Revista Brasileira de Ciências Agrárias
ISSN (on line) 1981-0997
v.14, n.2, e5644, 2019
Recife, PE, UFRPE. www.agraria.pro.br
DOI:10.5039/agraria.v14i2a5644
Protocolo 5644 - 30/04/2018 • Aprovado em 24/01/2019

Genetic diversity of cambui trees (*Myrciaria floribunda* (West ex Willd) O. Berg) differentiated by the color of the fruit

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ABSTRACT: The cambui tree (*Myrciaria floribunda* (West ex Willd) O. Berg), is a native fruit tree from Brazil, whose fruit present potential *in natura* and industrial consumption. Our objective was to evaluate the genetic diversity between individuals differentiated by fruit color - orange and purple, by using ISSR molecular markers. The plant material belongs to the Caju Private Natural Heritage Reserve, experimental field of Embrapa Coastal Tablelands, in the municipality of Itaporanga d'Ajuda, SE. Young leaves from 25 individuals (15 with orange color fruit and 10 with purple color fruits) were collected for DNA extraction and PCR-ISSR analysis with 15 primers, which produced a total of 93 bands, being 95.5% of them polymorphic. The mean similarity was 0.53, based on the Jaccard coefficient. The least similar individuals were FR5 and FL8; FR5 and FL9; FR1 and FR5. The most similar pairs of individuals were FL3 and FL4; FL4 and FL5. The UPGMA analysis clustered the individuals into two groups. Although no specific molecular marking that characterizes fruit coloration has been found, there is genetic variability among the evaluated individuals.

Key words: native fruits; ISSR; Myrtaceae

Diversidade genética de cambuizeiros (*Myrciaria floribunda* (West ex Willd) O. Berg) diferenciados pela coloração dos frutos

RESUMO: O cambuizeiro (*Myrciaria floribunda* (West ex Willd) O. Berg), é uma frutífera nativa do Brasil, cujos frutos apresentam potencial para consumo *in natura* e industrial. O objetivo do presente trabalho foi avaliar a diversidade genética entre indivíduos diferenciados pela coloração dos frutos - laranja e roxa, utilizando marcadores moleculares ISSR. O material vegetal pertence a Reserva Particular do Patrimônio Natural do Caju, campo experimental da Embrapa Tabuleiros Costeiros, no município de Itaporanga d'Ajuda, SE. Folhas jovens de 25 indivíduos (15 com frutos de cor laranja e 10 com frutos de cor roxa) foram coletadas para extração de DNA e análise PCR-ISSR com 15 primers, os quais produziram um total de 93 bandas, sendo 95,5% polimórficas. A similaridade média foi de 0,53, com base no coeficiente de Jaccard. Os indivíduos menos semelhantes foram FR5 e FL8; FR5 e FL9; FR1 e FR5. Os pares de indivíduos mais similares foram FL3 e FL4; FL4 e FL5. A análise UPGMA agrupou os indivíduos em dois grupos. Apesar de não ter sido encontrada uma marca molecular específica que caracterize a coloração dos frutos, há variabilidade genética entre os indivíduos avaliados.

Palavras-chave: frutas nativas; ISSR; Myrtaceae

Introduction

The Atlantic Forest biome is composed of native forest formations, assembling 35% of the plant species from Brazil. Its biodiversity is greater than that of some entire continents (MMA, 2018); however, it is also one of the most endangered on the planet. Several native fruit trees species with ample potential of use are found in this biome. In the state of Sergipe, for example, researches have been conducted with jenipapeiro (*Genipa americana* L.) (Silva et al., 2015; Silva et al., 2018), cambuizeiro (*Myrciaria tenella* O. Berg) (Santana et al., 2016) and mangabeira (*Hancornia speciosa* Gomes) (Santos et al., 2017, Soares et al., 2018).

Among the botanical families found, Myrtaceae stands out as one of the richest (Lourenço et al., 2012). They belong to the Myrteae tribe, which form a phylogenetically cohesive group (Wilson et al., 2001), divided into three subtribes: Eugeniinae, Myrciinae and Myrtinae, based on the embryos morphology (Berg 1857, 1858, 1859). The family has relevance in the restoration of altered areas, enrichment of secondary forests and phytotherapeutic uses (Gomes et al., 2017), in addition to its fruits being important sources of food for the wild fauna (Gressler et al., 2006).

There is a large number of Myrtaceae still not domesticated and underexplored, being among them the *Myrciaria floribunda* O. Berg species, known as cambuizeiro, a fruit tree native to Brazil. It presents hermaphrodite flowers, with cross pollination and possible autogamy. It also presents a great variability within the species, making it possible to differentiate them by the coloration of its fruit (Pio-Corrêa, 1984): glabrous and bright globose berries, with colors that varies from purple to orange (Pinheiro et al., 2011). Furthermore, the species also displays physical and physical-chemical characteristics according to the maturation of the fruit. (Silva et al., 2012a).

The researches on the species are recent. Pinheiro et al. (2002) reported the high content of vitamin C (129.43 mg of ascorbic acid, 100 g^{-1}), thus being the fruit appreciated in the production of juices, jellies and fermented with antioxidant properties (Rybka et al., 2011). Moreover, it is a considerable food source for ruminants due to its fiber content (48.65% of NDF - Neutral Detergent Fiber and 23.60% of ADF - Acid Detergent Fiber) (Voltolini et al., 2011).

While initially identifying the different colors of the fruit, there was a direct relation with the maturation stages (Pinheiro et al., 2011), however, the experience in expeditions of germoplasm collection and the constant visits to the natural population of the Caju Reserve, object of this study, allowed the identification of individuals that exclusively have purple or orange colored fruit. Therefore, the molecular characterization will be useful in order to evaluate the genetic diversity, to elaborate conservation strategies, and, when associated with botanical, chemical and functional data, may be decisive in the incorporation of the species into the regional productive systems.

The use of ISSR markers is rather widespread, with no prior genomic information required. Its usage has been established for several applicabilities, both for the conservation biology as well as for the molecular and systematic ecology (Gharbawi, 2015). In cambuizeiro, Santana et al. (2016) used 10 ISSR primers to characterize 50 plants of a natural population in Sergipe.

The aim of the current study was to evaluate the genetic diversity of cambuizeiro, differentiated by the coloration of the fruit - orange and purple, by using ISSR molecular markers.

Materials and Methods

Young leaves of the cambuizeiros with orange (Figure 1A) and purple (Figure 1B) colored fruit were collected from a natural population, *in situ* conserved, belonging to the Caju Private Natural Heritage Reserve (RPPN do Caju), experimental field of Embrapa Coastal Tablelands, in the municipality of Itaporanga d'Ajuda - SE (11.116585°S, 37.186742°W) (Figure 1C). The material was collected from 25 individuals, being 15 of them cambuizeiros with orange colored fruit (FL1 to FL15) and 10 of purple colored fruit (FR1 to FR10).

In order to extract the DNA, 15 leaf disks were used (Doyle & Doyle, 1990) adapted by Alzate-Marin et al. (2009). The quality of the extracted DNA was evaluated in electrophoresis with a 0.8% agarose gel. The gel was stained in ethidium bromide solution for 20 min, and photodocumented with the Gel doc L-pix HE (*Loccus* Biotechnology, Cotia, SP, Brazil). Subsequently, the DNA was quantified using the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The

Figure 1. Young leaves and fruit of orange colored cambuizeiro (B) Young leaves and fruit of purple colored cambuizeiro (C) Natural population of cambuizeiro (*Myrciaria floribunda* (West ex Willd) O. Berg). Caju Private Natural Heritage Reserve, Embrapa Coastal Tablelands, SE. (A) Photo: A.L.S. Nascimento.

samples were diluted at 10 ng μL^{-1} in a TE buffer solution (100 mM Tris-HCl pH 7.4 and 1 mM of EDTA) and stored at -20 °C for further use in the PCR reactions.

Moreover, 30 primers were tested in a 2% agarose gel, of which 15 were selected for DNA amplification by means of PCR-ISSR (Table 1).

For the PCR runs, the total reaction volume was 20 µL, containing: 1 µL of genomic DNA (10 ng μL^{-1}), 1 µL of each primer (25.0 pmol), 2 µL of buffer from the 10X reaction (100 mM Tris-HCl, pH 8.5; 500 mM KCl); 0.4 µL of dNTP (10 mM); 0.6 µL of $MgCl_2$ (50 mM) (4G Research and Development, Brazil/RS); 0.2 µL of Taq polymerase (5 U μL^{-1}) (4G Research and Development, Brazil/RS) and 14.8 µL of autoclaved ultrapure water. The DNAs were amplified using the ProFlex PCR System thermocycler (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA) programmed with the following protocol: initial denaturation at 94 °C for 4 min, followed by 37 amplification cycles; denaturation at 94 °C for 45 s; annealing at different temperatures for 45 s; extension at 72 °C for 2 min; and a final extension at 72 °C for 7 min, followed by cooling down at 4 °C. The amplification products were subjected to horizontal electrophoresis in a 2% agarose gel, then subsequently stained in ethidium bromide solution (0.5 µL mL^{-1}) during 20 min, and then photodocumented by the Gel doc L-pix (*Loccus* Biotecnologia, Cotia, SP, SP, Brazil). To standardize the fragments, the 100 bp molecular weight marker was employed (Ludwig Biotec, Brazil/RS).

After the agarose gels analysis, a binary matrix based on the absence (0) or presence (1) of bands from the selected primers was obtained. Estimates of genetic similarities between individuals were obtained using the Jaccard coefficient. The dendrogram was constructed with the aid of the NTSYS-pc 2.0 software, based on the genetic similarity matrix, using the UPGMA algorithm (Unweighted Arithmetic Mean Method) (Rohlf, 2001). The analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA) and genetic diversity parameters such as: the number of observed alleles (Na); number of effective alleles (Ne); expected heterozygosity (He); observed heterozygosity (Ho) and the Shannon index (I) were all obtained with the aid of the Genalex 6.5 software (Peakall & Smouse, 2012).

The Polymorphic Information Content (PIC), the expected heterozygosity under Hardy-Weinberg Equilibrium (HWE), and the correlation and stress values were all estimated using the GENES program (Cruz, 2016). In order to emphasize the clustering, the bootstrap resampling method with 10,000 replicates was employed from the GENES software.

The analysis of the genetic structure of *Myrciaria tenella* O. Berg individuals, based on Bayesian statistics, was estimated employing the Structure software (Pritchard et al., 2000). The Structure Harvester program (Earl & VonHoldt, 2012) was also employed to infer the number of clusters (ΔK).

Table 1. ISSR primers used to evaluate the genetic diversity between cambuizeiros differentiated by the fruit coloration – orange and purple - with their respective sequences and annealing temperature (Ta).

Primer	Sequence	Ta (°C)
UBC 807	AGA GAG AGA GAG AGA GT	47.0
UBC 809	AGA GAG AGA GAG AGA GG	57.2
UBC 811	GAG AGA GAG AGA GAG AC	46.8
UBC 813	CTC TCT CTC TCT CTC TT	44.6
UBC 815	CTC TTC TCT CTC TCT CTG	47.6
UBC 816	CAC ACA CAC ACA CAC AT	55.8
UBC 818	CAC ACA CAC ACA CAC AG	57.2
UBC 823	TCT CTC TCT CTC TCT CC	57.2
UBC 835	AGA GAG AGA GAG AGA GYC	50.2
UBC 845	CTC TCT CTC TCT CTC TRG	48.1
UBC 856	ACA CAC ACA CAC ACA CYA	56.5
UBC 857	ACA CAC ACA CAC ACY G	58.8
ISSR 2	CTC TCT CTC TCT CTC TAC	51.5
ISSR 3	CTC TCT CTC TCT CTC TTG	51.5
ISSR 5	CTC TCT CTC TCT CTC TGC	51.5

Results and Discussion

The 15 used primers provided 93 bands, 90 of them being polymorphic (95.5%). The number of bands ranged from 3 (UBC 815 and 856) to 9 (UBC 818 and 857) (Table 2).

In natural cambuizeiro population, Santana et al. (2016) used 10 primers and obtained 70 polymorphic bands (98.3%). For the characterization of jabuticaba trees (*Plinia* sp.), Cruz et al. (2016) used 18 primers, which resulted in 462 polymorphic bands (99.65%). In the study of genetic diversity among 28 individuals of canela-de-tabuleiro (*Myrcia lundiana* Kiaersk) originated from the Itabaiana National Park, Alves et al. (2016) tested 35 primers that generated 135 polymorphic bands (93.75%). Evaluating the genetic distance between accessions of guava and araçazeiros from the genus *Psidium*, Oliveira et al. (2014) obtained 216 polymorphic bands with the use of 17 primers.

Table 2. List of ISSR primers selected for evaluation of the genetic diversity between cambuizeiros differentiated by fruit coloration – orange and purple; total bands number (TBN); polymorphic bands number (PBN) and percentage of polymorphism generated by the PCR reactions.

Primers	TBN	PBN	% Polymorphism
807	07	07	100
809	04	04	100
811	07	07	100
813	05	05	100
815	04	03	75
816	05	05	100
818	09	09	100
823	07	07	100
835	07	07	100
845	06	05	83
856	04	03	75
857	09	09	100
ISSR 2	05	05	100
ISSR 3	07	07	100
ISSR 5	07	07	100
Total	93	90	95.5

The mean value of the observed alleles (Na) number was equal to 1.88 and the number of effective alleles (Ne) was 1.61. The mean observed heterozygosity (Ho) was 0.36, while the expected (He) was 0.34. These results suggest that there are more heterozygotes than the expected by the Hardy-Weinberg equilibrium due to the constant flux of genes from other nearby individuals. The mean value of the Shannon index (I) was 0.50 (Table 2). This index can vary from 0 to 1, and the diversity is considered lower when values are closer to zero (Silva et al., 2015). In this study, the cambuizeiros with fruits of distinct coloring displayed an intermediate level of genetic diversity, similar to that observed by Santana et al. (2016), which obtained (I) of 0.52.

In accessions of camu-camu (*Myrciaria dubia*), Nunes et al. (2017) also obtained an intermediate level of genetic diversity, (He) of 0.34 and (I) of 0.50. However, Almeida-Pereira et al. (2017), when studying the genetic diversity of native populations of *Croton tetradenius* Baill, found lower values: (He) of 0.30 and (I) of 0.45. The He value of should always be different than zero, since the individuals are susceptible to incorporation of new alleles by breeding, even in small or fragmented populations, as well as losses due to genetic drift (Silva et al., 2014).

The reliability of the results was verified by the stress value (E), which was 0.016, and the cophenetic correlation (0.997). Stability occurred upon reaching 90 amplified bands. A value of E <0.05 is indicative of great precision in the estimates (Kruskal, 1964).

The mean PIC value was 0.62. According to Xie et al. (2010), the PIC value ranges from 0 to 0.25 for markers that are considered as less informative; from 0.25 to 0.5 for those that indicates a moderate informative content; and above 0.5 for the highly informative ones. This value serves as the basis for the classification and selection of primers that were efficient in the discrimination of the individuals. Therefore, it can be pointed out that the polymorphism between the plants with fruits of orange and purple color was high.

Studying the genetic diversity in a natural population of mangaba (*Hancornia speciosa* Gomes) while using ISSR markers, Costa et al. (2015) obtained an mean PIC value of 0.37, classifying them as moderately informative. For the moringa (*Moringa oleifera* Lam), while using RAPD dominant markers, Silva et al. (2012b) obtained an mean PIC of 0.22, with the primers considered to be less informative.

By AMOVA means, it was observed that genetic diversity was higher within each coloration (90%) than among themselves (10%) (Table 4).

The diversity incidence among the studied cambuizeiros by ISSR molecular markers means demonstrated the efficiency of the technique in identifying diversity. The mean similarity was 0.53. The lowest similarity (0.25) was observed between the FR5 and FL8 individuals; FR5 and FL9; FR1 and FR5 due to them being more geographically distant. It was also possible to visualize that individuals with the same fruit color, such as FR1 and FR5, may be genetically different. The highest similarity (0.88) was equally found in individuals FL5 and FL6; FL12 and FL13. The most similar pairs of individuals were FL3 and FL4 (0.76); FL4 and FL5 (0.84), which had the same fruit coloration and very close distances, indicating interaction regarding the pollen dispersal normally carried out by insects (Table 5).

The UPGMA analysis resulted in the formation of two clusters, but it was not possible to obtain specific separation between the cambuizeiros with orange and purple colored fruits (Figure 2). Group 1 (G1) practically allocated all individuals, indicating wide diversity, and was subdivided into five groups (G1-A, G1-B, G1-C, G1-D, G1-E). Larger groups, composed by more individuals, cluster the pairs that present smaller distances, since the group size is delimited by a mean distance between pairs of individuals (Oliveira et al., 2009), and although the cambuizeiro have the autogamy mechanism, the diversity found can be explained by the tendency to allogamy (Oliveira et al., 2007).

In G1-A, the pairs of individuals FL5 and FL6; FL12 and FL13 were the most similar, presenting practically the same genetic material. In G1-B, FR7 and FR9 were the closest, genetically speaking. G1-C clustered individuals with fruit of distinct coloration, FL10 and FR10. This shorter distance, and consequently less variability, can be explained by the fact that

Table 3. Number of individuals. Number of observed alleles (N_A), Number of effective alleles (N_E), Shannon Index (I), expected heterozygosity (H_E), observed heterozygosity (H_O) and PIC (Polimorphic Information Content) for cambuizeiros with fruit of different colors – orange and purple obtained by ISSR markers.

Population	Number of individuals	N_A	N_E	I	H_E	H_O	PIC
Orange color fruit	15	1.87	1.59	0.49	0.33	0.35	-
Purple color fruit	10	1.88	1.62	0.51	0.35	0.37	-
Mean value	-	1.88	1.61	0.50	0.34	0.36	0.62
Total	25						

Table 4. Analysis of molecular variance (AMOVA) between and within the two groups of cambuizeiros identified by fruit coloration – orange and purple.

Variation source	Degrees of freedom	Sum of squares	Mean square	Variation component	Total (%)	P
Between groups	1	37.69	37.69	1.75	10	0.001
Within groups	23	382.63	16.64	16.64	90	
Total	24	420.32	-	18.39	100	

Table 5. Jaccard coefficient similarity matrix using 15 primers by the ISSR technique between 25 cambuizeiro individuals differentiated by the coloration of the fruit – orange and purple.

Plant	Plant																						
	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10	FL11	FL12	FL13	FL14	FL15	FR1	FR2	FR3	FR4	FR5	FR6	FR7	FR8	FR9	FR10
FL1	0.55	-																						
FL2	0.66	0.59	-																					
FL3	0.57	0.48	0.76	-																				
FL4	0.59	0.49	0.78	0.71	-																			
FL5	0.57	0.53	0.84	0.80	0.88	-																		
FL6	0.51	0.44	0.57	0.50	0.58	0.65	-																	
FL7	0.51	0.42	0.54	0.57	0.55	0.61	0.58	-																
FL8	0.55	0.48	0.58	0.63	0.57	0.60	0.48	0.69	-															
FL9	0.38	0.30	0.47	0.49	0.45	0.47	0.39	0.51	0.55	-														
FL10	0.40	0.43	0.41	0.37	0.41	0.43	0.33	0.41	0.50	0.36	-													
FL11	0.49	0.45	0.64	0.66	0.65	0.66	0.45	0.47	0.53	0.50	0.40	-												
FL12	0.55	0.46	0.65	0.67	0.66	0.67	0.48	0.50	0.54	0.49	0.39	0.88	-											
FL13	0.42	0.40	0.57	0.50	0.57	0.61	0.44	0.47	0.42	0.47	0.39	0.60	0.61	-										
FL14	0.44	0.36	0.55	0.55	0.56	0.64	0.62	0.58	0.53	0.42	0.31	0.50	0.53	0.47	-									
FL15	0.44	0.42	0.58	0.53	0.58	0.61	0.44	0.52	0.53	0.49	0.39	0.55	0.51	0.60	0.49	-								
FR1	0.47	0.50	0.60	0.51	0.54	0.62	0.47	0.51	0.49	0.42	0.42	0.50	0.53	0.54	0.55	0.51	-							
FR2	0.52	0.37	0.56	0.61	0.49	0.53	0.42	0.45	0.51	0.35	0.39	0.50	0.53	0.43	0.48	0.42	0.50	-						
FR3	0.41	0.41	0.55	0.53	0.49	0.57	0.39	0.49	0.53	0.44	0.36	0.54	0.55	0.51	0.50	0.55	0.52	0.48	-					
FR4	0.42	0.38	0.51	0.46	0.47	0.49	0.40	0.47	0.49	0.46	0.35	0.50	0.49	0.61	0.43	0.49	0.50	0.50	0.62	-				
FR5	0.29	0.34	0.33	0.34	0.32	0.32	0.25	0.25	0.36	0.33	0.33	0.27	0.23	0.30	0.25	0.33	0.28	0.33	0.27	0.29	-			
FR6	0.49	0.39	0.56	0.58	0.49	0.56	0.46	0.51	0.46	0.49	0.34	0.49	0.50	0.53	0.51	0.50	0.58	0.51	0.55	0.60	0.31	-		
FR7	0.38	0.29	0.46	0.46	0.47	0.48	0.37	0.49	0.44	0.55	0.31	0.49	0.48	0.57	0.45	0.52	0.51	0.37	0.53	0.49	0.29	0.65	-	
FR8	0.45	0.45	0.53	0.53	0.49	0.55	0.47	0.49	0.47	0.46	0.37	0.58	0.57	0.60	0.50	0.51	0.52	0.44	0.54	0.54	0.28	0.68	0.61	-
FR9	0.34	0.39	0.39	0.45	0.36	0.43	0.29	0.46	0.45	0.46	0.43	0.49	0.47	0.42	0.36	0.38	0.49	0.42	0.43	0.44	0.26	0.44	0.44	0.55

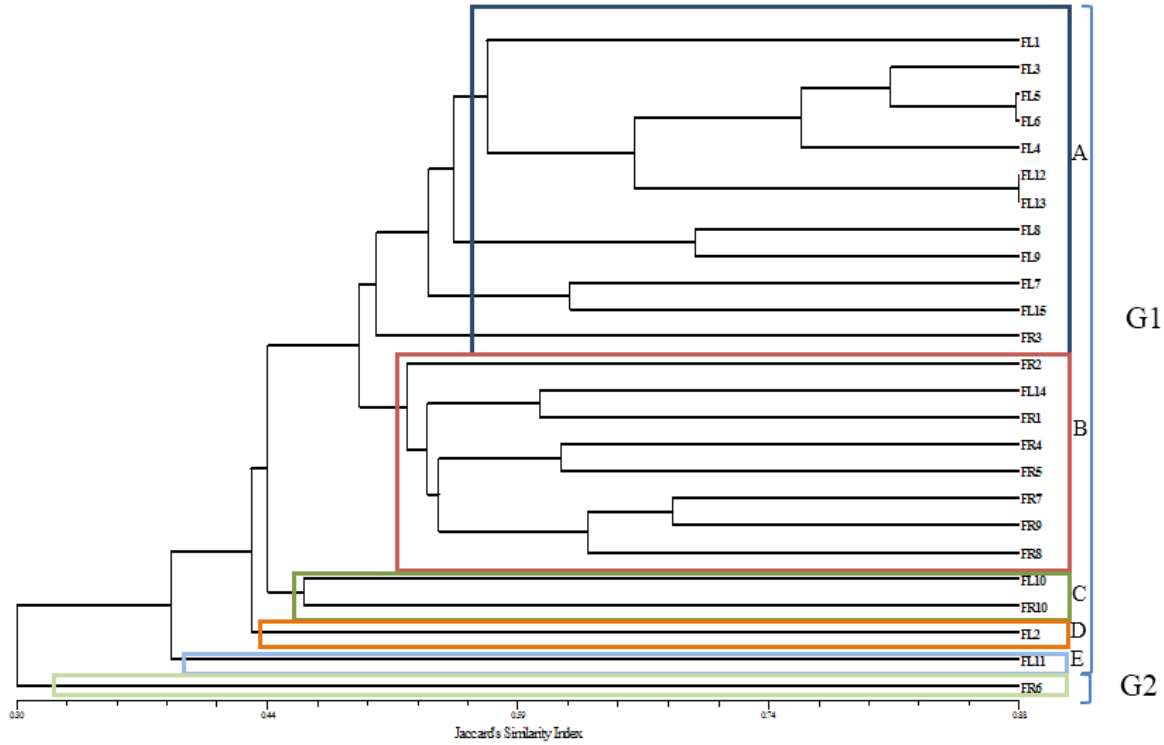

Figure 2. Dendrogram obtained by the UPGMA method based on the genetic similarity index by the Jaccard coefficient for 25 cambuizeiro individuals differentiated by the coloration of the fruit – orange and purple.

they may come from self-fertilization; and the subgroups as G1-D and G1-E were more isolated and composed by a single individual. Due to the greater genetic distance between the others, and the fact that they present the same fruit coloration, they could be promising in breeding.

Group 2 (G2) was only composed by the FR6 individual, the most genetically divergent. These isolated individuals have wide genetic variability and can be used for commercial purposes and be useful in the conservation and knowledge of available genetic resources for the formation of active germplasm banks and the development of genetic improvement of the species from controlled breedings.

The Principal Coordinates Analysis (PCoA) was employed in order to elucidate the genetic diversity patterns in a two-

dimensional plot (Figure 3) and it confirmed some groups by UPGMA. Eight groups were formed in this clustering model, where subgroups are highlighted, promoting a greater differentiation of individuals with fruit of distinct coloration.

The existence of high genetic variability was also found by both Pinheiro et al. (2011), while using RAPD markers in 20 cambuizeiro individuals, and by Santana et al. (2016) when using ISSR markers for the study of 50 individuals in a cambuizeiro natural population.

The employment of more than one clustering method, due to the differences in the hierarchy, optimization and ordering of the groups, allows the classification of the individuals to complement each other according to the criteria that each technique uses, and it prevents that erroneous inferences are adopted in the allocation of materials, within a given genotypes subgroup (Arriel et al., 2006).

The genetic structure was evaluated by the Bayesian analysis. This analysis groups the individuals based on genetic distinctions, without needing a pre-identification of individuals with fruit of orange or purple coloration. In the ΔK analysis, the maximum value occurred in K=3, with the individuals divided into three groups defined by the red, green and blue colors (Figure 4).

Results were noted to be concordant with the previous analyzes, where it was not observed clear genetic distinction between individuals with orange and purple fruit colors. Most individuals presented a part from each group, indicating that they may have a shared origin, resulting from a gene exchange between the analyzed individuals.

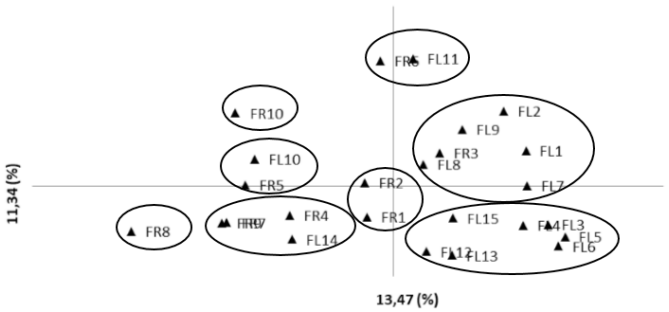

Figure 3. Principal Coordinates Analysis (PCoA) between the different cambuizeiros differentiated by the fruit coloration – orange and purple.

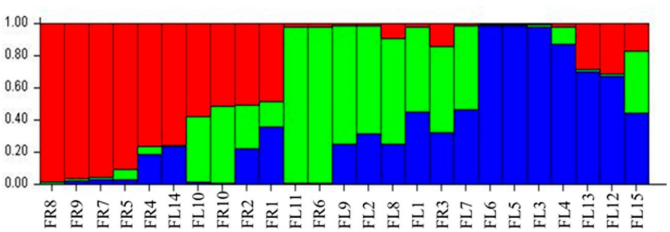

Figure 4. Representation of the 25 cambuizeiro individuals differentiated by the fruit coloration – orange and purple - into three groups defined by the Structure (ΔK = 3) using 15 ISSR markers.

Conclusion

No specific molecular marking was found for the fruit coloration – orange or purple - in cambuizeiro; however, there is high genetic diversity among the evaluated individuals.

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