Genetic Diversity and Population Structure of *Zingiber officinale* Roscoe in Northern Mato Grosso State, Brazil

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Abstract

The ginger (*Zingiber officinale* Roscoe) was introduced in Brazil in the 16th century and is currently cultivated for commercial purposes and for own consumption, in urban and rural backyards. Although the species reproduces vegetatively, the exchange of rhizomes among gardeners contributes to the maintenance of the genetic variability of the species, since they carry out the selection of genotypes adapted to the soil and climate conditions of the cultivation areas. Studies on the variability of ginger cultivated in Brazil are scarce, therefore this study aimed to evaluate the genetic diversity and population structure, using ISSR molecular markers, of 245 ginger specimens cultivated in rural and urban backyards of twenty municipalities (populations) in the state of Mato Grosso, Brazil. The ISSR primer set used was classified as moderately informative and it amplified a total of 105 fragments, of which 97.60% showed polymorphism, indicating the existence of genetic variability. The genetic diversity assessment separated the 245 specimens into 30 hierarchical groups. Group 1 was the most representative (72%), containing specimens from all populations sampled. The population-level assessment allocated 18 populations in a single group, while the populations of Apiacás and Peixoto de Azevedo formed exclusive groups. The Molecular Variance Analysis revealed that the genetic variation found within populations (76.58%) is greater than that found among populations (23.42%), which indicates a process of fixing of alleles and genetic structuring. The Bayesian analysis identified only two distinct genetic groups and some populations sampled showed a predominance of one of the groups, which evidences the process of genetic structuring. According to the genetic distance of Nei (h) and the Shannon Index (I), Apiacás was the population that presented the greatest genetic diversity, while Matupá was characterized as the population with less diversity.

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The genetic diversity found indicates that the cultivation, handling and selection performed by gardeners, as well as the exchange of rhizomes for propagation, are important factors for the conservation of the genetic diversity of the species. We also concluded that backyards are a source of genetic resources for programs that aim to identify traits of interest and promote the cultivation of the species in Brazil.

Keywords: On-farm conservation; ISSR; Genetic Resources; Genetic variability; *Zingiber officinale*.

1. Introduction

Ginger (*Zingiber officinale* Roscoe) was described by naturalists in the 17th century as a native Brazilian plant. It was found in a wild environment, it is of Asian origin and the varieties introduced in Brazil in the 16th century were probably brought from one of the Portuguese colonies (São Tomé Island), and initially cultivated in the state of Bahia [1;2]. Currently, the species is cultivated throughout Brazil, and the expansion to the national territory took place through the distribution and exchange of rhizomes among farmers, since, according to [3], the species has reduced flowering, it rarely produces fruits and seeds, and when it does, they are usually unviable and, therefore, its propagation is exclusively vegetative, which implies low variability. Intrapopulation genetic variability, arising from mutational processes and intensified, in species with sexual reproduction, by recombination processes, is fundamental for adaptability and, consequently, for natural and/or artificial selection [4]. Considering that ginger reproduces asexually, it is expected that the genetic variability of the species will be reduced, since this type of reproduction, still according to [4], is more efficient in preserving, over the generations, the alleles originated by mutations, on which the selection processes will act, as it does not have recombination and segregation mechanisms. Thus, the genetic variation present in ginger grown in Brazil is the result of natural mutation processes, the handling and exchange of rhizomes within families and communities, and the clonal selection carried out by producers in different soil and climate conditions. According to the latest agricultural production survey, the Brazilian production of ginger is approximately 24,000 megagrams, and the species is cultivated commercially, mainly by family farmers in Espírito Santo and São Paulo states, and in Paraná state, where cultivation is predominantly by non-family agriculture [5], with most of the production destined for export [6]. In addition to commercial production, the species is often cultivated in urban and rural backyards for personal consumption, both for culinary use and for the treatment of diseases, since its use as a herbal medicine is widespread in Brazil [7; 8; 9; 10; 11]. Thus, we can infer that the different forms of cultivation ensured the conservation of genotypes that contained alleles that expressed the characteristics of interest and/or that presented greater adaptability, productivity and resilience to local environmental conditions and therefore contributing to the conservation of the genetic diversity of the species. Considering the size of the Brazilian territory and that it has 12 distinct climatic zones [12] and 13 different soil classifications [13], it is to be expected that genotypes with different characteristics have been selected and cultivated over the years. In this context, in order to identify genotypes of interest for the composition of germplasm banks or use as matrices for planting, characterization studies and genetic diversity assessment are necessary, and they can be carried out with the use of phenotypic and/or molecular markers. Although Brazil is considered a major producer of ginger [5; 6], there are few studies that evaluate the national genetic material [14; 15; 16; 17]. The use of molecular markers for characterization and genetic diversity studies is recommended because they identify the polymorphism at the DNA level, are not influenced by environmental conditions and are used in different stages

of development of the target organism. Among the molecular markers commonly used are the ISSR (Inter Simple Sequence Repeats) markers, classified as dominant because they identify the polymorphism by the presence or absence of the allele (fragment) and composed, in general, by di- or trinucleotide sequences that may or may not, contain an anchor composed of two or more nucleotides that ensure that the primer matches in the same position every cycle [18]. ISSR molecular markers have been frequently used in characterization and genetic variability studies of ginger as described by [19; 20; 21; 22] and, in this context, this study aims to analyze the genetic diversity and population structure of ginger grown in urban and rural backyards of 20 municipalities located in Mato Grosso state, Brazil, using ISSR molecular markers.

2. Materials and methods

2.1. Study and sampling area

Table 1: Location of the 20 ginger populations and sample size (N).

Populations	Population Code	N	Latitude (S)	Longitude (W)
Terra Nova do Norte	TNN	12	10° 35' 50"	55° 06' 57"
Sorriso	SOR	10	12° 33' 06"	55° 43' 33"
Nova Santa Helena	NSH	11	10° 51' 04"	55° 11' 01"
Peixoto de Azevedo	PXT	18	10° 14' 41"	54° 59' 32"
Paranaíta	PAR	12	9° 40' 11"	56° 28' 44"
Novo Mundo	NMU	13	9° 58' 38"	55° 10' 19"
Nova Monte Verde	NMV	15	9° 58' 21"	57° 28' 21"
Nova Bandeirantes	NBS	11	9° 50' 35"	57° 49' 07"
Nova Mutum	NMT	11	13° 49' 58"	56° 06' 36"
Nova Canaã do Norte	NCN	12	10° 37' 52"	55° 42' 54"
Nobres	NOB	11	14° 43' 28"	56° 20' 03"
Matupá	MTP	13	10° 10' 20"	54° 55' 55"
Marcelândia	MAR	12	11° 04' 50"	54° 31' 10"
Lucas do Rio Verde	LRV	11	13° 04' 14"	55° 55' 18"
Guarantã do Norte	GTA	12	9° 57' 15"	54° 54' 58"
Diamantino	DIA	10	14° 24' 05"	56° 26' 01"
Colider	COL	12	10° 48' 15"	55° 27' 51"
Carlinda	CAR	9	9° 57' 59"	55° 49' 18"
Apiacás	APC	11	9° 33' 56"	57° 23' 43"
Alta Floresta	ALF	19	9° 52' 18"	56° 05' 30"

For the analysis of the genetic diversity of ginger, 245 specimens from urban and rural backyards of 20

municipalities located in the North mesoregion of the Mato Grosso state, and is distributed between the Amazon and Cerrado biomes were evaluated (Table 1 and Figure 1). Each municipality was considered as a population.
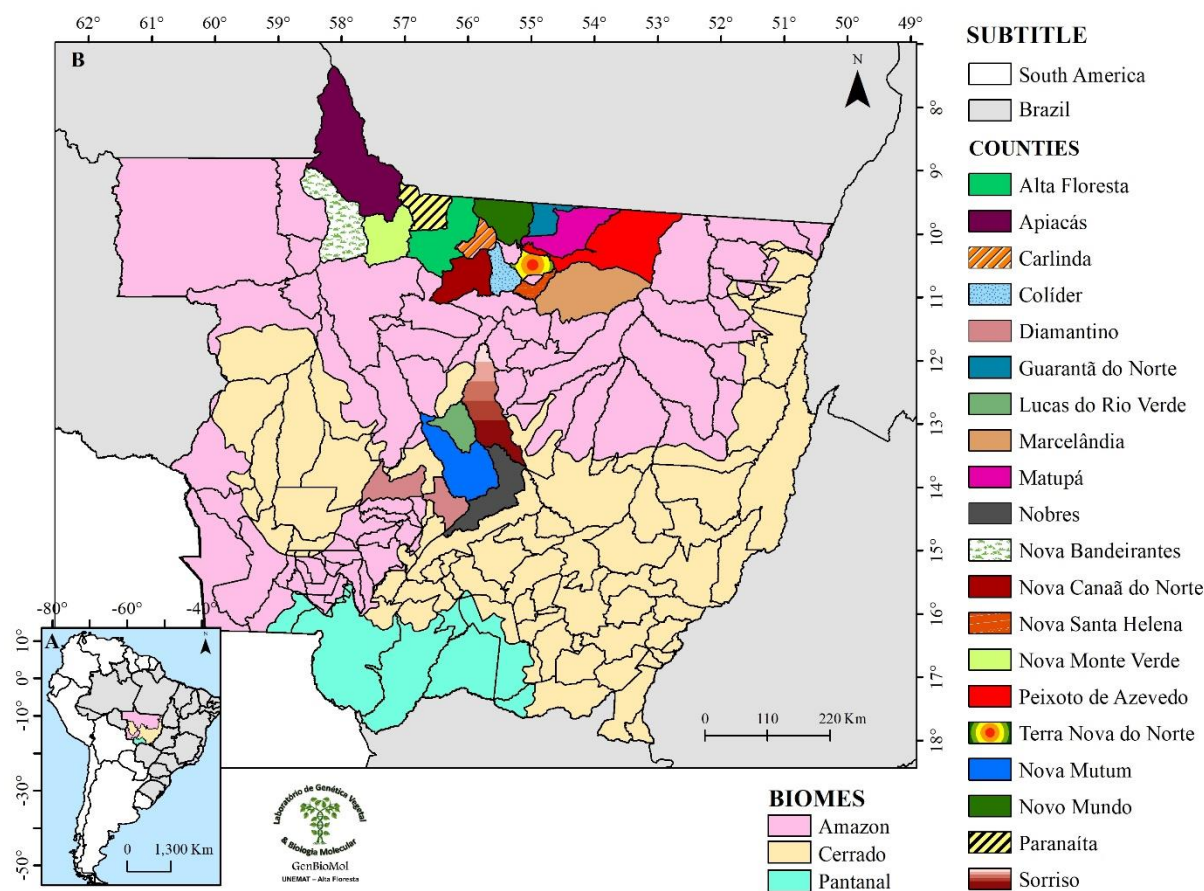

Figure 1: Geographic location of Mato Grosso municipalities where ginger specimens were collected. A: Location of the state of Mato Grosso in South America; B: Mato Grosso municipalities where the collections were carried out.

Among the municipalities represented in this study, 15 (75%) are located in the Amazon biome, with a humid or sub-humid tropical climate (Am), with an average rainfall of 3000 mm year^{-1}, while the other municipalities (SOR, LRV, NMT, NOB and DIA) are predominantly found in the cerrado biome, where the climate is characterized as tropical with dry winter (Aw) and the average precipitation is 2000 mm year^{-1} [12].

The collection of plant material was carried out through random visits to residents of rural and urban areas of each municipality, with young leaves being collected for molecular characterization studies and assessment of the diversity and population structure of ginger. The ginger leaves were collected, sanitized, wrapped in paper towels and placed in a plastic bag with an airtight seal, properly identified and containing silica gel.

The use of silica gel for the conservation of leaf material was chosen because it does not require refrigeration equipment during the collection and transport period, which, in addition to promoting dehydration, also prevents the proliferation of fungi and oxidation [23], contributing to the maintenance of the quality of the genetic

material. After dehydration, the leaf material was transferred to aluminum foil envelopes with their respective identifications and stored under refrigeration (-20 °C).

2.2. Molecular characterization

Total DNA extraction and amplifications were performed at the Laboratory of Plant Genetics and Molecular Biology (GenBioMol), Center for Research and Technology of the Southern Amazon (CEPTAM), of the State University of Mato Grosso Carlos Alberto Reyes Maldonado (UNEMAT), campus from Alta Floresta, Mato Grosso, Brazil.

For the extraction of total DNA, approximately 100 mg of leaf material were used, on which the protocol proposed by [24] was applied, with some changes: 1- grinding the leaf material in the presence of liquid nitrogen; 2- after the addition of ice-cold isopropanol, the solution was incubated for 60 minutes; 3- the pellets were washed three times, the first two with 70% ethanol and the last one with 95% ethanol; 4- addition of 0.24% RNase (10 mg mL^{-1}) in 0.1 mM TE buffer (tris HCl, EDTA, pH $\cong$ 8.0). The quality and integrity of the DNA were verified by electrophoresis in a 1% agarose gel stained with ethidium bromide (10 mg mL^{-1}), while the quantity and purity of the material were estimated using a Hercuvan ND-3800-OD Microspectrophotometer$^{®}$. The extracted DNA was diluted in milli-Q$^{®}$ water to a concentration of 10 ng µL^{-1}.

For the analysis of genetic diversity, 33 ISSR primers were tested, and those that resulted in a good amplification profile, with clear, intense and non-agglutinated fragments were selected. Amplifications via PCR (Polymerase Chain Reaction) were performed in a volume of 13.5 µL, containing 3 µL of 5X Colorless GoTaq$^{®}$ Reaction Buffer, 2.7 mM of MgCl$_2$, 0.11 mM of each dNTP, 0.22 µM of each primer, 0.6 U of taq DNA polymerase, 10 ng of DNA and Milli-Q$^{®}$ water to make up the total volume.

The amplifications were carried out in an Aeris$^{®}$ thermocycler, and the program consisted of initial denaturation for four minutes at 94 °C, 35 cycles of 94 °C for one minute, specific annealing temperature (TA) for each primer for one minute, in addition to another minute at 72°C for polymerization of the new DNA strand, followed by a final extension at 72°C for 10 minutes. To assist the analysis of the amplified fragments (bands), the Kapa Universal DNA Ladder marker (KK6302) was used. The amplification products were separated on 1.5% agarose gel and stained in ethidium bromide solution (0.6 µg mL^{-1}).

The visualization and photographic recording of the images of the gels with the extracted DNA and with the amplification products were performed in a transilluminator with UVB light (Loccus Biotecnologia$^{®}$ - LTB-STi), L-Pix Sti photodocumentator (Loccus Biotecnologia$^{®}$) and software L -Pic STi Image, respectively.

2.3 Data analyses)

From the visual analysis of the gels with the amplification products, a binary matrix was constructed indicating the presence (1) and absence (0) of the amplified fragments for each of the ISSR primers and the evaluated specimens. The binary matrix was then used to calculate the percentage of polymorphism of each primer (Equation 1) and the polymorphic information content (Equation 2).

%P = (nfp/ntf) x 100 (1)

In which, %P represents the percentage of primer polymorphism, nfp, the number of polymorphic fragments and ntf, the total number of amplified fragments

$$PIC = 1 - \sum_{i=1}^{n} fi^2 \qquad (2)$$

In which, fi is the frequency of the ith allele.

With the aid of the Genes program [25], the bootstrap analysis was performed, where the genetic similarity was estimated from simulations with resampling for different amounts of amplified fragments, also obtaining the correlation estimate between the original and simulated genetic similarity matrices, as well as the fit between these matrices (stress). Using the same program, Nei's dissimilarity matrices [26] were constructed, for evaluation at the population level, and, for evaluation at the specimen level, the matrix based on the arithmetic complement of the Jaccard coefficient (Equation 03), which, comparing specimens pair by pair, disregards the joint absence of fragments and compares the total number of fragments and the common presences [27]. The genetic similarity among the specimens and among the ginger populations was verified by means of the hierarchical grouping by the UPGMA method, while the determination of the number of groups was performed by the Mojena method [28].

$$D_{ij} = 1 - S_{ij} \quad \text{sendo que } S_{ij} = \frac{a}{a+b+c} \qquad (3)$$

Where, 'a' represents the fragment number in both individuals; 'b', the occurrence of the fragment only in individual i and 'c', the presence of the fragment only in individual j.

The number of genetic groups (K) was obtained using the "Structure" program [29], based on Bayesian statistics, with 20 runs for each value of K, 250,000 burn-ins and 750,000 Monte Carlo simulations of Chains. of Markov (MCMC). To define the most likely K, the criteria proposed by [30; 31].

The evaluation of the distribution of intra and interpopulation genetic diversity through Molecular Analysis of Variance (AMOVA), as described by [32], was performed with the aid of the Genes program, while the PopGene 1.32 software [33] was used to estimate the percentage polymorphism of each population, as well as the Nei genetic distance [34] and the Shannon Index [35].

3. Results and discussion

Among the 33 ISSR primers tested, only nine (27.27%) resulted in clear fragments that made it possible to perform genetic diversity analyzes (Table 2), and these amplified a total of 105 fragments, with 97.60% of polymorphism. The percentages of polymorphism produced by the primers UBC 808 and UBC 809 were 87.50% and 90.91%, respectively, while for the others, all fragments were polymorphic (%P = 100).

Table 2: ISSR primers used to amplify DNA fragments from 245 ginger specimens sampled in 20 populations from the North mesoregion of Mato Grosso state. Annealing temperature (TA), total number of amplified fragments (NTF), number of polymorphic fragments (NFP) and polymorphic content index (PIC).

Primer					
Code	**Sequence (5'--- 3')**	**TA (°C)**	**NTF**	**NFP**	**PIC**
UBC 807	$(AG)_8T$	52	9	9	0.39
UBC 808	$(AG)_8C$	52	8	7	0.22
UBC 809	$(AG)_8G$	52	11	10	0.35
UBC 825	$(AC)_8T$	50	14	14	0.59
UBC 830	$(TG)_8G$	55	11	11	0.45
UBC 834	$(AG)_8YT$	52	12	12	0.30
UBC 835	$(AG)_8YC$	52	16	16	0.50
UBC 880	$(GAGA)_3$	50	14	14	0.29
UBC 889	$DBD(AC)_7$	55	10	10	0.43
Total		---	105	103	---
Média		---	11.67	11.44	0.39

The set of primers used in this study is classified, according to [36], as moderately informative (0.25 < PIC < 0.50), since the average PIC was 0.39, and therefore it is indicated for future studies with the species. We emphasize the primers UBC 825 and UBC 835, once they were considered highly informative because they present ICP ≥ 0.50. The efficiency in detecting genetic variability was performed by calculating the PIC, which considers the number and frequency of amplified fragments [37], and by bootstrap analysis. Based on the bootstrap analysis, it was possible to define that at least 75 fragments would be necessary for the analyzes of genetic variability to be reliably estimated (Figure 2).
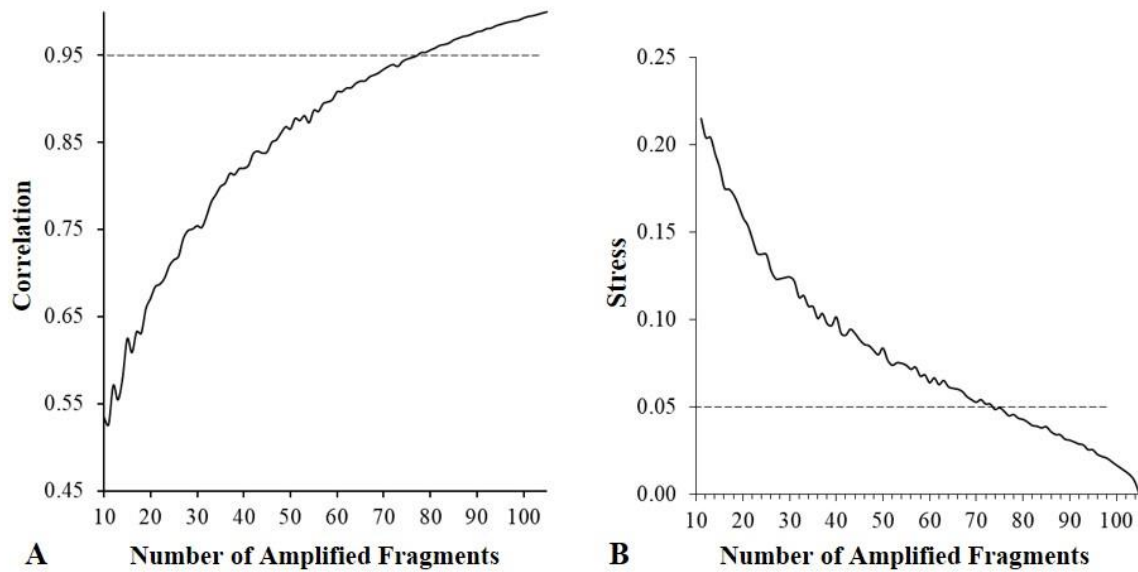
Figure 2: Result of bootstrap analysis for nine ISSR primers. Estimated correlation (A) and stress values (B) between the matrices (original and simulated) of genetic similarity, indicating the minimum number (75) of fragments necessary for genetic diversity analysis among the 245 ginger specimens sampled in 20 populations from North mesoregion of Mato Grosso state.

The determination of the minimum number of fragments is carried out by correlation analysis, which compares the original and simulated matrices of genetic distance, and by stress, which indicates the adjustment between them. When the stress value is equal to or less than 0.05, we can say that the matrices present a good fit [38]. In this sense, the number of fragments produced in this study (n = 105) was sufficient to reliably estimate the genetic variability of the populations sampled.

Genetic dissimilarity was estimated by the arithmetic complement of the Jaccard coefficient and varied between 0.0625 (SOR03 and SOR05) and 0.6447 (NMV02 and NMT03), with a mean of 0.3489. These values allowed us to infer that although the species reproduces exclusively by vegetative propagation, there is genetic diversity among the 245 specimens evaluated. Clustering using the UPGMA hierarchical method showed CCC (cophenetic correlation coefficient) of 0.82, distortion and stress of 1.82% and 13.47%, respectively.

These results reveal a good fit between the genetic and cophenetic dissimilarity matrices, since, according to [37], CCC > 0.80 is satisfactory for expressing similarity among specimens. The stress value indicated a good fit in the clusters, considering that values close to 10%, lower or higher, indicate efficiency in the clustering method used [38]. The 245 ginger specimens were divided into 30 groups, 72% of which (n = 176) were allocated in group 1 (G1), with specimens from all populations. Groups G5, G10, G11, G15, G16, G17, G18, G19, G20, G24, G25, G26, G29 and G30, on the other hand, are composed of a single specimen, while nine groups are composed of specimens from one of the sampled populations exclusively (G7 - DIA; G9 - NMU; G13 - ALF; G14 - ALF; G21 - LRV; G22 - PXT; G23 - PXT; G27 - NMV; G28 - NMT). The remaining groups (G2, G3, G4, G6, G8 and G12) are mixed, with specimens from more than one population and formed by two to eight specimens.

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The genetic diversity observed in this study highlights the importance of on-farm conservation for the maintenance of genetic resources caused by the evolutionary processes of the species. The material evaluated is cultivated in urban and rural backyards, with planting being carried out, every year, with rhizomes fragments harvested in the previous one. According to [39; 40], the conservation, management and use of genetic resources cultivated (on farm) contributes in the fight against genetic erosion, therefore, it is fundamental for the conservation of agrobiodiversity.

The evaluation of genetic diversity at the population level resulted in the formation of three distinct groups. Group I (GI) representing 90% of the populations (n = 18) (Figure 3), while the populations Apiacás (APC) and Peixoto de Azevedo (PXT), form the groups GII and GIII, respectively.
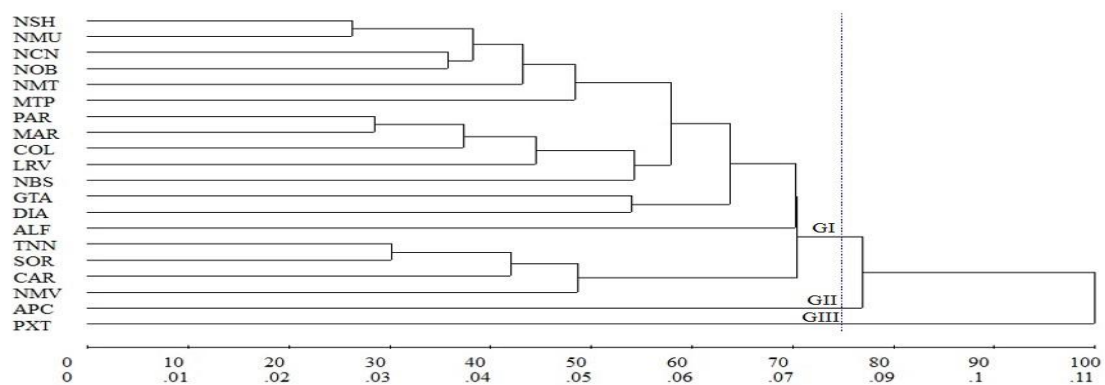

Figure 3: Dendrogram based on the genetic distance of Nei (1973) and obtained by the UPGMA method for the 20 Ginger populations from North mesoregion of Mato Grosso state. Cophenetic Correlation Coefficient (CCC): 0.76. Cut-off Point: 0.085 (74.85%).

The genetic distance between the populations showed greater similarity (0.0299) between the specimens cultivated in Nova Santa Helena (NSH) and Novo Mundo (NMU), while the greatest genetic distance (0.1518) occurred between Sorriso (SOR) and Peixoto de Azevedo (PXT). The evaluation of the genetic structuring, by means of the Molecular Variance Analysis (AMOVA) showed that most of the genetic variability (76.58%) is found within the populations (Table 3), in other words, there is greater genetic variation among the specimens of a given population than when compared it with those of another.

Table 3: Molecular Analysis of Variance (AMOVA) among the 20 ginger populations from North mesoregion of Mato Grosso state, based on nine ISSR primers.

Source of Variation	GL	QM	CV	VT (%)	F_{ST}
Between populations	19	57.96	3.74	23.42	0.2342[**]
Within populations	225	12.23	12.23	76.58	
Total	18	129.368	9.31748		

**Significant at 1% probability; GL: degree of freedom; QM: mean square; CV: variance component; VT: Total

Variance; FST: genetic divergence among populations.

The genetic differentiation among populations (FST), according to [41], is considered high, as FST values between 0.15 and 0.25 point to a process of allele fixation and genetic structuring, which may be directly related to the vegetative propagation of the species and the conservation of the rhizomes by the gardeners for later planting. The high differentiation among populations and/or groups of ginger was also observed by [42; 43; 21; 44], reinforcing the hypothesis that asexual reproduction and on-farm conservation contribute to the differentiation among populations.

The evaluation of the genetic structure, carried out through Bayesian analysis, revealed that 57.55% (n = 141) of the studied specimens presented genetic material from the group represented by the green color, while 35.92% (n = 88), were classified as belonging to the red group and 6.53% (n = 16) had genetic material from both groups in equal or very similar proportions (Figure 4A). Although two genetic groups were found, it is possible to identify the structuring process of some of the populations studied, considering that all, or most of their genotypes, represent one of the groups predominantly, such as for example, Paranaíta (PAR), Matupá (MTP) and Carlinda (CAR) (Figure 4B).
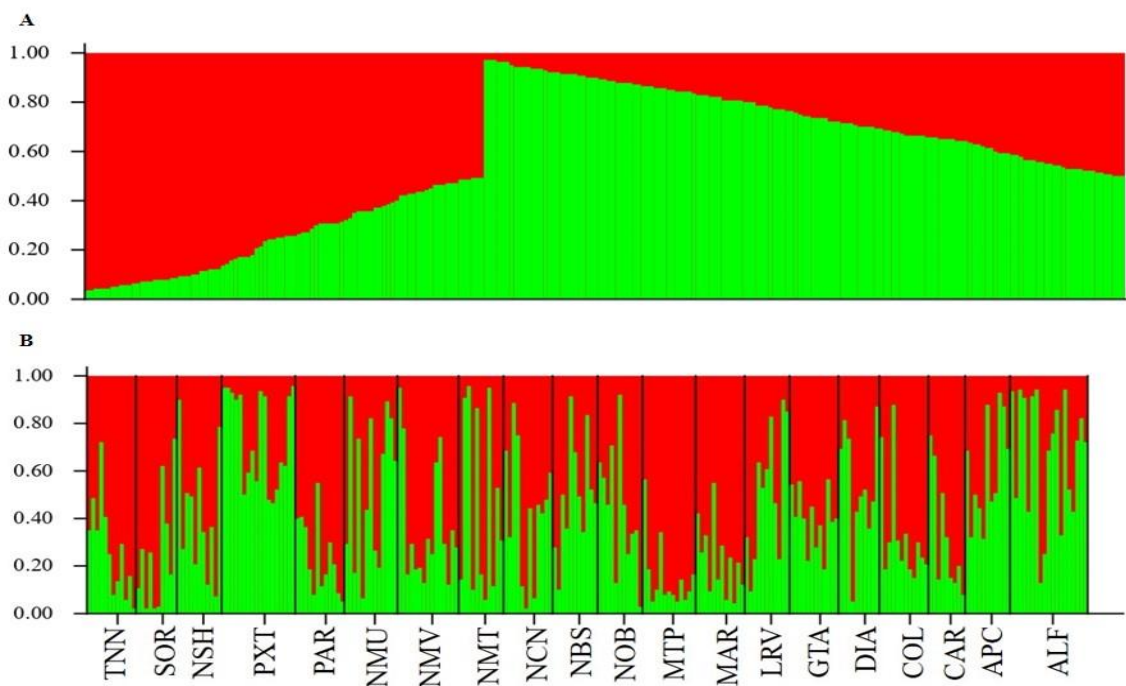

Figure 4: Genetic structure of 20 ginger populations from North mesoregion of Mato Grosso state, according to molecular bases with nine ISSR primers using Bayesian analysis. Individuals are represented by vertical bars colored according to the group they belong to (two groups, K = 2). A: graphical representation by K (groups); B: graphical representation by population.

Genetic structuring analysis using Bayesian statistics has been frequently used in genetic diversity studies once it is an alternative for solving complex problems. This methodology calculates the probability of a genotype being included in a group (a priori information (K)) and indicates the number of genetic groups that best

represents the data matrix whose graphic representation is easy to understand, as shown by [45; 46; 47; 48; 49]. The presence of only two genetic groups, as well as the presence of alleles from both groups in a single genotype (Figure 4B), may be related to the genetic basis of the species, since the reproduction process does not include the combination of parental gametes. In the 16th century few varieties of ginger were introduced in Brazil, which have been distributed and propagated vegetatively since then [2], therefore, the gene flow is carried out through the exchange and/or donations of rhizomes among producers. The evaluation of the genetic diversity within the ginger populations revealed that the populations of Apiacás (APC) and Alta Floresta (ALF) present high polymorphism (80% and 79.05%, respectively), while Matupá (MTP), with 45.71 %, Sorriso (SOR) and Paranaíta (PAR), both with 53.33%, are those with the lowest percentage of polymorphic fragments (Table 4).

Table 4: Number of specimens (N) and genetic parameters for the 20 ginger populations from North mesoregion of Mato Grosso state.

Populations	N	%P	h	I
TNN	12	57.14	0.25 (0.22)	0.36 (0.32)
SOR	10	53.33	0.24 (0.23)	0.34 (0.32)
NSH	11	72.38	0.32 (0.20)	0.45 (0.29)
PXT	18	65.71	0.26 (0.21)	0.38 (0.30)
PAR	12	53.33	0.23 (0.22)	0.33 (0.31)
NMU	13	76.19	0.32 (0.20)	0.46 (0.27)
NMV	15	64.76	0.29 (0.22)	0.41 (0.31)
NMT	11	73.33	0.31 (0.20)	0.45 (0.28)
NCN	12	69.52	0.29 (0.20)	0.42 (0.29)
NBS	11	60.95	0.24 (0.21)	0.35 (0.30)
NOB	11	70.48	0.30 (0.21)	0.43 (0.29)
MTP	13	45.71	0.17 (0.21)	0.26 (0.30)
MAR	12	60.95	0.25 (0.21)	0.37 (0.31)
LRV	11	71.43	0.32 (0.21)	0.45 (0.29)
GTA	12	61.90	0.25 (0.21)	0.37 (0.30)
DIA	10	61.90	0.26 (0.22)	0.38 (0.31)
COL	12	65.71	0.27 (0.21)	0.39 (0.30)
CAR	9	54.29	0.23 (0.22)	0.33 (0.32)
APC	11	80.00	0.33 (0.19)	0.48 (0.26)
ALF	19	79.05	0.30 (0.19)	0.44 (0.26)
Total	245	97.60	---	---

%P: Percentage of polymorphic fragments. h: Nei Genetic Distance (1972) and I: Shannon Index; ( ) Standard deviation.

The number and distribution of polymorphic fragments from each population are used to calculate the Nei (h) and Shannon (I) indices. This analysis reveal the genetic diversity within the populations, if the value is close to zero, the diversity between their genotypes will be lower (26; 35).

In this context, we can infer that the population of Matupá (MTP) is the one with the lowest intrapopulation diversity (h = 0.17 and I = 0.26), while the specimens collected in Apiacás (APC) are more divergent from each other (h = 0.33 and I = 0.48) (Table 4), which may be related to the origin of the material used in the cultivation. These results support those demonstrated by the Bayesian analysis, where populations with lower h and I (MTP, PAR and CAR) are more structured, with a predominance of one of the genetic groups (red or green).

4. Conclusion

The ISSR primers used were efficient in detecting the genetic variability present in the 245 ginger specimens evaluated in this study, pointing to a greater intrapopulation than interpopulation genetic diversity, revealing a structuring process among the populations evaluated.

The genetic diversity found in urban and rural backyards highlights the importance of this type of cultivation for the genetic conservation of the species, as well as characterizes them as places of on-farm conservation. Urban and rural backyards, therefore, are important sources of genetic resources for programs that aim to identify genotypes with a characteristic of interest, whether related to productivity, medicine or cuisine, and thus promote the cultivation of ginger in Brazilian territory.

Acknowledgements

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