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Estimation of genetic diversity in a natural population of cambui tree (*Myrciaria tenella* O. Berg) using ISSR markers

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ABSTRACT. Cambui (*Myrciaria tenella* O. Berg) is a native species from Brazil, which belongs to the family Myrtaceae. Molecular characterization is one of the most used tools for the study of the biotechnological potential of species because the diversity level between individuals can be inferred. Analysis of genetic diversity is fundamental to the direction of the strategies necessary to form and maintain a germplasm. This study aimed to evaluate the genetic diversity in a natural population of cambui using inter-simple sequence repeat (ISSR) molecular markers. The natural population, which provided the plant material, is found at the Private Reserve of Natural Heritage of Caju, which belongs to the experimental field of Embrapa Tabuleiros Costeiros, in the municipality of Itaporanga d'Ajuda, SE, Brazil.

Young leaves of each individual were collected for DNA extraction and analysis of PCR-ISSR. Thirty primers were tested and the top 10 were selected. The use of these primers resulted in 71 fragments with 98.3% polymorphism. Similarity of individuals ranged between 0.30 and 0.92. The most similar individuals were C13 and C17 and the most distant were C1 and C41. Through UPGMA, six distinct groups were identified. This information may be used for conservation of these genetic resources, germplasm exchange, creation of germplasm bank and in future studies with this species.

Key words: Myrtaceae; Native fruits; Molecular markers; Conservation

INTRODUCTION

Brazil has the distinction of possessing between 15 and 20% of all global biodiversity and has the largest number of endemic species. These species are mainly concentrated in the Amazon and in two of the nineteen world hotspots (Atlantic Forest and Cerrado) (Ganem, 2010). Although biodiversity is extremely important for the maintenance of natural ecological processes and for human activities, recent estimates show a significant decline in biodiversity and suggest that species extinctions will be concentrated in the hotspots where the threat of habitat loss is higher (Joppa et al., 2011).

Myrtaceae species are found on almost all continents. This family includes more than 140 genera and 5800 species of trees and shrubs (Govaerts et al., 2016). In Brazil, especially in regions dominated by the Atlantic Forest, trees of the Myrtaceae family are the woody plant of greater biological importance because they are predominant members of this biome (Gressler et al., 2006). However, ecosystem fragmentation has occurred because of the substantial increase of deforestation, the expansion of agricultural and livestock activities, the exploitation of trees, and population growth. This fragmentation is limited to small patches or isolated fragments that replace forest cover. Thus, species endemic to the Atlantic Forest biome have been rapidly disappearing (Morellato and Haddad, 2000).

In Sergipe, the forest cover of the Atlantic Forest currently is 7.47% of its original area (Jesus et al., 2014). Several native fruit trees have great potential for use, and some studies of these species have been conducted in Sergipe (Costa et al., 2011; Rabbani et al., 2012).

Several species of native plants have disappeared without any basic knowledge of them ever being known, such as some species of the Myrtaceae family, which are included in lists of endangered species in Brazil (Landrum and Kawasaki, 1997; Machado et al., 2005). Therefore, conservation strategies of these resources are crucial, and research on ecology, genetics, reproduction, physiology, biogeography, botany, and anthropology is required (Pádua and Ferreira, 2008).

Among the native fruits from Brazil, cambui (*Myrciaria tenella* O. Berg), which belongs to the Myrtaceae family, is a little known native fruit, and it mostly occurs in natural areas. Its potential has not yet been exploited, and it may be a source of food and various compounds (Pinheiro et al., 2011). Although Lorenzi (2000) suggests the species has potential for landscape and timber use, its production is not yet widespread. In contrast, there are other species of the Myrtaceae family, which have high economic value, such as guava (*Psidium guajava* L.), jabuticaba [*Myrciaria cauliflora* (Mart.) O. Berg.], and Surinam cherry (*Eugenia*

uniflora L.), which are commercially exploited (Landrum and Kawasaki, 1997).

The conservation of plant genetic resources is a complex multidisciplinary task, which involves several steps, such as the obtainment of germplasm, characterization, evaluation, documentation, conservation, and use of genetic resources (Pádua and Ferreira, 2008).

One of the means used to promote the conservation of genetic resources is the assessment of genetic diversity, which enables the degree of genetic variability to be known and evaluated. This information is fundamental in the formation of germplasms and in the identification of new genes of interest. Thus, molecular markers have been a biotechnological tool widely used for providing information at the DNA level. This study evaluated genetic diversity in a natural population of cambui trees using inter-simple sequence repeat (ISSR) molecular markers.

MATERIAL AND METHODS

Plant material

Young leaves of 50 cambui (Figure 1) were collected in a native population in May 2015. The population is located in the Private Reserve of Natural Heritage of Caju, which belongs to the experimental field of Embrapa Tabuleiros Costeiros in the municipality of Itaporanga d'Ajuda, SE (11.116585°S, 37.186742°W) (Figure 2). The area is typical restinga, and the soil is a Humiluvic Spodosol. Leaves were removed from the trees, wrapped in labeled plastic bags, and transported in coolers to the Laboratory of Molecular Biology of Embrapa Tabuleiros Costeiros in Aracaju. The material was stored at -80°C before DNA extraction.

Figure 1. Tree and fruits of cambui (*Myrciaria tenella* O. Berg). Embrapa Tabuleiros Costeiros, 2016.

Figure 2. Location of the studied natural population of cambui. Reserva Particular do Patrimônio Natural (RPPN) of Caju, Itaporanga d'Ajuda, SE, Brazil. Embrapa Tabuleiros Costeiros, 2016.

DNA extraction

Leaves (1 g) were used for DNA extraction according to the methodology of Romano and Brasileiro (1999). After preheating at 65°C, 1 mL 2% CTAB extraction buffer (5 M NaCl, 1 M Tris HCl, pH 8.0, 0.5 M EDTA) was added to the pulverized leaf material, along with 2 µL β-mercaptoethanol and 3 µL K proteinase (10 mg/mL). Samples were incubated at 65°C for 30 min and then allowed to rest for 30 min. Initially, extractions were performed with 500 µL chloroform/isoamyl alcohol (24:1), followed by 400 µL ice-cold isopropanol, and the stored overnight at -20°C. The obtained precipitate was washed three times, two washes with 70% ethanol and one with 100% ethanol. After drying, DNA was resuspended in 44 µL TE buffer (100 mM Tris-HCl, pH 7.4, 1 mM EDTA), treated with 6 µL RNAse (20 mg/mL), and incubated at 37°C for 30 min.

Electrophoresis, DNA quantification, and dilution

Evaluation of DNA quality was determined by electrophoresis on a 0.8% agarose gel, loaded with 2 µL each sample, 8 µL MilQ sterile water, and 2 µL loading solution (0.01% bromophenol blue, 40% glycerol). Horizontal electrophoresis was conducted for 45 min at

76 V, 38 mA, and 120 W. Subsequently, the gel was stained in ethidium bromide solution for about 40 min and then it was documented with a Gel Doc L-pix HE (Loccus Biotecnologia, Brazil). Concentrations of DNA samples were determined using the NanoDrop 2000c (Thermo Scientific, USA) software. DNA working solutions (10 ng/mL) were prepared by diluting the samples in TE buffer and then were stored at -20°C.

Testing and selection of ISSR primers

Thirty primers were tested on 2% agarose gels (Table 1). Each test reaction included 1 µL genomic DNA (10 ng/µL), 1.0 µL each primer (5 mM), 14.8 µL MilQ sterile water, 2 µL 10X reaction buffer, 0.6 µL $MgCl_2$, 0.4 µL dNTP (10 nM), and 0.2 Taq polymerase (5 U/µL) in a total volume of 20 µL. The ideal annealing temperatures varied between 44.6° and 57.2°C.

Table 1. ISSR primers, their respective sequences, and annealing temperatures (At).

Primers	Sequence (5'-3')	At (°C)
UBC 807	AGA GAG AGA GAG AGA GT	47.0
UBC 809	AGA GAG AGA GAG AGA GG	57.2
UBC 810	GAG AGA GAG AGA GAG AT	45.4
UBC 811	GAG AGA GAG AGA GAG AC	46.8
UBC 812	GAG AGA GAG AGA GAG AA	55.8
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 817	CAC ACA CAC ACA CAC AA	50.3
UBC 818	CAC ACA CAC ACA CAC AG	57.2
UBC 820	GTG TGT GTG TGT GTG TC	47.5
UBC 823	TCT CTC TCT CTC TCT CC	57.2
UBC 826	ACA CAC ACA CAC ACA CC	57.2
UBC 828	TGT GTG TGT GTG TGT GA	54.8
UBC 834	AGA GAG AGA GAG AGA GYT	45.6
UBC 835	AGA GAG AGA GAG AGA GYC	50.2
UBC 841	GAG AGA GAG AGA GAG AYC	48.5
UBC 843	CTC TCT CTC TCT CTC TRA	56.5
UBC 845	CTC TCT CTC TCT CTC TRG	48.1
UBC 848	CAC ACA CAC ACA CAC ARG	52.7
UBC 851	GTG TGT GTG TGT GTG TYG	49.2
UBC 855	ACA CAC ACA CAC ACY T	53.1
UBC 856	ACA CAC ACA CAC ACA CYA	56.5
UBC 857	ACA CAC ACA CAC ACY G	58.8
UBC 858	TGT GTG TGT GTG TGT GRT	56.5
UBC 864	ATG ATG ATG ATG ATG ATG	50.8
UBC 878	GGA TGG ATG GAT GGA	53.4
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

Polymerase chain reaction (PCR), electrophoresis, and documentation

PCR was performed with 1 µL genomic DNA (10 ng/uL), 1.0 µL each primer (5 mM), 14.8 µL Milli-Q sterilized water, 2 µL 10X reaction buffer, 0.6 µL $MgCl_2$, 0.4 µL dNTP (10 nM), and 0.2 Taq polymerase (5 U/µL) in a total volume of 20 µL. Templates were amplified in a Proflex® Applied biosystems thermocycler (Foster City, CA, USA) with denaturation at 94°C for 4 min, followed by 37 amplification cycles. Each cycle included denaturation at 94°C for 45 s, annealing for 45 s, and extension at 72°C for 2 min. After the reaction cycles, there was a final extension at 72°C for 7 min, followed by cooling at 4°C. Reaction

products were electrophoresed on 2% agarose gels at 250V, 145 mA, and 120 W for 3 h. For size standardization of bands, 10 µL 100-bp molecular weight marker was used. The gel was stained in ethidium bromide solution for about 40 min. Subsequently, results were documented using a Gel Doc L-pix HE (Loccus Biotecnologia).

Identification of the optimal number of bands

In order to verify if the number of generated markers was enough to analyze the sample group of 50 genotypes, bootstrap simulations were conducted with resampling of different sizes with each simulation replicated 5000 times using the DBoot software (Coelho, 2001).

Data analysis

The absence (0) or presence (1) of fragments on each gel was visually analyzed, and a binary matrix was generated. Binary data were used in all the following tests. Estimates of genetic similarities were obtained from the binary matrix using the Jaccard coefficient with the FreeTree software (Pavlícek et al., 1999). Genetic diversity parameters, such as number of observed alleles, effective number of alleles, Nei's genetic diversity, and Shannon index, were calculated using the Genalex 6.3 software (Peakall and Smouse, 2006). The total number of alleles (N_A), the effective number of alleles (N_E), and the expected heterozygosity (H_E) were obtained following the methodology of Lynch and Milligan (1994) and Maguire et al. (2002). The Shannon genetic diversity index (I) was obtained using the procedure of Brown and Weir (1983). The dendrogram was generated with the UPGMA algorithm (unweighted pair group method with arithmetic mean) using Treeview software based on the Jaccard coefficient. To examine clustering, 10,000 bootstraps were performed using TreeView software (Page, 1996). Principal coordinates analysis (PCoA) was performed with the Genalex 6.3 software, using the Jaccard coefficient obtained from the FreeTree software (Pavlícek et al., 1999).

RESULTS and DISCUSSION

Among the 30 tested primers, 10 provided informative and polymorphic bands. In the amplification of samples, 71 loci were generated of which 70 were polymorphic (98.3%), varying from 4 (UBC 820) to 10 loci (UBC 848) per primer (Table 2).

Table 2. List of ISSR primers, total number of fragments, number of polymorphic fragments, percentage of polymorphic fragments, and range of bp generated by PCR for the study of genetic diversity among individuals of a population of cambui.

Primers	Number of fragments	Number of polymorphic fragments	Polymorphism (%)	bp range
UBC 807	7	7	100	500-1650
UBC 813	7	7	100	850-2000
UBC 818	7	7	100	500-2000
UBC 820	4	4	100	850-2000
UBC 834	6	6	100	1000-3000
UBC 835	9	9	100	850-2000
UBC 841	7	7	100	500-1000
UBC 845	7	7	100	400-2000
UBC 848	10	10	100	500-3000
ISSR 3	7	6	85	850-2000
-	71	70	98.3	-

Several studies using ISSR markers have successfully obtained polymorphisms allowing the evaluation of genetic divergence. Silva et al. (2014) found 123 polymorphic bands using 12 primers in a study on the diversity and genetic structure of jenipapo (*Genipa americana* L.). Lorenzoni et al. (2014) evaluated the genetic divergence of 16 biriba accessions [*Rollinia mucosa* (Jacq.) Baill] and obtained 118 polymorphic bands using 13 primers. Santana et al. (2011) characterized the genetic variability between umbu-cajazeira accessions (*Spondias* sp) from the Germplasm Bank of Tropical Fruits of Embrapa Cassava and Fruits and obtained 201 polymorphic bands using 25 primers. Jimenez et al. (2015) evaluated the diversity of 38 mangaba (*Hancornia* speciosa Gomes) individuals from natural populations in the state of Pernambuco and obtained 83 polymorphic bands using six primers.

In Myrtaceae, Oliveira et al. (2014) analyzed the genetic distance between accessions of guava and araça trees of the *Psidium* genus, belonging to the Germplasm Bank of the Universidade Estadual do Norte Fluminense, and obtained 216 polymorphic bands using 17 primers. In an additional study with guava, Nogueira et al. (2014) used 18 microsatellite markers in populations from Espírito Santo and Minas Gerais and found that there is genetic diversity among and within populations. Mani et al. (2011), in studies carried out in India on the association of morphological and molecular characterization by ISSR and RAPD between species of the *Psidium* genus, obtained 234 polymorphic bands using 31 ISSR primers.

In other economically important crops, the use of ISSR markers was also successful. Dhanorkar et al. (2005) obtained 96 polymorphic bands in the analysis of genetic relationships between grape varieties (*Vitis* spp) using 13 primers. Kar et al. (2008) studied the variability of mulberry (*Morus* spp) and obtained 85 polymorphic bands using 14 primers.

The number of polymorphic bands is considered to be optimum when the coefficient of variation is less than 1%. According to Silva et al. (2013, 2014), there is a directly proportional relationship between the number of fragments generated and the coefficient of variation (CV%), with a decrease in variation associated with an increase in the number of fragments (Figure 3). In this study, CV% stabilized beyond 60 fragments suggesting that the fragments used in this study (71) can be used for diversity analysis.

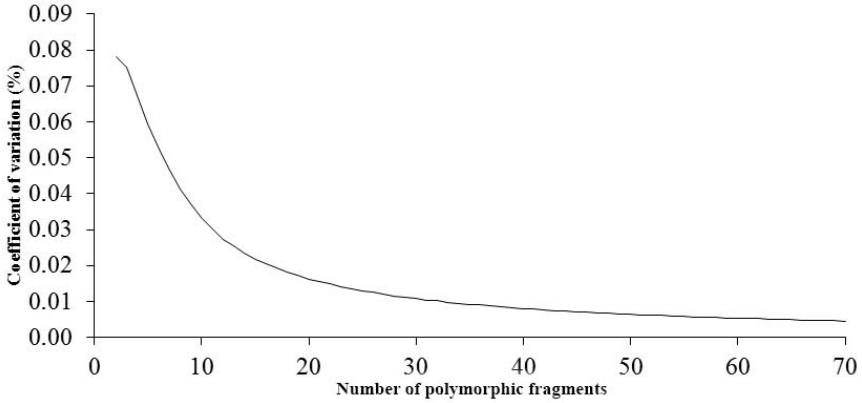

Figure 3. Coefficient of variation (CV%) as a function of the number of fragments in 50 individuals of cambui (*Myrciaria tenella* O. Berg) based on genetic similarities obtained by the bootstrap method with 5000 resamplings.

The total N_E ranged from 0.57 to 1.89, and the *I* values were 0.30 to 0.66. Genetic diversity (*H*) values ranged from 0.18 to 0.47 (Table 3). In this study, the *I* value obtained was 0.52. According to Padua and Ferreira (2008), the value of *I* may vary between 0 and 1, with a value of 1 indicating the maximum diversity of a population. Thus, the studied population possesses a considerable level of genetic diversity, which is a good candidate for population analysis using dominant (Dawson et al., 1995). In a study on genetic diversity in natural populations of buriti (*Mauritia flexuosa*), Rossi et al. (2014) found higher values for *I* and *H*, when evaluating the distribution of genetic diversity between versus within populations.

Table 3. Number of alleles (N_A), effective number of alleles (N_E), Shannon index (*I*), and genetic diversity (*H*) in the study of genetic diversity of a population of cambui (*Myrciaria tenella* O. Berg) using ISSR markers, where total represents the mean of each column.

Primer	N_A	N_E	I	H
UBC 807	2	1.80	0.63	0.44
UBC 813	2	1.89	0.66	0.47
UBC 818	2	1.42	0.43	0.27
UBC 820	2	1.69	0.54	0.38
UBC 834	2	1.73	0.60	0.42
UBC 835	2	1.55	0.51	0.34
UBC 841	2	1.52	0.46	0.30
UBC 845	2	1.56	0.52	0.35
UBC 848	2	0.57	0.57	0.39
ISSR 3	2	1.26	0.30	0.18
Total	2	1.50	0.52	0.35

In contrast to our results, Chagas et al. (2015) found a low mean value of *I* in the study of a palm population (*Elaeis guineensis* Jacq.) using ISSR markers and found low diversity between the analyzed individuals. Costa et al. (2011) observed low diversity between mangaba (*Hancornia speciosa* Gomes) accessions of Sergipe's germplasm bank, using the Shannon index based on molecular markers. Silva et al. (2016) evaluated diversity in commercial cupuaçu (*Theobroma grandiflorum* Schum.) and also found low values for the Shannon index and confirmed the uniformity of the crop.

Under natural conditions, the value of *H* is never zero since individuals may incorporate new alleles through crossing even in small populations or in fragments, in addition to losses due to genetic drift (Silva et al., 2014).

The occurrence of diversity among the individuals studied using ISSR markers suggests the efficacy of this technique for the identification of diversity with the most divergent individuals within a given group being C1 and C2. Overall mean similarity was 0.58. The lowest similarity was observed among individuals C1 and C41 (0.30), while the highest similarity was found among individuals C13 and C17 (0.92). The pairs of more similar individuals were C26 and C28 (0.80); C6 and C7, C36 and C38 (0.81); C12 and C13, C12 and C14, C13 and C14, C17 and C19, C34 and C35, C34 and C36 (0.82); C8 and C9 (0.83), C17 and C18 (0.84); C12 and C17, C14 and C17, C19 and C20, C36 and C39 (0.85); C18 and C19 (0.86), C7 and C8 (0,87); and C5 and C17, C27 and C28 (0.88).

Based on the Jaccard coefficient, six groups were identified by the UPGMA analysis (Figure 4). The most distant and different individuals are found in the G1 group, and C1 (0.31) and C41 (0.36) are totally isolated from other individuals. The largest group was G2 with 21 individuals, and the closest individuals were C13 and C17 (0.92). G3 had 12 individuals, and the pair of individuals C46 and C47 (0.88).

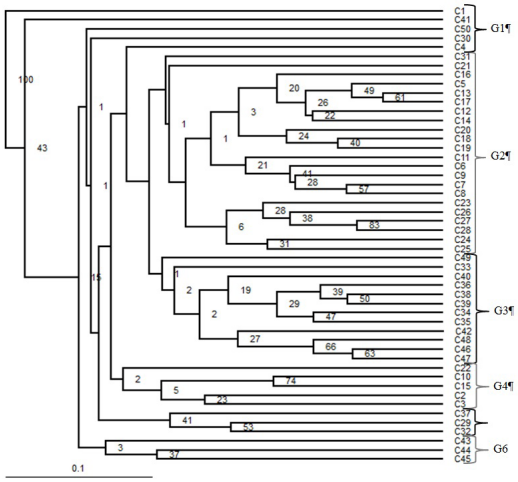

Figure 4. Phylogeny based on UPGMA estimated from the Jaccard coefficient (1908) of genetic similarity and bootstrap analysis (10,000X) for 50 individuals of a population of cambui (*Myrciaria tenella* O. Berg).

G4 has five individuals, with C22 (0.57 SJ) being the most distant. G5 and G6 groups were smaller with three genotypes each. A large distribution of genotypes was observed in most groups, indicating wide diversity between the evaluated genotypes. This high variability could be explained by the tendency of allogamy of species which have not yet been domesticated (Oliveira et al., 2009). This was also observed in the research of Santana et al. (2011), who found high variability between 17 umbu-cajazeira (*Spondias* sp) accessions, which is a species under domestication.

PCoA (Figure 5) was conducted to determine the genetic relationships between individuals minimizing variation. Eleven groups were formed in this clustering model and the subgroups were clear indicating greater differentiation of genotypes. Consistent with the UPGMA results, some pairs of individuals (C10/C15, C13/C17, C24/C25, C27/C28, C46/C47) were genetically close in the PCoA. This information is important for the establishment of an *ex situ* collection or a germplasm exchange, since in these cases, only one individual would be selected.

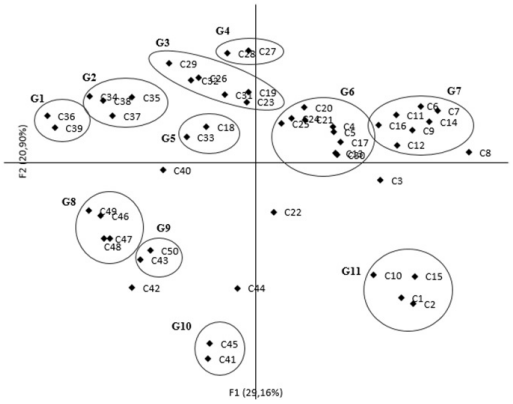

Figure 5. Principal coordinates analysis of 50 individuals of a population of cambui (*Myrciaria tenella* O. Berg).

The use of more than one grouping method, owing to the differences in hierarchical optimization and ordering of groups, enables the classification of individuals to be complemented by the criteria used by each technique, and prevents erroneous inferences from being adopted in the allocation of materials within a particular genotypic subgroup (Silva et al., 2011). The existence of high genetic variability was also observed by Pinheiro et al. (2011) using RAPD molecular markers in 20 individuals from a natural population of cambui in Sao Cristóvão, SE.

Knowledge of genetic diversity is fundamental for evolution, which results from changes in gene frequencies. In a germplasm collection, this information may provide useful data to assist in the conservation, management, and use of these genetic resources and may also help breeders to identify and select clones or relatives to establish a basic breeding program.

CONCLUSIONS

There is genetic diversity in the evaluated natural population of cambui. The level of genetic resolution and reliability obtained by the analysis using ISSR markers allowed the discrimination of genetically different genotypes, which may be used for the management of genetic resources of the species. For formation of *ex situ* germplasm collections and germplasm exchange, the genotypes C1, C41, C50, C30, and C4 are recommended owing to them being more genetically distant.

Conflicts of interest

The authors declare no conflict of interest.

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