

Genetic diversity of native populations of *Croton tetradenius* Baill. using ISSR markers

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ABSTRACT. Brazil has about 300 *Croton* species in different types of vegetation. *Croton tetradenius* Baill., which is endemic to the Northeast region and predominant in the Caatinga vegetation, stands out among the several species of this genus. Considering the importance of knowing the genetic variability of a species, the objective of this study was to analyze the genetic diversity of the genotypes of natural populations of *C. tetradenius* in the State of Sergipe, using ISSR molecular markers. Forty individuals were sampled in four natural populations of the State of Sergipe, Brazil. Thirteen primers were used for DNA amplification using ISSR-PCR, totaling 77 amplified fragments, of which 94.8% were polymorphic. Results of the cluster analysis obtained by the Jaccard's similarity index, using the UPGMA method, resulted in the formation of six distinct clusters. Analysis of molecular variance (AMOVA), used to estimate the genetic variability among populations, revealed significant genetic variance ($P < 0.01$) between and within the

studied populations, and most of the genetic diversity was found (87%) within populations. According to the Jaccard's similarity index, none of the studied plants was genetically identical. CTE210 and CTE305 presented high similarity index (0.76), while CTE105 presented low similarity index (<0.16) with all related individuals. ISSR markers were efficient and allowed the formation of a molecular profile, and had sufficient polymorphism to estimate the genetic variability between the accessions of the studied populations.

Key words: *Croton tetradenius*; Conservation strategies; Genetic diversity; ISSR

INTRODUCTION

Croton tetradenius Baill. is an endemic species of the Northeast region of Brazil, found in the Caatinga vegetation in the States of Alagoas, Bahia, Ceará, Paraíba, Pernambuco, Rio Grande do Norte, and Sergipe (Carneiro-Torres, 2009). It is disseminated in areas of sandy or stony soil, in shaded and humid environments (Lucena, 2009; Silva et al., 2009). It is popularly known as “caatinga-de-bode”, “zabelê”, “velandinho”, “barba-de-bode”, and has delicate leaves and a well-accented mentholated aroma (Lucena, 2009).

The genus *Croton* is one of the largest representatives of the family Euphorbiaceae (Webster, 1993). Some species of the genus *Croton* have been distinguished by their diverse pharmacological properties (Fontenelle et al., 2008). Despite the great diversity and significant importance of this genus, taxonomy, ecology, genetics, phytochemistry, and ethnobotany studies are still necessary, as well as the promotion of product development, and *in situ* and *ex situ* conservation strategies (Alves et al., 2012). The development of an essential oil-based formulation of *C. tetradenius* with biological potential was reported for the first time using the deposit of a patent (Blank et al., 2017).

The economic potential of medicinal and aromatic plants has contributed to the significant growth of the interest for germplasm characterization and conservation (Souza, 2015). Germplasm characterization allows the insertion of materials of interest in breeding programs and serves as a basis for the design of conservation strategies. The genetic characterization of natural populations using molecular markers has been reported in several studies (Estopa et al., 2006; Bertoni et al., 2010; Giustina et al., 2014; Soares et al., 2016).

Several molecular markers are available in the market, and the knowledge on the properties and applications of the species is necessary to choose the appropriate marker according to the profile of the study of interest (Souza, 2015).

Molecular markers, which are identifiable DNA sequences specific to the genome, are useful tools in the exploration of genetic diversity and provide information on genetic variability by eliminating possible environmental effects (Soares et al., 2016).

ISSR (inter-simple sequence repeat) markers were developed by Zietkiewicz et al. (1994) and are based on the amplification of regions between DNA adjacent microsatellite sequences via PCR (polymerase chain reaction). For being dominant markers, prior knowledge on the genome is not required (González et al., 2002). Moreover, ISSR markers have a high degree of polymorphism, high reproducibility, when compared with other markers, and can achieve results in a timely and cost-effectively manner (Borba et al., 2005). Therefore, ISSR

markers are very useful in studies that aim at determining the genetic distance between parents, as well as the performance of the hybrids for the construction of genetic linkage maps, and for the characterization of accessions and cultivars of several species (Santos et al., 2013; Soares et al., 2016).

ISSR markers are more reproducible than other dominant markers, such as random amplified polymorphic DNA (RAPD). The reproducibility of this marker is associated with two characteristics that provide high specificity to the ISSR primers: high annealing temperature and design based on the microsatellite regions, which makes ISSR semi-arbitrary markers (Sandes et al., 2016).

Several studies on genetic diversity in medicinal plants have been carried out using ISSR molecular markers, such as those in *Croton* (Lira Neto, 2011; Scaldaferrri et al., 2014; Rocha et al., 2016), in *Varronia curassavica* Jacq. (Brito et al., 2016), in *Capparis spinosa* L. (Liu et al., 2015), in *Rheum* (Tabin et al., 2016), in *Withania somnifera* (Khan and Shah, 2016), and in *Panax stipuleanatus* Tsai (Trieu et al., 2016).

The objective of this study was to analyze the genetic diversity of plants from four natural populations of *C. tetradenius* in the State of Sergipe, using ISSR molecular markers.

MATERIAL AND METHODS

Plant material

Three young leaves were collected from plants from four natural populations of *Croton tetradenius* in the municipalities of Lagarto (Povoado Antônio Conselheiro and Povoado Tiradentes), Porto da Folha and Poço Redondo, in the State of Sergipe, totaling 40 individuals (Figure 1 and Table 1). To prevent oxidation of the collected samples, leaves were wrapped in sterile gauze and stored on ice, and frozen at -80°C . Subsequently, the plant material was lyophilized in a LioTop (L101) and stored in a desiccator containing silica gel until DNA extraction.

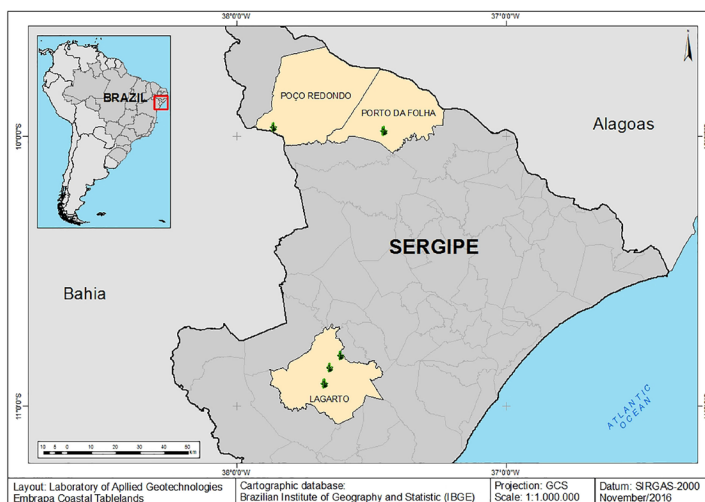


Figure 1. Map with the locations of the *Croton tetradenius* plants collected from natural populations in the State of Sergipe, Brazil.

Table 1. Identification of *Croton tetradenius* plants from four native populations, in the State of Sergipe, Brazil.

Plant code	Pop.	N	Origin (Municipality, village)	Georeferenced information
CTE101-110	1	10	Lagarto, Tiradentes	10°48'51.4"S 37°36'52.4"W; 10°48'51.5"S 37°36'52.4"W; 10°48'51.3"S 37°36'52.3"W; 10°48'52.5"S 37°36'52.4"W; 10°48'52.7"S 37°36'52.1"W; 10°48'52.7"S 37°36'51.7"W; 10°48'52.4"S 37°36'51.1"W; 10°48'52.2"S 37°36'51.3"W; 10°48'52.0"S 37°36'51.6"W; 10°48'53.0"S 37°36'52.5"W
CTE201-210	2	10	Porto da Folha, Lagoa do Rancho	9°58'54.3"S 37°27'11.9"W; 9°58'54.3"S 37°27'11.9"W; 9°58'54.4"S 37°27'11.3"W; 9°58'54.4"S 37°27'11.8"W; 9°58'54.3"S 37°27'12.4"W; 9°58'54.4"S 37°27'12.3"W; 9°58'54.5"S 37°27'12.5"W; 9°58'54.4"S 37°27'12.6"W; 9°58'54.3"S 37°27'12.6"W; 9°58'54.3"S 37°27'12.4"W
CTE301-310	3	10	Poço Redondo, Serra da Guia	9°58'07.0"S 37°51'49.0"W; 9°58'07.0"S 37°51'49.2"W; 9°58'07.0"S 37°51'49.1"W; 9°58'07.0"S 37°51'49.5"W; 9°58'06.9"S 37°51'49.3"W; 9°58'06.7"S 37°51'49.1"W; 9°58'06.2"S 37°51'49.0"W; 9°58'06.7"S 37°51'49.2"W; 9°58'06.9"S 37°51'50.0"W; 9°58'06.5"S 37°51'49.7"W;
CTE401-410	4	10	Lagarto, Antônio Conselheiro	10°55'14.1"S 37°40'28.7"W; 10°55'14.3"S 37°40'28.9"W; 10°55'14.1"S 37°40'28.8"W; 10°55'14.1"S 37°40'28.7"W; 10°51'38.1"S 37°39'18.1"W; 10°51'38.0"S 37°39'18.8"W; 10°51'38.2"S 37°39'18.8"W; 10°51'38.2"S 37°39'18.7"W; 10°51'37.8"S 37°39'20.0"W; 10°51'37.8"S 37°39'19.8"W

Pop. = population; N = number of plants.

DNA extraction and ISSR-PCR amplification

The previously lyophilized young leaves were subjected to DNA extraction, using approximately 1 g fresh leaves for each sample, based on the method described by Doyle and Doyle (1990), modified by Alzate-Marin et al. (2009). DNA was quantified using the Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). After dilutions at the desired concentrations, samples were stored at -20°C for further use in the PCRs.

The ISSR primers used to estimate the genetic diversity of the 40 individuals of *C. tetradenius* were obtained from the University of British Columbia, Vancouver, Canada (Table 2).

Thirteen primers were selected for DNA amplification using the ISSR-PCR. Each reaction was performed in a pre-sterile 12- μ L microtube containing 1 μ L genomic DNA (5 ng/ μ L), 0.2 μ L recombinant Taq polymerase from *Thermus aquaticus*, expressed in *Escherichia coli* (Ludwig Biotec, Brazil) (5 U/ μ L), 2 μ L 10X buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl), 0.6 μ L MgCl₂ (50 mM) (Ludwig Biotec), 0.4 μ L dNTP (2.5 mM), 2.0 μ L primer (25.0 pmol), and 5.8 μ L autoclaved ultrapure water.

DNAs were amplified using the thermocycler ProFlex PCR System (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA) programmed with the following protocol: an initial denaturation at 95°C for 5 min, followed by 35 amplification cycles; denaturation at 94°C for 40 s; annealing at different temperatures for 1 min (depending on the optimum primer temperature); extension at 72°C for 1 min; and a final extension at 72°C for 7 min.

Amplification products were subjected to horizontal electrophoresis on 2.0% agarose gel and then stained with ethidium bromide (0.5 μ L/mL), visualized under ultraviolet light, and photodocumented in Gel Doc L-pix (Loccus Biotecnologia, Cotia, SP, Brazil). Molecular weights were estimated using a 1-kb scale for each primer (Ludwig Biotec).

Data analysis

By analyzing the agarose gels, a binary matrix was obtained based on the presence (1) and absence (0) of bands for all selected primers. The binary data were used in all analyses.

Genetic similarities among individuals were obtained using the Jaccard's similarity index (Jaccard, 1908). For plant clustering, based on the genetic similarity, the unweighted

pair group method with arithmetic mean (UPGMA) was used to construct the dendrogram, with the aid of the NTSYS-pc 2.0 software (Rohlf, 2001). The values of correlation and stress, polymorphic information content (PIC), and expected heterozygosity (H_E) under the Hardy-Weinberg equilibrium (HWE) were also estimated using the GENES program (Cruz, 2001).

Percentage of polymorphic bands (PPB), number of observed alleles, effective number of alleles (N_E), Nei's genetic diversity (h), and Shannon index were estimated using the Genalex 6.5 software (Peakall and Smouse, 2012).

RESULTS

By the UPGMA dendrogram, based on the Jaccard's similarity index, six main clusters were formed using a mean distance of approximately 0.44 (Figure 2). Cluster I is composed mostly of natural individuals from the same geographical location (Lagarto, Antônio Conselheiro village), except for CTE101 (Lagarto, Tiradentes village) and CTE207 (Porto da Folha, Lagoa do Rancho village). Cluster II, the largest representative, consisted of 27 individuals from all the studied populations, emphasizing that all the individuals that represent the population 03 (Poço Redondo, Serra da Guia) were clustered together.

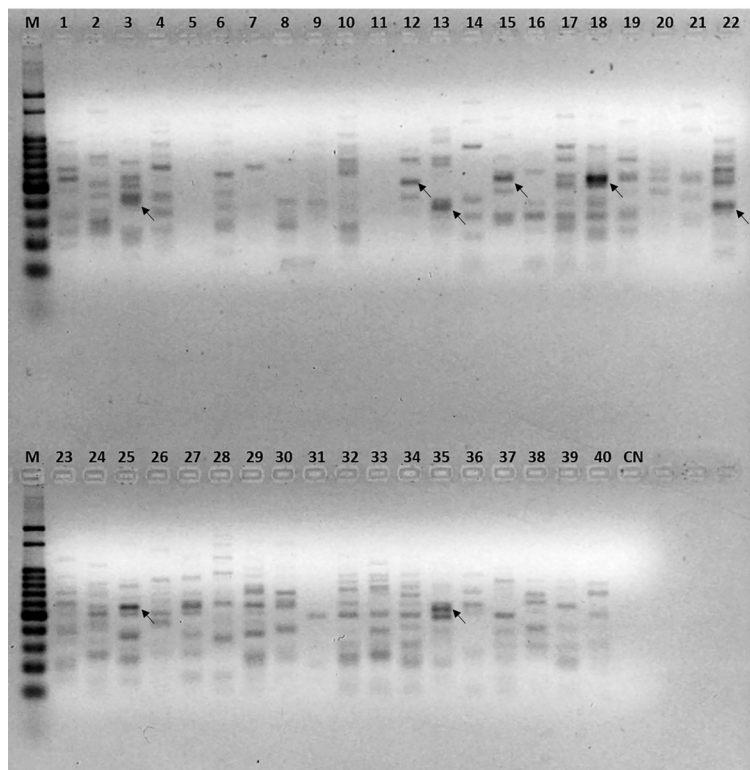


Figure 2. Agarose gels showing the electrophoretic profiles of the inter-simple sequence repeat markers amplified using the primer 842 in 40 *Croton tetradenius* plants from four native populations, in the State of Sergipe, Brazil. (Lane M: 1-kb molecular weight marker; lanes 1 to 10: Population 1; lanes 11 to 20: Population 2; lanes 21 to 30: Population 3; lanes 31 to 40: Population 4; CN: negative control). Arrows indicate specific bands.

Thirteen ISSR primers were used for the study on the genetic variability of the 40 samples of *C. tetradenius*, totaling 77 amplified fragments, of which 94.8% were polymorphic. The number of amplified fragments ranged from 3 to 11 bands per primer, totaling a mean of 5.9 bands per primer (Table 2).

Table 2. Annealing temperature, sequence, and amplified products used for genetic diversity analysis in *Croton tetradenius* plants from four native populations, in the State of Sergipe, Brazil.

Primer name	Sequence (5'-3')	Length (bp)	Annealing temperature (°C)	Total bands	Polymorphic bands	Polymorphism (%)
UBC851	GTG TGT GTG TGT GTG TYG	300-900	49.2	7	6	85.7
UBC817	CAC ACA CAC ACA CAC AA	300-1250	54.8	5	5	100.0
UBC812	GAG AGA GAG AGA GAG AA	300-1000	54.8	7	7	100.0
UBC816	CAC ACA CAC ACA CAC AT	300-800	54.8	6	6	100.0
UBC842	GAG AGA GAG AGA GAG AYG	200-1500	58.8	10	10	100.0
UBC864	ATG ATG ATG ATG ATG ATG	500-900	50.8	5	4	80.0
UBC888	BDB CAC ACA CAC ACA CA	200-700	56.4	5	5	100.0
UBC890	VHV GTG TGT GTG TGT GT	400-700	56.4	4	4	100.0
UBC809	AGA GAG AGA GAG AGA GG	300-800	57.2	3	2	66.6
UBC810	GAG AGA GAG AGA GAG AT	300-800	54.8	5	4	80.0
UBC811	GAG AGA GAG AGA GAG AC	400-700	46.8	3	3	100.0
UBC848	CAC ACA CAC ACA CAC ARG	200-1000	58.8	11	11	100.0
UBC815	CTC TTC TCT CTC TCT CTG	400-1500	47.6	6	6	100.0

The similarity matrix based on the Jaccard's similarity index among the 40 *C. tetradenius* showed a minimum value of 0.01 between the plants CTE105 and CTE210, and a maximum value of 0.76 between the plants CTE210 and CTE305. Greater genetic diversity was observed between two plants of different populations, CTE105 (Lagarto, Tiradentes village) and CTE210 (Porto da Folha, Lagoa do Rancho village), with a similarity index of 0.01. Conversely, CTE210 (Porto da Folha, Lagoa do Rancho village) and CTE305 (Poço Redondo, Serra da Guia), which also belong to different populations, presented the highest genetic diversity, with a similarity index of 0.76 (Table 3).

The reliability of the results was verified by the values of stress (0.0245), and correlation (0.997) for the 40 *C. tetradenius* studied plants, which confirmed the stability of the number of selected primers and the number of obtained fragments.

Genetic variability indices, such as H_E and the Shannon index, presented low means, 0.30 and 0.45, respectively, indicating low levels of genetic diversity (Table 4).

The clustering between individuals by the Jaccard's similarity index, using the UPGMA method, resulted in six distinct clusters: cluster I (CTE101, CTE207, CTE402, CTE403, CTE404, CTE405, CTE407, CTE408, and CTE410); cluster II (CTE102, CTE103, CTE104, CTE106, CTE108, CTE109, CTE110, CTE201, CTE202, CTE204, CTE205, CTE206, CTE208, CTE209, CTE210, CTE301, CTE302, CTE303, CTE304, CTE305, CTE306, CTE307, CTE308, CTE309, CTE310, CTE406, and CTE409); cluster III (CTE107); cluster IV (CTE203); cluster V (CTE401); and cluster VI (CTE105) (Figure 3).

Analysis of molecular variance (AMOVA), used to estimate the genetic variability among populations, revealed significant genetic variance ($P < 0.01$) between and within the studied populations, being most of the genetic diversity found within the populations (87%) (Table 5).

Table 3. Matrix generated based on the Jaccard's similarity coefficient of 40 *Croton tetradenius* plants from four native populations, in the State of Sergipe, Brazil.

Plant	Plant (C/F/E)																																							
	01	102	103	104	106	107	108	109	110	201	202	203	204	205	206	207	208	209	210	301	302	303	304	305	306	307	308	309	310	401	402	403	404	405	406	407	408	409		
CE1E105	0.64	0.63	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E104	0.66	0.53	0.48	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E105	0.16	0.07	0.10	0.11	0.07	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E106	0.54	0.37	0.52	0.51	0.14	0.33	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E108	0.43	0.58	0.54	0.50	0.07	0.63	0.32	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E109	0.46	0.50	0.45	0.45	0.05	0.44	0.33	0.46	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E110	0.38	0.46	0.50	0.45	0.05	0.44	0.33	0.46	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E120	0.33	0.61	0.54	0.42	0.06	0.56	0.28	0.58	0.52	0.54	0.53	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E204	0.33	0.59	0.44	0.43	0.06	0.47	0.32	0.56	0.37	0.52	0.47	0.51	0.25	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E205	0.49	0.61	0.51	0.50	0.09	0.56	0.33	0.58	0.46	0.52	0.64	0.64	0.26	0.33	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E206	0.45	0.64	0.57	0.51	0.08	0.49	0.37	0.67	0.47	0.53	0.47	0.56	0.28	0.44	0.53	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E207	0.45	0.64	0.57	0.51	0.08	0.49	0.37	0.67	0.47	0.53	0.47	0.56	0.28	0.44	0.53	0.61	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E208	0.46	0.58	0.50	0.41	0.09	0.59	0.28	0.64	0.39	0.60	0.54	0.54	0.17	0.55	0.54	0.61	0.60	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E209	0.42	0.58	0.52	0.48	0.08	0.54	0.34	0.56	0.40	0.58	0.57	0.58	0.25	0.41	0.56	0.47	0.55	0.62	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E302	0.42	0.53	0.49	0.43	0.07	0.59	0.37	0.53	0.42	0.55	0.50	0.56	0.36	0.56	0.54	0.58	0.50	0.61	0.50	0.53	0.64	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E303	0.40	0.57	0.50	0.46	0.09	0.56	0.32	0.49	0.48	0.54	0.48	0.52	0.29	0.52	0.52	0.51	0.53	0.57	0.57	0.60	0.60	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E304	0.40	0.57	0.50	0.46	0.09	0.56	0.32	0.49	0.48	0.54	0.48	0.52	0.29	0.52	0.52	0.51	0.53	0.57	0.57	0.60	0.60	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E305	0.39	0.55	0.49	0.41	0.08	0.49	0.32	0.53	0.37	0.55	0.53	0.56	0.23	0.56	0.59	0.59	0.55	0.64	0.53	0.76	0.68	0.56	0.44	0.56	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E306	0.47	0.59	0.52	0.46	0.11	0.54	0.34	0.51	0.47	0.55	0.53	0.59	0.25	0.52	0.59	0.49	0.45	0.59	0.58	0.54	0.59	0.59	0.64	0.03	0.54	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E307	0.47	0.53	0.47	0.46	0.10	0.57	0.35	0.58	0.50	0.58	0.45	0.60	0.33	0.52	0.59	0.51	0.50	0.61	0.55	0.48	0.59	0.63	0.61	0.60	0.56	0.02	0.67	--	--	--	--	--	--	--	--	--	--	--	--	
CE1E309	0.49	0.67	0.57	0.45	0.11	0.65	0.33	0.58	0.43	0.69	0.52	0.64	0.31	0.51	0.58	0.53	0.52	0.61	0.61	0.38	0.56	0.66	0.60	0.62	0.01	0.64	0.61	0.70	--	--	--	--	--	--	--	--	--	--		
CE1E310	0.36	0.60	0.46	0.43	0.09	0.49	0.33	0.63	0.47	0.56	0.56	0.62	0.25	0.49	0.48	0.37	0.40	0.51	0.50	0.48	0.62	0.59	0.58	0.55	0.49	0.69	0.57	0.59	0.41	--	--	--	--	--	--	--	--	--		
CE1E311	0.40	0.55	0.48	0.45	0.10	0.54	0.34	0.51	0.47	0.55	0.53	0.59	0.25	0.52	0.59	0.49	0.45	0.59	0.58	0.54	0.59	0.59	0.64	0.03	0.54	0.61	0.70	0.41	0.41	--	--	--	--	--	--	--	--	--		
CE1E402	0.48	0.43	0.38	0.43	0.16	0.41	0.28	0.40	0.22	0.51	0.33	0.33	0.32	0.47	0.33	0.47	0.54	0.46	0.45	0.40	0.40	0.40	0.40	0.40	0.32	0.58	0.37	0.38	0.31	0.29	0.68	--	--	--	--	--	--	--		
CE1E403	0.41	0.38	0.33	0.37	0.09	0.38	0.28	0.40	0.29	0.40	0.28	0.29	0.29	0.42	0.38	0.42	0.45	0.41	0.45	0.35	0.37	0.40	0.40	0.40	0.40	0.32	0.58	0.37	0.38	0.31	0.29	0.68	--	--	--	--	--	--		
CE1E404	0.57	0.36	0.42	0.42	0.14	0.48	0.31	0.49	0.36	0.55	0.50	0.40	0.40	0.34	0.53	0.44	0.56	0.48	0.53	0.50	0.48	0.51	0.52	0.49	0.48	0.48	0.50	0.50	0.45	0.38	0.50	0.58	0.62	--	--	--	--	--		
CE1E406	0.46	0.47	0.40	0.38	0.15	0.45	0.31	0.39	0.29	0.46	0.40	0.45	0.36	0.39	0.40	0.41	0.41	0.53	0.41	0.47	0.43	0.56	0.41	0.52	0.48	0.46	0.41	0.48	0.50	0.40	0.37	0.46	0.41	--	--	--	--	--		
CE1E407	0.45	0.43	0.43	0.43	0.12	0.46	0.25	0.42	0.37	0.50	0.44	0.44	0.44	0.44	0.44	0.44	0.46	0.53	0.54	0.47	0.39	0.49	0.42	0.51	0.49	0.41	0.44	0.54	0.46	0.45	0.38	0.44	0.41	0.54	0.72	0.38	0.39			
CE1E408	0.40	0.40	0.40	0.40	0.10	0.40	0.20	0.40	0.20	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40		
CE1E409	0.40	0.55	0.51	0.56	0.09	0.54	0.33	0.58	0.35	0.60	0.45	0.55	0.30	0.59	0.56	0.64	0.49	0.53	0.49	0.42	0.48	0.53	0.57	0.47	0.46	0.53	0.61	0.48	0.47	0.51	0.24	0.69	0.43	0.61	0.47	0.44	0.54	0.47		
CE1E410	0.47	0.46	0.43	0.36	0.15	0.37	0.29	0.37	0.33	0.46	0.41	0.37	0.26	0.36	0.37	0.29	0.38	0.37	0.44	0.45	0.41	0.32	0.45	0.39	0.41	0.38	0.39	0.39	0.43	0.23	0.26	0.51	0.37	0.51	0.52	0.36	0.42	0.33	0.37	

Table 4. Number of observed alleles (N), effective number of alleles (N_E), Shannon's index (I), expected (H_E) and observed (H_O) heterozygosities, and polymorphic information content (PIC) for the four native populations of *Croton tetradenius*, obtained by ISSR markers.

Population	N° of individuals	N	N_E	I	H_E	H_O	PIC
Pop. 1	10	1.78	1.58	0.49	0.33	0.37	0.23
Pop. 2	10	1.48	1.44	0.39	0.26	0.29	0.17
Pop. 3	10	1.85	1.58	0.50	0.33	0.37	0.24
Pop. 4	10	1.75	1.45	0.42	0.27	0.31	0.22
Total	40	1.71	1.51	0.45	0.30	0.33	0.22

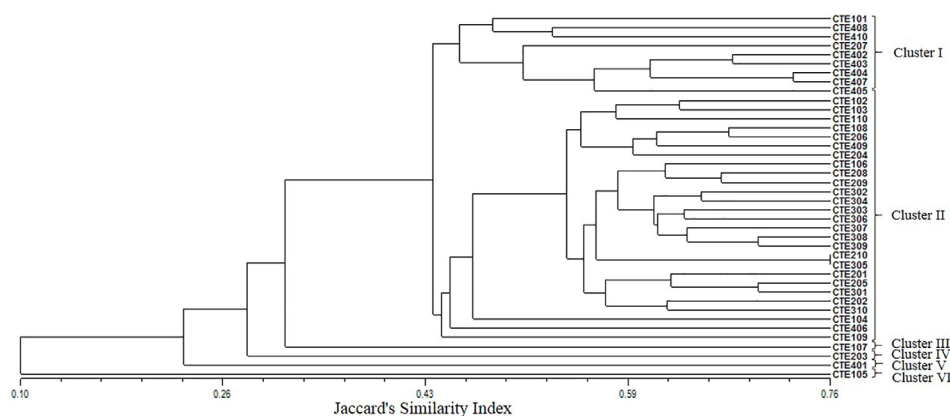


Figure 3. Dendrogram generated by the unweighted pair group method with arithmetic mean analysis (UPGMA) of Jaccard's similarity indices for 40 *Croton tetradenius* plants from four populations, in the State of Sergipe, Brazil.

Table 5. Analysis of molecular variance of 40 *Croton tetradenius* plants from four native populations, in the State of Sergipe, Brazil.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Components of variation	% of variation
Among populations	3	98.85	32.95	2	13
Within populations	36	465.80	12.94	12.93	87
Total	39	564.65	-	14.94	100

DISCUSSION

Considering the set of parameters of diversity evaluated, the genetic variability among the studied *C. tetradenius* individuals was moderate.

The primer UBC 848 presented the highest number of fragments (11), resulting in 100% polymorphism. The low percentage of monomorphic primers found in this study demonstrates the efficiency of the ISSR molecular marker.

Many studies have proved the effectiveness of this marker in articles on genetic diversity and characterization of accessions between and within populations, such as those with *Ocimum basilicum* (Aghaei et al., 2012), *Pitcairnia flammea* (Souza-Sobreira et al., 2015), *Croton heliotropiifolius* (Rocha et al., 2016), *Erythrina velutina* (Souza et al., 2016), and *Varronia curassavica* (Brito et al., 2016).

Values different from zero were expected in relation to the mean value of genetic diversity (0.30) and to the Shannon index (0.45), since under natural conditions, the Hardy-Weinberg equilibrium is not expected. This is because individuals are likely to incorporate new alleles using crosses, and lose alleles using genetic drift (Silva et al., 2012). The Shannon index may vary from 0 to 1, and lower genetic diversity is represented by values closer to zero (Silva et al., 2015). Several studies on natural populations indicate the percentage of the polymorphic locus as an important measure of genetic diversity; however, despite being commonly used, a variation in these values is observed (Soares et al., 2016). According to Nei (1987), the proportion of polymorphic locus is not a significant measure of the genetic variation, and thus the parameter of genetic diversity (H_E) is more appropriate.

This study was the first to use ISSR markers to evaluate genetic diversity in *C. tetradenius* plants. According to the Jaccard's similarity index, none of the studied plants is genetically identical, which indicates that the resolution level was sufficient to distinguish all genotypes (Silva et al., 2013). CTE210 and CTE305 showed greater genetic similarity (0.76), while CTE105 showed low similarity index (<0.16) with all related individuals.

High similarity indices suggest that individuals have a closer genetic relationship between each other, while lower indices reveal greater genetic distance (Sayed et al., 2009).

Probably, great genetic proximity occurs between the individuals of this population (Poço Redondo, Serra da Guia). Clusters III, IV, V, and VI are individually represented by the genotypes CTE105 (Lagarto, Tiradentes village), CTE107 (Lagarto, Tiradentes village), CTE203 (Porto da Folha, Lagoa do Rancho village), and CTE401 (Lagarto, Antônio Conselheiro village), respectively (Figure 2).

These genotypes have wide genetic variability, being, therefore, more divergent when compared with the other individuals. These results can be used in breeding and conservation programs of the species, and for commercial purposes.

The genetic structure of plant populations involves interactions of several distinct processes, such as habitat fragmentation and/or population isolation, changes in distribution, mutation, reproductive isolation, genetic drift, gene flow, selection, among others (Schaal et al., 1998; Thendral Hepsibha et al., 2010).

The separation of the 40 *C. tetradenius* individuals into six distinct clusters confirms the results obtained by AMOVA, which revealed greater genetic diversity within than between the studied populations.

The genetic study of natural populations allows the evaluation and quantification of genetic variability and its distribution in time and space. Thus, the greater the genetic variability in the population, the greater is the chance of perpetuation of the species. Besides, the characterization of the variability between and within natural populations allows the implementation of conservation strategies (Estopa et al., 2006).

ISSR markers have demonstrated their efficiency in the study of genetic variability for several other species. Lira Neto (2011) evaluated five DAF primers and six ISSR primers in 40 accessions from 27 *Croton* species, resulting in 186 bands produced by the ISSR marker, all of which were polymorphic. However, the number of primers must be increased to consolidate the clustering of the species.

Brito et al. (2016) studied the genetic diversity between *Varronia curassavica* accessions, using 14 primers, and obtained 149 bands with 97.98% polymorphism, Shannon index of 0.42, H_E of 0.27; values close to the ones found in the present study. Rocha et al. (2016), when evaluating 41 *Croton heliotropiifolius* individuals, used 18 RAPD primers,

which is also a dominant marker, and 15 ISSR primers, totaling 137 bands, of which 73 bands were produced by RAPD markers, and 64 bands were produced by ISSR markers, and the diversity found is relatively high. Soares et al. (2016) genetically characterized individuals from 10 *Hancornia speciosa* populations using 15 primers, generating 162 fragments with 100% polymorphism.

The authors state that genetic diversity among populations (77%) was higher than within the populations (23%), unlike the present study.

The flow between individuals may occur by pollinators and seed dispersers; thus, the knowledge on the seed pollination and dispersal mechanisms is important (Rocha et al., 2016). In addition, the knowledge of the reproduction system of the species of interest is fundamental, since this characteristic can influence the genetic variability, both by homogenizing and by increasing the divergence between individuals and populations (Zanella et al., 2012).

Genetic variation and genetic relationships were efficiently determined using the ISSR markers. Knowledge on the genetic diversity of the selected individuals is of ultimate importance, since it contributes to the information on the species and allows the selection of genotypes to be included in future conservation programs. Thus, the most divergent genotypes can be selected, as well as the most similar, according to the research interest.

The results of this study will help the research group to select plants for a collection of *C. tetradenius* in the germplasm bank of medicinal and aromatic plants of the Federal University of Sergipe.

Conflicts of interest

The authors declare no conflict of interests.

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