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Genetic diversity of a native population of *Myrcia ovata* (Myrtaceae) using ISSR molecular markers

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ABSTRACT. *Myrcia ovata* Cambess. (Myrtaceae) is a medicinal and aromatic plant that has analgesic, bactericidal and fungicidal properties. Even though this plant has economic potential, nothing is known about the variability and genetic diversity of this species. This information is necessary to establish conservation strategies and allow prospection of natural resources. The aim of this study was to evaluate the genetic diversity of *M. ovata* individuals of a native population in the municipality of Japaratuba, Sergipe State, Brazil, using Inter-Simple Sequence Repeat molecular markers (ISSR). Nine primers were tested, resulting in 99 polymorphic bands. The 24 individuals evaluated were clustered in two groups by the software Structure. The Jaccard similarity ranged from 0.21 (MYRO-034 and MYRO-159) to 0.82 (MYRO-178.1 and MYRO-178.2), with an average of 0.38. The genetic diversity of *M. ovata* was considered of medium level. The individuals MYRO-154, MYRO-175 and MYRO-175.1 presented the most variability.

Key words: Myrtaceae; Medicinal plant; Genetic variability

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INTRODUCTION

The *Myrcia* genus is composed of more than 300 species and belongs to the subtribe Myrciinae of the Myrtaceae family. *Myrcia ovata* is an aromatic and medicinal species originally from the South American tropics. In Brazil, it is popularly known as laranjinha-do-mato (small forest orange) and is used in traditional medicine against diseases such as gastritis and diarrhea (Limberger et al., 2004; Lucas et al., 2007, Cândido et al, 2010). *Myrcia ovata* has been documented in the Brazilian states of

Alagoas, Amazonas, Bahia, Ceará, Espírito Santo, Mato Grosso, Pará, Pernambuco, Rio de Janeiro, São Paulo (SpeciesLink, 2018) and Minas Gerais (Torres, 2012). In Sergipe, plants of this species were found in an area exposed to human disturbance and fire, therefore, at risk of becoming locally extinct.

Works involving *M. ovata* are quite recent, mainly after 2010, when it was proven that its essential oil has antibacterial activity, thus generating economic interest (Cândido et al., 2010). Since then, new studies discovered that it also has analgesic (Santos et al., 2014) and fungicidal properties (Sampaio et al., 2016).

Several species of the Brazilian flora have already been studied with the aim of obtaining bioactive molecules potentially useful to man. Unfortunately, many other species became extinct before their capabilities were evaluated. The anthropic destruction of forests and natural ecosystems, the habitat of numerous medicinal species, justifies the need to conduct research that will aid in the conservation of these resources currently in risk of genetic erosion. The establishment of conservation and management strategies to maximize the genetic variability within species is only possible through the measurement of the genetic variability in the populations (Lima et al., 2015a).

A study of the chemical diversity in *M. ovata* essential oil was already done in plants from the Brazilian state of Sergipe (Sampaio et al., 2016). However, this is the first study that characterizes the genetic diversity of the species. This characterization identifies the degree of polymorphism between individuals and populations, regardless of the phenotypic variation and the stage of development (Grattaplagia and Ferreira, 1998).

The analysis of genetic variation of individuals can be obtained by molecular characterization using molecular markers. Developed in the 90s, the molecular marker Inter Simple Sequence Repeats (ISSR) is a dominant marker (binary) that performs an amplification of the DNA chain by PCR, without a need of prior knowledge of the gene sequence, generating polymorphic standards (Zietkiewicz et al., 1994). The ISSR marker has already been used in studies of variability and genetic diversity in species of plants of the Myrtaceae family, such as *Eucalyptus* spp. (Ballesta et al., 2015), *Psidium* spp. (Oliveira et al., 2014), *Eugenia* spp. (Brunchault et al., 2014) and *Myrcia* spp. (Brandão et al., 2015; Alves et al., 2016).

Since assessment of genetic variability can be used for conservation and use in genetic resources programs, the aim of this study was to determine the genetic diversity of *M. ovata* found in the municipality of Japaratuba-SE, using ISSR molecular marker.

MATERIAL AND METHODS

Plant material

For the extraction of DNA, fresh leaves of 24 individuals of *M. ovata* were collected in silica from the municipality of Japaratuba, in the State of Sergipe, Brazil on 09/15/2016 (Figure 1 and Table 1). This region has rain forest and dune vegetation. The average of annual rainfall is 1400 mm (Sergipe, Semarh/SRH, 2014) and the rainy season is from March to August. The average annual temperature is 25.3°C and the climate is dry sub-humid mesothermal type (Sergipe, Seplantec/Supes, 2000).

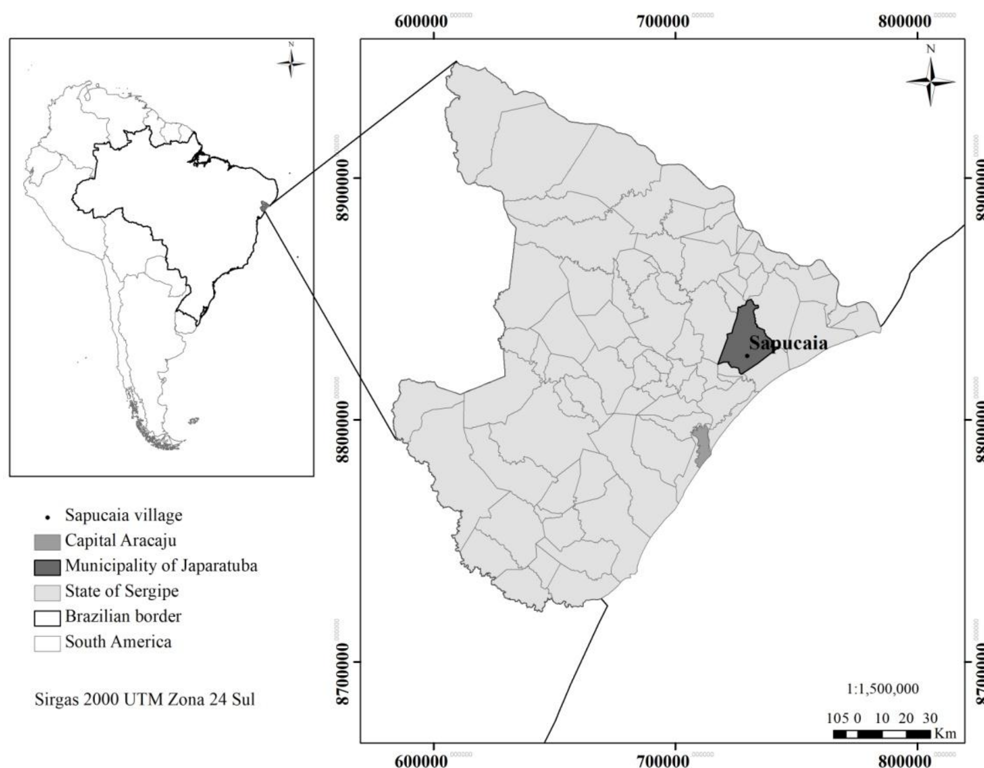

Figure 1. Map where individuals of *Myrcia ovata* were collected from a natural population located in the municipality of Japaratuba, in the state of Sergipe, Brazil.

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Table 1. Identification of 24 *Myrcia ovata* individuals from a native population located in the municipally of Japaratuba, in the state of Sergipe, Brazil.

Individuals	Classification	Longitude	Latitude
MYRO-154	Shrub	10°37’38.1”S	36°53’17.0”W
MYRO-155	Shrub	10°37’37.9”S	36°53’17.4”W
MYRO-178	Shrub	10°37’38.8”S	36°53’19.7”W
MYRO-178.1	Shrub	10°37’38.8”S	36°53’19.7”W
MYRO-178.2	Shrub	10°37’38.8”S	36°53’19.7”W
MYRO-156	Shrub	10°37’38.6”S	36°53’19.7”W
MYRO-157	Shrub	10°37’39.0”S	36°53’19.7”W
MYRO-160	Shrub	10°37’37.7”S	36°53’18.0”W
MYRO-813	Shrub	10°37’37.7”S	36°53’18.2”W
MYRO-159.1	Shrub	10°37’37.3”S	36°53’17.4”W
MYRO-159	Shrub	10°37’37.2”S	36°53’17.4”W
MYRO-159.2	Shrub	10°37’37.2”S	36°53’17.4”W
MYRO-093	Shrub	10°38’45.2”S	36°52’17.5”W
MYRO-162	Shrub	10°38’45.4”S	36°52’16.4”W
MYRO-174	Shrub	10°38’45.3”S	36°52’17.0”W
MYRO-175	Shrub	10°38’45.2”S	36°52’17.8”W
MYRO-175.1	Shrub	10°38’45.2”S	36°52’17.8”W
MYRO-176	Tree	10°38’44.1”S	36°52’19.4”W
MYRO-029	Shrub	10°37’37.8”S	36°53’17.3”W
MYRO-030	Shrub	10°37’37.8”S	36°53’17.3”W
MYRO-032	Shrub	10°37’38.7”S	36°53’20.1”W
MYRO-033	Shrub	10°37’38.7”S	36°53’20.2”W
MYRO-034	Shrub	10°37’38.8”S	36°53’20.4”W
MYRO-036	Tree	10°38’45.3”S	36°52’16.3”W

DNA extraction and ISSR amplification

DNA extraction was carried out as described by Nienhuis et al. (1995), with modifications. For the PCR-ISSR reaction, nine primers from Invitrogen were used (Thermo Fisher Scientific, Carlsbad, CA, USA) (Table 2).

Table 2. Primer information and amplified products from the genetic diversity analysis of *Myrcia ovata* individuals in a native population located in the municipality of Japaratuba, in the state of Sergipe, Brazil.

Name	Sequence (5’-3’)	Length (bp)	Annealing temp.	Total bands	Polymorphic bands	Polymorphism (%)
UBC807	(AG)8-T	1500 - 700	43°C	10	10	100%
UBC808	(AG)8-C	2000 - 400	47°C	12	12	100%
UBC809	(AG)8-G	2000 -400	48°C	14	14	100%
UBC810	(GA)8-T	1500 - 400	45.4°C	12	11	92%
UBC811	(GA)8-C	2000 - 500	45°C	9	9	100%
UBC813	(CT)8-T	2000 - 500	47°C	10	10	100%
UBC825	(AC)8-T	2000 - 500	47°C	13	13	100%
UBC827	(AC)8-G	2000 - 500	47°C	13	12	92%
UBC834	(AG)8-YT	2000 - 700	46°C	9	8	89%

R = purine (A or G) e Y = pyrimidine (C or T)

The amplifications were carried out in a PTC-100 Thermocycler (MJ Research Inc., Quebec, Canada) programmed under the following protocol: initial denaturation for 5 min at 94°C; 35 cycles each comprising denaturation for 40 s at 94°C, 30 s for each primer annealing temperature (Table 2) and extension for 60 s at 72°C; and, a final extension for 7 min at 72°C.

The fragments were subjected to electrophoresis on a 1.5% agarose gel (1X TBE: 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) in a horizontal electrophoresis system (Loccus Biotecnologia LCH 20 x 25) at 120 V for 2 h. Each sample was stained with 2µL of GelRed® dye (Biotium) and the amplification products were visualized under UV light.

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Data analysis

From the analysis and interpretation of the agarose gel, a binary matrix was constructed based on the presence and absence of the fragments, represented by “1” and “0” respectively. The optimal number of fragments was estimated by the GENES software (Cruz, 2001), in order to obtain the correlation and stress value. The average values of Polymorphic Information Content (PIC) (Botstein et al., 1980) and Hardy-Weinberg Expected Heterozygosity (H_E) (Nei, 1973) for dominant molecular markers, and the Jaccard similarity (Sneath and Sokal, 1973) and the Bootstrap analysis for 100 simulations analysis were also performed using GENES software. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram was constructed by the NTSYS-pc 2.0 software (Rohlf, 2001). The percentage of polymorphic loci, the number of different alleles (N_a), the number of effective alleles (N_e) and the Shannon Index were calculated using the GeneAlEx 6.5 version (Peakall and Smouse, 2012).

Another cluster analysis, using Bayesian method, was performed in the STRUCTURE software, version 2.3.4 (Pritchard et al., 2012). The admixture model was used with correlated allele frequencies, and simulations were carried out with a burn-in period and a MCMC number of 10^4 each. The choice for the best fit clustering number (K) was evaluated using ΔK, from the Evanno et al. (2005) method, in the on-line STRUCTURE HAVERSTER software (Earl and vonHoldt, 2012).

RESULTS

Primers analysis

The nine ISSR primers that were used generated 99 polymorphic bands. The fragments number varied from 9 to 13 per primer, with an average of 11.3 bands per primer (Figure 2).

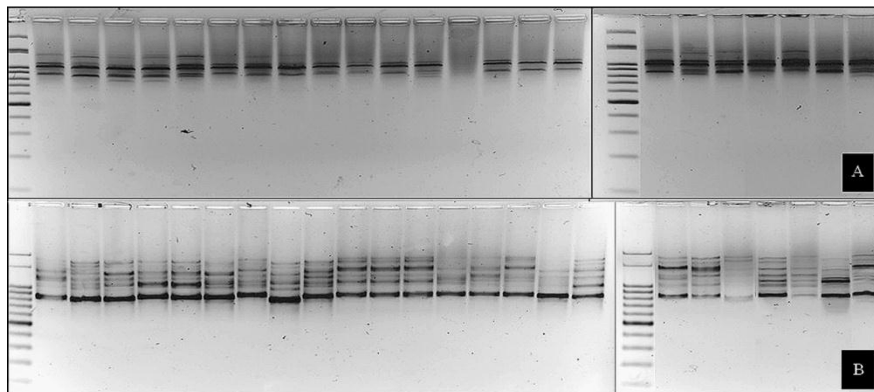

Figure 2. Electrophoretic profiles of the primers UBC807 (A) and UBC834 (B) amplified for 24 *Myrcia ovata* individuals from a native population located in the municipality of Japaratuba, in the state of Sergipe, Brazil.

The optimization analysis showed correlation and stress values of 0.9986 and 0.0127, respectively. These values confirm the stability among the number of primers and the number of fragments obtained. Furthermore, the content of PIC ranged from 0.1094 to 0.3469, with an average of 0.2594.

Genetic variability

The genetic variability for the population was estimated as moderate. The average number of different alleles (N_a) and number of effective alleles (N_e) were 1.971 and 1.412, respectively. The Shannon Information Index (I_S) was 0.4 and the Expected Heterozygosity (H_E) ranged from 0.1162 to 0.4466, with an average of 0.3097. The percentage of polymorphic loci was 97.06%.

Clustering analysis

The similarity coefficient of Jaccard between each pair of individuals ranged from 0.21 to 0.82, with an average of 0.38 (Table 3). The pair formed by the individuals MYRO-178.1 and MYRO-178.2 (0.82), followed by the pairs MYRO-032 and MYRO-033 (0.71), and MYRO-159 and MYRO-159.2 (0.69) presented the highest genetic similarity. Moreover, the pairs of individuals MYRO-034 and MYRO-159 (0.21), MYRO-029 and MYRO-093 (0.24), and MYRO-030 and MYRO-162 (0.25) presented the lowest genetic similarity.

Table 3. Jaccard similarity coefficient of 24 *Myrcia ovata* individuals from a native population located at the municipally of Japaratuba, in the state of Sergipe, Brazil.

	MYRO-154	MYRO-155	MYRO-178	MYRO-178.1	MYRO-178.2	MYRO-156	MYRO-157	MYRO-160	MYRO-813	MYRO-159.1	MYRO-159	MYRO-159.2	MYRO-093	MYRO-162	MYRO-174	MYRO-175	MYRO-175-1	MYRO-176	MYRO-029	MYRO-030	MYRO-032	MYRO-033	MYRO-034
MYRO-155	0.43	1																					
MYRO-178	0.45	0.59	1																				
MYRO-178.1	0.47	0.59	0.65	1																			
MYRO-178.2	0.48	0.55	0.57	0.82	1																		
MYRO-156	0.44	0.46	0.51	0.47	0.53	1																	
MYRO-157	0.38	0.41	0.49	0.41	0.51	0.58	1																
MYRO-160	0.35	0.52	0.51	0.53	0.63	0.59	0.51	1															
MYRO-813	0.37	0.48	0.50	0.40	0.43	0.46	0.41	0.41	1														
MYRO-159.1	0.35	0.30	0.33	0.31	0.36	0.32	0.27	0.33	0.41	1													
MYRO-159	0.33	0.30	0.31	0.35	0.38	0.26	0.27	0.29	0.28	0.67	1												
MYRO-159.2	0.40	0.33	0.37	0.38	0.44	0.31	0.33	0.36	0.33	0.67	0.69	1											
MYRO-093	0.28	0.26	0.27	0.28	0.34	0.32	0.28	0.30	0.29	0.43	0.36	0.50	1										
MYRO-162	0.36	0.40	0.39	0.40	0.43	0.33	0.32	0.33	0.35	0.50	0.40	0.50	0.54	1									
MYRO-174	0.29	0.31	0.30	0.33	0.38	0.36	0.31	0.32	0.38	0.60	0.51	0.51	0.51	0.51	1								
MYRO-175	0.35	0.31	0.33	0.30	0.34	0.40	0.28	0.33	0.37	0.38	0.28	0.36	0.32	0.39	0.37	1							
MYRO-175-1	0.30	0.38	0.40	0.35	0.35	0.36	0.36	0.37	0.35	0.33	0.32	0.38	0.33	0.37	0.30	0.37	1						
MYRO-176	0.37	0.38	0.42	0.41	0.44	0.34	0.36	0.37	0.36	0.34	0.38	0.44	0.31	0.29	0.33	0.34	0.38	1					
MYRO-029	0.33	0.32	0.33	0.34	0.35	0.30	0.29	0.33	0.32	0.35	0.34	0.31	0.24	0.36	0.30	0.33	0.34	0.37	1				
MYRO-030	0.37	0.46	0.42	0.38	0.41	0.44	0.36	0.44	0.38	0.29	0.30	0.30	0.29	0.25	0.29	0.37	0.33	0.50	0.37	1			
MYRO-032	0.29	0.43	0.39	0.33	0.36	0.41	0.38	0.41	0.51	0.41	0.33	0.30	0.31	0.32	0.40	0.40	0.41	0.41	0.57	0.46	1		
MYRO-033	0.25	0.38	0.36	0.27	0.30	0.41	0.38	0.39	0.48	0.36	0.27	0.27	0.33	0.37	0.35	0.39	0.38	0.43	0.45	0.43	0.71	1	
MYRO-034	0.32	0.34	0.38	0.39	0.39	0.40	0.31	0.35	0.42	0.30	0.21	0.31	0.29	0.30	0.32	0.38	0.33	0.37	0.41	0.39	0.44	0.47	1
MYRO-036	0.32	0.36	0.40	0.38	0.36	0.34	0.25	0.35	0.36	0.30	0.25	0.31	0.27	0.33	0.28	0.37	0.36	0.46	0.43	0.44	0.38	0.40	0.42

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The UPGMA dendrogram separated two groups (I and II) of individuals. According to this analysis, group I was formed by six individuals representing 25% of the population (MYRO-159, MYRO-159.1, MYRO-159.2, MYRO-093 and MYRO-162), and group II was formed by 18 individuals representing 75% of the population. The Bootstrap repeatability analysis showed a range from 19 to 100%. The junctions between MYRO-154 and MYRO-155 (100%); MYRO-178.1 and MYRO-178.2 (100%); and, MYRO-159 and MYRO-159.1 (97%) individuals showed higher consistencies (Figure 3).

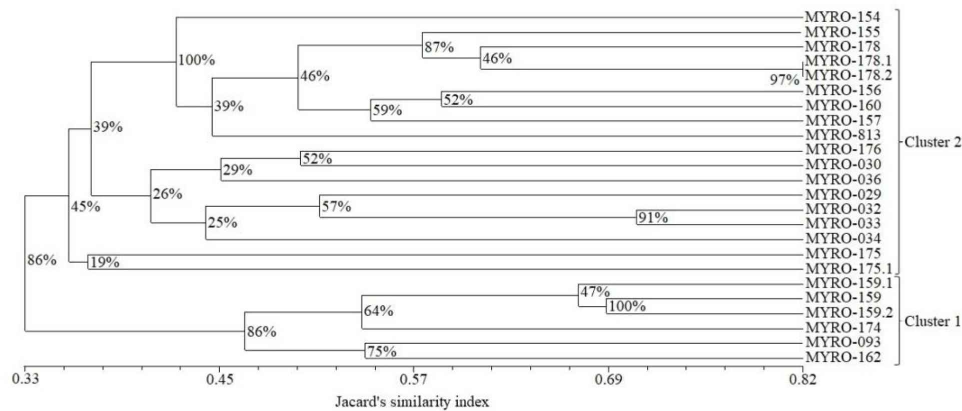

Figure 3. Dendrogram generated by the Unweighted Pair Group method with Arithmetic Mean (UPGMA) analysis of Jaccard similarity indices of 24 *Myrcia ovata* individuals from a native population located at the municipality of Japaratuba, in the state of Sergipe, Brazil.

The Bayesian cluster analysis from the STRUCTURE software divided the population into two groups. The individuals MYRO-154 and MYRO-175 had the most variability (Figure 4).

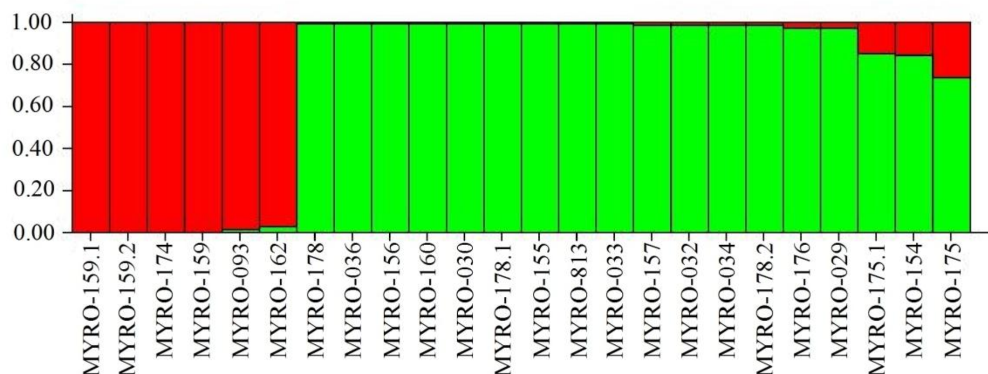

Figure 4. STRUCTURE clustering results with K=2 of 24 *Myrcia ovata* individuals from a native population located at the municipality of Japaratuba, in the state of Sergipe, Brazil.

DISCUSSION

The ISSR marker detected a relatively high level of polymorphism among the 24 plants of *M. ovata* in a native population located in the municipality of Japaratuba, in the state of Sergipe, Brazil. This is the first study to evaluate the genetic variability of *M. ovata*.

The number of fragments amplified by the ISSR markers was included in a lower range than would be expected for species of the Myrtaceae tribe (Lima et al., 2015b). In addition, the 99 polymorphic bands obtained with the nine primers were sufficient to find a reliable number of fragments to estimate genetic variability (Dudley, 1994). Also, using the optimization analysis, it was proved that the number of primers used were sufficient to evaluate the genetic variability of *M. ovata* individuals, with correlation and stress closer to 1.0 and below 0.05, respectively (Kruskal, 1964).

The PIC content represents the probability of finding each marker present and/or absent in each band, revealing allelic variation. It ranges from 0 to 0.5 and lower values can correspond to very rare or abundant markers (Roldan-Ruiz et al., 2000). In this paper, the PIC content (0.259) was considered to have moderate discriminatory power. In addition, the PIC content was lower than the H_E, as expected (Cruz, 2001).

Regarding the genetic variability, the mean number of different alleles (N_a) obtained for dominant markers was 1.97, close to the highest it can be, and among these 1.41 were considered as effective alleles (N_e). This means that 72% of the alleles can contribute to the construction of the genetic information of this native population of *M. ovata* in the Sergipe state.

The Shannon Index (I_S) measures the certainty of predicted genetic proximity between individuals, ranging from 0 to 1. The lower the number, the higher the certainty degree and the lower the population diversity (Estopa et al., 2006). The average H_E is associated with low diversity and consequently, reduced capacity of the remaining population for adaptation (Álvares-Carvalho et al., 2016).

The Shannon Index and the average H_E found for this native population (0.40; 0.30), was lower than those found by Brandão et al. (2015) (0.48; 0.33) and by Alves et al. (2016) (0.46; 0.30) who worked with *M. splendes* and *M. lundiana*, respectively. Knowing that the range of H_E is expected to be similar between species that present similar characters, including biological, reproductive and distribution characteristics (Lima et al., 2015b), the numbers of the Shannon Index and H_E can be, in part, explained because of the origin of the plants used in their research, which was a conserved vegetation. This contrasts with the *M. ovata* location, an anthropized vegetation (Santana et al., 2012), which could result in a lower gene flow.

The Jaccard analysis showed a moderate similarity (0.38), which can be influenced by a cross-pollination reproduction system (Kageyama et al., 2003; Sampaio et al., 2016) and the absent of domestication (Silva et al., 2011). Nevertheless, the existence of moderate genetic diversity does not justify the lack of conservation activities for this specie, mainly because it was not found in other locations of the state of Sergipe besides the study area, and the genetic variability shows a tendency to decay if no action is taken.

It's important to emphasize that the most similar pairs of individuals are shrubs, each located side-by-side, and clustered within the same group in the UPGMA and STRUCTURE analysis, implying that they probably have the same progenitors. This observation is applied to other shrubs that are side-by-side, for example MYRO-159 and

MYRO-159.1, MYRO-159.1 and MYRO-159.2 and MYRO-178 and MYRO-178.1. Furthermore, concerning the population genetic structure, the 24 individuals of *M. ovata* were clustered in two groups by the UPGMA and STRUCTURE analyses, which presented the same arrangement.

The choice of matrices to describe the variability and/or genetic diversity of individuals within and between populations is a prerequisite for genetic characterization of the species, since this characterization is a common procedure in the conservation of natural resources and genetic improvement programs. Based on this study, it was determined that the individuals MYRO-154, MYRO-175 and MYRO-175.1 of *M. ovata* present in the state of Sergipe should be selected as priorities for conservation of the species.

Regarding the comparison of chemical and genetic analyses, 12 individuals of *M. ovata* used in Sampaio et al. (2016) were also used in this paper. A match of the chemical and genetics groups described in the clustering analysis was not found. For example, each of these individuals: MYRO-174 and MYRO-176, and MYRO-159 and MYRO-160 were chemically grouped in the same clusters, but genetically clustered in different groups. This differentiation could be because different samples were used, from different collection periods for these studies. The chemical variation is commonly found in the chemical composition of plants, because it is influenced not only by gene composition, but also by dynamic factors such as rainy season, drought, temperature and pests, as well as by the extraction method (Scheffer, 1993; Ribeiro et al., 2016). This comparison could be improved with other molecular makers that also are influenced by dynamic factors, such as enzyme producers (Faleiro, 2007).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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