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RESEARCH ARTICLE

Genetic diversity and population structure of *Eplingiella* species (Lamiaceae) using ISSR markers

Anderson de Carvalho Silva · Lenaldo Muniz de Oliveira · José Floriano Barêa Pastore · Cassio van den Berg · Taliane Leila Soares · Everton Hilo de Souza

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Abstract The present study evaluated the population structure and genetic diversity using inter simple sequence repeat (ISSR) markers in 18 natural populations belonging to three species of *Eplingiella* (*E. cuniloides, E. fruticosa* and *E. brightoniae*), found growing naturally in the semiarid region of Northeast Brazil. Samples of 265 plants were analyzed using nine primer combinations, which generated 131 informative bands. *Eplingiella* spp. populations showed moderate genetic diversity (percentage of polymorphic bands, PPB = 75.6–96.9%, Nei's genetic diversity $He = 0.31–0.39$, Shannon's information index $I = 0.33–0.48$). Molecular variance analysis revealed that within populations, variations contributed more (74%) to the genetic diversity than between population variations (26%), with percentage of the genetic differentiation coefficient ($G_{ST} = 0.29$). The mean value of F_{ST} was 0.175, demonstrating good differentiation between populations. The analysis of the structure by the Bayesian method revealed the formation of two groups (K = 2), with many migrant individuals and a high level of miscegenation. The hierarchical cluster dendrogram grouped the 18 populations into two major clusters, with good support for the main clades (100%). According to principal component analysis, the two main principal components explained 21.06% of the total variation. The ISSR markers used were effective in identifying the variability of natural populations of *Eplingiella* spp., and population structure demonstrated recent diversification of species. The results shed more light on the genetic variation and evolutionary dynamics of *Eplingiella*, helping to formulate effective breeding strategies.

Keywords Genetic variability · Natural populations · "Alecrim de vaqueiro" · Molecular marker · Polymorphism

Abbreviations
ISSR Inter simple sequence repeat

A. d. Silva · L. M. de Oliveira · C. van den Berg · T. L. Soares (✉)
Universidade Estadual de Feira de Santana, Av. Transnordestina, s/n, Novo Horizonte, Feira de Santana, BA 44036-900, Brazil
e-mail: talialeila@gmail.com

A. d. Silva
e-mail: bio.anderson@gmail.com

L. M. de Oliveira
e-mail: lenaldo.uefs@gmail.com

C. van den Berg
e-mail: vcassio@uefs.br

J. F. B. Pastore
Universidade Federal de Santa Catarina, Rua Eng. Agronômico Andrei Cristian Ferreira, s/n, Trindade, Florianópolis, SC 88040-900, Brazil
e-mail: j.f.pastore@ufsc.br

E. H. de Souza
Universidade Federal do Recôncavo da Bahia, Rua Rui Barbosa, 710, Cruz das Almas, BA 44380-000, Brazil
e-mail: hilosouza@gmail.com

PPB	Percentage of polymorphic bands
He	Nei's genetic diversity
I	Shannon's information index
NPb	Number of polymorphic bands
G_{ST}	Genetic differentiation coefficient
F_{ST}	Wright's genetic differentiation coefficient between populations
PCA	Principal component analysis

Introduction

Lamiaceae is one of the most important families in terms of aromatic properties, comprising about 250 genera and more than 7000 species, which are common to the Mediterranean and subtropical regions (Bridi et al. 2021; Assaf et al. 2022; Fusani et al. 2022). Within this family, the genus *Hyptis* is considered to have great economic importance, and comprises about 280 species, which after a new circumscription based on molecular phylogenies have been split into 11 genera, including the genus *Eplingiella* Harley & J.F.B. Pastore (Harley and Pastore 2012).

Eplingiella comprises important aromatic species which are a relevant source of essential oils and secondary metabolites. They are used in food as well as in traditional and modern medicine, including the production of pharmaceuticals. Among these are *Eplingiella fruticosa* (Salzm. ex Benth) Harley & J.F.B. Pastore, *Eplingiella cuniloides* (Epling) Harley & J.F.B. Pastore, and *Eplingiella brightoniae* Harley (Harley 2014). These species occur in the semiarid region of the Brazilian Northeast, in impoverished sandy soils and are characterized by their shrub size, with small xeromorphic leaves and flowers with short pedicels (Harley and Pastore 2012; Harley 2014).

Eplingiella fruticosa (syn. *Hyptis fruticosa* Salzm. ex Benth.), known popularly as 'alecrim de vaqueiro', is native to the coastal region of the Brazilian Northeast (Silva et al. 2019). Pharmacological research has validated its analgesic (Melo et al. 2020), antinociceptive (Lima et al. 2013), anti-inflammatory (Andrade et al. 2010), antioxidant (Pinto et al. 2021) and vasorelaxant (Moreira et al. 2010) properties, as well as effects against neurodegenerative diseases (Beserra-Filho et al. 2019). *E. cuniloides* is a perennial shrub, endemic to dry and sandy areas in plateaus, found in the municipality of Morro do Chapéu, Bahia, Brazil. This species is also popularly known as 'alecrim de vaqueiro', but its potential use has not yet been scientifically investigated. *E. brightoniae* is a bushy shrub, up to 1–1.5 m tall, strongly aromatic, native to Serra do Curral Frio (Curral Frio Mountains), straddling the border between the municipalities of Umburanas and Sento Sé, in Bahia, Brazil. Its occurrence is so restricted that it is described as an endangered species, since its natural habitat is constantly subjected to fires and deforestation (Harley 2014).

The concern with the loss of variability and the extinction of species with medicinal potential has increased the demand for works involving collection, preservation and genetic conservation potential of these valuable species (Hashemifar and Rahimmalek 2018). Thus, plant breeders have been concentrating their efforts on maintaining variation in natural germplasm in an attempt to improve their breeding programs. Genetic variability studies represent an important tool for understanding the distribution relationships of species (Munda et al. 2022). For this purpose, the analysis of molecular markers is considered to be a robust tools for the evaluation of genetic diversity of several species of Lamiaceae (Triguero-Piñero et al. 2021; Koohdar and Sheidai 2022; Talebi et al. 2022). Among these markers, the ISSR (Inter Simple Sequence Repeat) markers have been widely used for the evaluation of genetic relationships of medicinal plants (Gupta et al. 2021; Feijó et al. 2022; Koohdar and Sheidai 2022; Peng et al. 2023). They are dominant markers, and have the advantages of being simple, quick, highly reproducible and highly polymorphic, besides requiring small DNA amounts and being highly informative (Gupta et al. 2021; Ghanbari et al. 2022).

However, the few molecular marker-based studies of *Eplingiella* species are mainly restricted to characterization of genotypes (Silva et al. 2017). To our knowledge, use of molecular markers for assessment of the structure and genetic diversity of natural populations of *E. fruticosa, E. cuniloides* and *E. brightoniae*, especially from the Northeast region of Brazil, has not been reported. This limits the implementation of reasonable conservation strategies and effective management practices of natural populations.

Based on these concerns, we conducted the present study with the following objectives: i) to evaluate for the first time the genetic variation within and among populations of selected species of *Eplingiella* (*E. fruticosa, E. cuniloides* and

E. brightoniae) using inter simple sequence repeat (ISSR) markers, ii) to determine the level of population differentiation and relationships between molecular and geographical distribution patterns of this species, and iii) to evaluate the genetic structure of the *Eplingiella* spp. populations and species studied. The information obtained provides new insights into the population genetic structure of these species to guide future actions for conservation and new collections for breeding.

Material and methods

Plant material

Two hundred and sixty-five samples from 18 natural populations of *Eplingiella* spp. (14 *E. fruticosa*, 3 *E. cuniloides* and 1 *E. brightoniae*) were collected in the states of Bahia and Sergipe, as listed in Table 1 and Fig. 1. These species were registered with platform of the National System of Genetic Heritage

Table 1 Location, voucher data and sampling size of 18 populations of three species of *Eplingiella* Harley & J.F.B. Pastore used in the present study

Population	Species	Municipality	Geographical coordinates	Sample size	Voucher
EF1850	*E. fruticosa*	Morro do Chapéu/BA	11°35′06.7" S 41°12′22.2" O	15	Pastore, J.F.B. 3260
EF1855	*E. fruticosa*	Santa Terezinha/BA	12°42′19.4" S 39°33′58.3" O	17	Pastore, J.F.B. 3270
EF1864	*E. fruticosa*	Rafael Jambeiro/BA	13°33′06.8" S 39°27′18.3" O	15	Pastore, J.F.B. 3272
EF1888	*E. fruticosa*	São Cristóvão/SE	10°55′33.6" S 37°11′56.1" O	15	Carvalho-Silva, A. 260
EF1893	*E. fruticosa*	Japaratuba/SE	10°35′91.4" S 36°59′28.4" O	15	Carvalho-Silva,A. 261
EF1921	*E. fruticosa*	Itaporanga d'Ajuda/SE	11°09′04.6" S 37°24′10.7" O	13	Carvalho-Silva,A. 262
EF1922	*E. fruticosa*	Esplanada/BA	11°45′24.8" S 37°56′32.9" O	10	Carvalho-Silva,A. 263
EF1932	*E. fruticosa*	Alagoinhas/BA	12°10′06.7" S 38°27′14.0" O	14	Carvalho-Silva,A. 264
EF1988	*E. fruticosa*	Simão Dias/SE	10°43′48.2" S 37°46′46.5" O	15	Carvalho-Silva,A. 266
EF1989	*E. fruticosa*	Itabaiana/SE	10°46′10.4" S 38°21′32.0" O	15	Carvalho-Silva,A. 265
EF2088	*E. fruticosa*	São Gonçalo/BA	12°23′30.6" S 38°53′17.5" O	15	Carvalho-Silva,A. 268
EF2089	*E. fruticosa*	Feira de Santana/BA	12°11′48.8" S 38°58′01.6" O	15	Carvalho-Silva,A. 269
EF2091	*E. fruticosa*	Saubara/BA	12°37′28.9" S 39°48′31.2" O	15	Carvalho-Silva,A. 267
EF2347	*E. fruticosa*	Umburanas/BA	10°23′27.1" S 41°19′10.7" O	15	Carvalho-Silva,A. 337
EC1842	*E. cuniloides*	Morro do Chapéu/BA	11°34′14.5" S 41°10′32.4" O	14	Pastore, J.F.B. 3250
EC1851	*E. cuniloides*	Morro do Chapéu/BA	11°37′40.3" S 41°00′01.8" O	15	Pastore, J.F.B. 3264
EC1856	*E. cuniloides*	Morro do Chapéu/BA	11°35′53.0" S 41°09′49.5" O	15	Pastore, J.F.B. 3267
EB2326	*E. brightoniae*	Umburanas/BA	10°24′10.6" S 41°18′40.3" O	17	Carvalho-Silva,A. 338

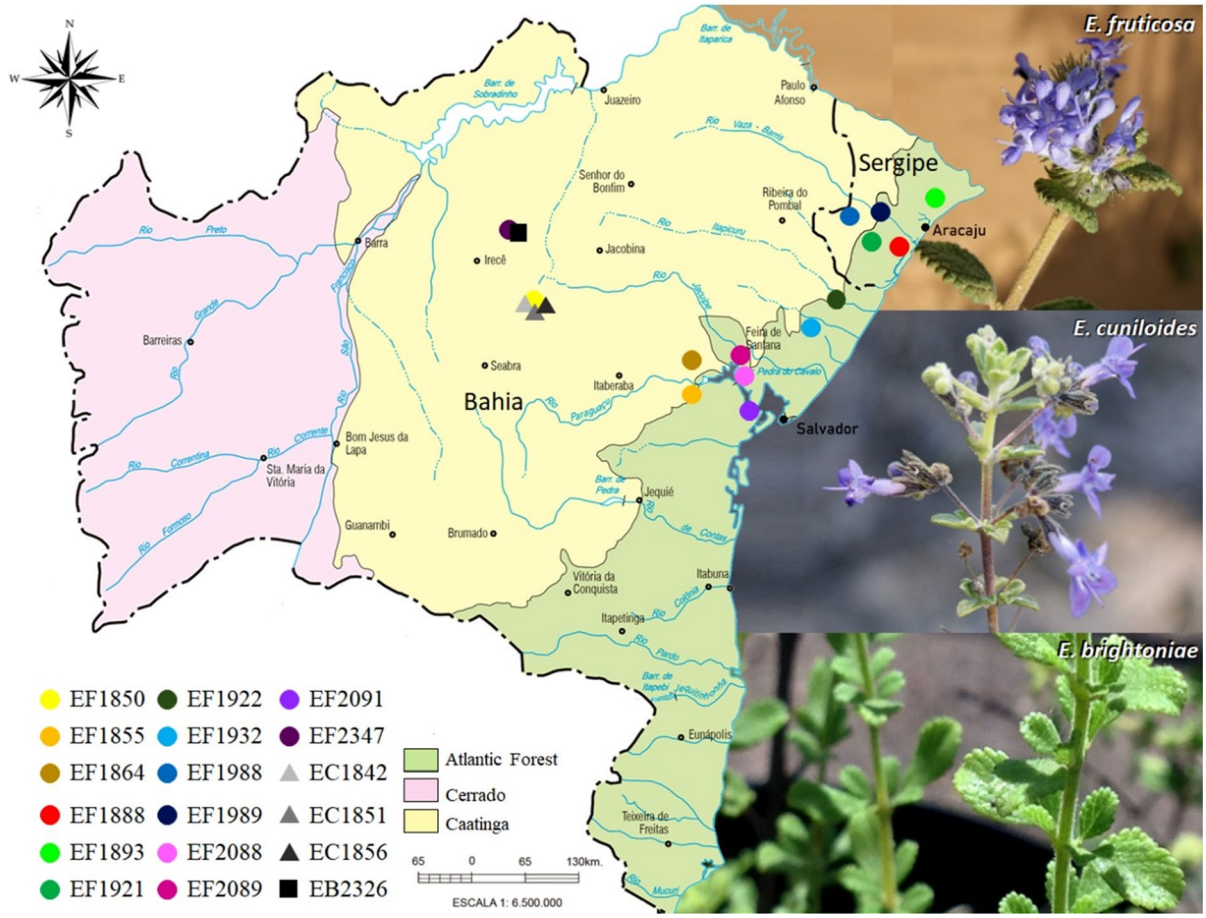

Fig. 1 Geographic locations of *Eplingiella fruticosa* (Salzm. ex Benth) Harley & J.F.B. Pastore (circles) *Eplingiella cuniloides* (Epling) Harley & J.F.B. Pastore (triangles) and *Eplingiella brightoniae* Harley (squares) populations sampled from the semiarid regions of Northeast Brazil

Management and Associated Traditional Knowledge (SISGEN) under reference number A1C1DEB.

Sampling was carried out considering the heterogeneous distribution of individuals in the habitats, with an average of 15 individuals per population. These were named according to the continuous numbering recorded on the extraction form. Extractions, quantifications and data capture were carried out at the Laboratory of Molecular Plant Systematics (LAMOL) of Universidade Estadual de Feira de Santana (UEFS). Geographic distances between populations ranged from 21 km (between EF2088 and EF2089) to 634 km (between EF1850 and EF1893).

The species were identified by Dr. José Floriano Barêa Pastore at UEFS and the voucher of each population were deposited in the Herbarium da Universidade Estadual de Feira de Santana (HUEFS), listed in Table 1.

It is interesting to note that *E. brightoniae* is a species restricted to two municipalities in Bahia (Umburana and Sento Sé), but in the present study we collected samples only in Umburana, since we were unable to locate this species in Santo Sé due to indiscriminate burning in that region at the time. Therefore, we only represent a single population of *E. brightoniae*.

DNA extraction

Aliquots of total DNA from each sample were used in analyses, with the remaining material incorporated in the DNA Bank belonging to the Laboratory

of Molecular Systems of Plants/UEFS. Fresh leaf samples (500 mg) were collected in CTAB gel (35% $NaCl_2$ and 3% CTAB) for DNA extraction using the 2X CTAB protocol (Doyle and Doyle 1987). The extracted DNA was quantified by electrophoresis in 1% agarose gel in comparison with a 100pb ladder marker (Invitrogen®).

PCR amplification

A total of 16 ISSR primers were tested. The polymerase chain reaction (PCR) was carried out with total reaction volume of 10 µl, containing 4.0 µL of the Top Taq Master Mix Kit (Qiagen Inc., Hilden, Germany), 2.0 µL of high-quality ultrapure H_2O (part of the amplification kit), 1.6 µL of TBT (Samarakoon et al. 2013), 2.0 µL of diluted sample DNA (5 mg/mL) and 0.4 µL of the primer (0.5 M). The amplification reactions were performed in an Esco Healthcare Swift® Max Pro thermocycler, starting with a denaturation cycle at 94 °C for 90 s, followed by 40 amplification cycles with denaturation at 94°C for 45 s, annealing at 45–52 °C for 45s, and extension at 72 °C for 90 s, plus two variations, the first at 94 °C and the second at 44 °C, both for 45 s, followed by a final extension at 72 °C for 7 min, for the 265 individuals.

The PC reaction products were separated and visualized in 2.0% agarose gels with SB buffer (Brody and Kern 2004), and compared to a 100-base-pair ladder marker with ethidium bromide staining. To verify the reproducibility of the results, each PCR and gel amplification run was repeated twice, and only the amplified ISSR fragments present in both lanes were considered. Gels were photographed and analyzed using the software GelCompar II (Applied Maths), to establish standardized comparisons between all samples in different gels and establish the homologous bands based on size.

ISSR-PCR data analysis

The ISSR bands were counted using the binary scoring system, recording the presence or absence of bands as 1 or 0, respectively. A binary matrix (1/0) was prepared with GelCompar II.

Genetic variation within and between populations was estimated using the following parameters: number of polymorphic bands (NPb); percentage of polymorphic bands (%PPb); and expected heterozygosity or genetic diversity of Nei (1973) (*He*). Genetic structure was estimated using the genetic differentiation coefficient (G_{ST}) (Culley et al. 2002), Shannon's information index (I) and the genetic distance between populations (F_{ST}). For the purposes of population analysis and conservation (Pearse and Crandall 2004), the latter two metrics are of great importance for assessing population structure and gene flow (Takahata and Nei 1984).

Analyzes were performed using POPGENE v.1.32 (Yeh et al. 1999) and GenAlEx. 6.5 (Peakall and Smouse 2012). Nei's genetic distance (1978) and genetic structuring (F_{ST}) were estimated using AFLP-Surv. 1.0 (Vekemans et al. 2002). From these distances (run of 1000 repetitions), cluster analysis was performed based on Nei's genetic distance matrix (1973) and the neighbor-joining algorithm, using PHYLIP v. 3.695 (Felsenstein 1989). One thousand bootstrap repetitions were performed to assess the statistical confidence of each branch.

Principal component analysis (PCA) and analysis of molecular variance (AMOVA) were performed using GenAlEx 6.5 (Peakall and Smouse 2012). AMOVA was carried out in two ways, first by assessing variability at one level, between populations and within populations, and then at two levels, partitioning the variability also between species.

The population structure was estimated using the STRUCTURE 2.2 software based on the Bayesian clustering algorithm (Pritchard et al. 2000), and the result was confirmed by Structurama (Huelsenbeck and Andolfatto 2007; Huelsenbeck et al. 2007; Huelsenbeck et al. 2011). Population structure was analyzed by setting the number of sub-populations (K-values) from 1 to 10, and each run was repeated five times. For each run, a burn-in period of 50,000 and a Markov chain Monte Carlo replication of 100,000 were set. The statistical parameter (ΔK) was used to determine the number of groups. Structure Harvester was used to determine the peak value of ΔK according to the method described by Evanno et al. (2005).

Results and discussion

Genetic diversity

Among the 16 primers tested, only nine produced amplification of polymorphic ISSR patterns (Table 2). A total of 131 polymorphic bands were

detected among nine ISSR primers from the 18 populations of *Eplingiella* obtained, with an average of 14.55 bands (loci) per primer (Table 2), indicating a high level of polymorphism.

Genetic diversity estimates from ISSR are summarized in Table 3. PCR amplification of the 265 individuals with the nine primers generated a total of 131 (100%) polymorphic bands (NPb), corresponding to an average number of 119 (90.8%) polymorphic bands per population. All primers produced high rates of polymorphism for the 18 populations, and the presence of exclusive bands between them was not identified. For the population of *E. fruticosa*, the proportion of polymorphic bands at the population level (%PPb) varied from 75.3% (EF1989) to 93.9% (EF1888, EF1893), with an average of 89.58% polymorphism. Silva et al. (2017), when evaluating the genetic diversity of *E. fruticosa* in the Brazilian state of Sergipe using ISSR markers, identified high polymorphism. They selected eight primers with 72 amplified bands, varying from 8 to 11 with a mean of nine bands per primer. The parameters that determine the degree of genetic diversity among individuals (expected heterozygosity –*He* and Shannon's information index—*I*) showed similar trend as the PPB. The *He* ranged from 0.30 (EF1989) to 0.38 (EF1850, EF1888) and the Shannon's index (*I)* tvaried from 0.37 (EF1989) to 0.47 (EF1850). The values found by us for *He*, *I* are comparatively higher than the levels reported for *E. fruticosa* by Silva et al. (2017), although the primers used to determine the genetic diversity of this species are not exactly the same in the present study.

Table 2 A list of nine Inter Single Sequence Repeat (ISSR) primers selected for the current study, their sequences and annealing temperatures

Primer	Primer sequence 5′–3′[a]	Annealing temperature (°C)	Polymorphic bands
MAO	$(CTC)_4$-RC	50	16
DAT	$(GA)_7$-RG	49	16
TERRY	$(GTG)_4$-RC	52	12
AW3	$(GT)_6$-RG	47	14
MANNY	$(CAC)_4$-RC	52	16
ISSR 901	$(GT)_6$-YR	52	13
ISSR 4	$(CA)_8$GT	49	15
ISSR 6	$(CTG)_8$G	51	17
ISSR 7	$(AG)_8$-YC	51	12
Total	–	–	131

[a]Y = C or T; R = A or G

Table 3 Genetic diversity parameters calculated for each ISSR primer used in the 18 natural populations of three selected species of *Eplingiella* Harley & J.F.B. Pastore. Number of polymorphic bands (NPb), percentage of polymorphic bands (%PPb), expected heterozygosity (He) and Shannon's Information Index (I)

Species	Population	NPb	%PPb	*He*	*I*
E. fruticosa	EF1850	120	91.6	0.38	0.47
	EF1855	117	89.3	0.35	0.43
	EF1864	122	93.1	0.37	0.46
	EF1888	123	93.9	0.38	0.46
	EF1893	123	93.9	0.36	0.44
	EF1921	117	89.3	0.36	0.43
	EF1922	116	88.5	0.37	0.41
	EF1932	119	90.8	0.35	0.46
	EF1988	117	89.3	0.36	0.43
	EF1989	99	75.6	0.30	0.37
	EF2088	120	91.6	0.35	0.43
	EF2089	119	90.8	0.35	0.41
	EF2091	118	90.1	0.35	0.43
	EF2347	113	86.3	0.35	0.44
Average		117	89.58	0.36	0.43
E. cuniloides	EC1842	112	85.5	0.32	0.39
	EC1851	121	92.4	0.38	0.46
	EC1856	119	90.8	0.35	0.42
Average		117	89.57	0.35	0.42
E. brightoniae	EB2326	127	96.9	0.39	0.48
Average	–	127	96.9	0.39	0.48

The population represented by *E. cuniloides* presented %PPb, ranging from 85.5 (EC1842) to 92.4 (EC1851), *He* values between 0.32 (EC1842) and 0.38 (EC1851), and Shannon index (*I)* ranging from 0.39 (EC1842) to 0.46 (EC1851). *E. brightoniae* presented %PPb (96.9), *He* (0.39) and I (0.48). The different levels of genetic diversity were probably due to the locations of the sampled populations. The results obtained in our study are in line with those reported by other authors who have worked with natural populations of different species of Lamiaceae. Gupta et al. (2021) identified 90% polymorphic bands among *Ocimum* populations, and Jedrzejczyk and Rewers (2018) found 100% polymorphic bands for natural populations of *Mentha* L. On the other hand, Kameli et al. (2013) observed low levels of diversity in populations of *Satureja* spp., with percentage

of polymorphic bands varying between 52.63% and 73.68%, although with high averages for Shannon's (0.46) and Nei's (0.42) indices. These species, however, have a restricted and fragmented distribution, which may have led to a narrowing of their genetic bases (Nybom 2004; Rodrigues et al. 2013).

Genetic structure

Analysis of molecular variance (AMOVA) revealed significant variations ($P < 0.01$), and the majority of the variance observed occurred within populations (71%), while the remaining 29% was variation between populations, indicating a strong genetic structure (Table 4). Generally, the presence of high variability within the population detected in the present study meant the divergence of the individual within a single population. This is in agreement with reports on other Lamiaceae plants that also indicated greater within-population variation, such as *Mentha cervina* L. (Rodrigues et al. 2013), *Perovskia abrotanoides* Karel and *P. atriplicifolia* (Hashemifar and Rahimmalek 2018), *Salvia rosemarinus* Schleid. (Zigeneet al. 2020), and *Melissa officinalis* L. (Koohdar and Sheidai 2022).

Considering two levels of partitioning, when populations were grouped by species, the proportion of within-population variance decreased (69%), as well as among-population variance (27%), with 4% of the variation being attributed to the differentiation among species. This can indicate there is very little differentiation at the species level, or that the method used was not sensitive to genetic differences between them, although the result was statistically significant. In addition, these rates reveal the great diversity among the sampled populations, confirming the values of *He* and *I*.

The average value of the genetic differentiation coefficient (G_{ST}) among populations of *Eplingiella* spp. was 0.29, indicating a high degree of genetic differentiation between populations, as well as Φ_{ST} shown in Table 4, which represents the value of individuals taken at random within each population in relation to individuals taken at random in the entire sample. Nei (1978) classified (G_{ST}) into three classes: low < 0.05), moderate ($0.05 < G_{ST} < 0.15$) and high (G $G_{ST} > 0.15$). In this study, the average value of the genetic differentiation coefficient (G_{ST}) among populations of *Eplingiella* spp. was (0.29), in a moderate class (total = 0.29), indicating a high degree of genetic differentiation between populations, as well as Φ_{ST} shown in Table 4, which represents the value of individuals taken at random within each population in relation to individuals taken at random in the entire sample.

A high and significant ($P < 0.01$) genetic differentiation, according to the Φ-statistic ($\Phi_{ST)}$, was also observed among species, among populations and within populations. The highest value Φ_{ST} was found within populations ($\Phi_{ST} = 0.449$) and the lowest was observed among species ($\Phi_{ST} = 0.279$). Similar patterns of genetic differentiation have been observed for other plant species (Flihi et al. 2022).

The AMOVA also revealed a moderate wright genetic differentiation between populations ($F_{ST} = 0.175$). If the F_{ST} value is between 0 and 0.05, genetic differentiation between populations is low; if it is between 0.05 and 0.15, differentiation is moderate; if it is between 0.15 and 0.25, it is high; and if it is above 0.25, differentiation is very high (Wright

Table 4 AMOVA results of ISSR data used to determine the genetic structure for different hierarchical levels of the 18 populations of *Eplingiella* Harley& J.F.B. Pastore

Source of variation	*DF	SQ	MSD	Variance	(%)	Φ_{ST}	*P*
Eplingiella *spp.*							
Among populations	17	2269.085	133.476	7.778	29%	0.290	< 0.01
Within populations	247	4699.503	19.026	19.026	71%		< 0.01
Taxa: *E. fruticosa*, *E. cuniloides* and *E. brightoniae*							
Among species	2	377.938	188.969	1.086	4%	0.040	< 0.01
Among populations	15	1891.147	126.076	7.346	27%	0.279	< 0.01
Within populations	247	4699.503	19.026	19.026	69%	0.307	< 0.01

**DF* degrees of freedom; *SQ* sum of squares; *MSD* mean square deviation; variance; (%): Percentage of variance; Φ_{ST} Genetic differentiation; *P* significance value

1978, Hartl and Clark 1997). A recent study carried out by Duan et al. (2022) stated that the genetic differentiation coefficient F_{ST} is a vital parameter to reflect the degree of genetic structure. The results obtained here are in line with those reported by Saidi et al. (2013) and Silva et al. (2017), who also found a moderate genetic diversity in *Satureja* L. (Lamiaceae) and *E. fruticosa* (Lamiaceae) species, respectively.

The genetic structure of the 18 populations of *Eplingiella* spp. was determined by employing the Bayesian clustering method based on STRUCTURE (version 2.2) analysis, resulted in the formation of three peaks (K = 2, K = 6 and k = 8), indicating three possibilities of assigning individuals to real populations (Figs. 2, 3). Applications of this method include demonstrating the population structure present, identifying genetically distinct populations, assigning individuals to populations, and identifying migrant or mixed populations and individuals (Pritchard et al. 2010). The peak K = 2, indicating the formation of two groups for the 18 populations of *Eplingiella* spp., was the largest, albeit with many migrant individuals, demonstrating a high level of miscegenation among them. This result was further confirmed by the STRU CTURAMA analysis (Huelsenbeck et al. 2011), strengthening the possibility of the best fit being formation of two large genetic groups among the sampled populations.

Despite the high level of miscegenation, populations of *E. fruticosa* (EF1850, EF1855, EF1864, EF1888, EF1893, EF1921), *E. cuniloides* (EC1842, EC1851, EC1856) and *E. brightoniae* (EB2326) have predominantly the same gene pool, therefore being allocated in the same group (Fig. 3), even though having a strong interspecific genetic mixture. This mixture involved populations of *E. fruticosa* (EF1850–EF1921), *E. cuniloides* (EC1842–EC1856) and *E. brightoniae* (EB2326). Archibald et al. (2006a) reported that it is possible for co-migrant bands to represent different loci, which appear homologous because they are approximately the same size, or loci that are identical due to convergences. In addition, allogamous species, with recent diversification, tend to present greater allelic variation within populations, enabling conserving much of the ancestral genetic load (Maia 2010).

The *E. fruticosa* populations (EF1988, EF1989, EF2088, EF2089, EF2091 and EF2347) predominantly shared the same gene pool, being gathered in the opposite group (Fig. 3). On the other hand, in populations EF1922 and EF1932, the gene pool was shared between characteristics of the two groups formed, making it impossible to analyze their relationship within each group individually. This was because in this method the sampled individuals are assigned (probabilistically) to populations, or together to two or more populations if their genotypes

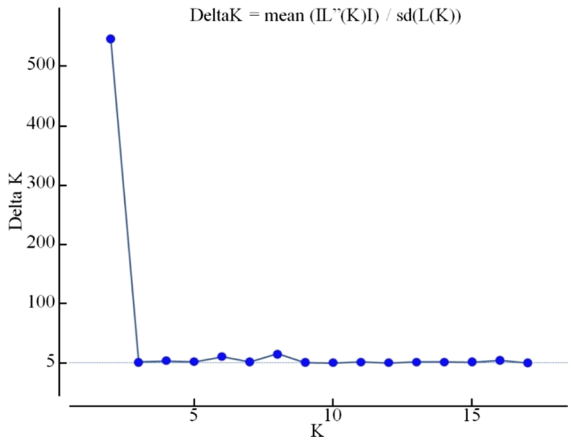

Fig. 2 Estimated ΔK of the 18 natural populations of *Eplingiella* Harley & J.F.B. Pastore during 2 runs of each K value

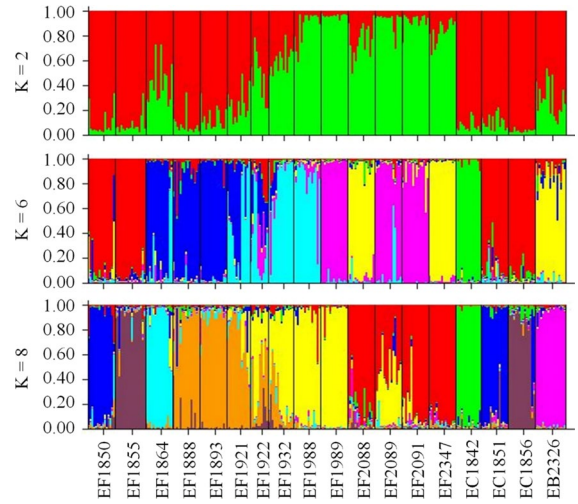

Fig. 3 Population structure inferred by the STRUCTURE software based on ISSR data at K = 2; K = 6; K = 8. Populations: *E. fruticosa* (EF1850, EF1855, EF1864, EF1888, EF1893, EF1921, EF1922, EF1932, EF1988, EF1989, EF2088, EF2089, EF2091, EF2347); *E. cuniloides* (EC1851, EC1842, EC1851, EC1856) and *E. brightoniae* (EB2326)

indicate they are mixed (Pritchard et al. 2010). It is also important to highlight that the studied species have very similar morphological characteristics, and also *E. cuniloides* and *E. brightoniae* present very restricted occurrences and share these occurrences with *E. fruticosa*. Based on this information, there may be genetic exchanges between them at some level. In addition, the pattern presented reveals plesiomorphism of traits close to the three species.

The second peak (K = 6) showed the possible formation of six populations, although with strong miscegenation (Fig. 3). The groups were arranged as follows: first, individuals from populations of *E. fruticosa* (EF1850, EF1855), *E. cuniloides* (EC1851 and EC1856), forming the group represented mostly in red; then individuals of *E. fruticosa* (EF1864, EF1888 and EF1893), represented in blue. In relation to the remaining populations (EF1921, EF1922, EF1932 and EF1988), they formed the predominantly cyan group, but with the first two showing a higher level of genetic mixing. Populations of *E. fruticosa* (EF1989, EF2089 and EF2091), with representation in lilac; EF2088, EF2347 (*E. fruticosa*) and EB2326 (*E. brightonia*), represented in yellow, and the isolated population EC1842 (*E. cuniloides*), in green. In this situation, the strong mixture identified may be related to the low intensity of the peak along with the distribution in a larger number of groups, which may have forced the grouping of weakly related individuals in the same population. However, there are cases where the grouping is justified by the ease of biological explanation, at a certain level disregarding the percentages of miscegenation.

Similar behavior was observed for the individuals grouped in the third possibility, where the peak K = 8 was also of low intensity and formed a greater number of groups. The individuals sampled were assigned to eight populations, also with a strong genetic mix, grouping individuals from population EF1850 (*E. fruticosa*), and EC1851 (*E. cuniloides*), marked with predominant blue color; EF1855 (*E. fruticosa*) and EC1856 (*E. cuniloides*), in purple; EF1864 isolated in cyan; EF1888, EF1893 and EF1921(*E. fruticosa*) in orange; and EF1922, EF1932, EF1988 and EF1989 (*E. fruticosa*) predominantly in yellow.

Cluster analysis based on neighbor joining methods classified the 18 populations into two main clades (Fig. 4) with good support (100%), which were analogous to the ones identified by STRUCTURE. Clade I consisted of five (27.8%) populations (EF1850, EF1855, EC1842, EC1851 and EC1856) belonging to two different species (*E. fruticosa* and *E. cuniloides*). Therefore, it is a mixed formation, and most of the populations (80%) allocated in this clade had the same geographic origin (Morro do Chapéu, BA). Within this clade, subgroups were formed, composed by EF1850 and EC1851 populations, which despite belonging to different species, apparently retain a very close genetic composition, so there is probably still a genetic exchange between them (both are from Morro do Chapéu). This can change the configuration of the species. Similar behavior was observed for the subgroup formed by the populations EC1842, EC1856 and EF1855. The data presented here suggest the occurrence of gene flow.

The isolation of the EB2326 population (100%) in clade II can be explained by the fact it belongs to the species *E. brightoniae* Harley, which is endemic and restricted to the rocky fields of Serra do Curral Frio, straddling the border between the municipalities of Umburanas and Sento Sé (Harley 2014). In clade II, the subgroup formed by EF1864 (from Jambeiro, Bahia), EF1888 (from São Cristóvão. Sergipe) and EF1893 (from Japaratuba, Sergipe) may be related to the places of origin, because despite the distance between these populations (Table 1), the edaphoclimatic characteristics of the three places are similar (data not shown). In addition, the inclusion of populations EF1864 and EF1888 in this clade may be related to the resilience factor, since they coexist in locations that are frequently subject to burning. In the subgroups formed by EF1921; EF1932 and EF1922, EF1989, the relationship between populations also tended to converge due to environmental pressures, since these were collected in places with similar soil and climate characteristics, with sandy-stony soil, full sunlight, and good water supply throughout the year (800 to 1300 mm). Finally, the last subgroup analyzed, with the populations EF1988, EF2088, EF2089, EF2091 and EF2347, converged due to the environmental characteristic of being collected in transitional sites (ecotones) between the Caatinga and Atlantic Forest biomes, with predominance of the second biome.

In general, the stratification of populations and subpopulations of the three species of *Eplingiella* spp. studied reinforce the idea of a common ancestor, of recent diversification in a short evolutionary

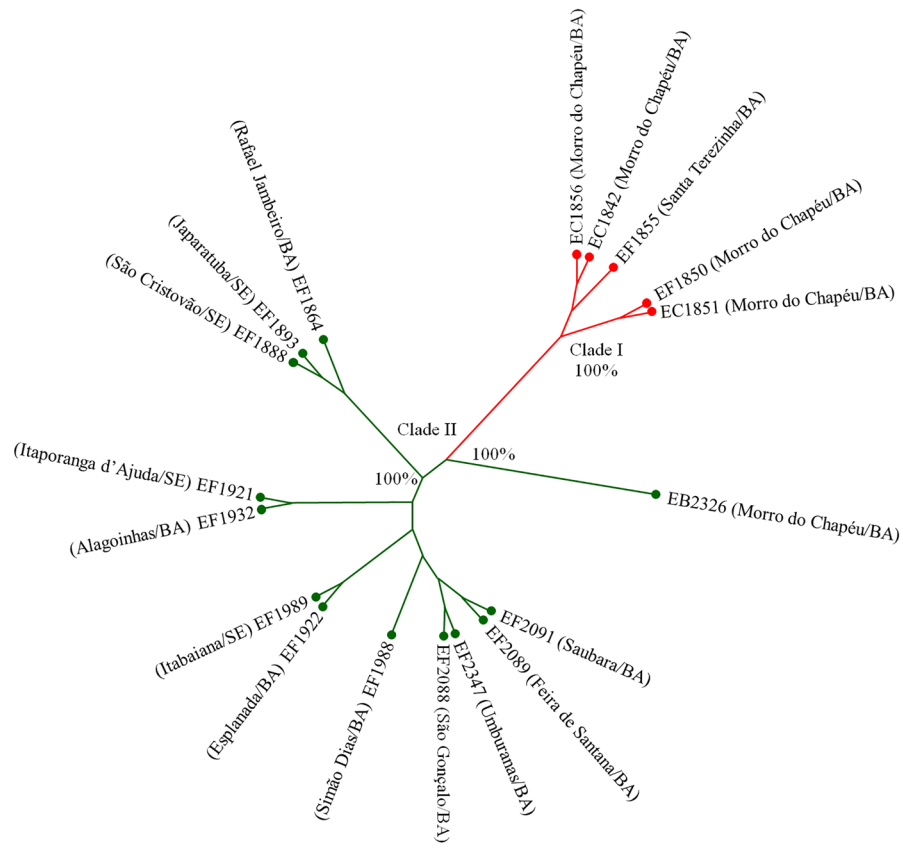

Fig. 4 Neighbor-joining dendrogram based on Nei's genetic distance matrix for the 18 populations of *Eplingiella* Harley & J.F.B. Pastore. Populations: *E. fruticosa* (EF1850, EF1855, EF1864, EF1888, EF1893, EF1921, EF1922, EF1932, EF1988, EF1989, EF2088, EF2089, EF2091, EF2347); *E. cuniloides* (EC1851, EC1842, EC1851, EC1856) and *E. brightoniae* (EB2326)

interval. In addition to the moderate genetic variability present, these species are possibly still in the process of genetic differentiation.

The results of the principal component analysis (PCA) generated by using genetic distance-based analysis were very consistent with the result obtained by NJ clustering, where first two principal coordinates explained only 21.06% of the total variation (Fig. 5), demonstrating the intermixed but integrated genetic relationship of the populations studied. In this case, it was not possible to observe, through the scatter plot (Fig. 5), the separation of populations into groups. The pattern demonstrated in this analysis suggests the putative existence of three species corresponding to a single group (one species), which is widely diverse.

Genetic variation based on molecular markers is an effective and functional method used in breeding programs (Zigene et al. 2020). It is interesting to note that the number of polymorphic bands identified (131 bands) in our study was sufficient to delimit the genetic structure of the groups formed. Furthermore, the recent diversification of this group may explain the level of homology identified here. The allogamy

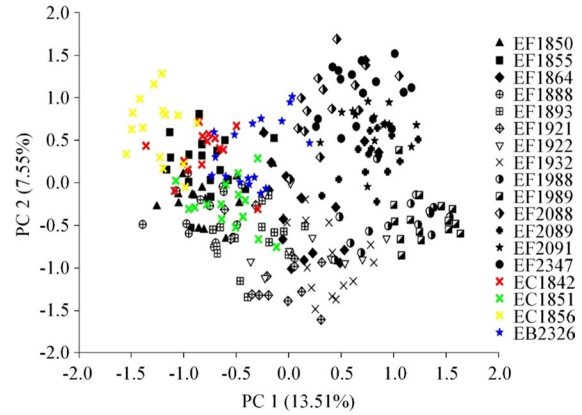

Fig. 5 Principal component analysis of the genetic distance matrix of 265 individuals from 18 populations of *Eplingiella* Harley & J.F.B. Pastore, based on 131 ISSR loci. Populations: *E. fruticosa* (EF1850, EF1855, EF1864, EF1888, EF1893, EF1921, EF1922, EF1932, EF1988, EF1989, EF2088, EF2089, EF2091, EF2347); *E. cuniloides* (EC1851, EC1842, EC1851, EC1856) and *E. brightoniae* (EB2326)

factor related to the recent radiation of taxa can generate a series of problems in defining the genetic structure of populations, a situation commonly reported in studies of native and endemic species of recent taxonomic evolution (Archibald et al. 2006a; 2006b).

Conclusions

The use of ISSR-like dominant markers proved to be an efficient method to identify the variability existing between natural populations of *Eplingiella* spp. It was possible to identify important traits of the genetic relationship between the populations studied, to support measures for the conservation and rational use of these genetic resources. Populations EB2326, EF1888 and EF1893 displayed the greatest diversity, indicating that protective measures and greater collection efforts can be directed to areas of their natural occurrence in order to safeguard the highest percentage of the species' gene pool. Furthermore, it was possible to question the population structure of the three species of the genus, and also their small genetic differences, supporting the hypothesis of recent diversification and persistent genetic link between them.

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Author contribution AS: Conceptualization, Methodology, Investigation, Data curation, Writing-Original draft preparation; LO: Conceptualization, Investigation, Methodology, Resources, Data curation, Writing—original draft, Writing—review & editing, Supervision, Funding acquisition; JFBP: Conceptualization, Formal analysis, Data curation, Writing—original draft, Supervision; CvdB: Conceptualization, Resources, Methodology, Data curation, Formal analysis, Writing—original draft, Supervision; TS: Conceptualization**,** Visualization, Formal analysis, Writing—review & editing; ES: Visualization, Writing—review & editing.

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Declaration

Conflict of interest The authors declare that they have no conflict of interest.

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