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# Analysis of genetic diversity of *Hyptis pectinata* (L.) Poit. plants using ISSR markers

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**ABSTRACT.** *Hyptis pectinata*, popularly known as 'sambacaitá' or 'canudinho', is a medicinal and aromatic species widely used in the Brazilian Northeast. In Sergipe, the excessive extraction of natural resources may reduce the genetic variability of native plants. Thus, molecular markers have frequently been applied to the characterization of genetic diversity as the basis for germplasm conservation and breeding programs. The objective of the present study was to evaluate the genetic diversity of *H. pectinata* plants collected in different municipalities of the State of Sergipe using ISSR molecular markers. Thirty-four primers were tested, nine of which were selected for providing reproducible and analyzable amplification products, resulting in 67 polymorphic bands. The expected heterozygosity ranged from 0.32 to 0.45, with a mean of 0.39. Polymorphism information content was of 0.49, which classifies the markers as moderately informative. A dendrogram was constructed

using unweighted pair group method with arithmetic mean, forming three clusters: Cluster I (79 plants); Cluster II (4 plants); and Cluster III (2 plants). Jaccard's similarity coefficients ranged from 0.06 to 0.98. The plants SAM-117 and SAM-119 presented greater similarity. Conversely, SAM-107 and SAM-171 were the most genetically distant. In general, *H. pectinata* plants collected in the State of Sergipe presented low to moderate genetic diversity.

**Key words:** *Hyptis pectinata*; Conservation strategies; Genetic diversity; ISSR

## INTRODUCTION

Inadequate exploitation of environmental resources has had negative consequences, such as the fragmentation of ecosystems and the loss of genetic diversity. Several plant species influenced by this fragmentation are important sources of biologically active natural products, and the reduction of the genetic variability of these plants limits the scientific discovery of products of social and economic importance (Gonçalves et al., 2014).

Medicinal and aromatic species have been widely used by civilizations from the earliest days of humankind to the present day, either as the main treatment or as a complement for industrialized chemicals.

The medicinal and aromatic plant *Hyptis pectinata* (L.) Poit. (Lamiaceae) has antidematogenic, antinociceptive, antimicrobial, insecticide, anti-inflammatory, and leishmanicidal activities (Arrigoni-Blank et al., 2008; Nascimento et al., 2008; Silva et al., 2008; Raymundo et al., 2011; Falcao et al., 2013). A recent study on the toxicity of the essential oil of *H. pectinata* plants against leaf-cutting ants resulted in the deposit of a patent (Arrigoni-Blank et al., 2016). The confirmation of the formicidal potential of the essential oils of *H. pectinata* qualifies this species as a promising raw material source for the formulation and commercialization of bioproducts to control leaf-cutting ants. This finding, together with the unsystematic exploitation, the intervention of human activity, and the consequent deforestation of native areas highlight the importance to study and to create conservation strategies to maintain the diversity of this species.

The study of the wide variability in native plants is a fundamental way to conserve the species and select genes and alleles of interest for future use in breeding programs (Oliveira et al., 2013). Molecular markers have been the most frequent strategy used to analyze such variability. The inter-simple sequence repeat (ISSR) markers are advantageous owing to their high reproducibility and low costs; moreover, they do not require prior knowledge of DNA sequences for the development of specific primers of the species under analysis (Coral et al., 2016).

ISSR markers have been successfully used in the analysis of the genetic diversity of several species, such as *Cunila menthoides* Benth (Agostini et al., 2010), *Mentha cervina* (Rodrigues et al., 2013), *Satureja bachtiarica* Bunge (Khadivi-Khub et al., 2015), and *Varronia curassavica* Jacq. (Brito et al., 2016).

The present study was carried out to evaluate the genetic diversity of *H. pectinata* in Sergipe-Brazil, using ISSR molecular markers.

## MATERIAL AND METHODS

### Plant material and location

Fresh and young leaves were collected from 86 native plants of *H. pectinata* in 17 municipalities in the State of Sergipe (Figure 1 and Table 1). Samples were wrapped in gauze and stored in ice to avoid oxidation. In the Laboratory of Molecular Biology of Embrapa Coastal Tablelands, they were stored in a freezer at -80°C until DNA extraction.

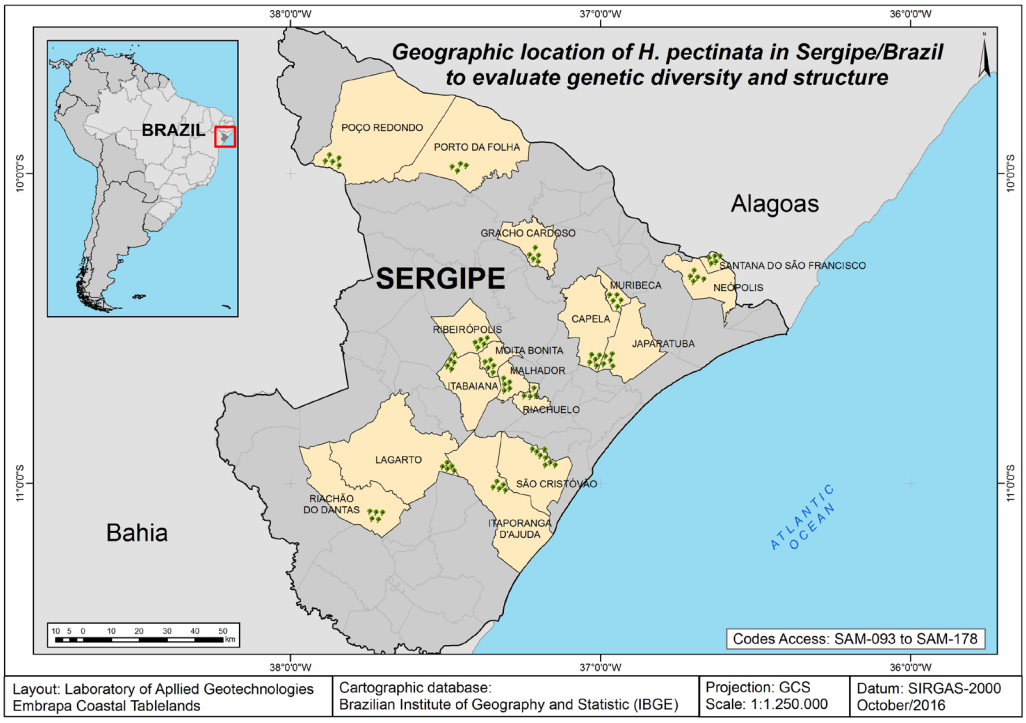


**Figure 1.** Location of collection points of 86 *Hyptis pectinata* plants in the State of Sergipe, Brazil.

### DNA extraction, quantification, and dilution

Three young leaves were used for DNA extraction, following 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. The extracted DNA was quantified using the NanoDrop 2000c (Thermo Scientific, Wilmington, DE, USA). Samples used in the reactions were diluted (5 ng/mL) in TE buffer solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored in a freezer at -20°C.

### Polymerase chain reaction (PCR), electrophoresis, and photodocumentation

Thirty-four ISSR primers were tested in this study (Eurofins MWG Operon - Operon

**Table 1.** Identification of 86 *Hyptis pectinata* plants collected in the State of Sergipe, Brazil.

| Plant code | N | Location of origin | Geographical coordinates |
|---|---|---|---|
| SAM093-SAM097 | 5 | Graccho Cardoso | 10°17'10.7"S 37°16'57.2"W; 10°17'11.8"S 37°16'58.3"W<br>10°17'12.3"S 37°16'58.4"W; 10°17'11.9"S 37°16'59.2"W<br>10°14'28.2"S 37°12'42.8"W |
| SAM098-SAM102 | 5 | Japaratuba | 10°35'00.8"S 36°57'51.7"W; 10°35'01.6"S 36°57'50.3"W<br>10°35'01.2"S 36°57'49.8"W; 10°35'01.7"S 36°57'50.0"W<br>10°35'02.0"S 36°57'49.3"W |
| SAM103-SAM110 | 8 | São Cristóvão | 10°54'44.1"S 37°11'46.1"W; 10°54'44.4"S 37°11'46.6"W<br>10°54'43.8"S 37°11'47.7"W; 10°53'33.4"S 37°10'50.9"W<br>10°53'32.9"S 37°10'52.3"W; 10°53'34.1"S 37°10'53.6"W<br>10°53'34.1"S 37°10'53.7"W; 10°53'33.8"S 37°10'53.6"W |
| SAM111-SAM114 | 4 | Porto da Folha | 09°58'11.2"S 37°27'12.0"W; 09°58'11.0"S 37°27'12.1"W<br>09°58'11.3"S 37°27'12.2"W; 09°58'11.4"S 37°27'12.3"W |
| SAM115-SAM119 | 5 | Poço Redondo | 09°57'45.2"S 37°51'51.2"W; 09°57'47.7"S 37°51'50.8"W<br>09°57'48.1"S 37°51'53.7"W; 09°57'46.0"S 37°51'53.3"W<br>09°57'48.7"S 37°51'53.5"W |
| SAM120-SAM124 | 5 | Capela | 10°35'29.8"S 36°59'08.5"W; 10°35'30.0"S 36°59'08.3"W<br>10°35'30.1"S 36°59'07.7"W; 10°35'30.0"S 36°59'08.5"W<br>10°35'29.9"S 36°59'09.1"W |
| SAM125-SAM129 | 5 | Muribeca | 10°24'34.6"S 36°57'27.2"W; 10°24'34.8"S 36°57'27.3"W<br>10°24'32.9"S 36°57'26.4"W; 10°24'32.5"S 36°57'26.3"W<br>10°24'32.4"S 36°57'26.5"W |
| SAM130-SAM134 | 5 | Santana do São Francisco | 10°16'04.8"S 36°36'53.0"W; 10°16'04.8"S 36°36'53.3"W<br>10°16'04.5"S 36°36'53.4"W; 10°16'03.4"S 36°36'54.5"W<br>10°16'04.6"S 36°36'55.5"W |
| SAM135-SAM139 | 5 | Neópolis | 10°20'11.3"S 36°41'16.5"W; 10°20'11.5"S 36°41'16.7"W<br>10°20'11.2"S 36°41'16.9"W; 10°20'12.3"S 36°41'18.1"W<br>10°20'13.7"S 36°41'20.2"W |
| SAM140-SAM-143 | 4 | Riachuelo | 10°43'04.8"S 37°12'41.6"W; 10°43'04.7"S 37°12'40.7"W<br>10°43'04.6"S 37°12'39.5"W; 10°43'03.7"S 37°12'39.1"W |
| SAM144-SAM148 | 5 | Malhador | 10°39'40.1"S 37°18'44.1"W; 10°39'39.9"S 37°18'44.1"W<br>10°39'39.8"S 37°18'44.2"W; 10°39'39.9"S 37°18'44.1"W<br>10°39'40.3"S 37°18'43.5"W |
| SAM149-SAM153 | 5 | Moita Bonita | 10°37'47.2"S 37°21'48.2"W; 10°37'47.3"S 37°21'47.8"W<br>10°37'47.3"S 37°21'47.6"W; 10°37'47.7"S 37°21'47.8"W<br>10°37'47.4"S 37°21'47.6"W |
| SAM154-SAM158 | 5 | Ribeirópolis | 10°33'34.1"S 37°22'23.7"W; 10°33'32.8"S 37°22'23.5"W<br>10°33'32.9"S 37°22'23.6"W; 10°33'33.5"S 37°22'24.4"W<br>10°33'33.7"S 37°22'24.5"W |
| SAM159-SAM163 | 5 | Itabaiana | 10°35'06.6"S 37°28'21.4"W; 10°35'05.3"S 37°28'20.9"W<br>10°35'05.1"S 37°28'20.7"W; 10°35'04.9"S 37°28'20.8"W<br>10°35'04.7"S 37°28'20.9"W |
| SAM164-SAM168 | 5 | Itaporanga d'Ajuda | 10°59'44.8"S 37°20'04.2"W; 10°59'44.9"S 37°20'04.3"W<br>10°59'45.5"S 37°20'04.4"W; 10°59'45.0"S 37°20'05.0"W<br>10°59'45.2"S 37°20'05.3"W |
| SAM169-SAM173 | 5 | Lagarto | 10°58'19.5"S 37°24'44.1"W; 10°58'19.3"S 37°24'44.2"W<br>10°58'19.6"S 37°24'44.3"W; 10°58'20.7"S 37°24'43.7"W<br>10°58'20.9"S 37°24'44.6"W |
| SAM174-SAM178 | 5 | Riachão do Dantas | 11°05'46.8"S 37°43'28.5"W; 11°05'46.2"S 37°43'28.6"W<br>11°05'45.5"S 37°43'28.5"W; 11°05'44.8"S 37°43'28.8"W<br>11°05'43.6"S 37°43'29.9"W |

N = number of plants.

Technologies, Louisville, KY, USA; IDT - Integrated DNA Technologies, Coralville, IA, USA; Invitrogen - Thermo Fisher Scientific, Carlsbad, CA, USA) on 2% agarose gel. PCRs were carried out in a total volume of 12 µL, containing 1 µL genomic DNA (5 ng/µL), 2.0 µL primer (25.0 pmol), 5.4 µL sterile MilQ water, 2 µL 10X buffer (100 mM Tris-HCl, pH 8.5, and 500 mM KCl) (Ludwig Biotec, Alvorada, RS, Brazil), 0.8 µL $MgCl_2$ (50 Mm) (Ludwig Biotec), 0.6 µL dNTP 5 nM), 0.2 µL Taq polymerase (5 U/µL) (Ludwig Biotec). The material was then amplified in a Proflex thermocycler (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA), programmed with the following protocol: initial denaturation at 94°C for 5 min, followed by 35 amplification cycles; denaturation at 94°C for 40 s; primer annealing for 1 min; extension at 72°C, for 1 min; and a final extension at 72°C for 7 min, followed by cooling at 4°C.

Amplification products were subjected to electrophoresis on 2% agarose gel. Molecular weights were estimated using 100-bp molecular weight marker (Ludwig) for each primer.

After electrophoresis, the gel was immersed in ethidium bromide solution for about 40 min and photodocumented with a Gel doc L-pix HE (Loccus Biotecnologia, Brazil).

## Data analysis

For the analyses, a binary matrix was constructed, according to the absence (0) or presence (1) of fragments, from the visualization of the bands on the gels.

Correlation estimates and the stress value were calculated using the Genes software (Cruz, 2006) for the analysis of fragment optimization.

Genetic diversity parameters, such as expected heterozygosity ($H_E$), polymorphism information content (PIC), and Shannon index were calculated using the GENALEX 6.5 software (Peakall and Smouse, 2012).

The Jaccard's similarity coefficient (Jaccard, 1908) was calculated, and a dendrogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA), using the NTSYSpc 2.0 software (Rohlf, 2001).

## RESULTS

A high level of polymorphism (100%) was found in ISSR markers among *H. pectinata* plants from the State of Sergipe. Fragments were visualized by the images generated by photodocumentation of the agarose gels (Figure 2).

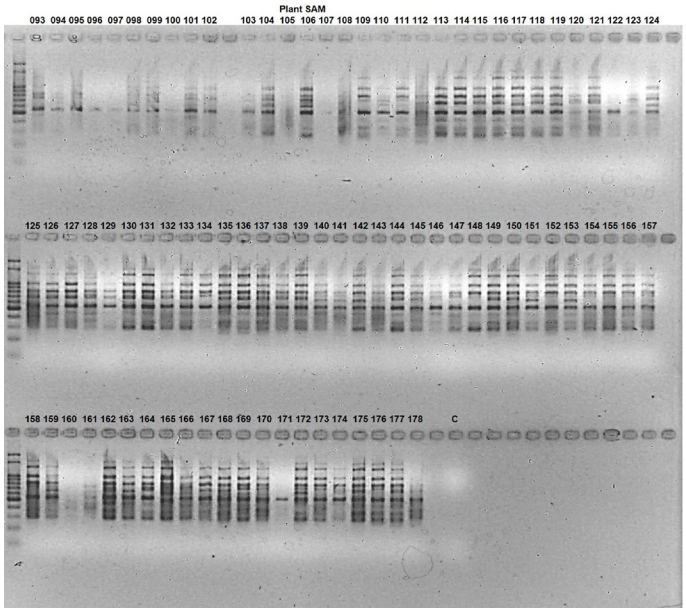


**Figure 2.** Agarose gel showing the electrophoretic profile of the inter-simple sequence repeat marker amplified using the primer UBC 807 for 86 *Hyptis pectinata* plants collected in different municipalities of the State of Sergipe, Brazil. C: negative control.

Of the 34 primers tested, nine provided reproducible and analyzable amplification products, totaling 67 fully polymorphic fragments, ranging from 4 (UBC 888) to 10 (UBC 861), and a mean number of 7.45 bands per primer (Table 2).

**Table 2.** Annealing temperature, Sequence, and amplified products used to analyze genetic diversity in *Hyptis pectinata* plants collected in the State of Sergipe, Brazil.

| Primer | Sequence (5'-3') | Length (bp) | Annealing temperature (°C) | Total number of fragments | Polymorphism (%) |
|---|---|---|---|---|---|
| UBC 807 | AGA GAG AGA GAG AGA GT | 100-1000 | 47.0 | 9 | 100.0 |
| UBC 809 | AGA GAG AGA GAG AGA GG | 100-750 | 57.2 | 9 | 100.0 |
| UBC 810 | GAG AGA GAG AGA GAG AT | 100-750 | 54.8 | 8 | 100.0 |
| UBC 835 | AGA GAG AGA GAG AGA GY | 100-750 | 58.8 | 9 | 100.0 |
| UBC 841 | GAG AGA GAG AGA GAG AYC | 100-750 | 58.8 | 7 | 100.0 |
| UBC 851 | GTG TGT GTG TGT GTG TYG | 150-1000 | 49.2 | 6 | 100.0 |
| UBC 861 | ACC ACC ACC ACC ACC ACC | 150-750 | 64.5 | 10 | 100.0 |
| UBC 862 | AGC AGC AGC AGC AGC AGC | 150-500 | 64.5 | 5 | 100.0 |
| UBC 888 | BDB CAC ACA CAC ACA CA | 150-300 | 56.4 | 4 | 100.0 |

In native plants of *H. pectinata*, the Shannon index ranged from 0.48 to 0.64, with a mean value of 0.58 per primer. For the $H_E$, values ranged from 0.32 (UBC 862) to 0.45 (UBC 809), with mean values of 0.39.

The Jaccard's similarity coefficient used to calculate the genetic similarity among the 86 *H. pectinata* plants by the ISSR markers ranged from 0.06 to 0.98, with a mean value of 0.65. SAM-117 and SAM-119, both from the Municipality of Poço Redondo, presented a greater similarity. Conversely, SAM-107 and SAM-171 were the most genetically distant. Most of the pairs with higher coefficients belonged to the same municipalities, and those with lower coefficients belonged to different municipalities. SAM-097 (Graccho Cardoso) presented, in general, the lowest similarity coefficient (Table 3).

**Table 3.** Pairs of plants with extreme values of the Jaccard's similarity coefficient.

| Greater similarity | | Lower similarity | |
|---|---|---|---|
| Pairs | Coefficients | Pairs | Coefficients |
| SAM-117 x SAM-119 | 0.9800 | SAM-107 x SAM-171 | 0.0600 |
| SAM-113 x SAM-114 | 0.9622 | SAM-107 x SAM-161 | 0.1052 |
| SAM-114 x SAM-115 | 0.9622 | SAM-097 x SAM-175 | 0.1206 |
| SAM-115 x SAM-116 | 0.9615 | SAM-094 x SAM-160 | 0.1315 |
| SAM-116 x SAM-119 | 0.9607 | SAM-100 x SAM-107 | 0.1363 |
| SAM-120 x SAM-121 | 0.9565 | SAM-107 x SAM-160 | 0.1428 |
| SAM-149 x SAM-150 | 0.9649 | SAM-097 x SAM-165 | 0.1428 |
| SAM-154 x SAM-155 | 0.9655 | SAM-097 x SAM-165 | 0.1451 |
| SAM-166 x SAM-167 | 0.9655 | SAM-097 x SAM-130 | 0.1500 |
| SAM-176 x SAM-177 | 0.9666 | SAM-110 x SAM-160 | 0.1500 |
| SAM-137 x SAM-138 | 0.9508 | SAM-101 x SAM-171 | 0.1522 |
| SAM-133 x SAM-139 | 0.9500 | SAM-097 x SAM-152 | 0.1525 |
| SAM-144 x SAM-145 | 0.9464 | SAM-160 x SAM-165 | 0.1525 |
| SAM-163 x SAM-167 | 0.9491 | SAM-097 x SAM-166 | 0.1551 |
| SAM-167 x SAM-168 | 0.9482 | SAM-097 x SAM-167 | 0.1552 |
| SAM-172 x SAM-177 | 0.9508 | SAM-097 x SAM-149 | 0.1579 |
| SAM-168 x SAM-170 | 0.9473 | SAM-097 x SAM-150 | 0.1579 |
| SAM-126 x SAM-127 | 0.9444 | SAM-097 x SAM-154 | 0.1579 |
| SAM-127 x SAM-128 | 0.9434 | SAM-097 x SAM-147 | 0.1632 |
| SAM-137 x SAM-139 | 0.9344 | SAM-097 x SAM-113 | 0.1698 |

The 86 *H. pectinata* plants were distributed into three groups based on the cluster analysis: Cluster I (79 plants); Cluster II (4 plants); Cluster III (2 plants) (Figure 3). SAM-107 was not associated with any of the clusters, suggesting greater genetic diversity when compared with the other plants.

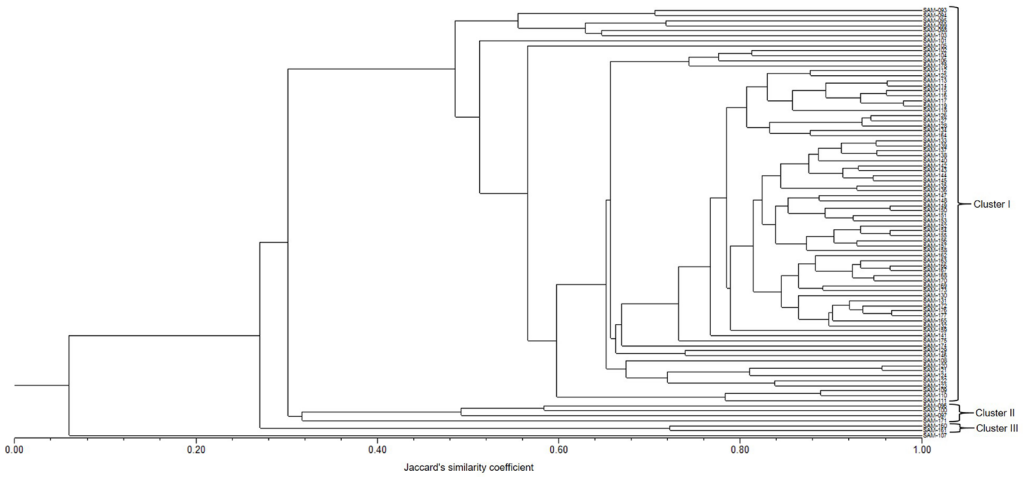


**Figure 3.** Dendrogram generated by the UPGMA analysis of the Jaccard's similarity coefficients for 86 *Hyptis pectinata* plants collected in different municipalities of the State of Sergipe, Brazil.

## DISCUSSION

Despite the several studies with ISSR used in plants of the family Lamiaceae, this is the first report on the genetic diversity of the species *H. pectinata*, which presented low to moderate diversity among the plants evaluated in the State of Sergipe. Fracaro and Echeverrigaray (2006) observed high genetic diversity in a study with *Hesperozygis ringens* Benth. plants collected in different regions of the South of Brazil. Agostini et al. (2010), when studying *Cunila menthoides* Benth. plants, found low genetic variability. Gadidasu et al. (2011) used 15 primers in a study with *Hyptis suaveolens*, which amplified a total of 123 fragments, ranging from 7 to 12 fragments per primer. Khadivi-Khub et al. (2015) studied individuals of *Satureja bachtiarica* Bunge. and observed high genetic diversity among them.

ISSR molecular markers have also been used as efficient tools to analyze the genetic diversity of many other medicinal plant species. Pillai et al. (2012) observed that 15 primers amplified a total of 91 fragments in *Rauvolfia serpentina* L., ranging from 2 to 11 fragments per primer. Tripathi et al. (2012) studied 25 primers in *Bacopa monnieri* L. and observed a mean amplification of 11 fragments. Brito et al. (2016) analyzed the genetic diversity of *Varronia curassavica* accessions and observed that 14 primers resulted in a mean amplification of 11 fragments. Xing et al. (2016) observed the mean value of 6.9 fragments from 15 primers in a study with *Toona sinensis* Roem.

The Shannon index may range from 0 to 1, with lower genetic diversity represented by values closer to zero (Silva et al., 2015). In the native plants of *H. pectinata* used in the present study, primers showed a mean value of 0.58, which corresponds to moderate diversity.

$H_E$ showed a mean value of 0.39, indicating low to moderate genetic variability among the studied plants.

PIC in the present study was of 0.49. Since this parameter defines the efficiency of the molecular marker in revealing the polymorphism between plants (Botstein et al.,1980), the markers used in the present study are considered as moderately informative (PIC > 0.5 - highly informative; 0.25 < PIC < and 0.5 - moderately informative; and PIC < 0.25 - poorly informative).

According to the dendrogram, 91.9% of the evaluated plants formed a single cluster, being very similar genetically. Plants more geographically isolated and with difficult access exhibit greater genetic differentiation (Gois et al., 2014). Several random *H. pectinata* plants occur in locations of high and easy accessibility, such as road borders and backyards, where the flow of transport, people, and animals is constant. The seed of this species is quite light and small and can be easily transported to different locations, which may explain the similarity found among plants.

Some plants collected in the same municipality were clustered separately. Different evolutionary factors may influence these genetic differentiations, such as migration, mutation, and natural selection (Loveless and Hamrick, 1984).

Faced with the increasing devastation of plant areas and with the use of medicinal plants by the population, studies that address the analysis of genetic diversity are fundamental to select priority genotypes that may serve to guide future pharmacological studies (Gonçalves et al., 2014). Genetic diversity of plants of the same species can result in the production of several active compounds, and consequently in several biological properties since genetic factors can influence the synthesis of these compounds.

Molecular markers allow quantifying the diversity between individuals of the same species and clustering the genetically similar ones. Therefore, they are efficient and extremely appropriate tools for the elaboration of conservation strategies, as well as for the use of plant resources in future breeding programs.

The genetic diversity found among native plants of *H. pectinata* of the State of Sergipe can be considered as low to intermediate. These results are important to guide the choice of conservation strategies of this species and demonstrate the need to use a greater number of primers and plants of *H. pectinata* for a better evaluation of the genetic diversity.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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