf (NL), leaf length (LL), leaf width (LW), length/width ratio (R) and leaf area (LA). The leaves were collected considering the four cardinal points.

For the analysis of variance, the traits assessed in both seasons were transformed into 12 variables which were analyzed to verify any differentiation between sample areas. The multivariate analysis of variance was used to verify the global genetic variability among populations, using Wilks's criterion. The analyses were done using General linear model (GLM) procedure of the software SAS (SAS Institute Inc. 1989). Differences between population means for each trait were verified by the Tukey test, using software Genes (Cruz 2001).

Principal component analyses were realized with standardized data for all individuals and considering the 12 traits on software Genes. The scores obtained for the first principal components were plotted in a graphic to verify genetic divergence in accessions. Correlations between the first principal components and the original traits were analyzed to verify the need to discard traits.

To verify genetic distances between sample areas we used the average Euclidean distance. The genetic similarities of sample areas were obtained from the genetic similarity means of two groups of individuals, each from different sample areas. The similarity clustering analyses were realized using UPGMA and system NTSYS-pc 2.0 (Rohlf 1992) to generate the dendrogram.

RESULTS AND DISCUSSION

The summarized analyses of variance for all traits are shown in Table 1. Since the cardinal points did not show any difference between trait means, they were not considered (data not shown). Experimental accuracy varied from 31.75% (DM in season 1) to 7.38% (R in season 2). In the experimental design we used, there was neither local control nor randomization what may have contributed to the higher estimates of experimental errors and less accuracy obtained. Nevertheless, significant differentiation among sample areas was observed for all traits (Table 1). The comparison means of each trait are shown in Table 2.

Table 1. Analysis of variance for the six traits sampled at two different seasons to evaluate the existence of genetic differentiation among the collection sites of *P. microphyllus*

Trait	Sample	Mean Squares	Total means	CV (%)
		Between		
DM (g)	1	0.06679**	0.255	31.75
	2	0.0100**	0.0800	29.80
NF	1	8.6667**	8.5800	13.91
	2	10.099**	8.6612	12.32
LL (cm)	1	3.48423**	2.6113	16.35
	2	3.5043**	2.600	16.45
LW (cm)	1	1.1015**	1.4746	17.53
	2	1.23775**	1.4465	17.39
R	1	0.06675**	1.791	8.36
	2	0.08371**	1.8199	7.38
LA (cm ²)	1	763.573**	24.69	28.14
	2	863.256**	24.40	30.80

**Significant at 1% probability level

Table 2. Test of means for six traits obtained for the 14 populations of *P. microphyllus* evaluated in two seasons

Collection site	DM1	DM2	NL1	NL2	LL1	LL2	LW1	LW2	R1	R2	LA1	LA2
Serra dos Carajás1, PA	0.17 b	0.17 b	9.50 abc	8.82 abc	2.10 c	2.23 bc	1.20 a	1.20 b	1.81 ab	1.87 a	18.88 bc	18.02 a
Serra dos Carajás2, PA	0.19 b	0.20 b	8.20 abc	8.11 abc	2.44 bc	2.42 bc	1.29 a	1.23 b	1.88 a	1.98 a	19.38 bc	17.50 a
Moju3, PA	0.32 ab	0.39 a	7.85 bc	7.85 abc	3.10 ab	3.23 ab	1.89 a	1.91 a	1.65 b	1.70 a	33.17 ab	37.01 a
Moju2, PA	0.27 ab	0.27 ab	9.04 abc	9.41 ab	2.56 bc	2.55 bc	1.51 a	1.48 b	1.73 ab	1.74 a	26.45 abc	26.76 a
Breu Branco, PA	0.17 b	0.18 b	9.72 a	9.76 a	1.97 c	2.00 c	1.11 a	1.09 ab	1.80 ab	1.84 a	16.11 c	15.95 a
Moju1, PA	0.18 b	0.18 b	9.16 abc	9.65 ab	1.94 c	1.96 c	1.12 a	1.10 b	1.75 ab	1.79 a	15.42 c	16.15 a
Moju4, PA	0.21 b	0.21 b	9.65 ab	9.78 a	2.19 bc	2.15 bc	1.27 a	1.22 b	1.73 ab	1.77 a	20.16 bc	18.94 a
Merck-MA	0.38 a	0.40 a	7.89 bc	7.70 abc	3.50 a	3.44 a	1.88 a	1.89 a	1.87 ab	1.83 a	39.13 a	36.80 a
Açailândia, MA	0.22 ab	0.21 b	7.94 abc	8.55 abc	2.51 bc	2.32 bc	1.39 a	1.31 b	1.85 ab	1.80 a	20.12 bc	18.84 a
Nina Rodrigues, MA	0.30 ab	0.32 ab	7.97 abc	7.53 abc	2.77 abc	2.68 abc	1.63 a	1.58 ab	1.71 ab	1.70 a	27.50 abc	23.56 a
Mata Roma, MA	0.36 ab	0.44 a	6.66 c	6.78 c	3.77 a	3.70 a	2.08 a	2.05 a	1.84 ab	1.84 a	37.40 ab	38.04 a
Brejo, MA	0.33 ab	0.36 ab	6.85 c	7.22 bc	3.23 ab	3.21 ab	1.73 a	1.70 b	1.83 ab	1.88 a	25.54 abc	30.24 a
Santa Quitéria, MA	0.21 ab	0.16 b	9.37 abc	9.12 abc	2.18 bc	2.07 bc	1.17 a	1.07 b	1.88 ab	2.00 a	17.91 bc	14.93 a
Dom Eliseu, PA	0.26 ab	0.23 ab	9.40 abc	9.70 ab	2.39 bc	2.28 bc	1.36 a	1.25 b	1.82 ab	1.83 a	22.13 bc	19.80 a

Means followed by the same letter within the columns do not differ from one another, according to Tukey's test, considering 1% significance

The genetic correlations (Table 3) between traits measured in each season varied from 0.51 (NF) to 0.80 (LF and CF). Yeater et al. (2004), who had assessed genetic variation in *Vicia villosa* also separated stem and leaf-related trait means according to the seasons winter and fall. They did not find high correlations between traits in each season.

With a λ value of 0.04892, multivariate analysis using Wilks's criterion indicated that there is genetic differentiation among sample areas, which corresponds to an F value of 2.98 ($P < 0.0001$), with 156 and 1150 degrees of freedom, respectively. The differentiation among the populations assessed by quantitative traits tends to be overestimated, since certain traits may be under natural selection and uninfluenced by gene flow rates. Sebben et

al. (2001) verified that certain traits had attained higher genetic divergence values in *Carciniana legalis* populations, concluding that these may have more adaptive importance for the species. The fact that all traits presented significant variation does not mean they are not redundant since one variable can indirectly represent the others.

Table 2 shows Tukey's test for all traits of 14 sample areas. Population from Merck, MA showed high means for DM (0.38 and 0.40 g) and LA (39.13 and 36.80 cm²). This population had probably already undergone selection cycles aimed to obtain higher pilocarpine production. However, natural populations from Mata Roma, MA and population 3 from Moju, PA also presented high means

for these traits. Further genetic studies should be done with these accessions for their use in *P. microphyllus* genetic breeding programs.

Genetic correlations between traits can be observed in Table 3. DM showed high correlations with LL, LW and LA. NL did not show consistent correlations with any of the traits, except for LL, which was significant and negative in all comparisons. NL showed a negative and low correlation

with LA, except between LA2 and NL1 (0.67). It also showed a negative and low correlation with DM. These results are not conclusive and more precise analyses are needed, considering replications and heritability measures to obtain more in-depth conclusions. But the results give us a clue that NL and DM are not highly correlated, so these traits can be selected together, and plants with high leaf dry matter and high number of leaflets per leaf can be obtained.

Table 3. Genetic correlations between leaf traits for *P. microphyllus* accessions obtained in two seasons

	DM1	NL1	LL1	LW1	R1	LA1	DM2	NL1	LL2	LW2	R2
NL1	-0.19*										
LL1	0.60**	-0.43**									
LW1	0.62**	-0.48**	0.89**								
R1	-0.08	0.18*	0.07	0.34**							
LA1	0.65**	-0.05	0.81**	0.85**	-0.15						
DM2	0.75**	-0.19*	0.63**	0.64**	-0.10	0.68**					
NL2	0.012	0.51**	-0.35**	-0.26**	-0.12	-0.09	-0.09				
LL2	0.65**	-0.36**	0.80**	0.71**	0.08	0.71**	0.71**	-0.36**			
LW2	0.62**	0.09	0.75**	0.80**	-0.19*	0.75**	0.70**	-0.36**	0.90**		
R2	-0.02	-0.18	-0.02	-0.30**	0.64**	-0.18	-0.06	0.09	0.09	-0.32**	
LA2	0.70**	0.67**	0.70**	0.73**	-0.16	0.79**	0.72**	0.01	0.79**	0.80**	-0.12

*, ** significant at 5% and 1% by t test, respectively

The eigenvalues, based on the 12 evaluated traits, as well as variances represented in individuals and accumulated percentages are shown in Table 4. The first two principal components explained 79.9% of total variance. This performance can be considered very good, considering that Ayana and Bekele (1999) obtained 79% of the total variance with the first five principal components using 15 quantitative traits of sorghum. According to Cruz and Regazzi (2001), if 80% of total variation is obtained with the two or three first principal components, it is possible to analyze genotype grouping using dispersion graphs. Using the first two principal components, the scores for the 152 accessions were obtained. The dispersion graph based on these scores is represented in Figure 2. The scores were plotted in a two-dimensional space, where the distance between plots is proportional to the genetic distances between sample areas. The dispersion graph does not show a clear separation of *P. microphyllus* accessions into groups according to their sample area. The accessions from Breu Branco, PA formed the clearest group. Accessions from populations of Maranhão State showed the highest dispersion on the graph. The non-formation of clear groups according to the individuals' origin can indicate the existence of a high portion of genetic diversity within groups. Since *P. microphyllus* was considered an allogamous species by Marques and Costa (1994), the occurrence of high genetic

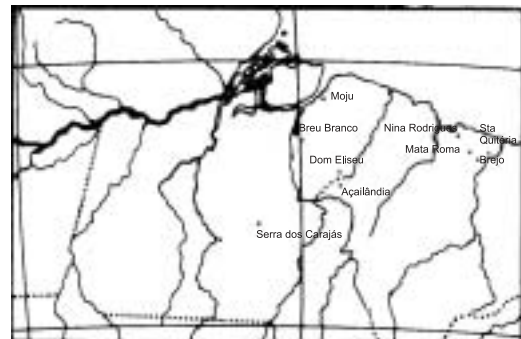


Figure 1. Map of the sample areas in two Brazilian states, Pará (right) and Maranhão (left)

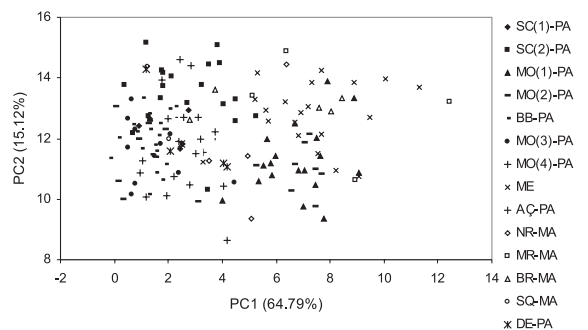


Figure 2. Bidimensional dispersion graph obtained with the first two principal components scores for the 152 *P. microphyllus* accessions from 14 sample areas. DE = Dom Eliseu, PA; SC = Serra dos Carajás, PA; MO = Moju-PA; BB = Novo Breu Branco, PA; ME = fazenda Merck, MA; AÇ = Açaílandia, MA; NR = Nina Rodrigues, MA; MR = Mata Roma, MA; BR = Brejo, MA; SQ = Santa Quitéria, MA

variance within groups is expected. Also, there was no formation of accession groups of agreeable to the states Pará and Maranhão. This would have been expected, since there are no physical barriers between the two states and gene flow among populations of both states probably occurs freely.

The dendrogram (Figure 3) based on Euclidean distances between sample areas showed the formation of two groups that did not discriminate the two states (Pará and Maranhão). It was already expected, as explained above. Besides, the dendrogram does not show clusters with different populations from Moju, PA and Serra dos Carajás, PA. Distances between populations from Moju, PA ranged from 1.6928 (Moju 1 and 3) to 0.8304 (Moju 3 and 4), averaging 1.2957, slightly under the mean genetic distance of all populations (1.3282). It is a curious result that populations that are so near geographically were genetically so distant from each other. The populations from Serra do Carajás did not group together on the dendrogram, and the distance between populations 1 and 2 was 0.9211. Such high distances between Moju, PA and Serra dos Carajás, PA populations can be attributed to human action at these locations, since *P. microphyllus* is a medicinal species and there may have been a mixture with plants from other regions. It could also be a sample size effect.

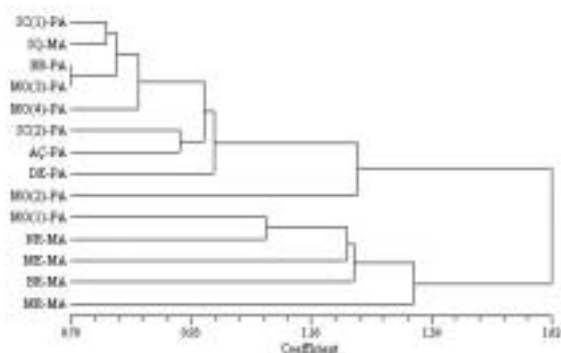


Figure 3. Dendrogram based on average Euclidean genetic distances between *P. microphyllus* sample areas. (See code in Figure 2)

According to Pearson's coefficient the correlation between genetic and geographic distances was not high, $r=0.2967$. Considering that phenotypic variation may be a result of natural selection and is sometimes uninfluenced by gene flow rates, a correlation with the climate conditions of each region rather than the geographical distance is expected. Steiner and De los Santos (2001) verified correlations between some quantitative traits of *Lotus corniculatus* and ecological characteristics of the collection sites. Boulli et al. (2001) observed correlations

between *Pinus hapelensis* traits and factors such as latitude, longitude and altitude. As it was not possible to correlate the accessions' differences with ecological conditions, it can be only assumed that ecological conditions may have had an influence on the selection of certain analyzed traits.

Correlations between traits and first principal components showed that DM, NL, LL, LW and LA are highly correlated to the first principal component and R had a high correlation to the second (Table 5). This may be explained by the fact that length/width ratio is related to leaf shape, which is a qualitative trait. Since all traits were highly correlated with the first two canonical variables, it can be concluded that the six traits are important to study genetic divergence between *P. microphyllus* accessions.

Table 4. Variance, percentage variance and accumulated variance of principal components, obtained from 12 measured traits for *P. microphyllus* accessions

Canonical variable	Eigenvalue	Variance (%)	Accumulated variance (%)
1	7.7752	64.7934	64.7934
2	1.8139	15.1166	79.9099
3	1.1157	9.2976	89.2075
4	0.4401	3.6680	92.8755
5	0.2454	2.0449	94.9204
6	0.2259	1.8823	96.8027
7	0.1731	1.4425	98.2453
8	0.1006	0.9054	99.1506
9	0.0554	0.4617	99.6123
10	0.3017	0.2514	99.8637
11	0.0126	0.1047	99.9684
12	0.0038	0.0315	100.00

Table 5. Correlation coefficients between the original traits and the first two principal components

Original trait	Season	Vector associated to first two eigenvalues	
		1	2
LM	1	0.8682	-0.152
	2	0.9188	-0.0449
NL	1	-0.6021	-0.2166
	2	-0.5956	-0.3826
LL	1	0.9449	-0.1106
	2	0.9558	0.1868
LW	1	0.9652	-0.1106
	2	0.9701	-0.0801
R	1	-0.1232	0.8874
	2	-0.2411	0.8486
LA	1	0.9232	-0.0687
	2	0.9249	-0.1161

CONCLUSIONS

Significant diversity was observed between *P. microphyllus* accessions. The germplasm bank from Embrapa Eastern Amazon contains jaborandi accessions with considerable genetic variation, considering important traits for a medicinal plant, such as leaf dry matter and leaf area. Other genetic parameters such as trait heritability, genetic correlations and genetic variance components need to be measured to provide improved information for *P.*

microphyllus genetic breeding.

So far it can be concluded that, in spite of records of *P. microphyllus* exploitation and natural population reduction, genetic variation in the species is still sufficient to ensure genetic breeding.

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Diversidade genética de acessos de *Pilocarpus microphyllus* usando caracteres de folha

RESUMO - *O jaborandi (Pilocarpus microphyllus) é uma planta medicinal que produz a pilocarpina, usada no tratamento de glaucoma, na indução de glândulas salivares e lacrimais e no tratamento capilar. O objetivo foi estudar a variabilidade genética existente em um banco de germoplasma na Embrapa Amazônia Oriental usando dados quantitativos e análises multivariadas. Folhas pertencentes a 152 indivíduos, de treze populações naturais dos estados do Pará e Maranhão e de uma população comercial, foram coletadas em duas épocas. Seis caracteres de folhas foram avaliados. As análises revelaram a existência de diferenciação genética entre as áreas de coleta. De acordo com o gráfico de dispersão, os acessos não se agruparam por seu local de coleta. Os dois primeiros componentes principais explicaram 79.9% da variação total e as seis variáveis originais tinham alta correlação com estes e, assim, nenhuma variável foi descartada.*

Palavras-chave: multivariada, componentes principais, planta medicinal, *Pilocarpus*.

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