ABSTRACT

The species Myrciaria floribunda O. Berg, popularly known as cambuí, belongs to the Myrtaceae family. Cambuí is a native, non-endemic species that occur in diverse environments in Central America and South America. They are slow-growing plants with a shrub or sub-shrub habit. The fruits, the product of interest of the species, are small, spherical berries orange or red in color turning to wine color when they are ripe. The exploitation of the species is still mostly extractivist, carried out by traditional local families who, in times of fruiting of the species, leverage their income by selling fruits at fairs. The fruits can be eaten fresh, in the form of jellies, liquor or wine. To study the genetic diversity of the species using ISSR-type molecular markers, it is necessary to first isolate DNA in sufficient quality and quantity. Here, leaves for DNA extraction were collected from the active germplasm bank of the Federal University of Alagoas, Brazil. The DNA of the species was extracted using CTAB detergent methodology with modifications adapted to the species. Twelve ISSR primers were tested on DNA from two cambuí genotypes. Of the twelve primers, eight were selected due to their polymorphism index above 50%, namely: UFAL-2, UFAL-3, UFAL-5, UFAL-6, UFAL-7, UFAL-8, UFAL-9 and UFAL-10.
Keywords: Marcadores moleculares; variabilidade; genética molecular.

1. INTRODUCTION

1.1 The Cambuí (Myrica floribunda O. berg)

The species Myrciaria floribunda belongs to the Myrtaceae family. With pantropical distribution, the Myrtaceae family comprises approximately 133 genera and over 3800 species. The centre of diversity in Australia, South Asia, the Neotropical region and a small portion in Africa [1]. The main botanical characteristics that distinguish the family are complete leaves with oil glands, semi-inferior to the inferior ovary, internal phloem, usually numerous stamens, and vessels with coated holes [1].

Cambuí is a native, non-endemic species that occur in diverse environments in Central and South America [2]. The plants grow slowly with a shrub or subbustive habit. Cambuí has cimosas? sympodial branching inflorescences composed of small white flowers. The fruits are berry type, small, spherical in color orange, red and wine color when ripe. In a characterization work carried out with populations along the coast of Alagoas, Araújo [3] fruit diameters can reach 12.18 mm, with an average weight of up to 1.43 g and pulp yield of 76.19%.

The exploitation of the species is mostly extractive. According to Gama et al. [4], the extraction of cambuí is generally performed by local traditional families and represents significant economic and social importance, composing an important part of the income of these families at harvest time. The fruits are consumed directly or used to manufacture juice, jellies, liqueurs and wines [5].

1.2 Extraction Protocol

The development of molecular studies depends directly on the quality of the extracted DNA, making optimization and establishment of extraction protocols necessary: A good extraction procedure should produce DNA of purity, quality and adequate quantities for manipulation (VTC, 1998).

The presence of polysaccharides, phenols and secondary compounds represent the main problem found in plant DNA purification, primarily due to the action these contaminants have on the activity of Taq DNA polymerase enzyme. Leaves of various plant species have varying levels of polysaccharides that are inhibitors of the PCR reaction and the protocols traditionally used for extracting plant DNA, although removing proteins, do not always effectively remove these polysaccharides [6].

Optimization of an extraction protocol is necessary when standard protocols that are widely used for many species, including CTAB-based protocols. The goal of optimization is to develop a fast, simple and well-reproducible protocol.

1.3 ISSR Markers

Molecular markers have been widely used in studies of diversity, characterization, phylogeny, population structure, among others. Dominant markers of the Inter Simple Sequence Repeat (ISSR) type are 100 to 3,000 base pair DNA segments and are based on the PCR technique [7]. These markers have a high degree of polymorphism, have good reproducibility and have low cost for development since it is not necessary to have prior knowledge of the DNA sequence to develop the primer used [8].

The regions amplified by ISSR markers are abundant and are distributed throughout the genome. Because they have low specificity and generally access different polymorphic loci, the ISSR markers are an important tool for genetic diversity studies, especially in species that are little explored in this respect, since, no prior knowledge of the DNA to be evaluated is required to generate multiple amplicons from varied loci [9].

2. MATERIALS AND METHODS

2.1 Material Collection

The material was collected at the active germplasm bank, located at the Center for Agricultural Sciences (CECA) of the Federal University of Alagoas (UFAL). The active germplasm bank is curated by Professor Eurico Eduardo Pinto de Lemos.

At the time of collection, plants with a higher presence of young leaves were prioritized. With the help of pruning shears, leaves were collected from the ends of the branches and soon after, the leaves were transferred to Falcon type tubes
(50 ml) and given an alphanumeric identification. After being identified, the tubes were placed in a thermal box in the presence of ice.

The collected material was transported to the Population Genetics Laboratory (LGP) of the Federal Rural University of Pernambuco (UFRPE). Upon arrival at the LGP, the tubes were transferred to a -20°C freezer and remained stored at the disposal of the research group (Fig. 1).

### 2.2 Optimization of DNA Extraction

For extraction of individuals collected in the field, 39 in total, 150 mg of leaf were macerated in a mortar in the presence of 1.3 µL of extraction buffer (2% CTAB). After a maceration of the samples, 2-Mercaptoethanol was added with proteinase K and the material was transferred to Eppendorf tubes and placed in a water bath at 60°C for one hour. After being removed from the water bath, the material cooled to room temperature and 650 µl ice-cold CIA (chloroform: isoamyl alcohol - 24: 1) was added and the tubes were gently shaken by hand until an emulsion formed. After this step, the tubes were centrifuged at 12,000 RPM for 5 minutes at room temperature. After centrifugation, the upper aqueous phase of each tube was collected and transferred to a new Eppendorf tube where 200 µL of extraction buffer and 650 µL of CIA were added, the tubes were shaken again, centrifuged at 12,000 rpm for 5 minutes and the upper aqueous phase collected again and transferred to a new Eppendorf tube. The previous step was repeated one more time and after transferring to a new Eppendorf tube the samples received the 650 µL volume of ice-cold Isopropanol and remained in a freezer at -20°C overnight.

The next day, the tubes were centrifuged at 12,000 rpm for 5 minutes and all surplus was discarded, leaving only the pellets in the tubes. Two washes were performed with one mL of 70% ethanol at room temperature and the samples were centrifuged for 3 min at 12,000rpm. After washing, the pellets were dried in the tubes at room temperature and resuspended in 40 µL of ultrapure water and placed in a freezer at -20°C (Fig. 2).

### 2.3 Electrophoresis

The extracted DNA was submitted to agarose gel horizontal electrophoresis to analyze the quantity and quality of the obtained material. The agarose gel was prepared to a final concentration of 1% in 0.5x TBE. Electrophoresis was for 20 minutes at 80 V.

The 39 extracted DNA samples were applied to the same 40-well gel. In each well 3.5 µL of DNA with 0.8 µL of Blue-Green dye was applied. The result of the electrophoresis was visualized with the aid of an LED-type transilluminator.

### 2.4 ISSR Primer Selection

A total of 12 available ISSR primers were used to select those whose markers were more polymorphic for the study of *M. floribunda* genetic diversity. For this, the DNA of nine preselected samples was submitted to PCR amplification with the primers.

![Fig. 1. Samples of leaves collected from cambui, kept in the freezer in LGP](image-url)
For each reaction, 25 ng template DNA, 1x Taq PCR buffer, 2 mM MgCl$_2$, 0.2 mM each dNTP (Synapse), 0.4 mM primer (Table 1) and 1 U Taq were used for each reaction. DNA Polymerase (Synapse) to a final volume of 15 µL. All reactions were performed in a Biogener thermocycler and underwent an initial denaturation of 94°C for 4 minutes, followed by 40 cycles composed of the denaturation steps at 94°C for 1 minute, annealing at 46°C for 1 minute and extension at 72°C for 2 minutes. Finally, a final extension step of 72°C was performed for 5 minutes. Negative controls consisting of all reagents except template DNA were performed in each experiment to verify possible contamination.

The amplified products were separated by horizontal electrophoresis in 2% agarose gel, submerged in 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA) under constant voltage of 80V for 70 minutes. The samples were directly stained with Blue Green loading dye I. At the end, they were visualized under LED light and photographed with a digital camera (Major Science). The Ladder 100 bp Plus molecular weight marker (Synapse) was used to compare the size of the produced bands.

### 3. RESULTS AND DISCUSSION

Quality of the genetic material isolated from the samples was assessed visually. Extraction did not work for all samples. A common factor was noted among the samples from which we did not obtain a clear and full band: These were samples of old and dark leaves. The remaining samples presented clear and integral bands (Fig. 3). In works related to DNA extraction from leaf tissue, the authors advise prioritizing young, tender and fresh-looking leaves in order to avoid higher concentrations of phenols and polysaccharides, which negatively affect the isolation process. DNA development [10].

From the 12 primers evaluated, three showed no amplification product. Of the nine primers that amplified, all were polymorphic (Fig. 4). The number of bands ranged from two to seven, with an average of four bands. The band length ranged from 2000 to 400 base pairs. Regarding polymorphism, the average of the primers that amplified was around 80%, a result similar to that found by Santana et al. [11] when working with umbu-cajazeira, applying ISSR markers to study the genetic diversity of the species.

In studies that are developed using dominant markers such as ISSR, the percentage of polymorphic loci is considered as a measure of genetic diversity [12]. Based on this information, primer selection is performed based on the percentage of polymorphic bands presented by the primer, these results can be seen in Table 1. Primers UFAL-2, UFAL-3, UFAL-7, UFAL-9, UFAL-10 had a 100% polymorphic amplification standard. The highest number of bands was obtained by amplifying the primers UFAL-5, UFAL-6, and UFAL-8 with 7 amplified bands with equal polymorphism index 57%, 71% and 71% respectively.
Fig. 3. Agarose gel electrophoresis of genetic material obtained from the DNA extraction protocol. Samples were compared against marker (M) with a 100 base pair ladder.

Fig. 4. Agarose gel electrophoresis with material amplified by eight of the twelve primers analyzed. The first and last wells of the gel received the 100 base pair ladder for comparative analysis of the band lengths. The first eight wells after the marker are for the 2G genotype, and the next eight are for the 4G genotype.

Table 1. Results of amplification of the ISSR markers used, with the number of bands amplified, number of polymorphic bands and polymorphism index calculated between the proportion of polymorphic bands and the number of bands amplified by the primer.

| Primers | Nº of alleles | Nº of alleles polymorphic | %P |
|---------|---------------|----------------------------|-----|
| UFAL-1  | 0             | 0                          | 0   |
| UFAL-2  | 4             | 4                          | 100 |
| UFAL-3  | 4             | 4                          | 100 |
| UFAL-4  | 6             | 1                          | 17  |
| UFAL-5  | 7             | 4                          | 57  |
| UFAL-6  | 7             | 5                          | 71  |
| UFAL-7  | 4             | 4                          | 100 |
| UFAL-8  | 7             | 5                          | 71  |
| UFAL-9  | 3             | 3                          | 100 |
| UFAL-10 | 2             | 2                          | 100 |
| UFAL-11 | 0             | 0                          | 0   |
| UFAL-12 | 0             | 0                          | 0   |
4. CONCLUSION

Primers UFAL-2, UFAL-3, UFAL-5, UFAL-6, UFAL-7, UFAL-8, UFAL-9 and UFAL-10 can be used as molecular markers for genetic diversity study with the M species floribunda O. Berg.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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