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RAPD markers in the discrimination of genetic variability in ornamental peppers

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Abstract

Genetic variability in pepper genotypes has been widely assessed using phenotypic traits and molecular markers, which are essential to estimate the genetic divergence of the species and thus recommend parental individuals for breeding programs. In this scenario, this study aimed to evaluate the genetic divergence between ornamental pepper accessions (*C. annuum* L.) using RAPD molecular markers. Seventeen ornamental pepper accessions and one plant of the commercial cultivar Calypso were used in the study. The accessions were subjected to RAPD analysis with 15 primers. The analysis revealed high variability between the accessions and evidenced that accessions UFPB-355 and UFPB-348 were the most dissimilar, whereas accessions UFPB-443 and UFPB-77.3 were the most similar. The accessions were grouped into three different groups, evidencing their genetic divergence The accessions characterized in this study are promising and can be used in future pepper breeding programs due to their high genetic variability. Moreover, the results obtained in this study allow recommending accessions UFPB-45, UFPB-77.3, UFPB-137, UFPB-356, and UFPB- 443 as parent individuals since they are the most divergent. Finally, the RAPD markers were highly sensitive in driscriminating pepper accessions and should be used as an auxiliary tool for choosing parents.

Keywords: *Capsicum* spp., dissimilarity, genetic variability, molecular analysis

Introduction

Ornamental plants are used to improve the beauty of different environments, e.g., interior decoration and landscaping (Pereira et al., 2022). Peppers stand out among the currently cultivated ornamental plants due to their growing and continuous acceptance by the consumer market, showing importance for several uses, both fresh (*in natura*) and processed, and providing good profitability with field cultivation (Rêgo et al., 2011; Rêgo & Rêgo, 2018; Finger et al., 2012).

Breeding programs of ornamental peppers have been conducted by hybridization or mass selection (Rêgo et al., 2016). In this scenario, the choice of parental individuals is crucial to genetic improvement and depends on the available genetic variability (Neitzke et al., 2010). Germplasm characterization using morphologic and agronomic traits and biochemical and molecular markers is essential to determine the genetic variability between and within accessions and make them available to plant breeders (Maciel et al., 2016; Carvalho et al., 2017; Oliveira et al., 2019).

The phenotypic and genotypic diversity of ornamental peppers can be used as essential information in breeding programs (Costa et al., 2021). This genetic variability between and within species provides aesthetic value for ornamental plants, e.g., leaf variegation, small sizes, erect flowers, and marked and small fruits, in addition to resulting in plants of relatively easy cultivation (Stommel & Bosland, 2007; Melo et al., 2014).

Molecular markers are basic tools for estimating the genetic variability between and within populations (Pessoa et al., 2019), and the identification of divergent parental individuals based on molecular markers has increased due to the satisfactory discrimination of genotypes according to the number of alleles detected (Buso et al., 2016). From this perspective, Silva et al. (2017)

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highlighted the importance of RAPD (Random Amplified Polymorphic DNA) markers for estimating genetic variability in ornamental peppers.

In this context, this study aimed to evaluate the genetic diversity between ornamental pepper accessions (*C. annuum* L.) using RAPD molecular markers.

Material and Methods

The experiment was developed in the Laboratory of Biotechnology and Plant Breeding at the Center of Agricultural Sciences of the Federal University of Paraíba (CCA-UFPB), state of Paraíba, Brazil. Seventeen ornamental pepper accessions (*C. annuum* L.) belonging to the Germplasm Bank of the CCA/UFPB were used in the experiment: UFPB-001, UFPB-004, UFPB-045, UFPB-046, UFPB-77.3, UFPB-099, UFPB-132, UFPB-134, UFPB-137, UFPB-340, UFPB-348, UFPB-355, UFPB-356, UFPB-443, UFPB-449, UFPB RED, UFPB ORANGE, and the ornamental pepper commercial cultivar Calypso (*C. annuum* L.), as an ornamental pepper ideotype.

The seeds were germinated in 180-cell polystyrene trays filled with the commercial substrate Plantmax®. When showing six true leaves, the plants were transplanted to 900 ml pots containing the same substrate, after which the morpho-agronomic characterization was performed (Pessoa et al., 2018), and young leaves were collected, identified, and stored in a freezer for later DNA extraction.

DNA quantification and extraction

For DNA extraction, 200 mg of young leaf tissue collected from each individual was used according to the protocol described by Doyle & Doyle (1990). The quality and quantity of DNA were analyzed in 0.8% agarose gel by applying aliquots from each DNA sample to the gel wells. The concentration of the samples was estimated by visually comparing the fluorescence intensity of the DNA bands against bands of known pattern. The running was performed in TAE 1X buffer (Tris-acetate 0.04 M and EDTA 1 mM) at 80 V, and the gel stained with ethidium bromide was photographed under UV light in a Gel Logic 112® imaging system.

Purification of the DNA samples

The *C. annuum* genomic DNA samples were incubated in a water bath at 37 °C for 12 minutes with a DNA concentration ratio of 1:0.5 RNAse (40 ng/mL) (v:v). After this process, NaCl was added at a ratio of 1:10, followed by 2/3 of the volume with cold isopropanol, after which the samples were kept at -20 °C for 2 hours. Subsequently, isopropanol was carefully discarded (and only the pellet was left in the microtube), and DNA washing was started in three stages: the first and second stages were the same and occurred by adding 1000 µL of 70% alcohol, pellet detachment from the bottom of the microtube, and then centrifugation for five minutes at 13,400 rpm. After double washing with 70% alcohol, the same procedure was repeated with 95% alcohol and centrifugation at 13,400 rpm. Next, the supernatant was carefully discarded, and the microtubes were kept at ambient temperature until total ethanol evaporation, after which the precipitate was resuspended in 40 µL of TE buffer.

RAPD reaction

The oligonucleotides for the RAPD reactions were selected based on the information available in the literature for *C. annuum* L. by synthesizing 15 RAPD (5'→ 3'): MEP–01 (AGACGGCTCC); MEP–02 (GTTACGGACC); MEP–03 (GGGCGACTAC); MEP–04 (GTGCGCAATG); MEP–05 (TCGCATCCAG); MEP–06 (CAGAAGCGGA); MEP–07 (CACAGCGACA); MEP–08 (CAAAGCGCTC); MEP–09 (TCCCCATCAC); MEP–10 (TGCGGGTCCT); MEP–11 (CAGGATTCCC); MEP–12 (GTGGAGTCAG); MEP–13 (AAGTCCGCTC); MEP–14 (CAGCACTGAC), and MEP–15 (GACAGGAGGT).

The amplification reactions were performed in a 25 µL solution containing 23 µL of Master Mix (1X buffer + 3 mM $MgCl_2$ + 200 mM dNTP + 1 mM primer + 1 U Taq DNA polymerase) and 2 µL of genomic DNA (50 ng). The reactions were performed in a thermocycler by observing the following working program: initial denaturation at 94°C for 3 min; 40 cycles, each consisting of the following sequence: 15s at 94°C, 30s at 34°C, and 60s at 72°C. After the 40th cycle, a final extension stage was performed at 72°C for 7 min.

The amplification products were separated by electrophoresis in a horizontal vat containing 0.5 X TBE buffer for one hour using 1.5% agarose gel at 80V. After the running, the gels were photographed under UV light in a Gel Logic 112 imaging system. At this stage, 5µL of the DNA molecular marker 100pb DNA Ladder (Invitrogen®) was used for the analysis.

Data systematization and statistical analysis

A binary matrix was constructed (0, 1) based on the gel band reading, in which (0) indicated the absence and (1) indicated the presence of bands. Then, the dissimilarity matrix was estimated based on the dissimilarity coefficient by Sokal (1962). The locus in which the frequency of the most common allele was equal or superior to 0.95 was considered polymorphic according to the criteria proposed by Nei (1979).

Furthermore, a dendrogram was generated based on the genetic distance matrices using the UPGMA hierarchical clustering (Unweighted Pair Group Method with Arithmetic Mean) described by Sokal & Michener (1958). The cophenetic correlation coefficient stablished by Mojena's criteria (1977) was estimated to check the consistency of the dendrogram. Tocher's clustering and principal component analysis were also performed based on the genetic distance matrices. All analyses were performed using the statistical software R Studio, version 3.2.0.

Results

Of the 15 primers used, 11 were effective for amplifying the samples (Table 1), generating 206 bands distributed into 40 loci, all polymorphic. Primers MEP-1 (22%), MEP-2 (38%), MEP-3 (67%), MEP-5 (15%), MEP-6 (20%), and MEP-7 (10%) showed the highest amplification numbers, with clear bands, intense coloration (Fig. 1), and a mean of 18.72% of bands, whereas primers MEP-9, MEP-10, MEP-11, and MEP-12 did not amplify the samples.

The most dissimilar accessions were UFPB-77.3 x UFPB-443 (0.855). In contrast, the most similar accessions were UFPB-348 x UFPB-355 (0.220) (Table 2).

Table 1. RAPD oligonucleotides, nucleotide sequences, total number of amplified bands, number of polymorphic and monomorphic loci, and percentage of polymorphic loci in ornamental pepper accessions (*C. annuum* L.).

Primer	Sequence 5'→ 3'	Total nº of amplified bands	Number of polymorphic loci	Number of monomorphic loci	Polymorfism (%)
MEP - 01	AGACGGCTCC	22	7	0	100%
MEP - 02	GTTACGGACC	38	8	0	100%
MEP - 03	GGGCGACTAC	67	7	0	100%
MEP - 04	GTGCGCAATG	9	3	0	100%
MEP - 05	TCGCATCCAG	15	3	0	100%
MEP - 06	CAGAAGCGGA	20	2	0	100%
MEP - 07	CACAGCGACA	10	3	0	100%
MEP - 08	CAAAGCGCTC	6	2	0	100%
MEP - 09	TCCCCATCAC	0	0	0	0
MEP - 10	TGCGGGTCCT	0	0	0	0
MEP - 11	CAGGATTCCC	0	0	0	0
MEP - 12	GTGGAGTCAG	0	0	0	0
MEP - 13	AAGTCCGCTC	9	1	0	100%
MEP - 14	CAGCACTGAC	3	3	0	100%
MEP - 15	GACAGGAGGT	7	1	0	100%
TOTAL		206	40	0	

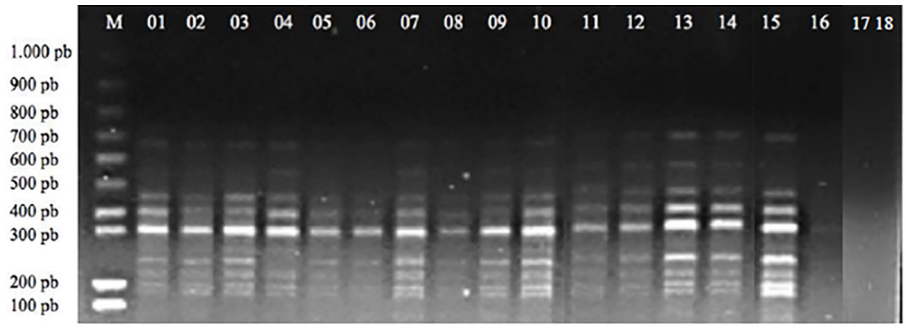

Figure 1. Electrophoretic profile of the RAPD amplification generated with the MEP-02 primer in 18 *Capsicum annuum* L. M accessions using the molecular weight marker Ladder 100 pb.

Table 2. Genetic distance estimates between 18 accessions and the ornamental pepper cultivar Calypso, all belonging to *C. annuum* L.

Accessions	Ac-001	Ac-004	Ac-045	Ac-046	Ac-77.3	Ac-99	Ac-132	Ac-134	Ac-137	Ac-340	Ac-348	Ac-355	Ac-356	Ac-443	Ac-449	Calypso	Ita Ora	Ita Red
Ac-001	0																	
Ac-004	0.624	0																
Ac-045	0.312	0.624	0															
Ac-046	0.468	0.604	0.563	0														
Ac-77.3	0.732	0.732	0.662	0.811	0													
Ac-99	0.413	0.604	0.517	0.315	0.811	0												
Ac-132	0.563	0.468	0.563	0.624	0.643	0.584	0											
Ac-134	0.584	0.624	0.493	0.680	0.541	0.643	0.604	0										
Ac-137	0.563	0.748	0.517	0.662	0.604	0.624	0.698	0.563	0									
Ac-340	0.541	0.541	0.584	0.604	0.698	0.517	0.349	0.624	0.715	0								
Ac-348	0.468	0.604	0.563	0.382	0.811	0.382	0.662	0.680	0.662	0.604	0							
Ac-355	0.468	0.604	0.563	0.382	0.841	0.312	0.662	0.643	0.662	0.604	0.220	0						
Ac-356	0.765	0.584	0.698	0.780	0.732	0.748	0.517	0.732	0.78	0.541	0.780	0.811	0					
Ac-443	0.441	0.584	0.541	0.468	0.855	0.349	0.604	0.698	0.643	0.584	0.413	0.413	0.732	0				
Ac-449	0.563	0.643	0.643	0.382	0.811	0.441	0.698	0.715	0.624	0.680	0.382	0.382	0.811	0.517	0			
Calypso	0.517	0.643	0.563	0.382	0.811	0.382	0.662	0.643	0.584	0.643	0.382	0.312	0.811	0.468	0.382	0		
Ita Ora	0.413	0.517	0.349	0.541	0.715	0.441	0.541	0.517	0.624	0.517	0.541	0.493	0.680	0.517	0.624	0.584	0	
Ita Red	0.563	0.468	0.643	0.382	0.780	0.382	0.584	0.604	0.698	0.517	0.382	0.382	0.715	0.468	0.441	0.441	0.541	0

According to the dendrogram obtained by UPGMA and the cut-off established by Mojena's criterion (1977), the accessions formed six genetic dissimilarity groups (Fig. 2). Group I was formed by accessions UFPB-45, UFPB-001, Ita Orange, and UFPB-134, all showing a trait of commercial interest in common, with yellow or orange ripe fruits (Fig. 3).

Grouped II comprised the largest number of accessions: UFPB-355, UFPB-348, Calypso, UFPB-099, UFPB-046, UFPB-449, Ita Red, and UFPB-443. Group III comprised accessions UFPB-004, UFPB-132, and UFPB-140, whereas the last three groups were formed by a single accession: group IV (UFPB-356), group V (UFPB-137), and group VI (UFPB-77.3). Accessions UFPB137 and UFPB 77.3 were strikingly different with regard to flower color, imature fruit color, mature fruit color, and leaf color (Fig.3). In spite of theses differences, Pessoa et al. (2018) stressed that these accessions have similar morphological traits, e.g., leaf length, chlorophyll a, number of petals, number of stamens, anther length, and dry matter content.

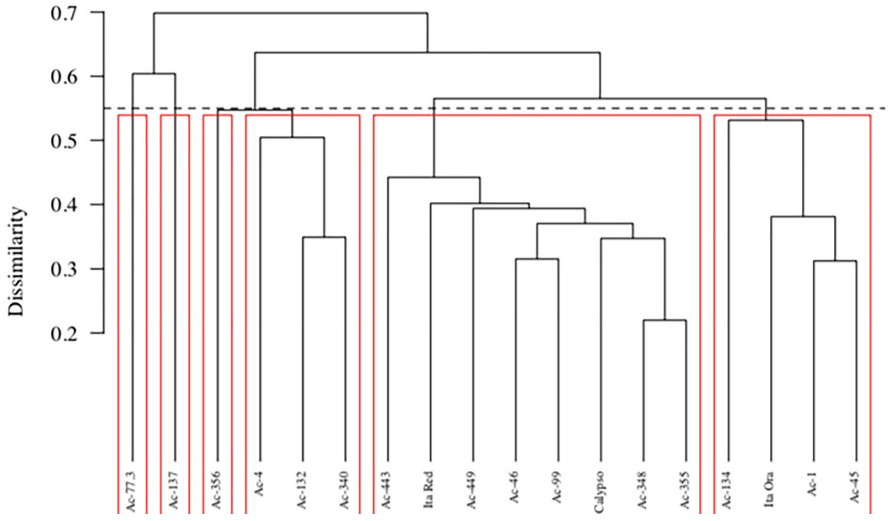

Figure 2. Dendrogram constructed by UPGMA based on the dissimilarity matrix of 18 *C. annuum* L. accessions using 15 RAPD markers.

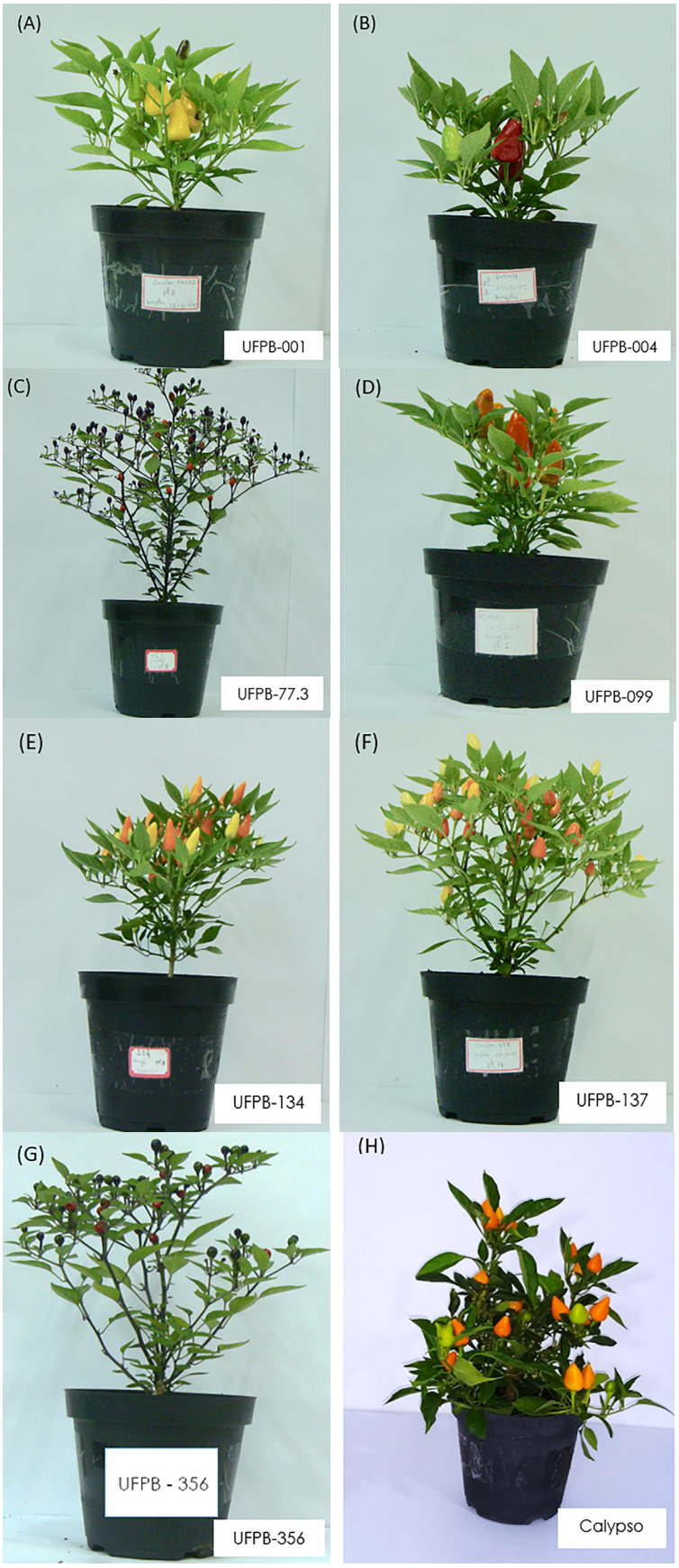

Figure 3. *Capsicum annuum* L. accessions selected in this study. (a) UFPB-01, (b) UFPB-004, (c) UFPB-77.3, (d) UFPB-099, (e) UFPB-134, (f) UFPB-137, (g) UFPB-356, and (h) Calypso.The cophenetic correlation coefficient (CCC) was significant and of high magnitude (Table 3). Furthermore, both distortion (1.57%) and stress (12.53%) showed very low estimates, which is consistent with the dendrogram obtained (Table 3).

Table 3. Cophenetic Correlation Coefficient between the distance matrices and the UPGMA hierarchical clustering generated based on the genetic divergence analysis between the 18 *Capsicum annuum* L. accessions through RAPD markers.

Cophenetic Correlation (CCC)	Degrees of freedom	T-test	Distortion (%)	Stress (%)
0.83**	151	18.48**	1.57	12.53

Tocher's optimization method allowed forming five distinct groups (Table 4). Group I included the largest number of accessions (11), representing 61.12% of the evaluated accessions and fusing the accessions of groups I and II in the dendrogram (underlined in Table 4): UFPB-001, UFPB-45, UFPB-46, UFPB-099, UFPB- 355, UFPB-348, UFPB-443, UFPB-449, UFPB-ITA Orange, UFPB-ITA Red, and Calypso. On the other hand, Group II was formed by only three accessions, UFPB-004, UFPB-132, and UFPB-340, representing 16.67% of the total. Group III was formed by two accessions, UFPB-77.3 and UFPB-134, representing 11.11% of the diversity. Therefore, Tocher's optimization method was once more ineffective in separating accessions UFPB-77.3 and UFPB-134, which are very different phenotypically (Fig. 3). Groups IV and V, in turn, were represented by only one accession each, UFPB-137 (5.55%) and UFPB-356 (5.55%)(Table 4).

Table 4. Representation of the clustering using Tocher's Optimization Method based on the dissimilarity between 18 *Capsicum annuum* L accessions.

	Accessions
I	UFPB-001, UFPB-45, UFPB-46, UFPB-099, UFPB-355, UFPB-348, UFPB-443, UFPB-449, Ita Orange, Ita Red, and Calypso
II	UFPB-004, UFPB-132, and UFPB-340
III	UFPB-77.3 and UFPB-134
IV	UFPB-137
V	UFPB-356

The first two principal components explained 57.8% of the existing genetic diversity between the 18 *C. annuum* L. accessions (Fig 4). There is a correspondence between the dendrogram obtained in the cluster analysis (UPGMA) and the map of principal components, forming six groups (Fig 4). The accessions that contributed most to the first two principal components were, in descending order of importance, UFPB-77.3, UFPB-356, UFPB-137, UFPB-132, UFPB-134, and UFPB-356 (Fig 4).

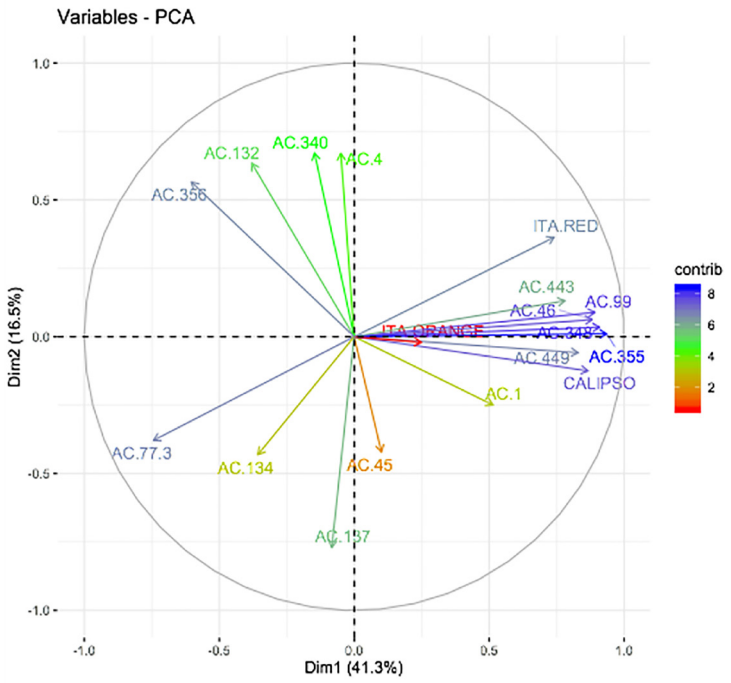

Figure 4. Contribution of the accessions for the first two principal components, which explained 57.8% of the whole genetic divergence among the 18 C. *annuum* L. accessions evaluated through 15 RAPD molecular markers. The shorter the length of the arrow and the closer to red, the lower the contribution. The larger the arrow and the closer to blue, the greater the contribution.

Discussion

The difference observed in the number of bands could be due to the competition between PCR products. This factor can generate distinct and unstable bands since each primer has a specific site of homologous recognition in the genome in which more bands shall be produced (Larekeng et al., 2019). The number of bands can also vary according to factors such as primer complementarity and secondary structures in the DNA template strand (Binneck et al., 2002).

The genetic diversity assessed in this research was superior to the diversity observed by Devi et al. (2018) using nine RAPD primers in C. annuum. These authors showed that it was possible to amplify 76 bands. These markers have been effectively used to evaluate genetic variability in different studies with *Capsicum* species (Gaudel, 2000; Costa et al., 2006; Renganathan et al., 2017; Devi et al., 2018).

The highest genetic dissimilarity was verified between accessions UFPB-443 and UFPB-77.3 (0.855) (Table 2). Dissimilar genotypes should be used to obtain gains in ornamental pepper breeding programs (Pessoa et al., 2019).

The RAPD markers were sensitive in separating accession UFPB-77.3 from UFPB-137 (Fig. 2), which are phenotypically different (Fig. 3c and 3f, respectively). These accessions were grouped into a same cluster in a study performed by Pessoa et al. (2018) based on qualitative and quantitative morphological traits. Fruit color was the main difference between accessions UFPB-001 (yellow) and UFPB-004 (red) (Fig. 3a and 3b, respectively) as well as accessions with different architectures, e.g., UFPB-134 and UFPB-137 (Fig. 3e and 3f, respectively). Accessions UFPB-001 (yellow) and UFPB-004 (red) were grouped in toa same cluster by Pessoa et al., 2018. These authors observed no separation between accessions UFPB-134 and UFPB-137 based on morphological data.

The variability observed by the clustering in genotype differentiation is essential for selection (Pessoa et al., 2019), which should be preferably applied to individuals belonging to distinct groups, which are more genetically dissimilar (Correa & Gonçalves, 2012). Rêgo et al. (2011) analyzed the genetic divergence of 29 *Capsicum* spp. accessions and reported the formation of eight different groups, demonstrating variability between the evaluated individuals. In another study, Costa et al. (2016) used UPGMA clustering analysis and separated *Capsicum* spp. individuals based on genetic diversity using RAPD into two distinct groups. Therefore, the number of groups formed through clustering is related to the variability of the analyzed individuals. The UPGMA method is also used in other studies, effectively categorizing *Capsicum* genotypes and determining genetic dissimilarity through other traits (Vasconcelos *et al.,* 2012; Carvalho *et al*., 2017).

The stress and distortion values should be the lowest possible in order to determine an adequate graphic projection, whereas cophenetic correlation coefficients should not be under 0.7 since vaues opposed to these would indicate inadequation of the clustering method (Rohlf, 1970). These parameters do not apply to this study since the coffenetic correlation value was above 80% and showed low stress and distortion values, confirming the consistency of the clustering pattern (Cargnelutti Filho et al., 2010; Cruz et al., 2014). Therefore, the statistical models used here were effectively detected dissimilarity between *Capsicum* accessions.

Optimization methods maintain the principle of group establishment in order to provide homogeneity within groups and heterogeneity between groups. Tocher's clustering (Table 4) showed a lower power in discriminating divergences between genotypes compared to UPGMA. This technique grouped the accessions of groups I and II in the cluster analysis, forming five groups instead of six. Another difference referred to accessions UFPB-77.3 and UFPB-134, which were clustered into the same group using Tocher's optimization (Table 4), even though they are phenotypically different (Fig. 3). Pessoa et al. (2018), working with the quantitative characterization of the same pepper accessions (*C. annuum* L.), obtained the same number of groups through Tocher's optimization analysis. However, the accessions were clustered in different manners, and accessions UFPB-001 and UFPB-099 and UFPB-77.3 and UFPB-134 remained together in the same group. These findings highlight the importance of molecular characterization for identifying identification of accessions, improving breeding programs, and as a phenotypical characterization toll, avoiding possible duplicates of parental individuals and increasing the sensitivity in the detection of genetic divergence between accessions.

The most divergent accessions through both cluster analysis (UPGMA) and through Tocher's method were UFPB-77.3, UFPB-137, and UFPB-356, which should be recommended as parental individuals to continue the genetic breeding program of this species.

Through Tocher's optimization method for clustering genotypes, the distances within groups are smaller than distances between groups (Cruz & Regazzi 1997; Vasconcelos et al., 2007). In this scenario, the

intergroup distance can be useful for parental choice since the cross between more divergent parents can increase the success in the selection of superior genotypes in segregating populations (Zuin et al., 2009). Faria et al. (2012) and Vasconcelos et al. (2014) studied the genetic diversity of pepper using Tocher's method and reported the formation groups with only one accession, as observed in the present research.

Principal component analysis (PCA) corroborated the results obtained by UPGMA and Tocher's analysis. However, high genetic variability was observed within groups (Fig. 4), corroborating the results found by Adeyemo &Lawal (2017) using PCA in the diversity analysis of *Capsicum* accessions. These authors highlighted the importance of genetic diversity in *Capsicum* for use in breeding programs.

Based on the analyses used in the present study, it was possible to assess the genetic divergence between the studied pepper accessions. The combination of different approaches in genetic diversity analysis (UPGMA, Tocher's method, and Principal Components Analysis) increases the sensitivity in the detection of divergence between accessions. These results allowed identifying accessions UFPB-77.3, UFPB-137, and UFPB-356 as the most genetically divergent. These accessions can be used as parental individuals in breeding programs of ornamental pepper.

The use of the RAPD technique was efficient to access the genetic divergence among the ornamental pepper accessions, based on the primers used. Bobadilla-Larios et al. (2017) and Devi et al. (2018) also reported the efficiency of RAPD markers in genetic variability studies.

Conclusions

The accessions characterized in this study should be used in breeding programs due to their genetic variability.

Accessions UFPB-77.3, UFPB-137, and UFPB-356 should be used as parental individuals in breeding programs of ornamental pepper.

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