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Genetic diversity of *Lippia origanoides* Kunth. in natural populations using ISSR markers

Diversidade genética por marcadores ISSR em populações naturais de *Lippia origanoides* Kunth.

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ABSTRACT

Lippia origanoides Kunth. is a medicinal plant that is widely available in the Northeast region of Brazil and is known as "alecrim-d'angola". However, there is no information available on the genetic variability of this species in the region. Thus, the current study was aimed to analyze the genetic diversity and structuring of *L. origanoides* populations occurring in the states of Bahia and Pernambuco, Brazil, using Inter-Simple Sequence Repeat (ISSR) molecular markers. The evaluated Nei's diversity index of the populations varied from 0.162 to 0.237, and the Shannon diversity index varied from 0.247 to 0.350. In molecular variance (AMOVA) analysis, a variation of 31% was observed among the populations, which denotes a high interpopulation structuring. The structure analysis and dendrogram indicated the possibility of classifying the 18 populations into four groups. As their genetic structure is extremely high, it is important to collect *L. origanoides* germplasm, including as many populations as possible. Since the region of Chapada Diamantina holds the most diverse populations of *L. origanoides* germplasm, it is a priority area to obtain the germplasm.

Keywords: Genetic variability; medicinal plant; Verbenaceae; germplasm conservation.

RESUMO

A espécie medicinal *Lippia origanoides* Kunth. tem ampla ocorrência no Nordeste brasileiro, sendo conhecida como alecrim-d'angola. Porém, dados sobre a variabilidade genética da espécie na região são inexistentes. O objetivo dessa pesquisa foi analisar os níveis de diversidade e estruturação genética das populações de *L. origanoides* ocorrentes no Estado da Bahia e Pernambuco, por meio de marcadores moleculares ISSR. Nas populações avaliadas o Índice de diversidade de Nei oscilou entre 0,162 e 0,237, o Índice de Shannon variou entre 0,247 e 0,350. A AMOVA identificou variação entre populações de 31%, sugerindo alta estruturação interpopulacional. A análise no Structure e o dendrograma gerado indicaram a possibilidade de separação das 18 populações em quatro grupos distintos. A coleta de germoplasma da espécie deve ser feita no maior número de populações possível, devido à alta estruturação genética encontrada. A região da Chapada Diamantina é uma área prioritária para obtenção de germoplasma de *L. origanoides*, pois detém as populações mais diversas.

Palavras-chave: Variabilidade genética; planta medicinal; Verbenaceae; conservação de germoplasma.

INTRODUCTION

The genus *Lippia* Linn. comprises many species that are known for their therapeutic applications (Stashenko et al., 2010; Trindade et al., 2021), including *Lippia origanoides* Kunth., popularly known as "alecrim-d'angola" and "salva-de-marajó". Due to the production of several essential oils, plants of this species have various confirmed medicinal applications, such as acaricidal, insecticidal, antioxidant, trypanocidal, anticancer, and antibacterial actions (Betancourt et al., 2019, Damasceno et al., 2018; Leal et al., 2019; Mar et al., 2018; Melo et al., 2020; Raman et al., 2018; Ribeiro et al., 2021; Stashenko et al., 2010).

The extractivism of medicinal plants results in a high risk of genetic variability loss because of fragmentation and anthropogenic intervention in their natural habitats and non-controlled extraction, making it necessary to adopt measures for quantification and conservation of these genetic resources (Costa et al., 2020). Therefore, the genetic diversity analyses of native species are important to define conservation strategies (Gois et al., 2018; Silva et al., 2017), through the selection of plants or populations for germplasm collections, as well as indicating the locations for in situ conservation and minimizing the occurrence of genetic erosions (Melo et al., 2018).

Although *L. origanoides* has medicinal importance, there is no information available on the genetic variability of this plant in Brazil. The species of plants grow naturally in arid and semiarid regions, and its populations cover a large area of the Northeast region of Brazil (O'Leary et al., 2012), including the state of Bahia, which is a favorable place to conduct the initial research on genetic diversity, to obtain data relevant for the conservation of the species (Silva et al., 2017).

Inter-Simple Sequence Repeat (ISSR) are molecular markers that have been useful for diversity genetic analysis of several medicinal plant species (Costa et al., 2020; Hadipour et al., 2020; White et al., 2018), including *L. origanoides* (Martínez-Natarén et al., 2014; Suarez et al., 2008; Vega-Vela; Delgado-Ávila; Chacón-Sánchez, 2013). Therefore, the present study was aimed to evaluate the interpopulation genetic diversity levels and distribution of *L. origanoides* populations occurring in the state of Bahia and Pernambuco, Brazil, using ISSR molecular markers, as well as generating data to assist the species germplasm conservation in the region.

MATERIAL AND METHODS

Plant material

A total of 18 natural populations of *L. origanoides* occurring in the states of Bahia and Pernambuco, Northeast region of Brazil, were collected from areas of Caatinga, Atlantic Forest, and in transition areas between these biomes (Table 1) in highly anthropized environments. The samples of young leaves were collected from 270 plants (15 of each population). The sampled plants were at least 5 meters (m) apart from each other to avoid collecting the plant material from clones originating from the vegetative propagation of species.

The fertile branches were also collected from each population for taxonomic identification, and exsiccates were housed in the Herbarium of the State University of Feira de Santana. The shortest distance between populations was 44.22 km (Jeremoabo and Santa Brígida), whereas the longest distance was 608.41 km (Jequié and Floresta).

Table 1: Population abbreviation, municipality, geographical coordinates, and altitude (m) of the locations of 18 *L. origanoides* Kunth. populations from the states of Bahia (BA) and Pernambuco (PE), Brazil.

Abbreviation	Municipality-State	Latitude and Longitude	Altitude
JEQ	Jequié-BA	13°45′54.0″S. 40°03′28.6″W	346
NIT	Nova Itarana-BA	12°58′22.0″S. 39°56′05.6″W	526
STE	Santa Terezinha-BA	12°35′46.8″S. 39°32′14.8″W	140
UTI	Utinga-BA	12°04′54″S. 41°05′40″W	863
PAL	Palmeiras-BA	12°26′47.7″S. 41°29′12.6″W	809
MCH	Morro do Chapéu-BA	11°36′19.5″S. 40°59′38.3″W	941
MUC	Mucugê-BA	12°59′42.3″S. 41°23′08.6″W	975
RCO	Rio de Contas-BA	13°28′53.8″S. 41°51′47.3″W	1065
JAG	Jaguarari-BA	10°05′20.4″S. 40°13′58″W	543
SSE	Sento Sé-BA	09°44′45″S. 41°53′06″W	400
SBA	Santa Bárbara-BA	12°01′04.5″S. 38°58′15.5″W	270
CNO	Casa Nova-BA	09°09′43″S. 40°58′15″W	417
FLO	Floresta-PE	08°41′0.6″S. 38°09′09.1″W	437
TUC	Tucano-BA	11°01′25.5″S. 38°49′23.7″W	354
JER	Jeremoabo-BA	10°13′35.7″S. 38°17′54.4″W	227
QUI	Quixabeira-BA	11°20′03.9″S. 40°07′39.1″W	396
SBR	Santa Brígida-BA	09°50′16.4″S. 38°15′43.9″W	397
SLU	Santaluz-BA	11°12′30.4″S. 39°24′50.0″W	348

DNA extraction and amplification

The leaf samples for genetic analysis were kept in cetyltrimethylammonium bromide (CTAB) gel (35% $NaCl_2$ and 3% CTAB) (Doyle; Doyle, 1987) at the time of collection and were stored in it till the DNA extraction process.

The genomic DNA was extracted according to Doyle and Doyle protocol (1987) and rescaled for 1-mL tubes for the stored samples. The tests were performed to verify polymorphisms using 13 primers. Among them, 9 primers showed good electrophoretic standards and polymorphism for the species and were selected for further analyses (Table 2). The DNA was amplified through Polymerase Chain Reaction (PCR), according to Wolfe et al. (1998) modified protocol, and using the TopTaq Master Mix Kit (Qiagen, Hilden, Germany). The reaction was performed employing the Esco Swift Max Pro thermal cycler (Esco Global, Portland, USA), and the same protocol was used for all primers.

Table 2: Name, sequence, annealing temperature (°C), and the number of polymorphic bands of primers selected for the analysis.

Name	Sequence	Annealing temperature (°C)	Number of polymorphic bands
Manny	$(CAC)_4$-RC	51.7	14
Jhon	$(AG)_7$-YC	46.9	15
ISSR6	$(AG)_8$-YT	50.2	18
ISSR7	$(AG)_8$-YC	50.2	16
Goofy	$(GT)_7$-YG	49.0	14
Chris	$(CA)_7$-YG	49.6	16
ISSR899	$(CA)_6$-RG	48.6	15
MAO	$(CTC)_4$-RC	50.9	14
OMAR	$(GAG)_4$-RC	49.9	13

The amplified PCR products were resolved on 2.0% agarose gels in Sodium Borate buffer (SB 1X) along with 100 base-pair markers. The gels were stained with 10% ethidium bromide solution for approximately one hour, followed by documentation in the Spectroline UV transilluminator (Fisher Scientific, Waltham, USA).

Data analysis

The GelCompar II 5.0 (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to analyze the gels and generate a binary matrix with values of zero (absence), one (presence) for the data obtained for each locus, and –1 (or –9, depending on the program used for the analyses) for missing data.

The binary matrix was used to estimate the parameters of genetic diversity, including the number and percentage of polymorphic loci, Nei's diversity index, Shannon diversity index, and genetic distance between populations. The analysis of molecular variance (AMOVA) and analysis of the correlation between matrices of genetic and geographical distances was used to evaluate the genetic structuring of populations through the Mantel test. In addition, Popgene 1.32 (Yeh; Yang; Boyle, 1997) and GenAlEx 6.5 (Peakall; Smouse, 2011) were also used.

To estimate the genetic pool through the correlation of allelic frequencies within populations, Structure 2.3.3 (Printchard; Stephens; Donnelly, 2000) was used for the genetic data analysis using the Bayesian method. Furthermore, Evanno's (2005) correction method was used to establish the best attribution peak (K), and for each attribution peak, 1000 runs were performed. AFLPsurv 1.0 (Vekemans, 2002) and Phylip 3.69 (Felsenstein, 2011) were used for the analyses using Nei's non-biased matrix of genetic distance, and the Neighbor-Joining grouping method with bootstrap support (Saitou; Nei, 1987). A dendrogram was developed and analyzed using the FigTree 1.2.2 (Rambaut, 2009).

RESULTS AND DISCUSSION

Genetic diversity

A total of 135 loci were obtained, with an average of 15 bands per primer. According to Colombo et al. (1998), an average quantity of 50 to 100 polymorphic loci is required for a secure evaluation using dominant markers such as ISSR. Several studies on *L. origanoides* using ISSR markers have shown similar numbers of polymorphic loci (Vargas-Mendonça et al., 2016; Vega-Vela et al., 2013). Thus, considering the individual populations, the average percentage of polymorphic bands was 60.08% (Table 3), and similar results (60.9%) were found for *L. origanoides* by Martínez-Natarén et al. (2014).

Regarding genetic diversity variables, Nei's diversity index in each population ranged from 0.162 (FLO) to 0.237 (RCO), with a mean of 0.191 (Table 3). The lowest Shannon diversity index (I) was found for FLO (0.247) and the highest for RCO (0.359), with a mean of 0.292 (Table 3). Thus, FLO showed the lowest genetic diversity, and RCO presented the highest.

The studies on the diversity of *L. origanoides* demonstrated different results. Suarez et al. (2008) and Vega-Vela et al. (2013) used the same molecular marker and found higher genetic diversity for the species with high Nei's diversity index, 0.453 and 0.367, respectively. However, similar studies presented a lower Nei's diversity index. For instance, Vargas-Mendoza et al. (2016) found a mean of 0.110 for 22 populations of *L. origanoides*, as well as Martínez-Natarén et al. (2014) found Nei's diversity index of 0.170 for 14 populations.

The genetic diversity found within populations can be correlated to environmental factors, such as relief and vegetation. The mountainous areas with diverse phytophysiognomies, such as Chapada Diamantina, have populations with higher genetic diversity (UTI, PAL, MCH, MUC, and RCO). According to Salimena et al. (2002), Chapada Diamantina is one of the diverse centers of *Lippia* L. in Brazil. The flatter reliefs and more uniform vegetation are found as the distance from this region increases; therefore, populations outside this region presented lower genetic diversity. The differences in relief and vegetation affecting the genetic diversity of *L. origanoides* were also found in Colombia (Vega-Vela; Sanchez, 2012).

According to Yang et al. (2012) and Gois et al. (2018), the occurrence of exclusive loci or private alleles indicates different populations. However, in the current study, the occurrence of private alleles was low; only the populations MUC, JAG, and SBR presented them (one each) (Table 2). Moreover, according to the distance between populations based on Nei's matrix of genetic identity (1978), the highest genetic similarity was found between the populations CNO and JAG (0.933), which were 112.44 km from each other. The lowest genetic similarity was found between JEQ and FLO (0.797), which were 608.41 km distant from each other. However, the results of the Mantel test showed no significant correlation between geographical and genetic distance matrices ($r = 0.0005$, $p = 0.384$). The report of Fajardo et al. (2018) demonstrated that these results indicate the fragmentation and spatial isolation of the analyzed populations are relatively new events and did not form standards of genetic geographical isolation.

Table 3: Number of polymorphic loci (*NL_p*), percentage of polymorphic loci (*% L_p*), Nei's diversity index *(NDI)*, Shannon diversity index (*SDI*), exclusive locus (*EL*), and genetic group (*GG*) in 18 populations of *L. origanoides* Kunth.

Population	NL_p	% L_p	NDI	SDI	EL	GG
JEQ	84	57.78	0.182	0.281	0	1
NIT	90	64.44	0.185	0.290	0	1
STE	90	63.70	0.170	0.269	0	1
SBA	79	54.07	0.169	0.260	0	1
QUI	88	61.48	0.199	0.304	0	1
SLU	82	58.52	0.185	0.284	0	1
UTI	99	68.89	0.226	0.343	0	2
PAL	94	65.19	0.211	0.322	0	2
MCH	86	59.26	0.188	0.288	0	2
MUC	96	65.93	0.207	0.317	1	2
RCO	99	71.11	0.237	0.359	0	2
JAG	96	65.19	0.214	0.325	1	3
SSE	89	58.52	0.207	0.309	0	3
CNO	81	54.07	0.180	0.272	0	3
FLO	78	51.11	0.162	0.247	0	4
TUC	81	54.07	0.175	0.266	0	4
JER	80	53.33	0.163	0.251	0	4
SBR	80	54.81	0.186	0.280	1	4
Mean	87.33	60.08	0.191	0.292	-	-

Population genetic structure

Since above 25% of the variability between populations denotes strong structuring, the Analysis of Molecular Variance (AMOVA) showed 69% of the genetic variability within populations and 31% between them, indicating the high genetic structuring. The genetic distance between populations indicates the genetic difference between populations, which can be classified as low (0.05 to 0.15), moderate (0.15 to 0.25), or high (>0.25) (Wright, 1978). The mean genetic distance between populations was obtained at 0.19, denoting a moderate difference between populations. Moreover, various studies on *L. origanoides* found moderate structuring between populations (Vega-Vela; Sanchéz, 2012; Vega-Vela et al., 2013).

Organization of genetic structure

The analysis of genetic structure by the Bayesian method pointed out the formation of four genetic groups (K = 4) based on the highest ΔK value (Figure 1).

According to the highest peak (K = 4), the populations were organized into four groups (Figure 2). The populations JEQ, NIT, STE, SBA, QUI, and SLU formed the first group (yellow); UTI, PAL, MCH, MUC, and RCO formed the second group (green); JAG, SSE, and CNO formed the third group (blue); and FLO, TUC, JER, and SBR formed the fourth group (red) (Figure 2). The analysis indicated that the highest mixing level occurred for the populations JEQ, TUC, and SBR.

The Neighbor-Joining dendrogram (Figure 3) also showed the formation of four clades, with similar arrangements to the one resulting from the Structure analysis. Among the four groups formed in the dendrogram, three (group 1, group 3, and group 4) presented low support values (less than 500). In genetic diversity studies, the occurrence of low support values is common in dendrograms originated by clustering methods, especially in the innermost nodes, as a consequence of instabilities in the chosen algorithm. However, the congruence with the result obtained in the Structure analysis indicates the plausibility of separating evaluated populations into four groups.

The MUC, UTI, MCH, PAL, and RCO populations formed the first clade, and these populations are from the Chapada Diamantina region, presenting the highest genetic diversity indexes. The SSE, JAG, and CNO populations were clustered as the second clade, and these populations are from the northern state of Bahia, and the two latter are in a subgroup (Figure 2). The third clade was formed by three subgroups; first by SBA and QUI (Figure 3); second by JEQ and NIT; and third by SLU and STE. Moreover, these populations are within and nearby the ecotone between the Caatinga and Atlantic Forest biomes. SBR, FLO, TUC, and JER, populations from the northeastern state of Bahia and near the Pernambuco border, composed the fourth clade, and these populations presented the lowest genetic diversity, and the latter two populations formed a subgroup (Figure 2).

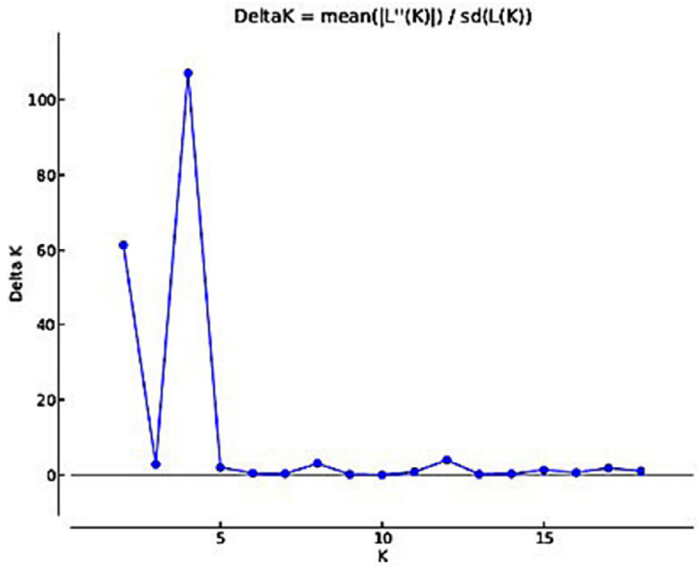

Figure 1: Values of Delta K according to the correction of Evanno (2005), showing participation peaks (K) for 18 populations of *L. origanoides* Kunth. (Highest K value = 4; and the other peaks: K = 2, K = 8, and K = 12).

Although some populations close to each other remained in the same group, the geographical distance did not affect the formation of these groups, according to the Mantel test. Therefore, these groups represent the genetic diversity of *L. origanoides* created by various environmental factors, such as relief, climate, and vegetation, indicating adaptive genetic differentiations (Coop et al., 2010). The obtained genetic structuring level in this study suggests the need for adequate planning for *L. origanoides* germplasm collections, considering the genetic diversity distribution between the studied populations for better conservation of the genetic variability (Dardengo, et al., 2021).

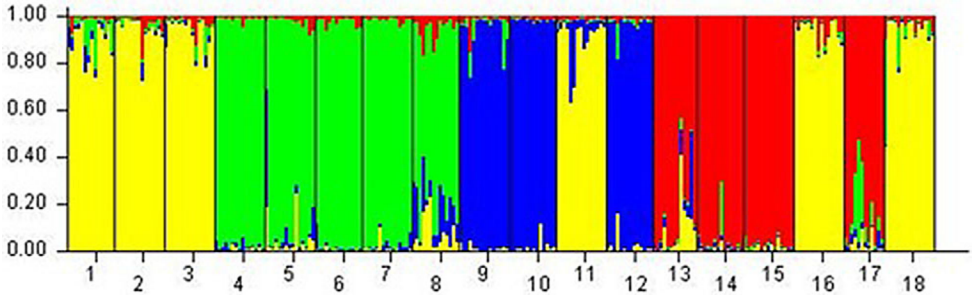

Figure 2: Proportions of Bayesian mixture, generated from the Structure program data for 18 populations of *L. origanoides* Kunth. collected in the states of Bahia and Pernambuco, Brazil. 1 = JEQ; 2 = NIT; 3 = STE; 4 = UTI; 5 = PAL; 6 = MCH; 7 = MUC; 8 = RCO; 9 = JAG; 10 = SSE; 11 = SBA; 12 = CNO; 13 = FLO; 14 = TUC; 15 = JER; 16 = QUI; 17 = SBR; 18 = SLU.

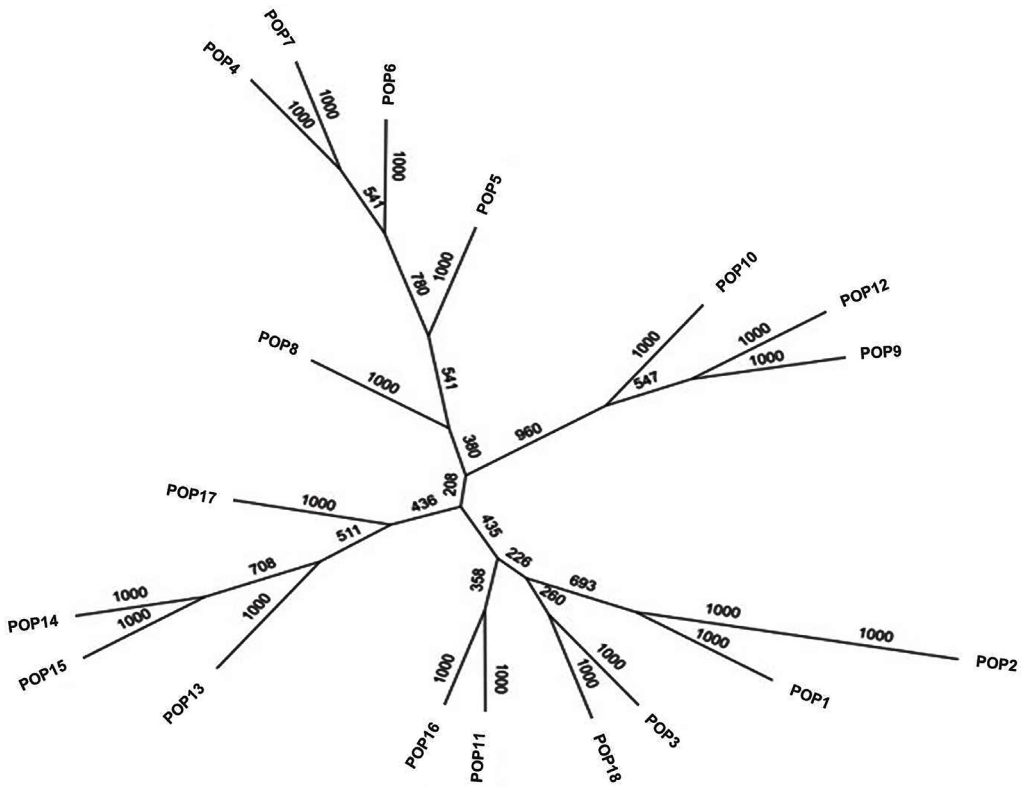

Figure 3: Neighbor-Joining dendrogram based on the Nei's matrix of genetic distance for 18 populations of *L. origanoides* Kunth., denoting the formation of four groups. Group 1: POP04 = UTI; POP05 = PAL; POP06 = MCH; POP07 = MUC; POP08 = RCO. Group 2: POP09 = JAG; POP010 = SSE; POP012 = CNO. Group 3: POP01 = JEQ; POP02 = NIT; POP03 = STE; POP011 = SBA; POP016 = QUI; POP018 = SLU. Group 4: POP013 = FLO; POP014 = TUC; POP015 = JER; POP017 = SBR.

CONCLUSIONS

The application of ISSR molecular markers facilitated the understanding of genetic diversity as well the genetic structuring level of species populations. The populations of *L. origanoides* had a mean value of 0.292 for the Shannon diversity index and 0.191 for the Nei's diversity index, with populations located in Chapada Diamantina showing the highest values. There was a high genetic structure among populations (31%). Moreover, the cluster analysis indicated the separation of populations into four genetic groups. The collection of *L. origanoides* samples should comprise germplasm from populations belonging to different genetic groups. The priority area to collect *L. origanoides* germplasm is the Chapada Diamantina region.

AUTHOR CONTRIBUTION

Conceptual idea: Feijó, E.V.R.S.; Berg, C.V.D.; Oliveira, L.M.; Methodology design: Feijó, E.V.R.S.; Berg, C.V.D.; Oliveira, L.M.; Data collection: Feijó, E.V.R.S.; Barbosa, B.L.; Oliveira, L.M.; Data analysis and interpretation: Feijó, E.V.R.S.; Barbosa, B.L.; Berg, C.V.D.; Writing and editing: Feijó, E.V.R.S.; Barbosa, B.L.; Berg, C.V.D.; Oliveira, L.M.

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