Analysis of genetic diversity of a native population of *Myrcia lundiana* Kiaersk. plants using ISSR markers

M.F. Alves[1], D.A.C. Nizio[1], F.A. Brito[1], T.S. Sampaio[1], A.V.C. Silva[2], M.F. Arrigoni-Blank[1], S.V.A. Carvalho[1] and A.F. Blank[1]

[1]Laboratório de Recursos Genéticos Vegetais e Óleos Essenciais, Departamento de Engenharia Agronômica, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil
[2]Laboratório de Biotecnologia Molecular, Embrapa Tabuleiros Costeiros, Aracaju, SE, Brasil

Corresponding author: A.F. Blank
E-mail: afblank@ufs.br

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ABSTRACT. *Myrcia lundiana* Kiaersk. is a tree of the family Myrtaceae found in tropical and subtropical areas of the southern hemisphere that produces essential oil. The aim of this study was to characterize the genetic diversity of *M. lundiana* plants from a native population of Parque Nacional de Itabaiana, using inter-simple sequence repeat molecular markers. Thirty-five primers were tested, 20 of which were polymorphic, resulting in 135 polymorphic and informative bands. Results of the cluster analysis, obtained using the unweighted pair group method with arithmetic mean, grouped plants into three clusters: Cluster I - MLU001, MLU002, MLU003, MLU004, MLU005, MLU006, MLU018, MLU019, MLU020, MLU021, MLU022;

MLU008, MLU011, MLU012, MLU014, MLU015, MLU017, MLU026, and MLU028; Cluster II - MLU007, MLU009, MLU010, MLU013, and MLU016; and Cluster III - MLU023, MLU024, MLU025, and MLU027. Jaccard similarity coefficients for pair-wise comparisons of plants ranged between 0.15 and 0.87. MLU014 and MLU015 presented low genetic diversity, with a similarity index of 0.87. Conversely, MLU007 and MLU019 presented high diversity, with a similarity index of 0.15. According to the structure analysis, three distinct clusters were formed. Genetic diversity of *M. lundiana* plants was intermediate, and expansion of its genetic diversity is necessary. MLU026 and MLU028 are the most suitable for selection in breeding programs, since they clearly represent all of the diversity present in these plants. Moreover, these results provide important information on the existing genetic variability, highlighting the importance of Parque Nacional de Itabaiana for the conservation of this species.

Key words: *Myrcia lundiana*; Conservation; Genetic diversity; ISSR

INTRODUCTION

Myrcia lundiana Kiaersk, popularly known as "canela-de-tabuleiro", is a medicinal species of the family Myrtaceae, found in tropical and subtropical areas of the southern hemisphere (Govaerts et al., 2008). In folk medicine, species of the *Myrcia* genus have been used as astringents, diuretics, and for the treatment of diabetes mellitus, hypertension, gastric diseases, and diarrhea (Russo et al., 1990). Although little is known about the biological activity of *M. lundiana*, a recent study showed the antifungal potential of its essential oil against *Lasiodiplodia theobromae* (Alves et al., 2016).

The search for new molecules and bioactive substances from natural sources has highlighted the need for studies aiming to improve the conservation and maintenance of the genetic diversity of potentially useful plant species. The development of new roads, the construction of hydroelectric plants, and extractive activities, among other human activities, have greatly impacted the diversity of many species of the fauna and flora. Thus, it becomes necessary to manage genetic resources in order to preserve the maximum levels of genetic diversity within species. However, without information on the distribution of this variability between or within populations, important decisions regarding the management and conservation of these resources cannot be made (Lima et al., 2015). Knowledge on genetic variability and its organization in plant species, represent important steps for the exploitation, definition of genetic conservation strategies, and breeding programs (Setotaw et al., 2010). Owing to the importance of several medicinal species, either for popular use or for the pharmaceutical industry, several studies on genetic diversity in native populations have been carried out, such as those on *Senna reticulata* (Lima et al., 2015), *Pilocarpus pennatifolius* (Bandeira et al., 2010), *Pothomorphe umbellata* (Valle et al., 2013), and *Annona crassiflora* (Telles et al., 2003).

The study of genetic diversity in plant species is usually carried out using molecular markers. Among them, the inter-simple sequence repeats (ISSR) should be highlighted. These markers are dominant and permit the analysis of multiple loci in a single reaction owing to their

abundance and dispersion within the genome. In addition, they exhibit high reproducibility, and can achieve results in a timely and cost-effective manner when compared with other markers (Rodrigues, 2010). ISSR markers are efficient at revealing genetic diversity among medicinal and agricultural plants, such as *Varronia curassavica* accessions and cassava landraces (Brito et al., 2016; Tiago et al., 2016).

Although limited information is available on the chemical diversity of the essential oil of *M. lundiana* in a native population from Sergipe (Alves et al., 2016), there are no reports in the literature regarding genetic studies of this species. Therefore, the objective of the present study was to analyze the genetic diversity of *M. lundiana* plants from a native population in the State of Sergipe using ISSR molecular markers.

MATERIAL AND METHODS

Plant material

Young leaves of 28 *M. lundiana* plants from of a native population of Parque Nacional de Itabaiana municipality of Areia Branca, State of Sergipe, Brazil, were collected (Table 1). After collection, leaves were wrapped in sterile gauze and stored on ice to prevent oxidation. The material was frozen at -80°C until lyophilization in a LioTop (Liobras, São Carlos, SP, Brazil). After lyophilization, samples were stored in a desiccator containing silica gel until DNA extraction.

Table 1. Identification of 28 *Myrcia lundiana* plants from of a native population of Parque Nacional de Itabaiana, in the State of Sergipe, Brazil.

Plant	Georeferenced information
MLU001	11°44'17.9"S; 37°52'0.80"W
MLU002	10°44'57.0"S; 37°20'24.6"W
MLU003	10°44'58.0"S; 37°20'26.2"W
MLU004	10°44'58.0"S; 37°20'25.0"W
MLU005	10°44'57.7"S; 37°20'24.6"W
MLU006	10°44'57.5"S; 37°20'26.0"W
MLU007	10°44'57.5"S; 37°20'26.1"W
MLU008	10°44'56.2"S; 37°20'26.3"W
MLU009	10°44'58.1"S; 37°20'26.1"W
MLU010	10°45'00.0"S; 37°20'26.0"W
MLU011	10°44'57.7"S; 37°20'24.3"W
MLU012	10°44'56.2"S; 37°20'26.5"W
MLU013	10°45'8.30"S; 37°20'27.5"W
MLU014	10°44'57.5"S; 37°20'26.5"W
MLU015	10°44'54.5"S; 37°20'27.9"W
MLU016	10°44'56.5"S; 37°20'24.0"W
MLU017	10°44'16.6"S; 37°52'00.5"W
MLU018	10°44'58.2"S; 37°20'26.1"W
MLU019	10°44'56.8"S; 37°20'24.0"W
MLU020	10°44'57.5"S; 37°20'26.0"W
MLU021	10°44'57.6"S; 37°20'26.1"W
MLU022	10°45'8.10"S; 37°20'17.5"W
MLU023	10°45'8.10"S; 37°20'18.5"W
MLU024	10°45'00.0"S; 37°20'26.0"W
MLU025	10°44'57.7"S; 37°20'24.3"W
MLU026	10°45'00.0"S; 37°20'26.0"W
MLU027	10°44'57.7"S; 37°20'24.3"W
MLU028	10°45'00.0"S; 37°20'26.0"W

DNA extraction and PCR-ISSR amplification

DNA extraction followed the procedures described by Doyle and Doyle (1990), modified as described by Alzate-Marin et al. (2005) to obtain DNA suitable for use in these experiments. DNA was diluted and subsequently standardized and quantified using the NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA). The ISSR primers used in this study were obtained from Eurofins MWG Operon (Operon Technologies, Louisville, KY, USA), IDT (Integrated DNA Technologies, Coralville, IA, USA), and Invitrogen (Thermo Fisher Scientific, Carlsbad, CA, USA). Thirty-five primers were tested for PCR amplification. PCR was carried out in a total volume of 20 µL, containing 1.0 µL genomic DNA (10 ng/µL), 0.2 µL recombinant Taq polymerase from *Thermus aquaticus*, expressed in *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA) (0.05 U/µL), and 2 µL 10X buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin) (Sigma-Aldrich), 0.4 µL dNTP (2.5 mM), 1.0 µL primer (25.0 pmol), and 15.4 µL autoclaved ultrapure water.

PCR amplification was carried out in a ProFlex PCR thermocycler (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA) programmed with the following protocol: 5 min at 94°C; 45 cycles of 40 s at 94°C; 30 s ranging from 50.4 to 53°C, according to the annealing temperature used for each primer (Table 2); 1 min at 72°C; and a final extension for 7 min at 72°C. Amplification products were subjected to electrophoresis on 1.5% agarose gel, stained with ethidium bromide, visualized under ultraviolet light, and photodocumented. Molecular weights were estimated using a Ludwig DNA 1-kb scale for each primer.

Data analysis

The fragments of amplified DNA were analyzed for the presence (1) or absence (0) of equal-sized bands, and a binary matrix was constructed. Based on this binary matrix, the Jaccard similarity coefficient between each pair of individuals was calculated (Jaccard, 1908). Similarity coefficients were used to construct a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA). These analyses were carried out using the NTSYSpc 2.0 software (Rohlf, 2001).

The Shannon index (I) and marker index (MI) were calculated using the software GENALEX 6.5 (Peakall and Smouse, 2012). STRUCTURE v.2.3.3 was used to analyze genetic structure using a Bayesian clustering method (Hubisz et al., 2009). The "admixture" model was used with correlated allele frequencies, and simulations were carried out with a burn-in of 100,000 generations and K values ranging from 2 to 6 clusters. The number of clusters (K) was determined using STRUCTURE HARVESTER (Earl and vonHoldt, 2012).

RESULTS

A high level of polymorphism was found in ISSR markers among the 28 *M. lundiana* plants from of a native population of Parque Nacional de Itabaiana, in the State of Sergipe, Brazil. Band locations can be visualized on the images generated by photodocumentation of the agarose gels (Figure 1). Of the 35 primers tested, 20 were polymorphic, since they presented good amplification patterns, with 144 amplified bands observed, of which 135 were polymorphic. This corresponds to 93.75% polymorphism, ranging from 3 (UBC826) to 17 (UBC815), and a mean number of 7.20 bands per primer (Table 2).

Table 2. Annealing temperature, sequence, and amplified products used to analyze genetic diversity in *Myrcia lundiana* plants from a native population of Parque Nacional de Itabaiana, in the State of Sergipe, Brazil.

Primer name	Sequence (5'-3')	Length (bp)	Annealing temperature	Total bands	Polymorphic bands	Polymorphism (%)
ISSR1	CAC ACA CAC ACA GG	500-2000	51.5°C	9	9	100.0
ISSR3	CTC TCT CTC TCT CTC TTG	750-2000	51.5°C	8	8	100.0
ISSR5	CTC TCT CTC TCT CTC TGC	1000-1500	51.5°C	6	6	100.0
ISSR7	CAC ACA CAC ACA GT	1500-2000	51.5°C	4	4	100.0
ISSR8	GAG AGA GAG AGA GG	1500-2000	52.8°C	9	9	100.0
ISSR9	GTG TGT GTG TGT GG	1500-2000	55.0°C	9	9	100.0
ISSR10	GAG AGA GAG AGA CC	500-1500	52.0°C	5	5	100.0
ISSR11	GTG TGT GTG TGT CCC	750-2000	52.0°C	8	8	100.0
UBC810	GAG AGA GAG AGA GAG AT	500-1000	50.4°C	8	8	100.0
UBC811	GAG AGA GAG AGA GAG AC	500-1000	53.0°C	4	4	100.0
UBC815	CTC TTC TCT CTC TCT CTG	500-2000	52.8°C	17	16	94.12
UBC817	CAC ACA CAC ACA CAC AA	750-2000	50.4°C	11	9	81.82
UBC823	TCT CTC TCT CTC TCT CC	750-1500	50,4°C	5	5	100.0
UBC826	ACA CAC ACA CAC ACA CC	750-1500	52.8°C	3	3	100.0
UBC834	AGA GAG AGA GAG AGY T	750-2000	52.8°C	6	0	00.00
UBC835	AGA GAG AGA GAG AGA GYC	1500-2000	55.0°C	4	4	100.0
UBC855	ACA CAC ACA CAC ACY T	500-2000	53.0°C	5	5	100.0
UBC856	ACA CAC ACA CAC ACA CYA	500-1500	50.4°C	8	8	100.0
UBC857	ACA CAC ACA CAC ACY G	750-2000	53.0°C	9	9	100.0
UBC858	TGT GTG TGT GTG TGT GRT	500-2000	53.0°C	6	6	100.0

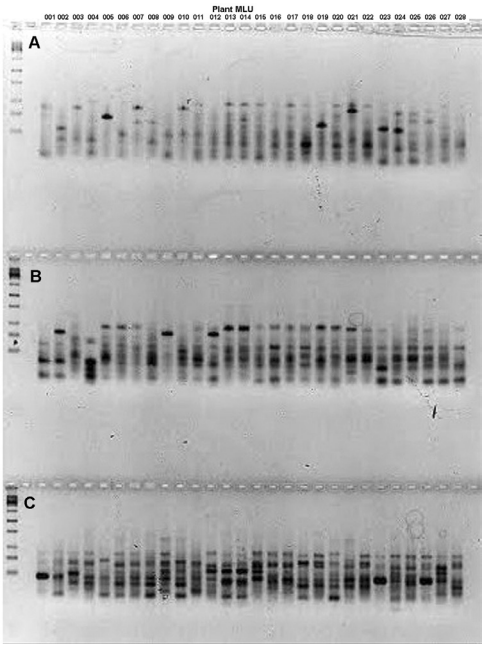

Figure 1. Agarose gels showing the electrophoretic profiles of the inter-simple sequence repeat markers amplified using the primers UBC856 (**A**), UBC823 (**B**), and UBC835 (**C**) in 28 *Myrcia lundiana* plants from of a native population of Parque Nacional de Itabaiana, in the State of Sergipe, Brazil.

The similarity coefficients used to calculate the genetic similarity between the 28 *M. lundiana* plants, as assessed by ISSR markers, ranged from 0.15 to 0.87, with a mean value of 0.49. Low genetic diversity was observed between MLU014 and MLU015, with a similarity index of 0.87, representing the plants that were genetically most similar among all pairs assessed. Conversely, MLU007 and MLU019 exhibited the highest diversity, with a similarity index of 0.15. There were 23 genotype combinations with intermediate genetic diversity (0.60 to 0.78), and other combinations with high genetic diversity, since their similarity values in the cluster were below 0.6 (0.24 to 0.59; Table 3).

By means of clustering analysis, three clusters were formed according to the genetic distances between plants (Figure 2). Cluster I (67.86% of plants) was formed by MLU001, MLU002, MLU003, MLU004, MLU005, MLU006, MLU018, MLU019, MLU020, MLU021, MLU022; MLU008, MLU011, MLU012, MLU014, MLU015, MLU017, MLU026, and MLU028, with Jaccard similarity indices between 0.4 and 0.87; Cluster II (17.86% of plants) presented similarity indices between 0.6 and 0.78, and was formed by the plants MLU007, MLU009, MLU010, MLU013, and MLU016; and Cluster III (14.29% of plants) showed similarity indices between 0.4 and 0.78, and was formed by MLU023, MLU024, MLU025, and MLU027.

To determine the genetic patterns of population differentiation, Bayesian analysis was carried out by the Structure software and, according to the ΔK method, the most likely number of clusters was K = 3 (Figure 3). Graphic visualization of the population structure permitted the identification of two plants, MLU026 and MLU028, which carried a mixture of the genetic material of the three clusters.

Table 3. Matrix generated based on the Jaccard similarity coefficient of 28 *M. lundiana* plants from of a native population of the Parque Nacional de Itabaiana, in the state of Sergipe, Brazil.

Plants	MLU 001	MLU 002	MLU 003	MLU 004	MLU 005	MLU 006	MLU 007	MLU 008	MLU 009	MLU 010	MLU 011	MLU 012	MLU 013	MLU 014	MLU 015	MLU 016	MLU 017	MLU 018	MLU 019	MLU 020	MLU 021	MLU 022	MLU 023	MLU 024	MLU 025	MLU 026	MLU 027	MLU 028
MLU001	-																											
MLU002	0.78	-																										
MLU003	0.69	0.69	-																									
MLU004	0.68	0.81	0.71	-																								
MLU005	0.70	0.80	0.74	0.84	-																							
MLU006	0.65	0.72	0.59	0.66	0.72	-																						
MLU007	0.26	0.30	0.24	0.33	0.26	0.34	-																					
MLU008	0.61	0.59	0.56	0.65	0.61	0.54	0.36	-																				
MLU009	0.24	0.27	0.26	0.31	0.28	0.34	0.66	0.38	-																			
MLU010	0.23	0.26	0.29	0.34	0.34	0.34	0.67	0.36	0.78	-																		
MLU011	0.55	0.61	0.51	0.59	0.66	0.69	0.35	0.60	0.37	0.38	-																	
MLU012	0.47	0.54	0.54	0.53	0.59	0.65	0.33	0.57	0.39	0.37	0.70	-																
MLU013	0.34	0.40	0.29	0.38	0.36	0.43	0.71	0.34	0.57	0.59	0.48	0.38	-															
MLU014	0.50	0.57	0.50	0.56	0.54	0.62	0.47	0.69	0.42	0.41	0.72	0.72	0.51	-														
MLU015	0.52	0.60	0.51	0.58	0.60	0.69	0.47	0.65	0.44	0.43	0.76	0.81	0.49	0.87	-													
MLU016	0.25	0.28	0.33	0.28	0.32	0.35	0.60	0.33	0.68	0.62	0.40	0.43	0.66	0.43	0.48	-												
MLU017	0.55	0.52	0.61	0.53	0.51	0.58	0.40	0.59	0.38	0.38	0.63	0.66	0.46	0.77	0.79	0.44	-											
MLU018	0.78	0.68	0.76	0.70	0.68	0.66	0.26	0.57	0.25	0.25	0.53	0.49	0.34	0.47	0.49	0.29	0.58	-										
MLU019	0.60	0.65	0.58	0.69	0.69	0.57	0.15	0.52	0.18	0.17	0.48	0.45	0.26	0.42	0.44	0.19	0.41	0.71	-									
MLU020	0.70	0.71	0.76	0.72	0.72	0.60	0.22	0.54	0.24	0.23	0.54	0.49	0.32	0.50	0.49	0.29	0.62	0.86	0.70	-								
MLU021	0.68	0.74	0.67	0.82	0.83	0.67	0.27	0.62	0.28	0.27	0.58	0.54	0.35	0.52	0.54	0.31	0.50	0.78	0.80	0.80	-							
MLU022	0.71	0.82	0.65	0.79	0.74	0.68	0.32	0.59	0.26	0.26	0.55	0.54	0.40	0.57	0.57	0.28	0.52	0.81	0.73	0.78	0.82	-						
MLU023	0.46	0.40	0.54	0.50	0.45	0.33	0.34	0.36	0.37	0.39	0.25	0.23	0.29	0.24	0.25	0.30	0.33	0.53	0.38	0.54	0.50	0.45	-					
MLU024	0.50	0.45	0.53	0.49	0.43	0.35	0.37	0.44	0.41	0.37	0.29	0.25	0.34	0.31	0.30	0.33	0.38	0.56	0.40	0.59	0.50	0.50	0.81	-				
MLU025	0.32	0.36	0.34	0.39	0.35	0.42	0.42	0.33	0.51	0.47	0.31	0.30	0.38	0.33	0.32	0.40	0.29	0.41	0.34	0.41	0.43	0.42	0.58	0.60	-			
MLU026	0.49	0.46	0.55	0.48	0.44	0.48	0.30	0.55	0.36	0.34	0.54	0.56	0.28	0.56	0.58	0.30	0.71	0.59	0.42	0.60	0.50	0.50	0.45	0.45	0.42	-		
MLU027	0.32	0.26	0.34	0.26	0.28	0.32	0.44	0.30	0.51	0.47	0.33	0.40	0.40	0.33	0.40	0.45	0.46	0.38	0.21	0.40	0.32	0.29	0.48	0.52	0.47	0.51	-	
MLU028	0.64	0.62	0.63	0.55	0.59	0.52	0.24	0.54	0.28	0.26	0.55	0.54	0.35	0.49	0.54	0.33	0.61	0.69	0.55	0.74	0.66	0.60	0.43	0.45	0.30	0.63	0.51	-

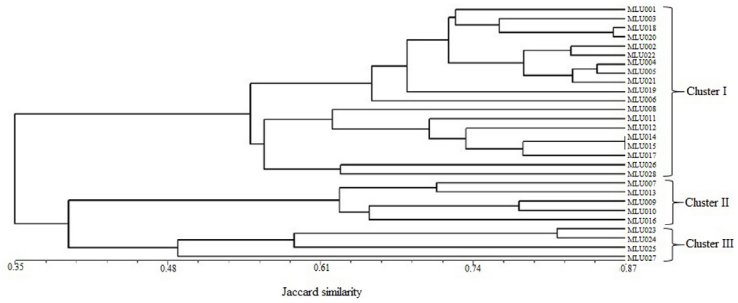

Figure 2. Dendrogram generated by the unweighted pair group method with arithmetic mean (UPGMA) analysis of Jaccard similarity indices for 28 *Myrcia lundiana* plants from of a native population of Parque Nacional de Itabaiana, in the State of Sergipe, Brazil.

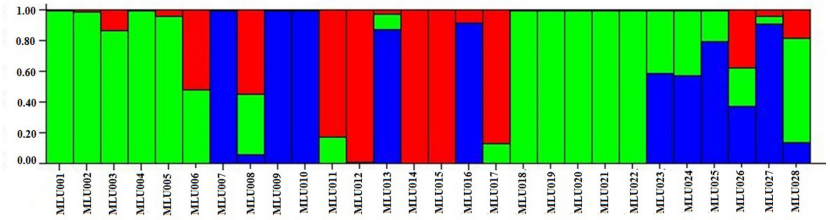

Figure 3. STRUCTURE results for 28 *Myrcia lundiana* plants from of a native population of Parque Nacional de Itabaiana, in the State of Sergipe, Brazil, with K = 3. Each vertical bar represents one accession, with color indicating cluster membership.

DISCUSSION

The native populations of *M. lundiana* were found to possess intermediate genetic diversity based on the values of the diversity parameters assessed, and by the division of the 28 plants into three clusters. The genetic diversity results obtained in this study were similar to those reported in a study on the chemical diversity of a population of *M. lundiana* by Alves et al. (2016), who detected high chemical diversity, and division into three chemical clusters. This indicates that studies using ISSR molecular markers should be extended in order to identify greater variability.

The Shannon index (I) presented a low mean value (0.46), which was below 0.5. This index of genetic diversity may vary from 0 to 1, with lower genetic diversity represented by values closest to zero (Silva et al., 2011). Heterozygosity, or the Nei index, which measures the genetic variability that estimates the variation between related individuals, was considered low, with a mean value of 0.30.

The bar graph obtained by the Bayesian analysis using the Structure software showed the formation of three clusters defined by the colors blue, red, and green. MLU026 and MLU028 presented part of each cluster, which indicates that these plants are probably located within a transition point, and/or a region of intense exchange of genetic material. Such plants should be prioritized for preservation since they may be useful in future studies aimed at the genetic improvement of the species.

Little information is available on the history of the study area; however, vegetation corridors in the studied landscape are of great importance. The intermediate level of genetic

diversity observed in the present study can be explained by the pollination type of *M. lundiana* (entomophily), by the dispersion of the diaspores (zoochory), by the proximity of the plants, and by the fact that they are interconnected by vegetation corridors.

Owing to the importance of many medicinal plant species for popular use, or even for use in the pharmaceutical industry, many studies on the genetic diversity of native populations have been carried out. In studies with *S. reticulata* and *P. umbellata*, high genetic diversity was observed within native populations (Valle et al., 2013; Lima et al., 2015). Bandeira et al. (2010) observed low genetic diversity between native populations of Jaborandi.

Few studies using ISSR markers have been reported in plants of the Myrtaceae family. When studying the spatial genetic structure in fine-scale of *Myrcia splendens* in environments with fragments and in their connections, Brandão et al. (2011) observed high levels of genetic diversity within populations. Oliveira et al. (2014), when studying the genetic distance between plants of the genus *Psidium* using ISSR markers, observed high variability between guava genotypes, which permits the crossing of superior genotypes with high divergence. Mani et al. (2011), when assessing the genetic distances in different species of *Psidium* spp, obtained a total of 234 polymorphic bands in 31 ISSR markers. Silva et al. (2011) assessed the variability of plants of the *Manihot* genus, and obtained a total of 154 polymorphic bands using 20 primers.

The use of modern techniques, such as those involving molecular markers, is extremely important in breeding programs. This is owing to the accuracy of the information present in the genome, since it is not affected by the environment.

According to Celestino et al. (2015), molecular tools are crucial for the characterization of genetic diversity in aromatic and medicinal plants, i.e., although the agronomic and chemical characteristics distinguish the genotypes of the clusters, the composition of essential oils can be altered by environmental factors.

Genetic diversity of *M. lundiana* plants from a native population of Parque Nacional de Itabaiana, in the State of Sergipe, Brazil, is intermediate. Plants MLU026 and MLU028 were the most suitable for selection in breeding programs of this species, since they carry all of the genetic diversity present in these plants. The results found in this study are important for the conservation of this species, and highlight the need to increase the number of molecular markers, as well as the number of samples for the study of genetic diversity in Parque Nacional de Itabaiana.

Conflicts of interest

The authors declare no conflict of interest.

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