

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.

RESUMO

Chrysobalanus icaco L. (Chrysobalanaceae) é uma espécie medicinal amplamente utilizada no Brasil, principalmente no tratamento do diabetes. Apesar da importância medicinal de *C. icaco*, as informações genéticas desse gênero ainda são limitadas. Assim, nosso objetivo foi avaliar a influência da base genética de *C. icaco* pela determinação de seus quimiotipos. 25 genótipos de *C. icaco* foram coletados em 15 municípios localizados em três das mesorregiões do estado do Pará, Brasil: Metropolitana de Belém, Marajó e Nordeste Paraense. Os genótipos foram selecionados avaliando-se as características morfológicas da planta, como cor dos frutos e hábito da planta. A impressão digital de DNA foi realizada utilizando a técnica de RAPD baseada em PCR e os dados foram tratados por métodos estatísticos adequados. Marcadores RAPD foram utilizados para avaliação da diversidade genética e caracterização molecular de *C. icaco*, utilizando um total de 18 primers decâmeros. Esses primers produziram 85 produtos de amplificação, com média de 4,7 bandas por primer e 99,2% de polimorfismo. Os genótipos são geneticamente distintos, formando agrupamentos que variam em número e constituição, por diferentes métodos. Pelas características morfológicas consideradas, existe uma tendência de agrupamento em função da cor do fruto maduro. Descobrimos que o conteúdo de metabólitos secundários produzido pelos quimiotipos não está relacionado às condições ambientais, mas sim ao genoma de *C. icaco*. Portanto, isso pode ter implicações diretas no uso etnofarmacológico desses quimiotipos.

Palavras-chave: Medicina tradicional brasileira, Ajuru, Quimiotipo, RAPD, Diversidade genética.

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 phytoequivalent 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 $-20\text{ }^{\circ}\text{C}$ 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.

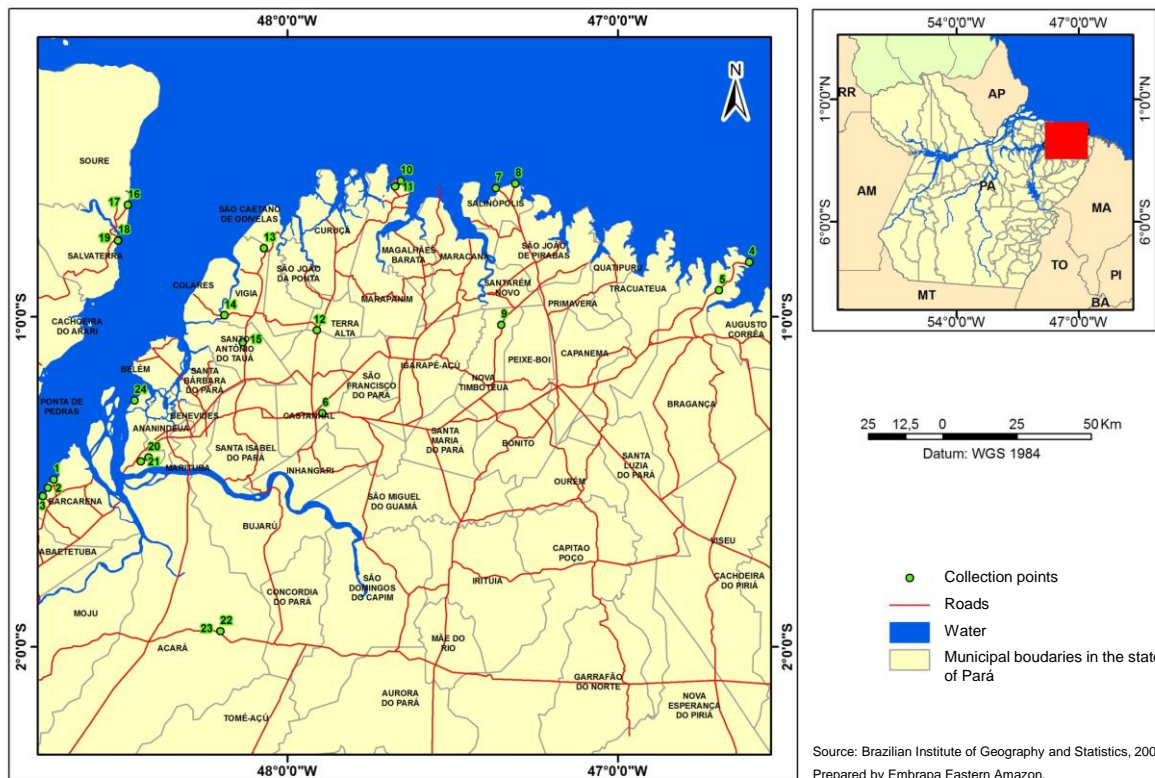


Table 1. Information on 25 *Chrysobalanus icaco* L. genotypes collected from 15 different sites in the northeast of Pará, Brazil (see Figure 1 for mapping of the collection points).

Genotype	Voucher number	Collection site	Geographical location		Fruit color	Common name	Plant habit
			Latitude	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	Red	Red ajuru	Low-sized shrub
10	192706	Acará	1° 57' 10,188" S	48° 12' 10,195" W	Pale-yellow	White 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	Low-sized shrub
19	191987	Terra Alta	1° 2' 27,168" S	47° 54' 39,718" W	Red	Red ajuru	Medium-sized shrub
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. $\square\text{L}^{-1}$, 3.47 μL ultrapure water, 0.6 μL MgCl_2 50 mM, 1.04 μL dNTPs (dATP, dCTP, dGTP, dTTP) 1 mM, 3.5 μL primer 0.1 nmol. $\square\text{L}^{-1}$, 0.2 μL *Taq* DNA polymerase 50 un. μL^{-1} , 1.04 μL BSA 10 $\mu\text{g}.\mu\text{L}^{-1}$. 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 (Loccus 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 GenAIEx 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).

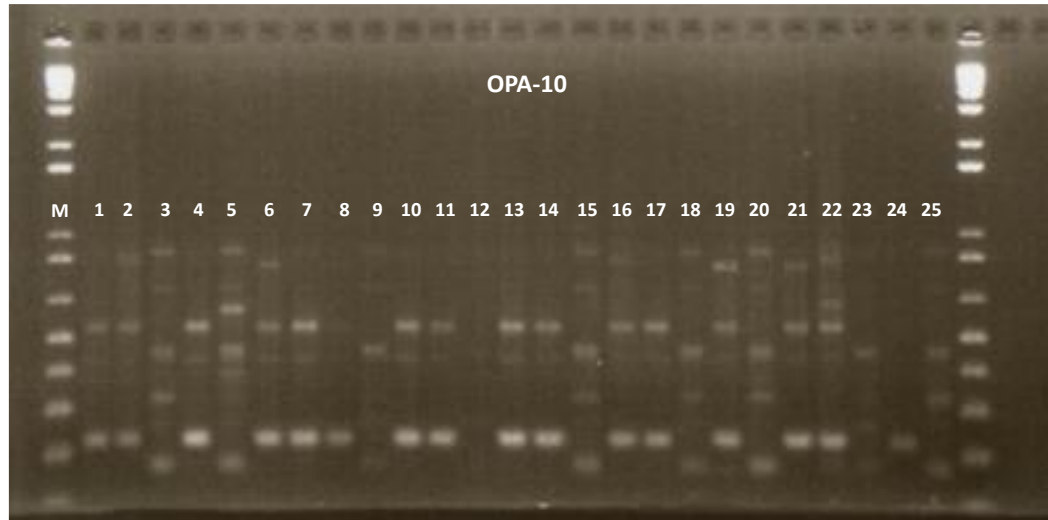
3 RESULTS AND DISCUSSION**3.1 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) FINGERPRINTING PROFILE**

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 (5' → 3')	Number of bands			Polymorphism (%)
		Monomorphic	Polymorphic	Total	
OPA-03	5' AGTCAGCCAC 3'	0	4	4	100
OPA-10	5' GTGATCGCAG 3'	0	10	10	100
OPA-11	5' CAATCGCCGT 3'	0	6	6	100
OPAB-03	5' TGGCGCACAC 3'	0	2	2	100
OPAR-11	5' GGGAAAGACGG 3'	0	2	2	100
OPAZ-03	5' GGCTGTGTGG 3'	0	5	5	100
OPAZ-04	5' CCAGCCTCAG 3'	0	5	5	100
OPB-01	5'GTTTCGCTCC 3'	0	3	3	100
OPB-17	5' AGGGAACGAG 3'	0	2	2	100
OPBA-03	5'GTGCGAGAAC 3'	0	6	6	100
OPBA-05	5' TGCGTTCCAC 3'	0	5	5	100
OPBA-07	5' GGGTCGCATC 3'	0	5	5	100
OPBA-08	5' CCACAGCCGA 3'	1	6	7	85.7
OPL-07	5' AGGCGGGAAC 3'	0	7	9	100
OPM-04	5' GGCGGTTGTC 3'	0	3	3	100
OPU-02	5' CTGAGGTCTC 3'	0	5	5	100
OPU-03	5' CTATGCCGAC 3'	0	4	4	100
OPU-08	5' GGCGAAGGTT 3'	0	2	2	100
Total		1	84	85	
Mean		0.1	4.6	4.7	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).

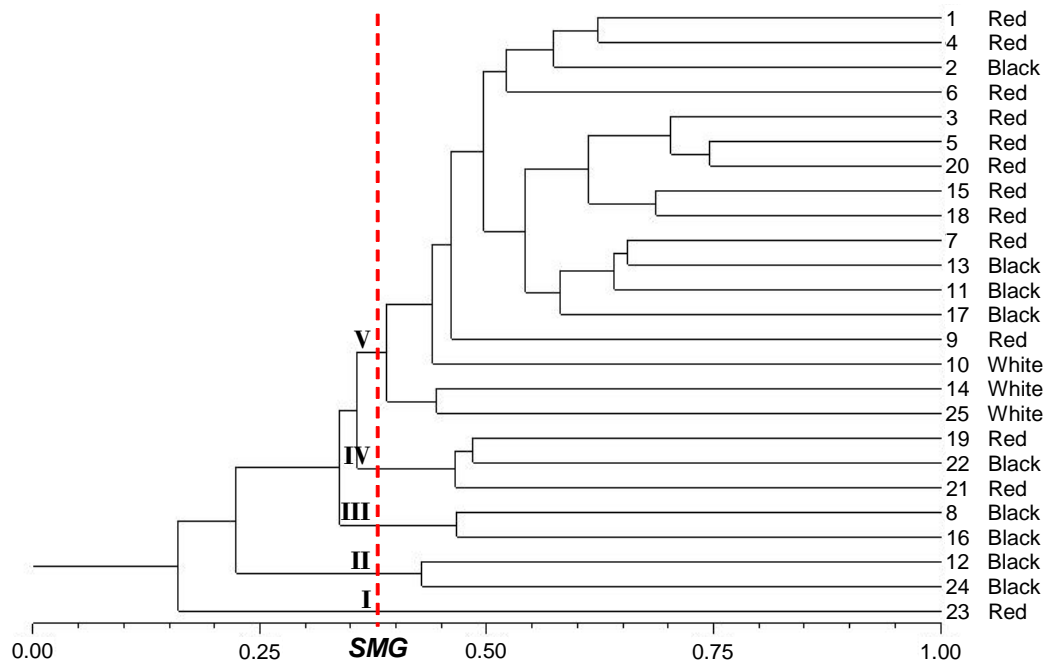
Table 3. Clustering of the 25 *Chrysobalanus icaco* L. genotypes collected from 15 sites in the northeast of Pará, by the Tocher method, using the genetic similarity of Jaccard index, based on 84 polymorphic bands. Arabic numbers represent the samples of *C. icaco*, as shown in Table 1, and were identified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

Group	Genotypes of <i>Chrysobalanus icaco</i> 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,

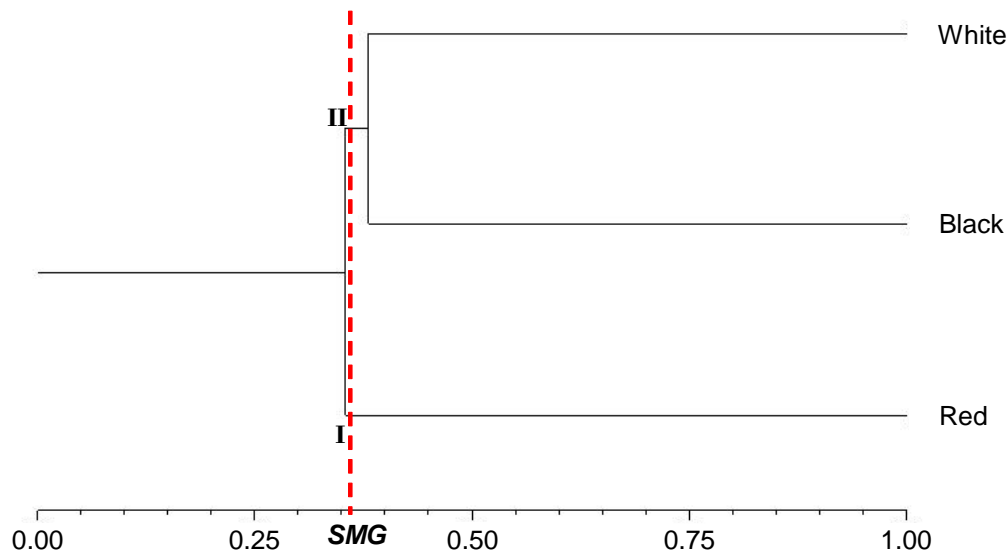
suggesting a good representation of the similarity matrix generated by Jaccard's coefficient (SILVA, 2007).

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 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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