Genetic diversity in sacaca (*Croton cajucara* Benth.) accessed by RAPD markers

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ABSTRACT: Genetic diversity in sacaca (*Croton cajucara* Benth.) accessed by RAPD markers. In 1971, sacaca (*Croton cajucara* Benth.) was identified as a source of linalool which can be extracted from leaves and tiny branches, without the need to harvest the whole plant. Linalool is priced at US$ 13 - 15.00, per flask with 100 ml of a 97% solution. In purer forms, such as DL-linalool, tested by gas chromatography, may cost up to US$ 326.00 per gram. In view of its economic potential, a *Croton* Germplasm Bank that holds 16 sacaca accessions, collected since 1994 up to 1999, on the States of Acre, Amazonas and Pará is maintained in Embrapa Western Amazon, Manaus/AM. The accessions are morphologically classified as red and white, according to the predominant color perceived in the young leaves and branches. The objective of this work was to estimate the genetic divergence(s) between the red and the white morphotypes, using RAPD patterns produced with five 10mer primers. The software GENES (Federal University of Viçosa) was used to estimate the diversity coefficients and to group the accessions hierarchically. The values found for the diversity index were higher when the red sacaca accessions were confronted to the white sacaca accessions (inter-morphotypic diversity) than when accession of red or accessions of white sacaca were confronted to each other (intra-morphotypic diversity). The hierarchical organization of the red sacaca accessions corresponded better to the geographic localization of the collect points than did the organization of white sacaca accessions. We suggest that red and white sacaca are different genotypes as well as morphotypes.

Key words: sacaca, *Croton*, germplasm, RAPD, genetic diversity

INTRODUCTION

Linalool is a terpenic alcohol continuously demanded by perfumery industries. Along the past decades, rosewood (*Aniba rosaeodora* Ducke and *A. duckey* Kosterm.) trees were explored as the principal source of this substance and in reason of the non-planed exploitation, these species became menaced of extinction. Seeking to reduce this menace, about thirty years ago, a group of plants found in the Amazon region was screened as putative linalool providers (Araújo *et al.*, 1971; Kalil-Filho *et al.* 1998), and sacaca (*Croton cajucara* Benth.) was considered an interesting alternative to rosewood because it is easily cultivated and presents relatively high percentages of linalool in the essential oil taken from the leaves (Lemos *et al.*, 1999; Lopes *et al.*, 2000). Sacaca plants are known, in addition, by the healing properties which has been, in part, scientifically demonstrated, more specifically, the reduction of glucose and lipids levels in blood of rats and mice (Farias *et al.*, 1996 e 1997; Costa *et al.*, 1999; Grynberg *et al.*, 1999; Hiruma-Lima *et al.*, 1999; Maciel *et al.*, 2000; Silva *et al.*, 2001 a e b).

A collection with sixteen accessions of sacaca is maintained at Embrapa Western Amazon (Embrapa Amazônia Ocidental, Manaus/AM). The results of the analysis conducted in 1995 pointed to values above 30% for the linalool contents in the essential oil extracted from the leaves of cultivated sacaca plants (Sá Sobrinho *et al.*, 1998). This sort of experiment is part of the compromises of Embrapa with conservation and sustainable exploitation of biodiversity, what shall be performed through the development of forestry and agroforestry management systems adequate to the Amazon Brazilian region, including identification and domestication of species that can turn to be or to produce new commercially valuable items (pharmaceuticals, colorants, insecticides, aromatics, etc) (Kitamura *et al.*, 2002). In addition, it would be a goal for plant germplasm collections, in general, to have their importance evaluated and the access to the already maintained plants facilitated (Berretta, 2001). That is what, therefore, is being done for the collection of sacaca plants. The preliminary studies about genetic diversity for the collection of sacaca plants maintained at Embrapa Western Amazon are described bellow.

Recebido para publicação em agosto/2004
Aceito para publicação em julho/2006

FIGURE 1. Collect points of sacaca (*C. cajucara* Benth.) plants introduced as accessions at the *Croton* Germplasm Bank in Embrapa Western Amazon (Manaus/AM). **1** - Rio Branco/AC (9º 58' 29'' S - 67º 48' 36'' W); **2** - Iranduba/AM (3º 17' 05'' S - 60º 11' 10'' W); **3** - Manaus/AM (3º 06' 07'' S - 60º 01' 30'' W); **4** - Presidente Figueiredo/AM (2º 02' 04'' S - 60º 01' 30'' W); **5 -** Rio Preto da Eva/AM (2º 41' 58'' S - 59º 41' 59'' W); **6** - Belterra/PA. (2º 38' 11'' S - 54º 56' 14'' W); **7** - Santarém/PA (2º 26' 35'' S - 54º 42' 30'' W); **8** - Belém/PA (1º 27' 21'' S - 48º 30' 16'' W).

MATERIAL AND METHOD

Collection and maintenance: sacaca (*C. cajucara* Benth.) plants included in the *Croton* Germplasm Bank at Embrapa Western Amazon (Manaus/AM) were collected, from 1994 to 1998, on the States of Acre, Amazonas and Pará (Figure 1). Plants are registered as white or red following specially the color of young leaves and petioles found in newly formed branches, disposed in three blocks related to the date of introduction as accessions (1997, May and 1998, February and July), spaced by 1.5 m and manually provided of manure, watering and pruning.

DNA extraction, purification and quantification: leaves were taken from plants that represented the 16 sacaca accessions accessions and carried immediately to the Plant Biotechnology Laboratory for nucleic acid extraction, using the method developed by Edward (1991). Columns with silica membranes were used for DNA purification. Quantification was performed by spectrophotometry.

PCR (polymerase chain reaction) conditions and RAPD (random amplified polymorphic DNA) band patterns documentation and analysis: PCR reactions were made as follows: dNTPs 100 mM each; decamer primers 400 nM; $MgCl_2$ 2 mM; BSA 0.1%; KCl 50 mM; Tris-HCl 20 mM; Taq DNA polymerase 1.5 U and DNA 30 ng. Five different 10mer primers with GC content around 60% (5´-ACCCGACCTG-3´; 5´-AATCGGGCTG-3´; 5´-AGCGCCATTG-3´; 5´-GTTTCGCTCC-3´ and 5´-GTAGACCCGT-3´) were used for DNA amplification in a Perkin Elmer 2400 thermocycler adjusted to perform 94 ºC for 5 min; 25 x (94 ºC for 30 sec; 33 ºC for 45 sec; 72 ºC for 45 sec); 72 ºC for 7 min and e 4 ºC forever. PCR products were resolved in 1.5% agarose gels with ethidium bromide (0.5 mg/ml). The gels were exposed to UV light and the RAPD patterns were registered using a Kodak digital camera (DC120), as black and white images, saved as Kodak Application files (.pib extension). Kodak Gel Documentation software was settled to acquire "faint bands" stained with ethidium bromide (camera diaphragm opened 2.5 grades for 1 second). The identification of the RAPD bands was performed using the Kodak 1D Image Analysis software "find lanes" and "find bands" tools, without modification of the default options. "Profile width" ranged from 65 to 67% as informed by the software in response to the specific characteristics of the different registered agarose gels. Data acquired and registered as described above were transformed in binaries and processed by the GENES software – version 2001.0.0 for calculation of Sorenso/Nei & Li diversity coefficient for pairs of plants (Cruz, 2001). The values found for the diversity coefficient among sacaca plants were distributed in classes of frequency and the differences detected for the distribution of the coefficients calculated by confrontation of pairs of red sacaca, pairs of white sacaca and pairs of red x white sacaca plants were tested by chi-square for statistic significance. In addition the diversity coefficient matrix was submitted to a bootstrapping procedure using FITCH (Fitch-Margoliash method for distance calculation, Felsenstein, 2004), with 500 cycles of global rearrangements, being the bootstraped data submitted to CONSENSE (Felsenstein, 2004) and clustered as a network (an unrooted tree) using DRAWTREE (Felsenstein, 2004).

RESULT AND DISCUSSION

The sixteen accessions of sacaca branca (white sacaca) and sacaca vermelha (red sacaca) from the *Croton* Germplasm Bank organized at Embrapa Western Amazon were collected in the territorial limits defined for eight cities in the Amazon region (Figure 1), being most of the collects made in Manaus, at the Federal University of Amazonas, at particular properties and in the margins of the Manaus-Itacoatiara (AM 010) highway. Data referring to the plants sampled for genetic diversity analysis including

TABLE 1. Accessions identification, morphotypes, codes for the collect points and reference numbers to the dendrogram for sacaca (*C. cajucara* Benth.) plants introduced in the *Croton* Germplasm Bank at Embrapa Western Amazon (Manaus/AM).

ACCESSION CODE	MORPHOTYPE	COLLECT POINTS *	DENDROGRAM REF. NUMBER **
A3 – 3rd. introduction	red	6 - Belterra/PA	1
A5 – 3rd. introduction	red	7 - Santarém/PA	2
A6 – 3rd. introduction	red	7 - Santarém/PA	3
A7 – 3rd. introduction	red	1 - Rio Branco/AC	4
A8 – 3rd. introduction	red	1 - Rio Branco/AC	5
BE/PA – 1 st. introduction	red	8 - Belém/PA	6
A1 – 2nd. introduction	white	4 - Pres. Figueiredo/AM	7
A2 – 2nd. introduction	white	2 - Iranduba/AM	8
A3 – 2nd. introduction	white	5 - Rio Preto da Eva/AM	9
A4 – 2nd. introduction	white	3 - Manaus/AM	10
A5 – 2nd. introduction	white	3 - Manaus/AM	11
A6 – 2nd. introduction	white	3 - Manaus/AM	12
A7 – 2nd. introduction	white	3 - Manaus/AM	13
A8 – 2nd. introduction	white	3 - Manaus/AM	14
A9 – 2nd. introduction	white	3 - Manaus/AM	15
BE/PA – 1 st. introduction	white	8 - Belém/PA	16

* numbers refer to collect points, according to Figure 1
** references to the dendrogram, Figure 3

A total of 79 RAPD bands were produced and 65 of them were found to be polymorphic. At least four bands were detected exclusively in the red sacaca plants tested and other two were encountered just in white sacaca accessions. This specificity was statistically significant ($P < 0.05$).

The diversity coefficient values found among white sacaca plants ranged from 0.06931 (accession 8 *vs*. 11) to 0.19588, for accession 13 *vs.* 16. Among red sacaca plants, the lowest value found was 0.14000, for accession 5 *vs.* 6 and the highest was 0.25490, for accession number 1 *vs*. number 4. The lowest value calculated for the diversity coefficient by confrontation of white and red sacaca plants was 0.12871 (for accession 6 *vs.* 15) and the highest value found was 0.30000, for 1 *vs.* 7.

The values found for the diversity coefficient were distributed in nine classes of amplitude 0.02590 and this frequency distribution (upper and lower limits cited above) demonstrated that the analyzed red sacaca plants were more diverse from each other than were found to be white sacaca plants (Figure 2).Values estimated for the diversity coefficient among plants of the red morphotype were grouped mostly in the classes of intermediary values of diversity, while the values found by confrontation of white sacaca accessions were allocated mainly in the classes of low values of diversity. Once plants of the red and the white

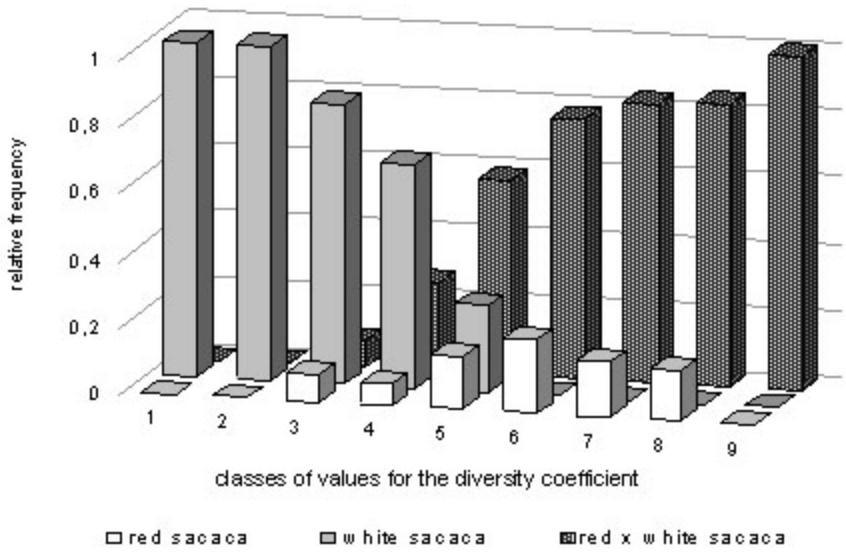

FIGURE 2. Frequency distribution for the values of the diversity coefficient calculated for pairs of red, white and red x white sacaca (*C. cajucara* Benth.) plants introduced as accessions in the *Croton* Germplasm Bank at Embrapa Western Amazon (Manaus/AM).

morphotypes were compared to each other (inter-morphotypic diversity) most of the diversity coefficients took places in classes of high values (Figure 2). The chi-square for the whole distribution was 78.08 ($P < 0.001$) and there was statistic significance ($P = 0.05$) for individual classes, except by classes 1, 5 and 9.

The results described above are represented with enough graphical fidelity by the network pictured in Figure 3. It resulted from a clustering process that presented 9.685 as the average percent standard deviation for the distances between accessions. Red sacaca plants, among which were found mostly intermediary values for the diversity coefficient (Figure 2), grouped as the more dispersed cluster found in one of the network extremes (Figure 3, accessions 1 to 6). Despite dispersion, plants of red sacaca collected in Santarém/PA (accessions 2 and 3) clustered together.

White sacaca accessions grouped more tightly in a separate cluster found in the opposite extreme of the network (Figure 3, accessions 7 to 16). This result is in agreement with the diversity coefficient frequency distribution as well, once the values for white sacaca occupied the lower classes in the distribution (Figure 2), meaning that these plants were less divergent.

Since this species, and principally the white morphotype, is frequently cultivated in backyards as a healing plant, the elected collect points would not necessarily be representative of the genetic structure of natural populations. The anthropological influence could, therefore, explain at least in part the absence of a higher correlation between the collect points and the subgroups of plants found inside the two principal clusters dominated, each of them, by one of the two morphotypes (Figure 3).

When networks were generated for red and white plants separately (results not shown), that correlation increased specially for the red morphotype, with plants from Rio Branco (accessions 4 and 5) grouping together and those from Santarém (accessions 2 and 3) keeping grouped.

Thus, we suggest that red and white sacaca could be considered different genotypes as well as morphotypes. The divergence is reinforced by the fact that the samples of essential oil taken from leaves of red sacaca plants and analyzed by gas chromatography presented hydroxy-calamenene as the major component while linalool was the principal constituent of the oil taken from leaves of white sacaca plants (Chaves *et al.*, 2003). This difference was maintained even when both morphotypes were cultivated in Manaus, at Embrapa Western Amazon, as it was the color of the young leaves and branches that is used to designate the plants as red or white.

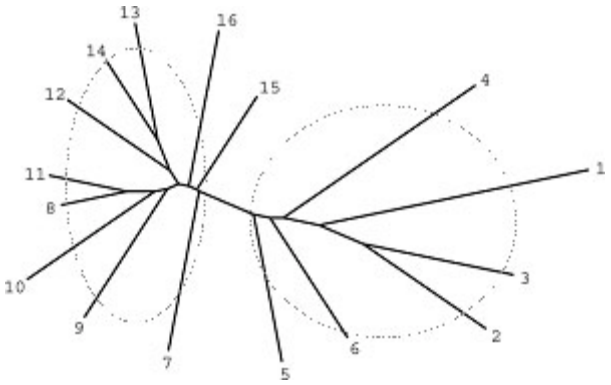

FIGURE 3. Network obtained after the bootstrapping process using the software FITCH. Numbers 1 to 16 refer to the sacaca (*C. cajucara* Benth.) plants introduced at the *Croton* Germplasm Bank in Embrapa Western Amazon (Manaus/AM) listed in Table 1.

CONCLUSION

- the values for the diversity coefficient were found to be higher when accessions of red sacaca were confronted to accessions of white sacaca (inter-morphotypic diversity) than when accessions of red or accessions of white sacaca were confronted to each other (intra-morphotypic diversity);

- two quite distinct clusters were produced with bootstrapped data revealing that red and white sacaca plants could be distinguished by RAPD markers.

ACKNOWLEDGMENT

We are thankful to Nelcimar Reis Sousa and to Katia Emidio da Silva, Researchers at Embrapa Western Amazon; to RENARGEN (Genetic Resources National Program); Embrapa Genetic Resources & Biotechnology (Embrapa Recursos Genéticos e Biotecnologia) and MCT (Ministry of Science & Technology) for the financial support and to IBGE for the georeferences.

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