Genetic fingerprinting of the Brazilian medicinal plant *Chrysobalanus icaco* L. (Chrysobalanaceae)

Impressão digital genética da espécie medicinal brasileira *Chrysobalanus icaco* L. (Chrysobalanaceae)

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ABSTRACT

*Chrysobalanus icaco* L. (Chrysobalanaceae) is a medicinal species widely used in Brazil mainly to treat diabetes. Despite the medicinal importance of *C. icaco*, genetic information of this genus remains limited. Thus, our aim was to evaluate the influence of the genetic basis of *C. icaco* by determining its chemotypes. 25 *C. icaco* genotypes were collected from 15 sites in Belém, Marajó and Northeastern mesoregions of Pará state, Brazil. The genotypes were selected by
evaluating the plant morphological characteristics such as fruit color and plant habit. The DNA fingerprinting profile was performed using PCR based RAPD technique and appropriate statistical methods were used. RAPD markers were used for evaluation of genetic diversity and molecular characterization of the *C. icaco*, using a total of 18 decamer primers. These primers produced 85 amplification products, with an average of 4.7 bands per primer and 99.2% polymorphism. The genotypes are genetically distinct, forming variable clusters in number and constitution by different methods. By the morphological characteristics considered, there is a tendency of clustering based on the color of the ripe fruit. We found the secondary metabolite content depends not on environmental condition, but rather on *C. icaco* genome. Therefore, it may have implications for ethnopharmacological use of the chemotypes.

**Keywords:** Traditional Brazilian medicine, Coco-plum, Chemotype, RAPD, Genetic diversity.

1 INTRODUCTION

*Chrysobalanus icaco* L. (Chrysobalanaceae) is a medicinal species mainly used for treating diabetes (AGRA et al., 2007; COELHO-FERREIRA, 2009; SILVA & PEIXOTO, 2009) that occurs naturally in municipalities of Pará state, Brazil. The individuals show great variation in morphological characters, especially for the size and color of ripe fruit (pale-yellow, red and dark-purple), size and shape of leaf, and growth habit (shrub and tree). It is popularly known as “ajuru-branco”, “ajuru-vermelho” or “ajuru-preto”, according to morphotype associated with the ripe fruit color: white, red and black, respectively. Although there is a
considerable amount of research evaluating biological activities of *C. icaco*, including hypoglycemic effect (BARBOSA et al., 2013; FERREIRA-MACHADO et al., 2014; WHITE et al., 2016), studies that elucidate this morphological variability remain scarce.

A multivariate analysis of chemical data by our group (PARACAMPO et al., 2017) showed that the *C. icaco* red morphotype differs from the white and black demonstrating that there are two chemotypes of *C. icaco* that are not phytotoxic equivalent to each other. However, we know the chemical constituents are susceptible to environmental factors (GOBBO-NETO & LOPES, 2007). Thus, it was necessary to evaluate the genetic structure of *C. icaco* because DNA markers are reliable for information on genetic polymorphism, regardless of the age, physiological condition or part of the plant used (SHARMA & HEMALATHA, 2017).

Studies on genetic variability and divergence are important to establish the genetic diversity within a species and among populations of plant genetic resources, to guide management actions in areas of natural occurrence, in rational crops, as well as possible parents for breeding programs (CRUZ et al., 2011).

Nowadays, DNA fingerprinting has been widely used for documenting genetic diversity of medicinal plants (GANIE et al., 2015; SHARMA & HEMALATHA, 2017). Techniques based on the polymerase chain reaction (PCR) have been described for the authentication of medicinal plants. Among different DNA markers available, Random Amplified Polymorphic DNA (RAPD) is the most popular because of its rapidity, simplicity and low cost (MILACH, 1998; BORÉM & CAIXETA, 2016). In addition, no knowledge of the DNA sequence is required (SINGH et al., 2014). There are many studies applying RAPD markers to access genomes of medicinal species (AGUIAR et al., 2015; ZONGRAM et al., 2017).

For this work, we evaluated the DNA profiles of the 25 *C. icaco* samples collected from 15 different sites in Pará state, Brazil. RAPD and appropriate statistical methods were used. To the best of our knowledge, there are no previous studies assessing the genetic diversity of *C. icaco*.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

Samples were collected from 25 *C. icaco* genotypes from 15 sites in the Belém, Marajó and Northeastern mesoregions of Pará state, Brazil (Figure 1, Geographic localities of *C. icaco* specimens used in this study). Fresh young leaves of wild *C. icaco* were collected between September and October of 2014. The genotypes were selected by evaluating the plant morphological characteristics such as fruit color and plant habit. All plant material was
authenticated by experts and voucher specimens were deposited at IAN Herbarium of Embrapa Eastern Amazon in Belém, Pará, Brazil. Information on the collection of *C. icaco* samples is shown in Table 1.

### 2.2 DNA EXTRACTION

Genomic DNA was individually extracted from the fresh young leaves of the 25 *C. icaco* samples using the cetyltrimethylammonium bromide (CTAB) method (DOYLE & DOYLE, 1990; COSTA & OLIVEIRA, 2002). 1.0 g of fresh leaf material was ground to a fine powder in liquid nitrogen with polyvinylpyrrolidone (PVPP). The DNA obtained was run 0.8% agarose gel, stained with ethidium bromide and photographed under UV light in the Loccus L-Pix photodocumentation device (Loccus Biotecnologia, SP, Brazil), using the software LabImage 1D L-340. Genomic DNA were diluted with TE (Tris-EDTA) buffer to make the final concentration of 10 ng·μL⁻¹ and stored at −20 °C for use in amplification reaction.

Figure 1. Geographic localities of the 25 wild specimens of *Chrysobalanus icaco* L. used in the study for assessing their genetic diversity using molecular markers. Map illustrating the 24 georeferenced points in 15 sites in the northeast of Pará, Brazil. Geographical coordinates of collection sites are shown in Table 1.
Table 1. Information on 25 *Chrysobalanus icaco* L. genotypes collected from 15 different sites in the northeast of Pará, Brazil (see Figure 1 for mapping of the collection points).

| Genotype | Voucher number | Collection site | Geographical location | Fruit color | Common name | Plant habit |
|----------|----------------|-----------------|-----------------------|-------------|-------------|-------------|
|          |                | Latidude | Longitude |                |             |             |             |
| 1        | 192359         | Belém     | 1° 25' 34,711" S | 48° 25' 13,171" W | Red         | Red ajuru  | Medium-sized shrub |
| 2        | 192357         | Belém     | 1° 15' 8,523" S  | 48° 27' 43,527" W | Dark-purple | Black ajuru | Tree         |
| 3        | 184904         | Belém     | 1° 26' 17,851" S | 48° 26' 36,758" W | Red         | Red ajuru  | Medium-sized shrub |
| 4        | 191984         | Salinópolis | 0° 35' 44,600" S | 47° 18' 36,761" W | Red         | Red ajuru  | Low-sized shrub   |
| 5        | 191982         | Salinópolis | 0° 36' 36,939" S | 47° 22' 8,952" W  | Red         | Red ajuru  | Medium-sized shrub |
| 6        | 191980         | Bragança  | 0° 55' 9,263" S  | 46° 41' 36,137" W | Red         | Red ajuru  | Medium-sized shrub |
| 7        | 191979         | Bragança  | 0° 50' 0,362" S  | 46° 36' 7,990" W  | Red         | Red ajuru  | Low-sized shrub   |
| 8        | 192465         | Soure     | 0° 39' 41,361" S | 48° 28' 55,902" W | Dark-purple | Black ajuru | Medium-sized shrub |
| 9        | 192464         | Soure     | 0° 39' 36,602"S  | 48° 28' 57,895" W | Pale-yellow | White ajuru | Low-sized shrub   |
| 10       | 192706         | Acará     | 1° 57' 10,188" S | 48° 12' 10,195" W | Dark-purple | Black ajuru | Tree          |
| 11       | 192705         | Acará     | 1° 57' 10,188" S | 48° 12' 10,195" W | Dark-purple | Black ajuru | Tree          |
| 12       | 192358         | Acará     | 1° 57' 8,125" S  | 48° 12' 9,752" W  | Dark-purple | Black ajuru | Tree          |
| 13       | 191978         | Barcarena | 1° 31' 5,360" S  | 48° 43' 27,981" W | Dark-purple | Black ajuru | Medium-sized shrub |
| 14       | 191976         | Barcarena | 1° 29' 33,354" S | 48° 42' 26,005" W | Pale-yellow | White ajuru | Medium-sized shrub |
| 15       | 192463         | Salvaterra | 0° 46' 5,001" S  | 48° 30' 41,708" W | Red         | Red ajuru  | Low-sized shrub |
| 16       | 192462         | Salvaterra | 0° 46' 7,600" S  | 48° 30' 43,622" W | Dark-purple | Black ajuru | Tree          |
| 17       | 191986         | Marapanim | 0° 36' 18,419" S | 47° 40' 28,736" W | Dark-purple | Black ajuru | Low-sized shrub |
| 18       | 191985         | Marapanim | 0° 35' 14,883" S | 47° 39' 25,993" W | Red         | Red ajuru  | Medium-sized shrub |
| 19       | 191987         | Terra Alta | 1° 2' 27,168" S | 47° 54' 39,718" W | Red         | Red ajuru  | Tree          |
| 20       | 191981         | Castanhal  | 1° 17' 28,595" S | 47° 53' 39,480" W | Red         | Red ajuru  | Tree          |
| 21       | 192019         | Vigia      | 0° 59' 41,782" S | 48° 11' 26,450" W | Red         | Red ajuru  | Tree          |
| 22       | 191983         | Nova Timboteua | 1° 1' 25,749" S | 47° 21' 9,598" W  | Dark-purple | Black ajuru | High-sized shrub |
| 23       | 192020         | Santo Antônio do Tauá | 1° 4' 34,782" S | 48° 8' 6,597" W  | Red         | Red ajuru  | Medium-sized shrub |
| 24       | 191977         | Abaetetuba | 1° 36' 59,651" S | 48° 48' 37,583" W | Dark-purple | Black ajuru | Medium-sized shrub |
2.3 RAPD-PCR ANALYSIS

RAPD analysis was initially screened using 100 commercial primers from Operon Technologies (Alameda, California, USA). The PCR reaction was carried out in 15 µL reaction containing: 3.5 µL DNA 10 ng. µL⁻¹, 3.47 µL ultrapure water, 0.6 µL MgCl₂ 50 mM, 1.04 µL dNTPs (dATP, dCTP, dGTP, dTTP) 1 mM, 3.5 µL primer 0.1 nmol. µL⁻¹, 0.2 µL Taq DNA polymerase 50 un. µL⁻¹, 1.04 µL BSA 10 µg. µL⁻¹. PCR amplification was performed with a thermocycler AmpliTherm TX96 (Axygen, NY, USA) under thermal conditions (OLIVEIRA et al., 2007). The amplified fragments were separated on 1 % agarose gel electrophoresis along with 100 bp DNA ladder (Invitrogen, Brazil) as DNA markers. Gels were stained with ethidium bromide, visualized and photographed under UV light (Locus L-Pix). After primary screening on five randomly selected C. icaco samples, only those primers giving polymorphic bands were selected for further use.

2.4 DATA ANALYSIS

The number of RAPD bands/DNA fragments were represented as present (1) or absent (0) in the genotypes for cluster analysis. The data collected was used to estimate the similarity on the basis of the number of shared amplification products. The Jaccard similarity coefficient (CRUZ & CARNEIRO, 2003) was calculated for all 25 C. icaco genotypes and estimates of genetic divergence among them were analyzed by NTSYS-pc version 2.1 (ROHLF, 2000), Genes (CRUZ, 1997) and GenAlEx version 6.503 (PEAKALL & SMOUSE, 2012) software. The similarity coefficients were utilized to generate dendrograms by using UPGMA (Unweighted Pair Group Method of Arithmetic means) and Tocher method. Analysis of Variance (ANOVA) was performed to compare the means (p < 0.00001). The reliability of clusters formed by dendrogram was evaluated by cophenetic correlation (Mantel test).
3 RESULTS AND DISCUSSION

3.1 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) FINGERPRINTING PROFILE

A total of 18 decamer primers were used in RAPD analysis; 17 of them provided at least two polymorphic bands, the remaining primer was monomorphic (Table 2). These primers produced 85 amplification products, with an average of 4.7 bands per primer and 99.2% polymorphism (Table 2). The polymorphic bands ranged from two in the primers OPAB-03, OPAR-11, OPB-17 and OPU-08 to ten bands in primer OPA-10. Primer OPA-10 was considered very informative due to its high efficiency in detecting polymorphisms among the C. icaco genotypes (Figure 2).

Table 2. List of the 18 randomly amplified polymorphic DNA primers used in this study and polymorphism of the PCR products.

| Primer  | Sequence          | Number of bands | Polymorphism (%) |
|---------|-------------------|-----------------|------------------|
|         | (5’ → 3’)         | Monomorphic     | Polymorphic      | Total |
| OPA-03  | 5’ AGTCAGCCAC 3’  | 0               | 4                | 100   |
| OPA-10  | 5’ GTGATCGCAG 3’  | 0               | 10               | 100   |
| OPA-11  | 5’ CAATCGCCGT 3’  | 0               | 6                | 100   |
| OPAB-03 | 5’ TGGCGCACAC 3’  | 0               | 2                | 100   |
| OPAR-11 | 5’ GGGAAAGACGG 3’ | 0               | 2                | 100   |
| OPAZ-03 | 5’ GGCTGTGTGG 3’  | 0               | 5                | 100   |
| OPAZ-04 | 5’ CCAGCCTCAG 3’  | 0               | 5                | 100   |
| OPB-01  | 5’ GTTTTCGCTCC 3’ | 0               | 3                | 100   |
| OPB-17  | 5’ AGGGAACGAG 3’  | 0               | 2                | 100   |
| OPBA-03 | 5’ GTGGGAGACG 3’  | 0               | 6                | 100   |
| OPBA-05 | 5’ TGGTGTTCCAC 3’ | 0               | 5                | 100   |
| OPBA-07 | 5’ GGGTGTCATC 3’  | 0               | 5                | 100   |
| OPBA-08 | 5’ CCACAGCAGA 3’  | 1               | 6                | 85.7  |
| OPL-07  | 5’ AGGCGGAGAC 3’  | 0               | 7                | 100   |
| OPM-04  | 5’ GCCGTTTGTC 3’  | 0               | 3                | 100   |
| OPU-02  | 5’ CTGAGGTCTC 3’  | 0               | 5                | 100   |
| OPU-03  | 5’ CTATGCGGAC 3’  | 0               | 4                | 100   |
| OPU-08  | 5’ GGCGAAGGTT 3’  | 0               | 2                | 100   |
| Total   |                   | 1               | 84               | 85    |
| Mean    |                   | 0.1             | 4.6              | 99.2  |
Figure 2. Randomly amplified polymorphic DNA fingerprints of 25 *Chrysobalanus icaco* L. genotypes amplified with OPA-10 primer. The amplified fragments were separated on 1% agarose gel electrophoresis. The gel was stained with ethidium bromide. Arabic numbers represent the samples of *C. icaco*, as shown in Table 1. M, 100 bp DNA ladder.

3.2 GENETIC RELATIONSHIP AMONG THE 25 *CHRYSOBALANUS ICACO* GENOTYPES BASED ON RAPD ANALYSIS

The cluster analysis obtained by Tocher method (Table 3) allowed the separation of the 25 genotypes into eight distinct groups, and group II had the largest number of genotypes, including genotypes of the three fruit color morphotypes (white, black and red).

Table 3. Clustering of the 25 *Chrysobalanus icaco* L. genotypes collected from 15 sites in the northeast of Pará, by the Tocher method, using the genetic similarity of Jaccard index, based on 84 polymorphic bands. Arabic numbers represent the samples of *C. icaco*, as shown in Table 1, and were identified by the color of the ripe fruits.

| Group | Genotypes of *Chrysobalanus icaco* L. |
|-------|-------------------------------------|
| I     | 1 (Red) 2 (Black) 23 (Red)          |
| II    | 3 (Red) 4 (Red) 8 (Black) 9 (Red) 14 (White) 16 (Black) 17 (Black) 21 (Red) 24 (Black) |
| III   | 5 (Red) 6 (Red) 12 (Black)          |
| IV    | 7 (Red) 10 (White)                  |
| V     | 11 (Black) 13 (Black) 15 (Red) 18 (Red) |
| VI    | 20 (Red) 22 (Black)                 |
| VII   | 25 (White)                          |
| VIII  | 19 (Red)                            |

Figure 3 shows that analysis obtained by UPGMA clustering method allowed the division of the 25 *C. icaco* genotypes into five groups, where group V was formed by the majority of genotypes, including genotypes of the three fruit color morphotypes. Average genetic similarity (SMG) among the 25 genotypes was 0.38. The cophenetic correlation ($r$) was 0.8914,
suggesting a good representation of the similarity matrix generated by Jaccard's coefficient (SILVA, 2007).

Figure 3. Dendrogram of genetic similarity among the 25 Chrysobalanus icaco L. genotypes collected from 15 sites in the northeast of Pará obtained by unweighted pair group method with arithmetic mean (UPGMA), using the genetic similarity of Jaccard index, based on 84 polymorphic bands. Arabic numbers represent the samples of C. icaco, as shown in Table 1, and were identified by the color of the ripe fruits. Average genetic similarity (SMG), 0.38. Cophenetic correlation (r), 0.8914; p < 0.00001.

In addition, the analysis obtained by UPGMA verified the formation of two groups by the cut-off criterion adopted (SMG = 0.36) (Figure 4). Group I consisted of the red C. icaco morphotype and group II grouped the white and black morphotypes. The cophenetic correlation was 0.9971, guarantees the reliability of these clusters.
Figure 4. Dendrogram of genetic similarity among the 25 Chrysobalanus icaco L. genotypes collected from 15 sites in the northeast of Pará obtained by unweighted pair group method with arithmetic mean (UPGMA), using the genetic similarity of Jaccard index, based on the three C. icaco morphotypes identified by the color of the ripe fruits. Average genetic similarity (SMG), 0.36. Cophenetic correlation (r), 0.9971; p < 0.00001.

Fingerprinting based on RAPD markers was an efficient method to study the genetic variability and divergence of Chrysobalanus icaco. The polymorphism identified among the samples showed that the C. icaco genotypes from 15 different sites in the state of Pará are genetically distinct, forming variable clusters in number and constitution by the different methods.

From the morphological characteristics considered, we were able to identify clustering based on the color of the ripe fruit. Consequently, this finding is agrees closely with the chemical result described in the literature (PARACAMPO et al., 2017).

4 CONCLUSION

Our results demonstrated that RAPD markers can easily discriminate the Chrysobalanus icaco genotypes and that there is a correlation between chemotypes of C. icaco, reported by our group, and their genetic structure. This unprecedented correlation to the taxonomy of C. icaco suggests the existence of a genetic basis (ripe fruit color) for the chemical fingerprinting. This shows that the differences in the chemical composition of the C. icaco extracts depend more on the genetic character and less on the environmental factors. Furthermore, the combined analysis (genetic and chemical fingerprinting) could be a useful tool to develop C. icaco genotype selections with high medicinal value.
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