ISSN 1807-1929
Revista Brasileira de Engenharia Agrícola e Ambiental
Brazilian Journal of Agricultural and Environmental Engineering
v.29, n.2, e278261, 2025
Campina Grande, PB – http://www.agriambi.com.br – http://www.scielo.br/rbeaa

DOI: http://dx.doi.org/10.1590/1807-1929/agriambi.v29n2e278261 ORIGINAL ARTICLE

Diversity among *Capsicum annuum* L. genotypes based on phenotypic and molecular markers and parental selection[1]

Diversidade entre genótipos de *Capsicum annuum* L. com base em marcadores fenotípicos e moleculares e seleção parental

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HIGHLIGHTS:

Pepper genotypes were differentiated by phenotypic and genotypic traits.
Divergent pepper genotypes were indicated as parents for breeding.
Genotypes can be selected by means of qualitative traits.

ABSTRACT: Ornamental pepper plants have genetic variability, which can be accessed through morphological and molecular traits. Genotype selection to form the base population for breeding can be performed through the joint analysis of several types of data, providing greater selection accuracy. From this perspective, this study aimed to evaluate the diversity among pepper accessions based on analysis of phenotypic traits and molecular markers and to select the best ones to use as parents in breeding programs. The study was carried out at the Centro de Ciências Agrárias of the Universidade Federal da Paraíba, Paraíba, Brazil. Sixteen ornamental pepper genotypes were used and characterized for eight quantitative traits, nine qualitative traits, and 18 pairs of microsatellite primers. Simultaneous variable analyses were performed using Tocher's clustering method, Ward's clustering algorithm, and the dissimilarity matrix. The clustering methods were efficient in separating the genotypes, identifying genetic variability, and accuracy in the selection through the joint analysis of quantitative, qualitative, and molecular data. Different groups were formed among the genotypes by Tocher's method (six groups) and Ward's method (three groups). There is genetic variability among ornamental pepper genotypes considering the joint analysis of quantitative, qualitative, and molecular data. Qualitative traits are important in the identification of genetic divergence among ornamental pepper accessions. The UFPB genotypes 46, 134, 137, 443 and 449, the mini pepper Akamu, and the cultivar Calypso are indicated for selection and can be used to carry out crosses and continue the breeding program.

Key words: multivariate analysis, ornamental peppers, genetic variability

RESUMO: As pimenteiras ornamentais apresentam variabilidade genética que pode ser acessada por meio de caracteres morfológicos e moleculares. A seleção de genótipos para formação de população base para o melhoramento pode ser realizada por meio de análise conjunta de vários tipos de dados, proporcionando maior precisão na seleção. Nessa perspectiva, este estudo teve como objetivo avaliar a diversidade entre acessos de pimenta com base na análise de características fenotípicas e marcadores moleculares e selecionar os melhores para serem utilizados como parentais em programas de melhoramento. O trabalho foi realizado no Centro de Ciências Agrárias da Universidade Federal da Paraíba, Paraíba, Brasil. Foram utilizados 16 genótipos de pimenteiras ornamentais, e caracterizados quanto a oito caracteres quantitativos, nove qualitativos e 18 pares de iniciadores microssatélites. As análises simultâneas das variáveis foram realizadas usando o método de agrupamento de Tocher, agrupamento do algoritmo de Ward e a matriz de dissimilaridades. Os métodos de agrupamentos foram eficientes na separação dos genótipos, identificando variabilidade genética e precisão na seleção por meio da análise conjunta de dados quantitativos, qualitativos e moleculares. Diferentes grupos foram formados entre os genótipos pelo método de Tocher (seis grupos) e pelo método de Ward (três grupos). Existe variabilidade genética entre os genótipos de pimenteiras ornamentais, considerando análise conjunta de dados quantitativos, qualitativos e moleculares. Características qualitativas são importantes na identificação de divergência genética entre acessos de pimenteira ornamental. Os genótipos UFPB 46, 134, 137, 443 e 449, o mini pimentão Akamu e a cultivar Calypso são indicados para seleção, podem ser utilizados para realizar cruzamentos e dar continuidade ao programa de melhoramento genético.

Palavras-chave: análise multivariada, pimenteiras ornamentais, variabilidade genética

• Ref. 278261 – Received 06 Sept, 2023
* Corresponding author - E-mail: angelapessoapb@gmail.com
• Accepted 05 Aug, 2024 • Published 28 Aug, 2024
Editors: Lauriane Almeida dos Anjos Soares & Hans Raj Gheyi

Introduction

The genus *Capsicum* comprises more than 200 species with wide variability in fruit size, color, shape, and chemical composition (Vázquez-Espinosa et al., 2023). Both sweet and pungent, peppers (*C. annuum* L.) are one of the most popular vegetables worldwide and are widely used in human nutrition, with variable contents of carotenoids and/or capsaicin (Wu et al., 2023). In addition to the food potential, peppers are also used for ornamental purposes due to the variability of phenotypic traits, especially those related to fruits (Pessoa et al., 2019a).

The great variability of pepper plants can be used in genetic improvement programs to select genotypes for the formation of base populations (Pessoa et al., 2018). There are various ways of accessing the genetic variability of germplasm banks, including phenotypic and genotypic characterizations (Walle et al., 2019), which provide useful information to planning future breeding strategies aimed at obtaining new cultivars.

Genotype characterization can be performed by using molecular markers in addition to morphological ones to eliminate undesirable aspects in selection, thus improving the process' efficiency. Since molecular markers are not influenced by the environment, it is possible to select individuals earlier (Oliveira et al., 2023).

Polymorphism can be explored through microsatellites or Simple Sequence Repeats (SSRs), considered ideal markers to assess genetic diversity in germplasm (Lira-Ortiz et al., 2022). In this scenario, the evaluation and selection of ornamental peppers have been often performed through morphological (Pessoa et al., 2019a; 2021; Rêgo et al., 2022) and molecular markers (Pereira et al., 2022). However, there are few studies using the joint analysis of these data.

Phenotypic and genotypic data and the joint analysis of variables can provide a better indication of the potentiality of genetic variability in accessions present in germplasm banks (Pessoa et al., 2019b). From this perspective, this study aimed to evaluate the diversity among pepper accessions based on analysis of phenotypic traits and molecular markers and to select the best ones to use as parents in breeding programs.

Material and Methods

The study was conducted in a plant nursery at the Plant Biotechnology of the Centro de Ciências Agrárias (CCA) of the Universidade Federal da Paraíba (UFPB), Areia (6º 57' 48" S, 35º 41' 30" W, and altitude of 618 m), State of Paraíba, Brazil. The plant material consisted of 16 ornamental pepper genotypes, belonging to the Germplasm Bank of the genus Capsicum at UFPB: 134, 137, 390, 77.3, 356, 45, 46, 132, 443, and 449, three mini hybrid peppers ISLA: Kaiki (PA001), Kalani (PL009) and Akamu (PV004), peppers etna (IVER, and ILAN), and Calypso variety.

The experiment was set up in a completely randomized design, with 16 treatments (pepper plant genotypes) and eight replications, with one plant per plot.

The accessions were sown in 128-cell polystyrene traits filled with a commercial substrate (Plantmax®). When the seedlings of all treatments had at least six true leaves, 35 days after sowing, they were transplanted to 0.9 dm^3 plastic pots containing the same substrate. The plants were irrigated on alternate days, receiving a nutrient solution that was prepared based on Mesquita et al. (2016). During the experimental period, the monitoring of pests and diseases was performed, along with phytosanitary measurements with the preventive objective of minimizing possible damage to the plants caused by pests and diseases.

Morpho-agronomical characterization was performed according to the recommendations of the descriptors of the genus *Capsicum*, proposed by IPGRI (1995). Plant and fruit characteristics were evaluated when 50% of the plants had ripe fruits, and flower characteristics were evaluated when 50% of the plants had at least one open flower. Eight quantitative traits (plant height, first bifurcation height, flower size, number of fruits per plant, fruit weight, fruit length, number of seeds per fruit, and fruit dry matter content) and nine qualitative traits (growth habit, type of branching, leaf density, leaf color, leaf shape, corolla color, anther color, unripe fruit color, and ripe fruit color) were analyzed.

Samples with 200 mg of young leaf tissue from each genotype were subjected to DNA extraction following the protocol described by Doyle & Doyle (1990). The DNA volume was analyzed in 0.8% agarose gel, aliquots from each DNA sample were applied to the gel wells, and the concentration of the samples was estimated by visually comparing the fluorescence intensity of the DNA bands with those of a known standard. The run was performed in 1X TAE buffer (0.04 M tris-acetate and 1 mM EDTA) at 80 V, and the ethidium bromide gel was photographed under UV light with a Gel Logic 112® molecular image camera.

For DNA purification, the samples were incubated in a water bath at 37 ºC, with DNA at a proportion of 1:1/2 RNAse (40 ng mL^{-1}; v/v) for 12 min. Subsequently, 1:10 5 M NaCl was added, followed by 2/3 of the cold isopropanol volume, and the samples were kept at -20 ºC for 2 h. After incubation, the samples were centrifuged for 10 min at 14,000 rpm. Then, the supernatant was removed and the microtubes were washed twice with 70% ethanol, once with 95% ethanol, and centrifuged at 14,000 rpm for 2 min per washing. Next, the supernatant was carefully discarded, and the microtubes were kept at ambient temperature for total ethanol evaporation. Then, the precipitate was resuspended in 40 µL of TE buffer.

The microsatellite markers were selected based on information available in the literature for *C. annuum* (Minamiyama et al., 2006; Portis et al., 2007), and 18 pairs of microsatellite primers were used (Table 1).

The amplification reactions were conducted in a final volume of 25 µL containing the following reagents: 2 µL of genomic DNA, 0.2U of Taq DNA polymerase, 2.5 µmol L^{-1} of 10X enzyme buffer (500 mM KCl, 100 mM Tris-HCl), 1.5 µmol L^{-1} of $MgCl_2$ (50 mM), 0.5 µmol L^{-1} of dNTP (deoxyribonucleotides), and 0.4 µM of the forward primer and 0.5 µM of the reverse primer, in addition to 17.4 µL of ultrapure water. Next, 2 µL of DNA was added, followed by 23 µL of the mix described before.

Table 1. Loci of microsatellites (SSR) and primers (forward and reverse)

Loci	SSR	Primer (Forward/Reverse)
EPMS-596	(A)19	CTCGTGCCGTATTTCTGTCA/AAGGGCGTGTTTGGTATGAA
EPMS-642	(AT)8	CAACTTCGCGTTATTGTCCA/AGGGCGGACAAAGAAGATTT
EPMS-643	(CT)17	CCAAGATCAACTCTTACGCTAT/CCCCTCAAGAATTCCCTCCAT
EPMS-649	(TA)12	AAGGGTTCTCGAGGAAATGC/TCAATCCCAAAACCATGTGA
EPMS-650	(TA)19	CATGGGTGAGGGTACATGGT/AGAGGGAAGGGTTATTTGCC
EPMS-654	(AAC)5	TTCCACTCTTCGAAGCACCT/GGTAGGGTTTAACACCGCCT
EPMS-657	(AAG)5	CTGATCGTGGATGTGGATTG/TAGAATTGCTGTGAGTGCGG
EPMS-658	(AAG)5	CCTTGAGTAGGCGCACAAAT/TTCCTCATTGCTTTTCCCAC
EPMS-677	(ATA)8	ATCTGCCCTTATCGATGCAC/CCGAATTGTGGAGGAAACAT
EPMS-680	(ATT)6	TGGAATTCACATGGTGAAAAA/TGAAACTTTGTGGGCTATGG
EPMS-683	(CAA)7	AAATGGATCCCAACAACCAA/GGAGTTGAAAACGGTGGAGA
EPMS-694	(CCA)8	CTAGTACGAGGCAGGGGAGG/CCAGATCCCGCTTTTGACTA
EPMS-703	(GAA)5	AAGATTTGGCGGAGACTTCA/TGCACCAACTTTGTCTCTGC
EPMS-705	(GAA)5	TCAACTAGATCCACCACGCA/TAACCCGTTGCTCACACTCA
EPMS-709	(GAG)6	ACGCCGAGGACTATGATGAC/TTCTTCATCCTCAGCGTGTG
EPMS-712	(GCA)6	CCACAAAGGGTTTAAGCAGC/AAGGCAGGAGCAGAGTTCAA
EPMS-924	(CT)6.(TA)9.(TA)9.(GTA)5	GCCGTCGTCAGAAAAGGTAG/TGCATTTCTGTCAGAGGCTG
EPMS-925	(AT)8	CTCACAAGCAGAAGTGGACC/CCCAGTAAAACTTAACCGCAC

All PCR amplifications were performed in a thermal cycler (model TC-Plus Techne Bibby Scientific Ltd®), and the amplification reactions were conducted as follows: 3 min at 94 ºC for initial denaturation, followed by 35 cycles, each consisting of 94 ºC for 1 min, 52-58 °C for 1 min (depending on the primer used), 72 ºC for 1 min, and a final extension at 72 ºC for 7 min. Later, the temperature was reduced to l0 °C.

The fragments amplified were then separated in 3.5% agarose gel in TAE 1x buffer and stained with ethidium bromide (10 mg mL^{-1}) by using 3 µL of ethidium bromide per 200 mL of TAE buffer. After amplification, each 10 µL of the amplified sample received 2 µL 1x of DNA Loading Blue I 10x, homogenized and applied at 10 µL aliquots into each gel well using 5 µL of the standard molecular weight of 1 Kb Plus DNA Ladder - 1 µg $µL^{-1}$. The run was carried out in an electrophoresis tank at 70 v for approximately 1 hour. The gels were visualized in a Geo-Logic 212 Pro-Carestream® Imaging System.

The images of the gels were captured for later analysis. The 1Kb Plus DNA ladder marker was used as a molecular weight reference to estimate the sizes of the amplification products. In the SSR loci analyzed, the frequency of alleles in each category in all samples was scored as either present (1) or absent (0).

The matrix of distances between genotypes (d) was obtained by adding the values of the Mahalanobis (mah) (quantitative traits), Gower (go) (qualitative traits) and Jaccard (ja) (binary data) distance matrices, observing the importance ratio of the traits in the divergence. Thus, d = mahp + gop + jap, where mahp = mah/max(mah), gop = go/max(go), and jap = ja/max(ja). Tocher's method was used based also on the mixed distance matrix. The cophenetic correlation coefficient was determined to validate the Tocher's clusters (Sokal & Rohlf, 1962). A non-metric multidimensional scaling (nMDS) was used for the graphic representation in the two-dimensional space of the distance matrices. All analyses were performed with R software, version 3.0.3 (R Development Core Team, 2014).

Results and Discussion

The clustering by Tocher's method enabled us to gather the genotypes into six different groups (Table 2). The data analysis was efficient to separate the genotypes, facilitating the indication of the most divergent ones to serve as parents in breeding programs in order to obtain a base population with wide genetic variability. Fortunato et al. (2019) and Carvalho et al. (2021) also reported the existence of genetic variability in ornamental pepper plants using Tocher's method to separate genotypes into groups, indicating individuals to advance generations, as carried out in this study.

Table 2. Clustering of 16 genotypes based on different phenotypic (quantitative and qualitative) and molecular traits of ornamental peppers according to Tocher's method

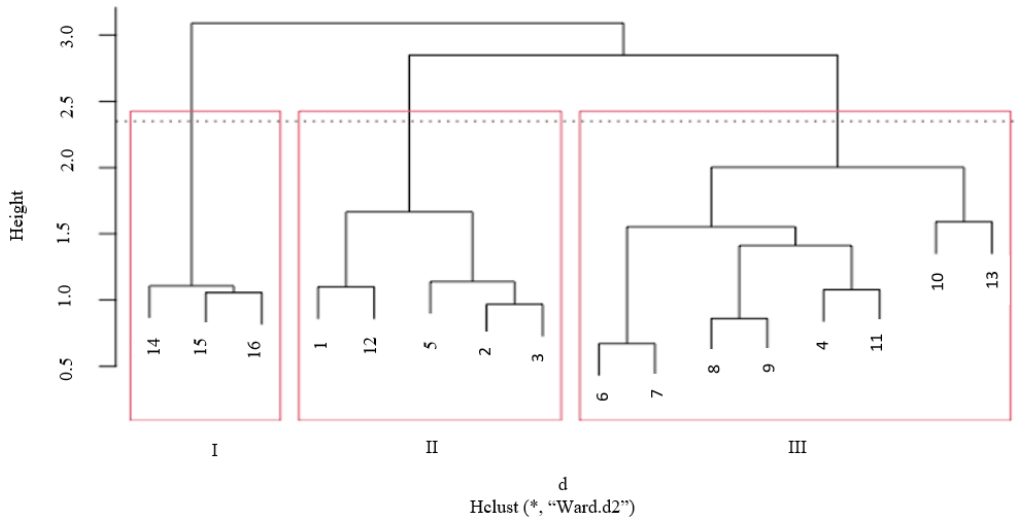
Groups	Genotypes
I	PV004, PL099, 356, 45, 132, 77.3
II	137, 390, PA001
III	IVER, ILAN, Calypso
IV	134, 443
V	46
VI	449

Group I was formed by six genotypes (Table 2), corresponding to 37.5% of the total individuals evaluated. Groups II and III were composed of three genotypes each, forming 18.75% of the total. Group IV was formed by two genotypes (12.5%). Groups V and VI contained one genotype each, corresponding to 6.25% of the genotypes evaluated.

The individuals that remained in the same group possibly showed similar phenotypic and genotypic traits. In an F_3 population of ornamental peppers, Pessoa et al. (2019a) reported genetic variability in 44 individuals through joint data analysis, with the formation of ten different groups by Tocher's method. Similar to the present study, variability was identified as the raw material of genetic improvement.

When beginning a breeding program, it is essential to use parents with wide genetic diversity to identify genotypes with the potential to advance generations. From this perspective, genotypes PV004, 137, 134, 46, and 449 are recommended to be used as parents in hybridization, for belonging to different groups showing diversity. According to Ikegaya et al. (2023), genetic diversity can favor the utilization of phenotypes with favorable traits for agronomic purposes.

The Cophenetic Correlation Coefficient (CCC) was 0.8059, highly significant by the t-test ($p \leq 0.01$), which reveals



Genotypes: 1 (134), 2 (137), 3 (390), 4 (77.3), 5 (PA001), 6 (PV004), 7 (PL009), 8 (356), 9 (45), 10 (46), 11 (132), 12 (443), 13 (449), 14 (Calypso), 15 (IVER), and 16 (ILAN)

Figure 1. Dendrogram obtained by Ward's algorithm based on the mixed distance matrices using phenotypic (quantitative and qualitative) and molecular traits of ornamental pepper genotypes (*Capsicum annuum* L.). The 16 genotypes were grouped into three groups: I, II and III

variability in the consistency of the cluster pattern. When the CCC values are greater than 0.80%, they are considered efficient and correlate the distance matrix and the grouping matrix (Sokal & Rohlf, 1962), as observed in this study.

Ward's clustering method revealed the formation of three different groups based on the genotypes evaluated (Figure 1), not agreeing with Tocher's clustering method, which was more effective in genotype separation. Even with a lower number of groups, Ward's method was efficient to identify genotypes and compose parental groups for hybridization. This statistical procedure is a useful tool to detect genetic divergence and cluster pepper genotypes using morphological and molecular markers simultaneously.

Group I was composed of three genotypes (Calypso, IVER, and ILAN), forming the smallest group (Figure 1). The clustering of genotypes based on quantitative (formed by various genes), qualitative (formed by a few genes), and molecular (DNA level) traits implies more reliability in group formation and subsequently in genotype selection, ensuring contrasting individuals for the formation of a base population for breeding. Luz et al. (2016) reported that one of the fundamental steps in plant improvement is identifying divergent and superior genotypes that allow obtaining segregating populations with higher genetic variability, as observed in this study.

The second group was formed by five genotypes (134, 443, PA001, 137, and 390). In turn, group III was formed by the highest number of genotypes, totaling eight (PV004, PL009, 356, 45, 77.3, 132, 46, and 449) (Figure 1). These two groups possibly revealed high similarity between the genotypes of each group based on their traits. The presence of genetic variability among genotypes from different groups is higher than that observed among genotypes belonging to the same groups, allowing genetic gain in the selection process of individuals from different groups.

Joint data analysis in pepper genotypes is essential in selection studies for using traits influenced by the environment and molecular data, having more accuracy in the selection process since genetic diversity is the main response factor to selection in breeding programs (Yao et al., 2023).

The dissimilarity matrix between ornamental pepper genotypes ranged from 2.2835 to 0.6704 (Table 3), with a ratio

Table 3. Dissimilarity matrix between ornamental pepper accessions (*Capsicum annuum* L.) revealed by quantitative, qualitative, and molecular traits

Accessions	134	137	390	77.3	PA001	PV004	PL099	356	45	46	132	443	449	Calypso	IVER	ILAN
134	0															
137	1.1238	0														
390	1.3804	0.9669	0													
77.3	1.9098	1.7328	1.4583	0												
PA001	1.3835	0.9841	1.2021	1.5742	0											
PV004	1.3815	1.4679	1.3495	1.2765	1.2736	0										
PL099	1.3137	1.5670	1.4625	1.1434	1.5806	0.6704	0									
356	1.5597	1.6622	1.5696	1.2223	1.4143	0.9874	1.1665	0								
45	1.8536	2.0358	1.7874	1.2587	1.9389	1.2863	1.4904	0.8604	0							
46	1.9180	2.0683	1.7556	1.4294	1.5613	1.5332	1.7865	1.4602	1.2298	0						
132	1.8764	1.7661	1.7556	1.0776	1.8312	1.1741	1.2238	1.3300	1.0182	1.5702	0					
443	1.0984	1.3760	1.5303	1.7766	1.2881	1.4490	1.4624	1.3126	1.3869	1.6047	1.5504	0				
449	1.8403	2.0805	1.8598	1.9570	1.9155	1.7074	1.6731	1.5680	1.4851	1.5900	1.5725	1.2909	0			
Calypso	1.6191	1.9879	1.7502	2.1145	2.0709	1.8252	1.6145	1.9344	1.9268	2.1020	1.7509	1.4040	1.8245	0		
IVER	1.7715	1.8094	1.7105	1.9341	2.0951	1.6396	1.3961	1.5649	1.5393	1.6182	1.3250	1.3880	1.7166	1.0873	0	
ILAN	2.0370	1.9491	2.0142	2.2835	2.1064	2.1503	1.7625	2.0299	2.0809	2.2145	1.8425	1.3679	2.0866	1.1006	1.0555	0

between the highest and lowest distances corresponding to 3.4061, confirming variability among the genotypes evaluated. Cruz et al. (2012) used a dissimilarity matrix to identify the most similar pair of genotypes, which will form the initial group, followed by the inclusion of new genotypes into the group.

The lowest distance was observed between genotypes PL099 and PV004 (0.6704), showing higher similarity for the traits evaluated. Conversely, genotypes ILAR and 77.3 (2.2835), and ILAR and 46 (2.2145) were considered the most divergent (Table 3) as they showed the highest genetic distances. Through distance analysis, it is possible to indicate genotypes for hybridization, aiming to maximize genic combinations and generate hybrids with higher heterosis. Qualitative and molecular traits have higher discriminating potential with regard to genetic diversity between genotypes than quantitative variables (Figure 2).

The genetic diversity of ornamental peppers has been studied using mainly quantitative variables (Costa et al., 2020; Carvalho et al., 2021; Mesquita et al., 2021) and molecular markers (Pereira et al., 2015; Pereira et al., 2022), which enable the use of DNA information in selection, thus allowing high selective efficiency, fast genetic gains with selection, and low costs (Resende et al., 2008). However, qualitative traits are rarely used, despite their importance in the study of genetic diversity, as observed in this study.

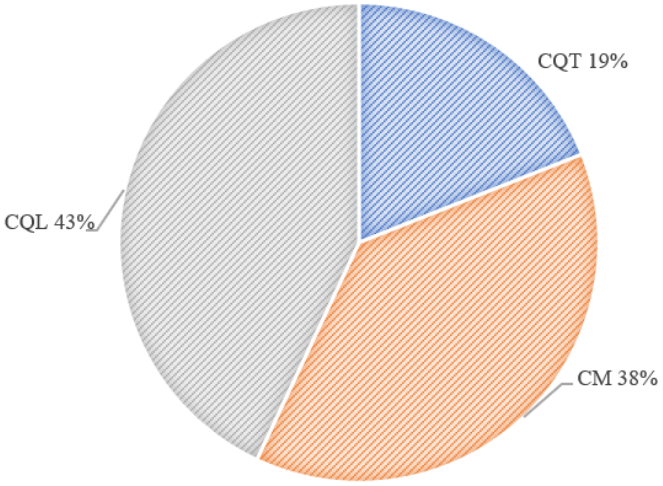


Figure 2. Relative importance of quantitative (CQT), qualitative (CQL), and molecular (CM) traits in the discrimination of genetic diversity

Conclusions

1. Different groups were formed among the genotypes studied by Tocher's method (six groups) and Ward's method (three groups).

2. There is genetic variability among the ornamental pepper genotypes studied considering the joint analysis of quantitative, qualitative, and molecular data.

3. Qualitative traits are important in the identification of genetic divergence between ornamental pepper accessions.

4. The UFPB genotypes 46, 134, 137, 443 and 449, the mini pepper Akamu, and the Calypso variety are indicated for selection and can be used to carry out crosses and continue the breeding program.

Contribution of authors: Angela M. dos S. Pessoa - Research design, data collection, analysis and interpretation, manuscript preparation, and literature review. Elizanilda R. do Rêgo - Research design, data analysis, and work supervision. Ana Paula G. da Silva - data collection, analysis, and interpretation. Mailson M. do Rêgo - Research design, data analysis, and work supervision.

Supplementary documents: There are no supplementary documents.

Conflict of interest: The authors declare no conflict of interest.

Financing statement: There was no financing.

Acknowledgement: This study was carried out with support from the Coordination for the Improvement of Higher Education Personnel – Brazil (CAPES) through the Postgraduate Program in Agronomy (PPGA).

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