MORPHOLOGIC AND MOLECULAR CHARACTERIZATION OF *Myrciaria* spp SPECIES[1]

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ABSTRACT - The *jabuticaba* tree is considered one of the most typical Brazilian fruit trees. However, few studies of this plant are found in the literature and even its botanical classification is very controversial. The present research reports some comparisons between *jabuticaba* species, using morphologic (organography) and molecular markers techniques. The morphologic characteristics of the plant used as markers were compared with specimens of the herbaria from São Paulo and Minas Gerais States and with the descriptions reported in the literature. Molecular differences between the species were identified by the use of RAPD markers. The experiment was made in Piracicaba, Jaboticabal and Ituverava municipal districts in São Paulo State, Brazil. Morphologic and molecular differences between the studied plants were identified and four groups of species were defined: *Myrciaria cauliflora* (Mart.) O. Berg, *M. coronata* Mattos, *M. jaboticaba* (Vell.) O. Berg. and *Myrciaria phytrantha* (Kiaersk.) Mattos. Both molecular and morphologic markers techniques showed to be important tools for the identification of *jabuticaba* tree species.
Index Terms: *jabuticaba* trees, biological identification, morphologic markers, molecular markers, RAPD.

CARACTERIZAÇÃO MORFOLÓGICA E MOLECULAR DE ESPÉCIES DE *Myrciaria* spp

RESUMO - A jabuticabeira é considerada uma das fruteiras mais típicas do Brasil. Entretanto, há poucos estudos sobre esta planta na literatura, e mesmo sua classificação botânica é muito controvertida. Este trabalho faz comparações entre as espécies de jabuticabeiras, usando as técnicas de marcadores morfológicos (organografia) e moleculares RAPD. As características morfológicas das plantas, usadas como marcadores morfológicos, foram comparadas com espécimes presentes nos herbários dos Estados de São Paulo e Minas Gerais e com as descrições obtidas em revisão de literatura especializada. As diferenças moleculares entre as espécies foram determinadas por meio do uso de marcadores RAPD. O experimento foi realizado nas cidades de Piracicaba, Jaboticabal e Ituverava do Estado de São Paulo, Brasil. Diferenças morfológicas e moleculares entre as plantas estudadas foram identificadas, e quatro grupos distintos de espécies foram definidos: *Myrciaria cauliflora* (Mart.) O. Berg, *M. coronata* Mattos, *M. jaboticaba* (Vell.) O. Berg. e *M. phytrantha* (Kiaersk.) Mattos. A técnica de marcadores moleculares, aliada à técnica de marcadores morfológicos, mostrou ser uma ferramenta importante na identificação de espécies de jabuticabeiras.
Termos para indexação: Jabuticabeiras, identificação biológica, marcadores morfológicos, marcadores moleculares, RAPD.

According to Mattos (1983), the *jabuticaba* trees have never been extensively studied under the taxonomic point of view. There is a lot of confusion concerning these plants popular names and sometimes the same name is used to different species and even to different genera, depending on the region. Many morphologic characteristics are considered to identify these species (Donadio, 2000). Undoubtedly such descriptors are more restricted, since the use of controlled conditions is necessary to minimize the natural variability. The development of genetic techniques based on DNA markers might be a useful tool in this case.

Individual organisms differ in its sequences of DNA and such variation can be considered at the level of individual genes or genotypes. The processes that affect the individuals, affect also the population, influencing the species and interfering in the taxonomic hierarchy (Sunnucks, 2000). According to the same author, the selection of the most adequate technique for genetic analysis is essential for the success of the individual's molecular identification study. The great usefulness of molecular techniques is the possibility of detecting small differences with high-resolution level, which could not be known when only phenotypic characteristics are used and the procedures to be used require appropriate techniques, like the polymerize chain reaction (PCR), which is one of the most important.

According to Williams et al. (1993), the RAPD method (Random Amplified Polymorphic DNA) actually introduce a democratization in the molecular polymorphism analysis, allowing the accomplishment of genetic analysis studies in species not previously contemplated.

For the exposed, and trying to contribute to the *jabuticaba* tree identification, the present research aimed to identify *jabuticaba* tree species using techniques of morphologic markers (organography) as well as molecular markers.

The botanical material used in the identification of the *jabuticaba* species (*Myrciaria* spp.) was collected using the procedure recommended by the São Paulo Botany Institute (Instituto de Botânica, 1989).

The *jabuticaba* tree flowers were conserved, due to its delicate texture, in Hammarlund liquid [saturated copper sulfate solution + 40% formaldehyde (= formalin) + distilled water in the proportion of 3:0.1:1]. Fruits from some plants were collected and conserved in 5 %-aqueous copper sulfate solution, in 5-6 %-aqueous sulfurous acid solution and glycerin.

The flowers were dried between paper towels, pressed and taken to dry up in a forced air oven in the Botany Laboratory at the "Dr. Francisco Maeda" (FAFRAM) College, Ituverava, São Paulo State, Brazil. The dried flower sample was put in the paper bag of the herbarium specimen assembly cardboard, which received an identification label on the lower right corner and a small envelope fastened on the left higher corner that contained the dried plant parts that might eventually be broken during the drying process to complete the study.

In a total of 31 individual plants were collected and identified as follow: samples from the Horticulture Section Orchards of the Plant Production Department - University of São Paulo (USP), at Piracicaba (SP) received the codes Pira1, Pira2, Pira3, E01, E02, E03, E04, P01, and P02; samples from the Engineering Section orchards of the Plant Production Department - USP, at Piracicaba (SP), received the codes P1A, P2A, P3A, P4A, P5A, P6A, P7A, P8A, P9A, and P10A; and the samples from the Fructiferous Orchard Production Section – FAFRAM, Ituverava (SP), the codes 1I, 2I, 3I, 4I, 5I, 6I, 7I, 8I, 9I, 10I, 11I and 12I.

The collected and dried herbarium specimens material was

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compared with other specimens of the chosen herbaria list and with the literature.

Approximately 180 mg of young leaf fresh tissue of 31 plants were collected for molecular analysis, from each plant, and placed in small paper bags, properly identified with the plant code and stored in containers with liquid nitrogen at – 86 ºC. Afterwards, samples were transported to the Applied Biotechnology and Bacterial Genetics Laboratory at UNESP-Jaboticabal (SP), for DNA extraction.

The DNA extraction of the leaves followed the procedures described by Lodhi et al. (1994), modified by the Applied Biotechnology and Bacterial Genetics Laboratory at UNESP- Jaboticabal (SP) staff, where, 0.1 g of fresh plant tissue was grinded under liquid nitrogen. Afterwards, 1 mL of extraction buffer (20 mM EDTA; 100 mM Tris-HCl, pH adjusted to 8.0, using HCl also containing 1,4 M NaCl and 2% (v/v) of CTAB) was added together with 0.2% of β-mercaptoethanol and the mixture was transferred to a 2 mL microcentrifuge tube. Ten mg of PVP (polyvinylpolypirrolidine) (Sigma, P6755) was added and gentle tube mix was carried out with further incubation at 60°C during 65 min and latter left at room temperature.

After this incubation 1 mL of a mixture of chloroform/isoamylic alcohol (24:1) was added with gentle tube shaking, followed by a centrifugation at 10,621 x g during 15 min at room temperature. The supernatant was transferred to a new centrifuge tube where 0.5 vol of a solution of NaCl (5M) was added and 1 mL of cold (-20°C) ethanol 95%. The solution was kept at -80°C for 20 min for total nucleic acid precipitation and latter centrifuged at 4,460 x g during 5 min and, then centrifuged at 10,621 x g for 5 min at 4°C so as to collect the pellet. The supernatant was discharged and the pellet was washed using cold (4°C) ethanol 75% during 1 min and latter centrifuged at 10,621 x g for 5 min at 4°C.

After discharging the ethanol the nucleic acid pellet was dried and latter resuspended in 50 µl of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8,0), and treated with 10 µl of RNAse at a concentration of 10 mg/mL and incubated at 37°C during 30 min. The DNA samples were saved at -20°C for latter use.

The DNA content samples were quantified in the spectrophotometer DU 640B (Beckman), diluted in the proportion of 2 ìL of the DNA stock solution in 98 ìL of TE 10:1 (v/v).

The quantification using the spectrophotometer method allows estimating the DNA purity by the average readings taken at 260 and 280 nm. Pure DNA preparations have coefficient values in the range of 1.8 to 2.0. Values below 1.8 indicate contamination of the nucleic acid with protein (Sambrook et al., 1989). To evaluate the amount of total DNA obtained it was used a standard solution, for which an absorbance unit at 260 nm is equivalent to 50 mg of DNA per ìL of solution. The final concentration used as work solution was 10 ng $ìL^{-1}$, necessary for the RAPD reactions.

The extracted DNA quality analysis was run in electrophoresis of agarose gels at 0.8 %, to which 10 ìL of sample-DNA and 3 ìL of buffer (Tris - 0.1 mol L^{-1} HCL, pH 6.8; 0.02% bromophenol blue; 50% glycerol) were added. The electrophoresis band pattern comparisons were made using 8 ìL of GIBCO/BRL "1Kb Plus DNA Ladder". The buffer used in the preparation of gel electrophoresis was Tris-borate-EDTA 1X (89 mmol L^{-1} Tris, 1 mmol L^{-1} H_3BO_3, 2.5 mmol L^{-1} EDTA, pH 8.2) containing 0.5 mg ì L^{-1} of ethyl bromide. The electrophoresis time was approximately 2 hours under 48V tension. The gels were stained with ethidium bromide and the genomic DNA fragments were visualized under UV-light and registered in a photorecorder model GEL DOC 2000 (BIO RAD).

The proceedings for DNA amplification reactions, as well as the Polymerase Chain Reaction (PCR) analysis with selected primers, were the same as described by Williams et al. (1990). The primers used in this work came from the collection of the University of British Columbia - Nucleic Acid - Protein Service Unit (Canada), whose access numbers to the collection, as well as the respective sequences, are described in Table 1.

The reactions were run in a 20 µL-volume containing: 30 ng of the DNA to be amplified, 0,2mM dNTPs solution, 1.0 mM $MgCl_2$, 10mM Tris-HCl, pH 8.3; 50mM KCl, 15 ng of each initiator, 1.0 U Taq DNA-polymerize and distilled water degree Milli Q (previously sterilized). The

TABLE 1 - Arbitrary sequences of the initiators used and respective accesses numbers.

201.	CTG GGG ATT T	226.	GGG CCT CTA T
202.	GAG CAC TTA C	227.	CTA GAG GTC C
203.	CAC GGC GAG T	228.	GCT GGG CCG A
204.	TTC GGG CCG T	230.	CGT CGC CCA T
205.	CGG TTT GGA A	231.	AGG GAG TTC C
206.	GAG GAC GTC C	232.	CGG TGA CAT C
208.	ACG GCC GAC C	233.	CTA TGC GCG C
209.	TGC ACT GGA G	234.	TCC ACG GAC G
212.	GCT GCG TGA C	235.	CTG AGG CAA A
213.	CAG CGA ACT A	236.	ATC GTA CGT G
218.	CTC AGC CCA G	247.	TAC CGA CGG A
219.	GTG ACC TCA G	248.	GAG TAA GCG G
220.	GTC GAT GTC G	279.	AGA CAT TAG A
223.	GAT CCA TTG C	286.	CGG AGC CGG C
225.	CGA CTC ACA G	296.	CCG CTG GGA G

reactions were performed in a thermocycler MJ Research, model PTC 100, equipped with "Hot Bonnet" circuit. The program adopted for this analysis step was as follow: 4 min at 92 ºC and after, 48 cycles of 1 min at 92 ºC; 1 min 30 s at 37 ºC; 1 min 30 s at 72 ºC; and, in the end, 5 min at 72 ºC.

The fragments amplified by PCR and separated by electrophoresis were stained with ethidium bromide and photographed under UV light (GEL DOC). The molecular weight DNA ("1 Kb ladder") was used and the absence or the presence of electrophoresis migration of the same fragments as well as their distances, were evaluated.

The RAPD marker profiles were determined by direct comparison of the amplified DNA electrophoretic profiles and the data obtained were analized in the form of binary variables (band presence or absence). Each locus can be treated as a two-allele system, with only one of the allele per locus being amplifiable by the PCR. We also assumed that marker alleles from different loci do not comigrate to the same position on a gel (Lynch and Milligan, 1994).

Genetic similarity dendrograms among the populations of each river section were constructed by using the Jaccard (J) coefficient and the UPGMA cluster analysis algorithm in the NTSYS-PC (Rohlf, 1992) computer program.

Comparing the collected specimens using both the specialized descriptors of Mattos (1983) and the specimens conserved in the visited herbaria, it was possible to group the studied plants in four species: *M. jaboticaba*, *M. coronata*, *M. cauliflora*, *M. phitrantha* and one genus *Psidium*. It was evidenced, from the taxonomic point of view, that the species of *jabuticaba* trees are not well known, once just two species were found in the dried herbarium specimens of all consulted collections: *M. jaboticaba* and *M. cauliflora*.

The revision research evidenced also that the collections have a lot of material of the *Myrciaria* genus, however the described species of *jabuticaba* trees were restricted into two groups in the herbaria of São Paulo State.

The Pira 1 coded plants were initially identified as *Myrciaria* by the field-workers at Piracicaba (SP), and later were identified as *Psidium.* In spite of some herbaria have in their older collections of *Mirciaria* some *jabuticaba* trees with divergent classifications, there is a great confusion between the species *M. cauliflora* and *M. jaboticaba*.

Even in the literature this confusion can be noticed. Thus, in a research with *jabuticaba* trees collection belonging to Viçosa, Minas Gerais (MG) State, Mendonça (2000) classified *M. cauliflora* (Mart.) O. Berg. as *Cultivar Açú* and *M. jaboticaba* (Vell.) O. Berg as *Cultivar Sabará.* Pio Corrêa (1984), in the classic dictionary of brazilian useful plants, has classified *M. jaboticaba* as *Cultivar Açú* and *M. trunciflora* O. Berg. as *Cultivar Sabará*.

Samples belonging to the species *M. jaboticaba* (Vell.) O. Berg,

presented bicarpel ovary, infra-axillary placentation, pilose on the base, style surpassing the stamens, captured stigma, numerous stamens, flat terminal branches, green superior and light green lower leaf surfaces, pentamerous actinomorphe corolla, and globular 1.6 to 2.2 cm-diameter fruits, with smooth and black surface when ripe, containing one to four seeds. These characteristics are in agreement with the descriptions of Mattos (1983).

The species *M. cauliflora* (Mart.) O. Berg presented bicarpel ovary, infra-axillary placentation, glabrous, 6 mm-length style, peltate stigma, flat terminal branches and leaf central veins slightly engraved in the higher leaf surface and prominent in the lower leaf surface, pentamerous actinomorphe corolla, glabrous floral buds, 2.2 to 2.8 cm-length and 2.2 to 2.9 cm-diameter globular fruits.

The *M. phitrantha* (Kiaersk.) Mattos, presents bicarpel ovary, infra-axillary placentation, long and glabrous style, pentamerous actinomorphe corolla, large "neck" fruits (3 to 4 cm diameter) and large leaves as main characteristic and pendants.

The specimens of the species *M. coronata* presented a captured stigma, bicarpel sericeous ovary, with axillary placentation, flat and grizzly terminal branches, main leaf vein engraved in a higher surface and prominent in a lower leaf surface, globular fruit and whitish contour of the apex disk.

The 11 selected primers generated 45 polymorphic bands in the RAPD analysis. The most polymorphic primers were 203 (figure 1) and 226.

Two main groups (A and B) are observed in Figure 2: Group B had just two plants, with an 80 % genetic dissimilarity, approximately.

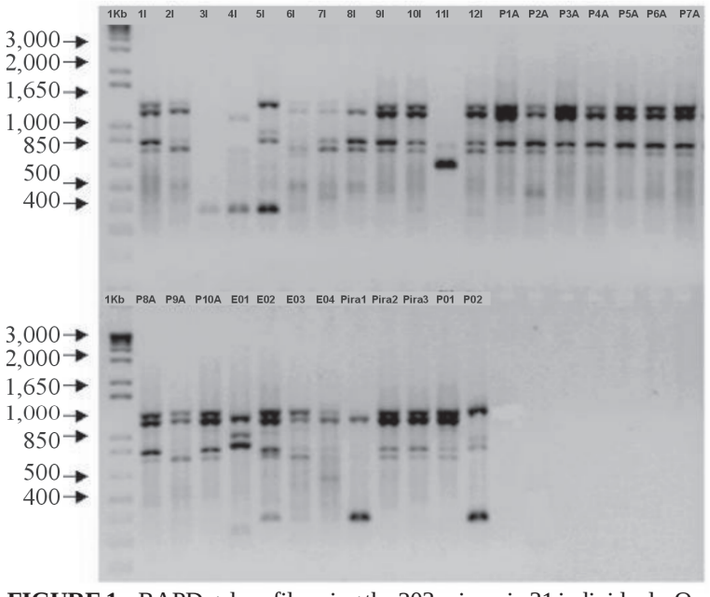

FIGURE 1 – RAPD gel profile using the 203 primer in 31 individuals. On the left in the molecular weight pathern (Ladder 1 Kb).

The plant of code "4I" was morphologically classified as *M. coronata* Mattos; the plant of code "Pira 1", was at first collected as a *Myrciaria* and later identified as belonging to the Myrtaceae family of the genus *Psidium* spp., confirming the taxonomic divergence for these families.

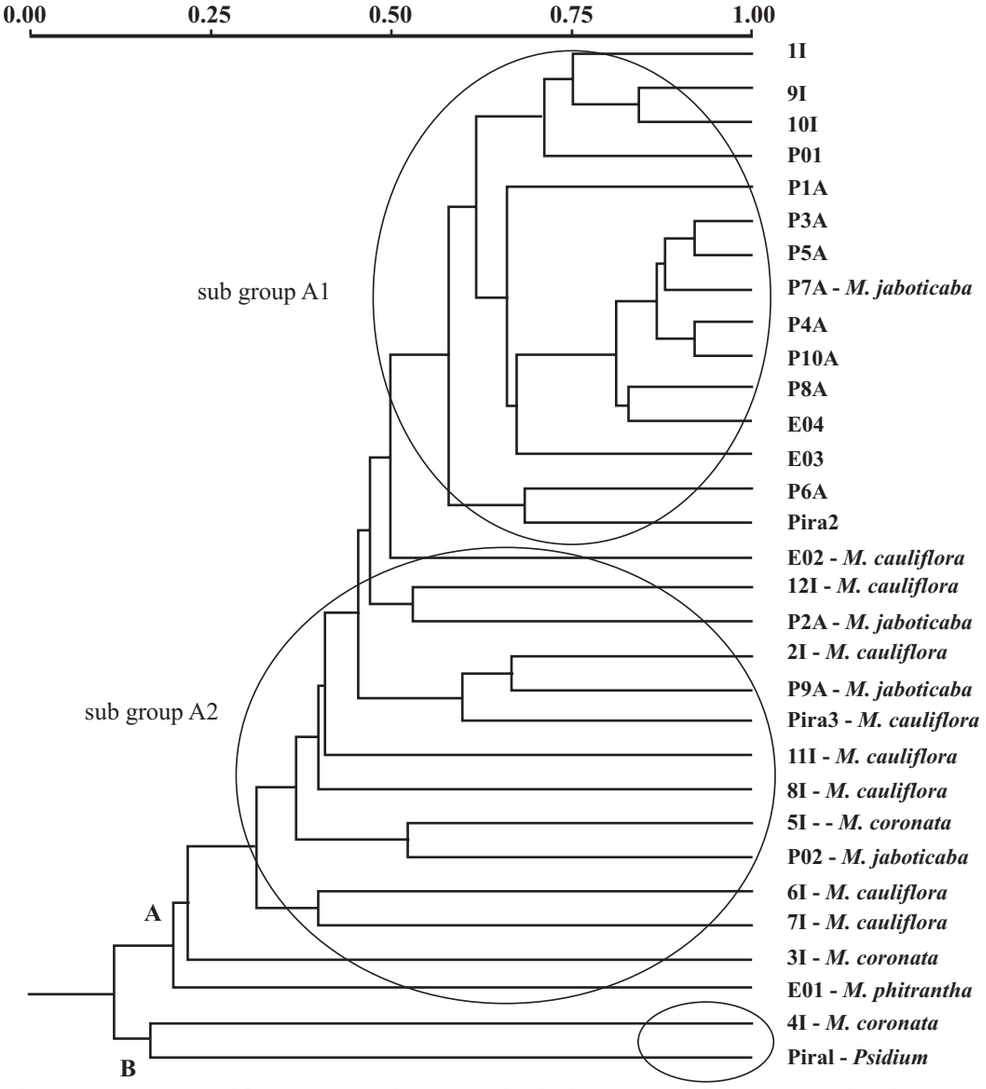

FIGURE 2 – Genetic similarity pattern obtained for 31 "jabuticaba" tree individuals. UPGMA clustering based on the Jaccard similarity index.

Other species (*M. jaboticaba*, *M.cauliflora*, and *M. phitrantha*), beyond of *M. coronata* are distributed in grupo A. Inside this group, the formation of a sub-group is observed (A1), including the majority of plants classified as *M. jaboticaba*, with a similarity degree varying from 60 % to 90 %. Another sub group (A2) was formed with species of *M. cauliflora*, *M. coronata*, and *M. jaboticaba*, with non-similarity degrees, varying from 80 % to 30 %, however, with very close genetic characteristics.

It was observed in the dendrogram that the species *M. phitrantha* Mattos presents a genetic divergence of 80 % in relation to other species, showing an isolated branch separated from the other groups.

In this context, it was concluded that RAPD markers, did not allow the grouping of plants at the species level, but it allowed to analyze, by means of the dendrogram, the degrees of genetic similarity among them. Thus, the species *M. phitrantha* was farther distant from the other species, as well as the plant identified as *Psidium* spp., initially identified as *Myrciaria.*

The studied material was compared with older collections of *Myrciaria*, in which divergent classifications were found, mainly between *M. cauliflora* and *M. jaboticaba*. This corroborated the analysis of the subgroup A2 that showed most of the grouped plants belonging to the species *M. cauliflora*, except for the P2A, P9A, and P02 plants, morphologically classified as *M. jaboticaba*.

The species *M. coronata* (3I, 4I, and 5I) were morphologically classified just based on the literature descriptors (Mattos, 1983), since no dried herbarium specimens of this species were found in the visited herbaria.

Observing the dendrogram, it was evidenced the largest genetic distances among the plants of subgroup A2, confirming the difficulty of an actual morphologic classification.

The data obtained in this research with the recent molecular techniques allowed suggesting the need for a revision on the *Myrciaria* genus classification, as well as on the dried herbarium specimens deposited in the visited herbaria.

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