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Genetic diversity of *Lippia rotundifolia* Cham. in Minas Gerais, Brazil

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ABSTRACT. *Lippia rotundifolia* is an aromatic species, endemic from Campos rupestres and isolated by mountains. We aimed with the research to study the genetic variability of chá-de-pedestre (*Lippia rotundifolia Cham.)* with natural occurrence in ten places of the State of Minas Gerais, from molecular markers of ISSR type. The places of collection were: Parque Estadual de Serra Nova; Parque Estadual Veredas do Peruaçu; Abóboras community; Gigante community; edge of Rio do Peixe; environmental preservation area of Olhos d´água; private property in Joaquim Felício; Parque Estadual do Rio Preto; São Gonçalo do Rio das Pedras and brook of Rio Tigre. After the establishment of the extraction and amplification of DNA fragments with primers ISSR, an array of genetic distance was built. From this matrix, we analyzed the genetic diversity index as the allelic frequency (Na and Ne), Shannos´s index (H´), polymorphism (PLP), heterozygosity (He) and gene flow (Nm). The result showed higher genetic variability within the population (93%). The genetic diversity index was low (He = 0.132; H´= 0.214; Na = 1.111; Ne = 1.183; PLP = 56.67%). In general the species have low genetic diversity. The greatest diversity in populations occurred with an average temperature of 20°C. The Rio Tigre showed higher genetic distance and isolation.

Keywords: pedestrian tea, genetic variability, medicinal plant, conservation.

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Introduction

Cerrado has one of the highest rates of endemism of flora. With approximately 30% of the local vegetation intact, it is considered one of the hotspots for conservation. In Minas Gerais these environments are located in the rupestrian field, one of the physiognomies of the Brazilian Cerrado. The rupestrian fields are characterized by altitudes above 800 m, xeromorphism and the presence of rock outcrops (Gastauer, Messias, & Neto, 2012).

The floristic composition in this phytophysiognomy is predominant of species in the Verbenaceae family. The genus *Lippia* Linn. is the second largest of this family, in which concentrates the majority of endemic species in these altitudes (Carvalho et al., 2012). Among the endemic species of the rupestrian fields is *Lippia rotundifolia* Cham. popularly known as pedestrian tea, due to popular use as hot foot bath by the Tropeiros of the Estrada Real and by the inhabitants of the Vale Jequitinhonha since the XVIII century. It is a shrub from 0.5 to 2 m high, from restricted populations and with few individuals. Its flowers are grouped in large bunches ranging from pink-lilac to magenta or fake pink. In the natural environment this species is confused with others of the same genus due to the great morphological similarity and reproductive synchronism, making it difficult to identify it (Salimena & Silva, 2009).

Studies of genetic diversity in Cerrado native species have concentrated efforts on endangered tree and fruit tree species (Silva et al., 2016). In aromatic species of medicinal interest, studies of genetic structure have concentrated on germplasm of domesticated species or those which chemotypes have already been identified and have consolidated space in the market. Among the species studied are lemon balm (*Lippia alba*), pepper rosemary (*Lippia sidoides*) and Mexican oregano (*Lippia origanoides* Hunth and *Lippia graveolens*) (Manica-Cattani, Zacaria, Pauletti, Atti-Serafini, & Echeverrigaray, 2009; Vega-Vela & Sánchez, 2012; Rocha et al., 2015).

However, identifying populations with greater genetic diversity is one of the mitigating measures for conservation. From this knowledge it is possible to develop management techniques in the local community, such as establishing enrichment plantations and germplasm banks, contributing to the conservation of *in situ* and *ex situ* genetic resources of these native populations (Collevatti, Lima, Soares, & Telles, 2010). In this context, we aimed to study the genetic variability of tea-pedestrian (*Lippia rotundifolia* Cham.) naturally occurring in ten locations in Minas Gerais, Brazil.

Material and methods

The research was accomplished from August 2014 to December 2015. In the period 193 matrices were selected from 10 accessions located in eight municipalities of Minas Gerais which matrices were propagated through cuttings and cultivated in a greenhouse. All populations were georeferenced with Garmin GPS Oregon (Global Position System) receiver and registered in SisGen (National System of Management of Genetic Heritage and Traditional Associated Knowledge). The characterization and geographical coordinates of each population are available in Table 1.

Table 1. Characterization of ten environments of natural occurrence of *Lippia rotundifolia* in Minas Gerais, Brazil.

City Code	N	Coordinates		Climate conditions			Herbarium Deposit
		Latitude	Longitude	Alt. (m)	Hum. (mm)	Temp. (C°)	
SNO - Rio Pardo de Minas	15	-15°36'S	-42°44'W	790	700	20 ± 1	PAMG 58096
PVP - Cônego Marinho	15	-14°55´S	-44°38´W	729	700	23 ± 1	PAMG 58090
ABO - Montes Claros	17	-16°56'S	-43°55'W	700	1100	22.5 ± 1	PAMG 58101
GIG - Botumirim	20	-16°35´S	-42°55´W	726	1350	22.5 ± 2	PAMG 58097
RPE - Botumirim	24	-16°52´S	-43°28´W	722	1100	22.5 ± 2	PAMG 58094
ODA - Olhos D'água	18	-17°26´S	-43°37´W	691	1100	22.5 ± 2	PAMG 58095
JFE - Joaquim Felício	24	-17°44´S	-44°11´W	1010	1350	22.5 ± 3	PAMG 58093
PRP - São Gonçalo do Rio Preto	17	-18°06´S	-43°20´W	901	1350	< 19	PAMG 58091
SGS - Serro	25	-18°25´S	-43°28´W	1020	1350	18 ± 2	PAMG 58100
RTI - Gouveia	18	-18°33´S	-43°49´W	1020	1350	20 ± 2	PAMG 58092

SNO: Parque Estadual de Serra Nova; PVP: Parque Estadual Veredas do Peruaçu; ABO: Community Abóboras; GIG: Community Gigante; RPE: Margins of the Rio do Peixe; ODA: PPA of the Olhos d´água (PPA); JFE: Serra Geral in Joaquim Felício; PRP: Parque Estadual do Rio Preto; SGS: São Gonçalo do Rio das Pedras; RTI: Stream of Rio Tigre; N: Number of individuals collected at each location; SP: State Park; PPA: Environmental Preservation Area; Alt.: Altitude in meters; Hum.: Precipitation is annual day in millimeters; Temp.: Average annual temperature in degrees.

Genomic DNA was extracted from young leaves from each accession according to the methodology of cetyltrimethylammonium bromide CTAB adapted from Doyle and Doyle (1990). Material selection and methodological procedure were carried out at the Biotechnology Laboratory of the *Instituto de Ciências Agrárias* of the *Universidade Federal de Minas Gerais* in Montes Claros city. The quality of the extracted DNA was assessed on a 10.7% agarose gel, subjected to electrophoresis for 1 hour at 90 v. DNA of the phage lambda (λ) (Invitrogen, Carlsbad, CA, USA) was used to estimate the resulting DNA concentration. The gel was stained using Gel Red Biotium® safe (Uniscience), visualized under ultraviolet light, and photographed using an imaging system. Subsequently the DNA was standardized at 50 ng μL^{-1} for the ISSR reactions. For the detection of polymorphism, UBC ISSR in triplicates of parent plants was assayed (Adhikari, Saha, Bandyopadhyay, & Ghosh, 2015). Amplification reactions were done in a volume of 25 µl containing 10 mM Tris-HCl pH 8.3; 50 mM KCl; 2.0 mM $MgCl_2$; 0.2 mM of each dNTP; 0.25 µM ISSR primer, 50 ng template DNA, 1 unit Taq polymerase (Invitrogen®) and sterile H_2O. q.s.

The reactions were subjected to 35 cycles of amplification after initial denaturation at 95°C for 5 minutes. Each cycle consisted of 30 seconds at 94°C, 45 seconds between 47 and 56°C (temperature gradient test) and 2 minutes at 72°C. At the end of 35 cycles, a final extension of 7 minutes at 72°C was performed. The PCR reactions for all ISSR primers were performed on Eppendorf Master Cycler® gradient cycler (AG Flexlid, 22331 Hamburg). The amplification products were electrophoresed (5v cm^{-1}) on 2% (w / v) agarose gels, the molecular weight marker was used along it, using the TBE 1X run buffer. The gels were stained with 2% w / v of Red Gel visualized under UV light and photographed on a digital camera coupled to Photo Doc It 65 Imaging System photodocumentator.

A binary matrix was generated from the reading of the gels. The individuals were genotyped for the presence (1) and absence (0) of bands. With this matrix, variability analysis based on structure, gene flow

and genetic distances were obtained by clustering techniques, discriminant analysis and genetic and geographic distance correlation.

The allelic frequency was estimated by the percentage of polymorphism (P) by the formula:

$$P = \frac{nbp}{nbt}$$

where nbp is the number of polymorphic bands and nbt is the total number of bands. The expected heterozygosity (He) He= $1 - \sum Pi^2$ where *Pi* is the estimated frequency of the ith allele. The diversity index of Shannon (H ') by the formula: H'= $\sum_{i=1}^{s} pi \operatorname{Ln} pi$ where *pi* is the band frequency and *n* is the number of markers evaluated (Brown & Weir, 1983); the number observed and expected alleles as well as the mean and total of heterozygosity and the genetic distance of Nei were also observed.

The study of genetic structure among and within populations was obtained by Molecular Variance Analysis (AMOVA). The interpopulation variance component was extracted by mean squared equations (QMD), used to estimate the PhiPT. The significance associated with each of these estimates was obtained by means of 5000 permutations. To obtain a null distribution, without differentiation, of these statistics, randomization procedures were used, performed by the total decomposition of the components between and within the populations using squared distances, as described by Excoffier, Smouse, and Quattro (1992). For this analysis we used the free software GenAlEx v. 6.3 (Peakall & Smouse, 2012).

From the value of PhiPT, the gene flow (Nm) among the population was indirectly estimated assuming the island model proposed by Crow and Aoki (1984) by the formula:

$$Nm = \frac{1}{4a}\left(\frac{1 - PHIPT}{PHIPT}\right)$$

The intensity of the flow was obtained by formula:

$$PHIPT = \frac{1}{1 + 4Nm}$$

where *N* is the effective size of each population and *m* is the rate of migration between populations.

The cluster analysis was performed using the closest neighbor pairing (UPGMA) based on the Jaccard (j) and Nei (D) (Nei, 1972) similarity coefficient, adopting the routine SAHN (*Sequencial Agglomerative Hierarchical and Nested Clustering*). The similarity of the Jaccard was obtained by the formula:

$$S_{ij} \frac{a}{a + b + c}$$

where *a* is the number of cases in which band occur in all individuals, simultaneously, *b* is the number of cases in which bands only occur in the individual *i* and *c* is the number of cases in which band only occurs in individual *j*. Genetic distances were obtained based on the binary data calculated by Genalex 6.502 from the formula proposed by Nei (1972):

$$\text{D}=\left\{\delta_{xy}^{2}\right\} = 100\left[1 - \frac{2n_{xy}}{n_{x+n_y}}\right]$$

where, n_x e n_y are the number of markers observed in the *x* and *y* individuals, respectively, and $2n_{xy}$ is the number of marks in all individuals.

The representativeness of the dendrogram was tested by correlation between the original genetic distances and the distances between populations in the dendrogram with the help of the NTSYS-pc package (Numerical Taxonomy System), version 2.11 (Rohlf, 2000).

To verify the geoclimatic relationship with the genetic variables, a correlation analysis was performed between the genetic and geographic distances matrices (km in a straight line). For this analysis, the Pearson correlation coefficient (r) was applied between the matrices. The significance of this matrix correlation was tested by the Mantel test, using 1000 random permutations. The environmental variables: altitude, precipitation and temperature, were also correlated with the genetic structure.

Results and discussion

Out Of the 18 primers selected by the annealing temperature assay, ten of these presented a high reproducibility standard with polymorphism above 50%. Therefore they were chosen for the study of genetic diversity in the accessions of the populations of *Lippia rotundifolia*.

The ten primers used were: BECKY (CA)7-YC P = 50; CHRYS (CG)7-YG P = 50; TERRY (GTC)4-RC P = 70; UBC 810 (GA)8-C P = 100; UBC 812 (GA) 8-A P = 100; UBC 820 GT)8-C P = 52; UBC 830 (TC)8-G P = 75; UBC 864 (CT) 7-VDV P = 52 e UBC 890 (GT)-VHV P = 90, respectively. The criterion of the primers is according to Manica-Cattani et al. (2009) that obtained 65% of polymorfic bands for the *Lippia alba* for 17 primers ISSR, being considered reproducible and of high quality (Bhawna et al., 2014). These primers were generated 253 polymorphic bands in the analyzed individuals (Table 2). The genetic diversity (He) of individuals was greater than the number of individuals observed (Ho). Only for the accessions (1-GIG and 7-SNO), the Ho was slightly larger than the He, although the population (1-GIG) has low He, it is considered in the Hardy Weinberg (EHW) equilibrium. In general, for the populations studied, there was little difference between the expected and observed values of heterozygosity (0.003). This fact may be due to the natural selection that increases the frequency of heterozygotes during recruitment, this being a common occurrence in some tropical species (Gonçalves, Reis, Vieira, & Carvalho, 2010).

Table 2. *Lippia rotundifolia* indexes of genetic diversity using ISSR marker.

Place	Source	Sample N	Parameters of genetic diversity					
			Na	Ne	H'	He	Ho	PLP
1	GIG	20	0.667	1.078	0.112	0.063	0.065	44.44
2	RPE	24	0.889	1.220	0.211	0.139	0.136	44.44
3	RTI	18	1.333	1.312	0.309	0.204	0.198	66.67
4	JFE	24	0.889	1.191	0.198	0.129	0.126	66.67
5	SGS	25	1.778	1.265	0.313	0.191	0.185	88.89
6	ABO	17	1.333	1.218	0.261	0.160	0.157	55.56
7	SNO	15	1.778	1.258	0.321	0.189	0.195	88.89
8	ODA	18	0.889	1.119	0.158	0.094	0.092	44.44
9	PVP	15	0.889	1.103	0.150	0.087	0.084	33.33
10	PRP	17	0.667	1.067	0.103	0.058	0.056	33.33
Mean		19.3	1.111	1.183	0.214	*0.132	*0.129	56.67
Standard deviation			± 0.35	± 0.59	± 0.068	± 0.042	± 0.041	± 0.2
Total		193				*≠ 0,003	253	

N = number of individuals, Na = n° observed alleles, Ne = n° effective alleles, He = expected heterozygosity, H' = Shannon information index, Ho = observed heterozygosity, PLP = Percentage of polymorphic loci.

The percentage of polymorphic loci for the ten environments ranged from 33.33 in 9-PVP and 10-PRP to 88.89% in 5-SGS and 7-SNO, with an average percentage of 56.67%. In studies of genetic diversity developed with ISSR for medicinal native species, the percentages of detected bands were above 50% for *Lippia alba* Mill. (Manica-Cattani et al., 2009) and greater than 80% for *Lippia origanoides* H.B.K. (Suárez, Castillo, & Chacón, 2008).

The mean number of alleles (Na) per population ranged from 0.67 to 1.78 with a mean of 1.11. The number of effective alleles (Ne) at all sites averaged 1.18. The expected mean heterozygosity (He) and the Shannon index (H') for all population was 0.132 and 0.214 (Table 2). These mean indexes of genetic diversity are considered moderate to low values, because they are below 0.5 (Botstein, White, Skolnick, & Davis, 1980). For Maurya and Yadav (2016) the He below 0.22 is considered low. The *Phyla scaberrima* (Juss. ex Pers.) Moldenke from the same family, presented genetic variability lower than that obtained in the present study (PLP = 46.62, Hs = 0.0695 and H´ = 0.119). This fact is due to the reproductive behavior similar to that of *Lippia rotundifolia* (Androcioli et al., 2015). Already the *Lippia graveolens* H.B.K. specie, was presented high variability with heterozygosity of (H_T = 0.225) (Vargas-Mendoza, Ortegón-Campos, & Calvo-Irabién, 2016). As well as *Lippia origanoides* Hunth which presented diversity indexes of (H´ = 0.44 and 0.45) (Súarez et al., 2008).

Melo Júnior, Carvalho, Vieira, and Oliveira (2012) stated that the index of genetic diversity varies according to species and molecular marker. For the *Lippia rotundifolia*, this value was varied according to genetic structure with the place of occurrence of the species. When comparing the variability of the genetic structure with the environmental factors of each population, it was observed that the genetic structure has a strong correlation with temperature. The annual mean temperatures of 20°C have the best adapted populations, with higher indexes of genetic diversity.

The highest diversity indexes were for the 3-RTI, 5-SGS and 7-SNO populations, whose annual mean temperature are 20°C (Table 1). The individuals of the 3-RTI population had the highest number of effective alleles (1.312) and genetic diversity (He = 0.204). The individuals of the 5-SGS and 7-SNO population presented (1.25) and (1.258) of effective alleles and (He = 0.191) and (He = 0.189) of expected heterozygosity, with proximity between the parameters, which loci number polymorphism was 88.89%. The lowest genetic diversity was obtained in 10-PRP (He = 0.058), just like He and PLP, the others parameters evaluated (Na, Ne, H´, Ho) were also lower in this population.

The low genetic variability can be explained by the difficulty of crossing the species. The *Lippia rotundifolia* was presented pollen with a high percentage of abnormality, with 64. 98% pollen infertility. In a recent experiment we observed that the reproductive system of the species is facultative autogamic, with low pollen ovum (unpublished data). This result was corroborated by Reis et al. (2014) for *Lippia alba* Mill. It presented tetraploid and myxoploid chemotypes which meiotic irregularities caused high rates of unfeasible pollens. Another factor that may have contributed was the endemism due to the geographic isolation by small hills. Therefore, infertility and isolation contribute to the occurrence of crossover among related individuals, compromising the seed bank (Costa, Vieira, Fajardo, & Chagas, 2015). Thereby, it is believed that the main form of reproduction of this species is by budding xylopodia, because, according to *in situ* observation, the individuals are distributed close to each other, which can lead to a low genetic variability of the species (Meira, Martins, & Resende, 2016).

The largest structure of genetic variability performed by AMOVA occurred within populations, 93%, and only 7% occurred among populations (Table 3). In this analysis, the estimation of the differentiation index was F_{ST} = 0.073. This differentiation was considered moderate, because this amplitude was between 0.05 and 0.15 (Hartl & Clark, 2010). In *Lippia origanoides* H.B.H., this index was 0.179, also showing that the greatest variation occurs among individuals within the population, with 82% (Vega-Vela & Sánchez, 2012). This outcome is important for the conservation of genetic resources, once the greater the variability among individuals, the more alleles will be conserved in active germplasm banks.

In the structure of genetic variability, the genetic differentiation fixation index (F_{ST}) was inversely proportional to the gene flow (Nm). Therefore, this index estimated a movement of genes (Nm) from one population to another of 3.198. As Nm is greater than one (1.0), there is an evolutionary factor in the population that maintains the allelic frequencies, homogenizing of the populations. This value shows that genetic drift does not act in the differentiation between the population and t, in population, there is no risk of migrant alleles forming a different population from the one that originated it (Hartl & Clark, 2010).

The genetic distance between the individuals of each population is in accordance with the results of the genetic structure, in which the greatest diversity among and within populations was obtained by the same population (3-RTI). Although heterozygosity within the population is

moderate, the values were representative, corroborating the results of the genetic structure within each population (Table 3). The consistency of these results is in the similarity of genetic structure and in the minimum difference of variability (≠ He > Ho = 0.003). The moderate value of the genetic structure, besides the proximity among the individuals, can also be explained by the mixed mode of propagation as one of the reproductive strategies, which does not estimate any damage of the population structure for this species (Gonçalves et al., 2010; Hartl & Clark, 2010).

This information is important for the persistence of the species in unfavorable environmental conditions such as fires. However, as analyzes of variability based on allelic frequencies, as well as an estimate of the genetic structure of population and de genetic of Nei (1972) among the resources within each population contribute to the understanding of the space arrangement of the species from the random distribution of alleles on a time scale (Table 2 and 3).

Table 3. Analysis of molecular variance (AMOVA) and estimation of the gene flow (Nm) of the *Lippia rotundifolia* population.

Source of variation	FD	SQ	QM	Est. Var	% variance	F_{ST}	P	Nm
Among Population	9	20.458	2.273	0.071	7%	0.073	0.001	3.198
Within Population	183	166.195	0.908	0.908	93%	0.798	0.001	
Total	192	186.653		0.979	100%			

Freedom degree (FD), Sum of squares (SQ), Middle square (MQ), Components of variance (Est. Var.), Total variance (%), FST (index of fixation or proportion of maximum genetic differentiation (total variance) of the allelic frequencies occurring between and within population and the reduction of heterozygosity due to genetic drift analogous to PhiPT), P (Probability of having a component of variance greater than the values observed at random for 5.000 Nm = gene flow between populations by indirect mode.

Regarding the genetic distances they varied from 0.001 to 0.030 and the geographical distances varied from 39 to 443 km. The Mantel test didn't show correlation between genetic and geographic distances (r = -0.064, p > 0.04). The greatest genetic distance was obtained between 3-RTI and 1-GIG population, equidistant 223 km. The populations with the greatest geographical distance were 8-ODA and 7-SNO with 443 km, in which these were the closest genetically (Table 4).

Table 4. Genetic distance matrix of Nei (D) based on Euclidean distance (diagonal lower) and geographical straight line per kilometer (upper diagonal) of *Lippia rotundifolia* in ten naturally occurring populations in Minas Gerais, Brazil.

	DGG	GIG	RPE	RTI	JFE	SGS	ABO	SNO	ODA	PVP	PRP
DG		1	2	3	4	5	6	7	8	9	10
GIG	1	-	67	223	122	218	49	190	330	191	191
RPE	2	0.012	-	208	160	181	100	143	300	280	141
RTI	3	0.03	0.004	-	100	39	180	348	205	414	73
JFE	4	0.004	0.012	0.028	-	108	93	283	107	317	99
SGS	5	0.008	0.005	0.01	0.02	-	172	323	120	409	40
ABO	6	0.016	0.006	0.015	0.016	0.002	-	196	86	238	143
SNO	7	0.015	0.004	0.018	0.01	0.005	0.005	-	443	218	284
ODA	8	0.012	0.006	0.018	0.015	0.004	0.005	0.001	-	295	159
PVP	9	0.018	0.006	0.023	0.013	0.007	0.008	0.001	0.001	-	380
PRP	10	0.018	0.005	0.022	0.012	0.006	0.007	0.001	0.001	0.001	-

Lippia rotundifolia of population: 1: Comunity Gigante in Botumirim city; 2: Margins of the Rio do Peixe in Botumirim city; 3: Stream of Rio Tigre in Gouveia city; 4: Serra Geral in Joaquim Felício city; 5: São Gonçalo do Rio das Pedras in district of Serro; 6: Comunity Abóboras in Montes Claros city; 7: Parque Estadual de Serra Nova in Rio Pardo de Minas city; 8: PPA from Olhos D´água city; 9: Parque Estadual Veredas do Peruaçu in Cônego Marinho city; 10: Parque Estadual do Rio Preto in São Gonçalo do Rio Preto city.

The original distance matrices, with Nei distance, Jaccard similarity and 999 permutations, showed a high cophenetic correlation, where the correlation between resampling distance and Nei distance was of r = 0.90 and the correlation between Jaccard similarity and Nei distance was of r = 0.94. The similarity coefficient varied from 0.53 to 0.86 with 80% genetic similarity (Figure 1). The high correlation indicates that the dendrogram presented a good adjustment between the original data and the dissimilarity matrix (Vega-Vela & Sánchez, 2012).

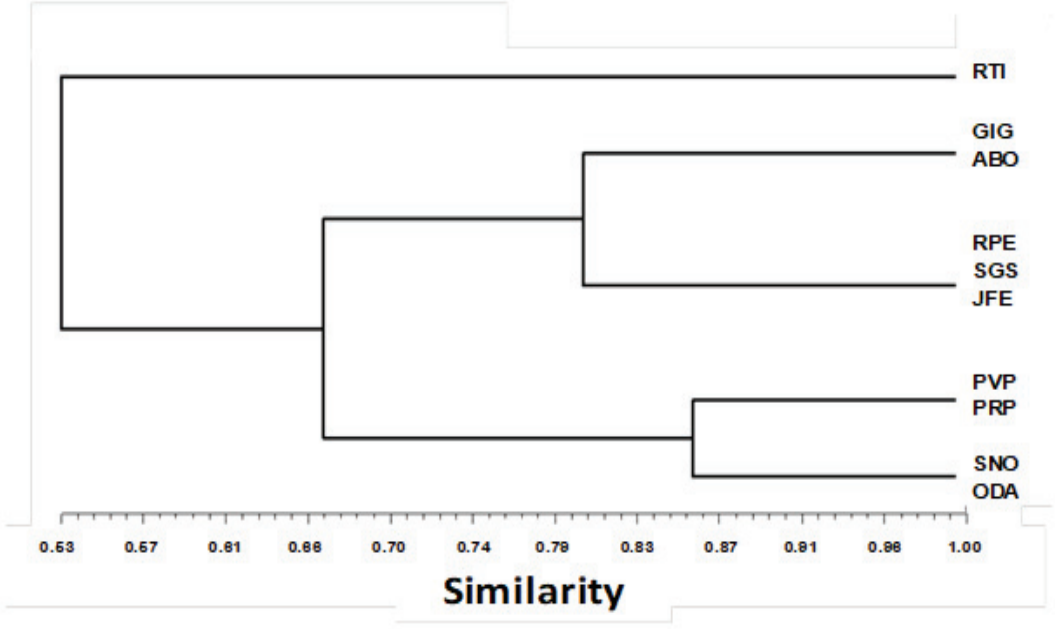

Figure 1. Genetic distance pattern among individuals from ten naturally occurring populations of the *Lippia rotundifolia* based on ISSR markers. Hierarchical grouping analysis defined by the UPGMA method based on the genetic distance of Nei. *Lippia rotundifolia* of population : GIG: Comunity Gigante in Botumirim city; RPE: Margins of the Rio do Peixe in Botumirim city; RTI: Stream of Rio Tigre in Gouveia city; JFE: Serra Geral in Joaquim Felício city; SGS: São Gonçalo do Rio das Pedras in district of Serro; ABO: Comunity Abóboras in Montes Claros city; SNO: Parque Estadual de Serra Nova in Rio Pardo de Minas city; ODA: PPA from Olhos D´água city; PVP: Parque Estadual Veredas do Peruaçu in Cônego Marinho city; PRP: Parque Estadual do Rio Preto in São Gonçalo do Rio Preto city.

Regarding the grouping, the connections in the dendrogram correctly reflected the multivariate patterns of genetic distance between the accesses. The grouping by the UPGMA hierarchical method allowed the visualization of three large groups. The first with the accesses of the 3-RTI population, the second grouped the accesses of populations 1-GIG, 6-ABO, 2-RPE, 5-SGS and 4-JFE and the third grouped the accesses of populations 9-PVP, 10-PRP, 7-SNO and 8-ODA (Figure 1).

The grouping is in accordance with the genetic variability among the population, in which the mean genetic distance was considered low (He = 0.132), with differentiation between the subpopulations of (F_{ST} = 0.07). Both considered the 3-RTI population as the most genetically distant (He = 0.214 e D = 0.03), as well as the 7-SNO, 8-ODA, 9-PVP and 10-PRP populations as the closest to each other (He = 0.189; 0.092; 0.087; 0.058 and D = 0.001) despite the geographic distance (Figure 1; Table 2 and 4).

The genetic distance of the 3-RTI population presented in the cluster analysis corroborates the isolation observed. This analysis is very important to develop measures of conservation of this environment because according to the *Record of the species in the flora and fungo virtual herbarium* (INCT & HVFF, 2019), this population was located more than 40 years ago, in 1971 under registration: MBM 18648 located as Tigre stream, Gouveia, Minas Gerais, Brazil. Despite the fragmentation of habitat and the low abundance, besides location of some individuals according to local observation, the environment is preserved and protected from human interference due to this environment being located in the river bed, under a highway in a dangerous curve which access is difficult (Rodrigues et al., 2013). These environmental characteristics make this genotype promising to be included in breeding programs. It justifies our *ex situ* conservation iniciative through the construction of the germoplasm bank.

This research is the first contribution of genetic diversity to *Lippia rotundifolia* with molecular markes-ISSR aiming to determine the allelic relationships among the different populations of natural occurrence in Minas Gerais. The low genetic diversity observed can be explained by the small population density, which made it impossible to keep sampling with the same distance between the individuals as sampled for *Lippia origanoides* (Suárez et al., 2008), where the authors reported that the sampling between individuals equidistant 1.2 Km, results in different genotypes. Another factor observed in this study was that the temperature may also have contributed to the genotypic adaptation of the plant (Vega-Vela & Sánchez, 2012; Meira, Martins, & Resende, 2017).

Conclusion

The *Lippia rotundifolia* species presents low genetic variability. The geographic isolation and temperature contribute to better allelic distribution of the species. The greatest diversity occurred in the population of Rio Tigre where the annual mean temperature was of 20°C.

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