



Genetic diversity of popcorn genotypes using molecular analysis

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ABSTRACT. In this study, we analyzed dominant molecular markers to estimate the genetic divergence of 26 popcorn genotypes and evaluate whether using various dissimilarity coefficients with these dominant markers influences the results of cluster analysis. Fifteen random amplification of polymorphic DNA primers produced 157 amplified fragments, of which 65 were monomorphic and 92 were polymorphic. To calculate the genetic distances among the 26 genotypes, the complements of the Jaccard, Dice, and Rogers and Tanimoto similarity coefficients were used. A matrix of D_{ij} values (dissimilarity matrix) was constructed, from which the genetic distances among genotypes were represented in a more simplified manner as a dendrogram generated using the unweighted pair-group method with arithmetic average.

Clusters determined by molecular analysis generally did not group material from the same parental origin together. The largest genetic distance was between varieties 17 (UNB-2) and 18 (PA-091). In the identification of genotypes with the smallest genetic distance, the 3 coefficients showed no agreement. The 3 dissimilarity coefficients showed no major differences among their grouping patterns because agreement in determining the genotypes with large, medium, and small genetic distances was high. The largest genetic distances were observed for the Rogers and Tanimoto dissimilarity coefficient (0.74), followed by the Jaccard coefficient (0.65) and the Dice coefficient (0.48). The 3 coefficients showed similar estimations for the cophenetic correlation coefficient. Correlations among the matrices generated using the 3 coefficients were positive and had high magnitudes, reflecting strong agreement among the results obtained using the 3 evaluated dissimilarity coefficients.

Key words: Genetic diversity; Random amplification of polymorphic DNA; *Zea mays*

INTRODUCTION

Brazil is one of the major producers of corn worldwide, and according to Bordallo et al. (2005), enormous potential exists for the production of specialty corns such as popcorn. Although popcorn is very popular in Brazil, its production and marketing have been understudied, and there is little official information regarding planting areas, production amounts, and import quantities (Galvão et al., 2000; Pereira and do Amaral Jr., 2001; Ribeiro et al., 2012; Pena et al., 2012; Vittorazzi et al., 2013), particularly for domestic varieties in the improvement phase (Pipolo et al., 2002).

The national popcorn market remains dependent on the importation of both seed and grain because of the small number of breeding programs for this crop in Brazil (Sawazaki, 2001; Rangel et al., 2008; Freitas Jr. et al., 2009; Vittorazzi et al., 2013). Santos et al. (2012) and Silva et al. (2013) described the shortage of registered cultivars with the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento) that combine good agronomic characteristics with high scalability for producers. According to Scapim et al. (2002), the small number of cultivars limits the expansion of popcorn cultivation in Brazil, as packaging companies will not work with local popcorn because of its limited supply in the marketplace, which is attributed to the lack of seed availability. To improve popcorn, several factors related to popcorn quality, including texture and softness, as well as yield and agronomic characteristics, should be taken into account. Farmers are most interested in high yields, whereas consumers are interested in popping expansion, which gives the popcorn a better and softer texture.

According to Hallauer et al. (2010), corn breeding and popcorn improvement have common goals in that both are focused on selecting populations of interest. Populations with high potential for use as progenitors in intrapopulation selection programs should have high means and high genetic variability regarding characteristics of interest. For improvement, molecular markers that provide a reliable measure of genetic diversity are used to determine

the degrees of relatedness among lines and varieties and are also used to protect intellectual property rights, among other uses. Similarly, molecular markers can be linked to alleles of interest, helping to increase the understanding of the biology and structure of numerous characteristics, particularly quantitative characteristics (Borém and Miranda, 2013). Currently, in breeding programs for various crops, molecular markers are used to guide crop improvements (Sakiyama et al., 2014).

Several studies have used molecular markers as tools at various stages of plant breeding programs. Moeller and Schaal (1999) used random amplification of polymorphic DNA (RAPD) marker analysis to identify genetic variation among 15 accessions of corn native to the Americas and reported high levels of polymorphism among the genetic material analyzed. These results revealed a strong correlation between the genetic relatedness and the geographical location of accessions native to the Americas. The same authors concluded that RAPD can be used to increase the understanding of intraspecific genetic differentiation. Lanza et al. (1997) indicated that RAPD is an important tool for determining the extent of variability among corn lines and for grouping various genotypes into distinct heterotic groups. Carvalho et al. (2004) analyzed 81 corn accessions and found that RAPD is a suitable marker analysis method for establishing germplasm collections. For this analysis, DNA amplification is based on the use of a single small primer that anneals to random genomic regions, and the DNA is amplified by polymerase chain reaction (Welsh and McClelland, 1990; Williams et al., 1990). Thus, RAPD markers are frequently used to evaluate genetic diversity among corn genotypes (Thormann et al., 1994; Lanza et al., 1997; Moeller and Schaal, 1999; Carvalho et al., 2004; Vilela et al., 2008; Munhoz et al., 2009; Leal et al., 2010; Souza et al., 2012).

The aim of the present study was to compare the results obtained using 3 dissimilarity coefficients to quantify genetic distance based on binary molecular data from RAPD molecular marker analysis. In addition, genetic diversity among 26 popcorn genotypes was estimated.

MATERIAL AND METHODS

Genotypes analyzed

In the present study, 26 varieties of popcorn were analyzed, including 4 hybrids (Jade, Zélia, IAC-112, and IAC-125), a genotype with an unknown pedigree (Chile), and 21 open-pollinated varieties (BRS-Angela, Composto V1, Composto V2, Iguatemi 1, Iguatemi 2, Composto Barreto, Composto Matheus, P1 sintético, Composto Fracaro, Composto Aelton, PR-023, IAC-112 F2, Argentina, UNB-2, PA-091, Viçosa, CMS-42, SE-013, Laranjeiras do Sul, UEM-M2, and RS-20). The pedigree of each variety is shown in Table 1.

For DNA extraction, leaves were collected in bulk from 78 plants of each of the 26 cultivars at approximately 15 days after germination. Genomic DNA was isolated following the method described by Hoisington et al. (1994). Genomic DNA was quantified using gel electrophoresis on a 0.8% agarose gel. DNA extracted from each sample was compared to DNA standards (phage λ) of known concentrations (50, 100, and 150 ng). After electrophoresis, the gel was stained in 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide and imaged on a High-Performance Ultraviolet Transilluminator (EDAS 290; UVP, LLC, Upland, CA, USA) using the program Kodak 1D 3.5 (Rochester, NY, USA).

Table 1. Genealogy of popcorn maize genotypes evaluated in this study.

Genotypes	Genealogy
Jade	Three-way hybrid obtained from temperate and tropical germplasm
Zélia	Three-way hybrid obtained from temperate and tropical germplasm
IAC-112	Simple hybrid-variety SAM with inbred lines originated from hybrid intervarietal Guarani and Yellow Viçosa
IAC-125	Topcross hybrid from the Agronomic Institute of Campinas (Instituto Agronômico de Campinas - IAC)
Chile	Material unknown genealogy
Composto V1	Recombination crossovers between IAC-112 and Zélia
BRS-Angela	CMS-43
Iguatemi 1	Crosses involving the S5 lines Zélia, CMS-42, CMS-43, UEM-J1, and RS-20, with an American genotype and a material called Catedral
Iguatemi 2	Crosses involving the S5 lines Zélia, IAC-112, CMS-42, CMS-43, UEM-J1, Viçosa, and RS-20 with an American genotype and a material called Catedral
Composto V2	Crossovers between IAC-112, Zélia, and BRS-Angela, with subsequent recombination
Composto Barreto	Crossing of five inbred lines extracted from Zélia, with five inbred lines of the local population UEM-J1
Composto Matheus	Crossing of eight populations of white grains: PR-038, PR-079, RR-046, SC-016, PR-017, BRS-Angela, SC-002, and PR-009
P1 sintético	Crossing of lines drawn from an American hybrid, with the lineages obtained IAC-112
Composto Fracaro	Crossing between hybrid topcrosses BRS-Angela, IAC-112, and Zélia with lines S2 of CMS-43
Composto Aelton	Crossing of five lineages elite S4 of Zélia
PR-023	Obtained from advanced generations of an American hybrid
IAC-112 F2	F2 generation of hybrid IAC-112
Argentina	Genotype originating from Argentina of unknown pedigree
UNB-2	Crossing the population UNB-1 population with popcorn American
PA-091	Advanced generation of American hybrid
Viçosa	Crosses between local populations and American hybrids
CMS-42	Cross between 25 yellow grain materials
SE-013	Advanced generation of an American hybrid
Laranjeiras do Sul	From Taiwan (Republic of China)
UEM-M2	Unknown pedigree
RS-20	Unknown pedigree

Molecular analysis using RAPD markers

To analyze diversity using RAPD molecular markers, 15 primers with a high degree of polymorphism and strong band intensity were selected, including OPA12, OPA13, OPB01, OPB07, OPB08, OPC01, OPC04, OPC06, OPC07, OPC08, OPC09, OPC10, OPC12, OPC13, and OPC16. To amplify the DNA of various bulk samples, the primers OPA12, OPB01, OPB07, OPB08, OPC04, OPC06, and OPC16 were used at a concentration of 0.2 μ M and the primers OPA13, OPC01, OPC07, OPC08, OPC09, OPC10, OPC12, and OPC13 were used at a concentration of 0.27 μ M. These conditions were necessary to improve the clarity of the amplified fragments. The patterns and quality of the DNA amplification for these genotypes, following standardization, are shown in Figure 1, which shows the DNA fragments amplified using primer OPC09.

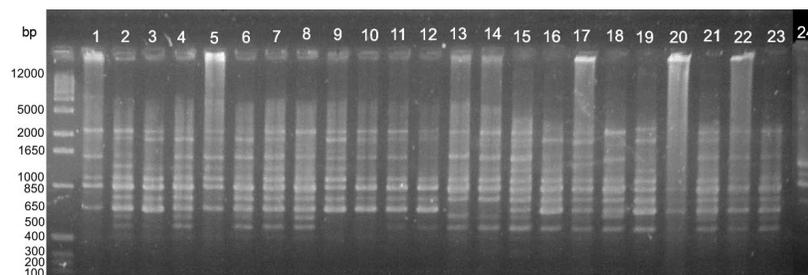


Figure 1. Agarose gel used to separate DNA fragments. Samples 1 to 24 were amplified with the OPC-09 primer to 0.8 μ L and the enzyme Platinum Taq DNA Polymerase. The well before sample 1 corresponds to the 1-kb DNA ladder.

The amplification reactions were performed in a Techne TC-512 thermocycler (Bibby Scientific, Ltd., Staffordshire, UK) and prepared in 0.2-mL microtubes. The reactions were standardized to a final reaction volume of 20 μ L and included 1 U TaqDNA polymerase, 2.0 μ L 10X reaction buffer (Invitrogen, Carlsbad, CA, USA), 0.1 μ M of each dNTP, 2.0 mM $MgCl_2$, 0.2 or 0.27 μ M primer, 20 ng genomic DNA, and autoclaved milli-Q water. The following program was used: 96°C for 5 min, followed by 45 amplification cycles at 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min. A final 7-min extension step was performed at 72°C.

The amplification products were separated by 1.7% agarose gel electrophoresis in 0.5X TBE buffer, pH 8.0 (0.045 M Tris-borate, 0.001 M EDTA). Electrophoresis was conducted for approximately 5 h at 60 V. The gels were stained in 0.5 μ g/mL ethidium bromide and imaged using a High-Performance Ultraviolet Transilluminator (EDAS 290) with the program Kodak 1D 3.5. To determine the lengths of the amplified fragments, 1-kb DNA Ladder (Invitrogen) molecular weight marker was used.

Genetic and statistical analysis

The data generated using the RAPD primer amplification were converted into numerical code, with the presence of a band assigned as 1 and the absence of a band assigned as 0, resulting in a binary matrix. To calculate the genetic distances among the 26 cultivars, the complements of the Jaccard, Rogers and Tanimoto, and Dice similarity coefficients were calculated using the program GENES (Cruz, 2013).

Based on each of the distance measures, a matrix of D_{ij} values (dissimilarity matrix) was constructed and a dendrogram was generated using the unweighted pair-group method with arithmetic average method in the program Mega, version 5 (Kumar et al., 2009), to visually represent the genetic distances among genotypes in a more simplified manner.

RESULTS AND DISCUSSION

RAPD analysis of 26 popcorn cultivar samples using the 15 selected primers yielded a total of 157 amplified fragments. Of these, 65 were monomorphic (41.4%) and 92 were polymorphic (58.6%), generating a mean of 6.13 polymorphic bands per primer. The primer that yielded the highest number of bands and most polymorphisms was OPA-12, which generated 17 bands, 13 of which were polymorphic (76.47%). Moreover, the primer with the fewest polymorphisms was OPC-01, which generated 11 bands, only 3 of which were polymorphic (27.27%) (Table 2).

The level of polymorphism detected in the samples analyzed was lower than the values (74.3, 62, 89.24, and 82.54%) reported by Vilela et al. (2008), Munhoz et al. (2009), Leal et al. (2010), and Souza et al. (2012), respectively. This lower level of polymorphism can be explained by the fact that several of the genetic samples analyzed in this study shared common genotypes in their pedigree, indicating small genetic distances among these samples. This result was confirmed by comparison with a study by Munhoz et al. (2009), who analyzed many of the same samples included in this study and obtained a mean of 6.75 polymorphic bands per primer, a value that was 9.2% higher than that observed in this study.

According to the distance matrices generated from the RAPD markers, for all coefficients evaluated, the greatest genetic distance was observed between UNB-2 and PA-091. The distance between these 2 cultivars was 0.65 using the Jaccard coefficient, 0.74 using

the Rogers and Tanimoto coefficient, and 0.48 using the Dice coefficient, indicating that among the genetic material analyzed, these cultivars were more distinct, likely because they do not share common genotypes in their genetic constitutions. The PA-091 genotype is the result of an advanced generation of an American hybrid. In turn, the genotype UNB-2 is an open-pollinated variety that originated from the selection of an indigenous composite from the Luiz de Queiroz College of Agriculture/University of São Paulo. This indigenous composite was donated to the National University of Brasília, giving rise to UNB-1. This genotype was crossed with an American population of popcorn, and selected progenies were then crossed with a population of popcorn with yellow grains that was resistant to *Exserohilum turcicum*. After 2 cycles of mass selection, a population of resistant plants with high yields and yellow grains was obtained. This population was backcrossed 3 times with the American cultivar, yielding UNB-2.

Table 2. Number of amplicons (NA) and number and frequency of monomorphic (NMF) and polymorphic (NPF) fragments obtained for each primer used to amplify the DNA of different genotypes of popcorn.

Primers	NA	NMF	(%)	NPF	(%)
OPA12	17	4	23.52	13	76.47
OPA13	8	3	37.50	5	62.50
OPB01	14	6	42.85	8	57.14
OPB07	13	4	30.76	9	69.23
OPB08	7	4	57.14	3	42.85
OPC01	11	8	72.72	3	27.27
OPC04	8	5	62.50	3	37.50
OPC06	6	3	50.00	3	50.00
OPC07	12	3	25.00	9	75.00
OPC08	13	6	46.15	7	53.84
OPC09	10	3	30.00	7	70.00
OPC10	13	6	46.15	7	53.84
OPC12	8	4	50.00	4	50.00
OPC13	11	3	27.27	8	72.72
OPC16	6	3	50.00	3	50.00
Total	157	65	41.40	92	58.59

Regarding the smallest genetic distance between 2 genotypes, the results using the 3 coefficients were not in agreement. When using the Dice coefficient, the smallest distance (0.12) was calculated between PR-023 and IAC-125, while using the Jaccard coefficient, the smallest genetic distance (0.21) was observed between IAC-112 and BRS-Angela. Finally, using the Rogers and Tanimoto coefficient, the smallest genetic distance (0.33) was observed between BRS-Ângela and Composto V2. The genotype PR-023 is an open-pollinated population obtained from advanced generations of an American hybrid of unknown pedigree. The genotype IAC-125 is a topcross hybrid from the Agronomic Institute of Campinas. The composite Matheus originated from the crossing of 8 populations with white grains (PR-038, PR-079, RR-046, SC-016, PR-017, Angela, SC-002, and PR-009). Except for BRS-Angela, all cultivars sampled were advanced generations of American hybrids. The composite Fracaro was originated by crossing topcross hybrids between Angela, IAC-112, and Zélia with the S2 lines derived from CMS-43.

For cluster analysis considering the Dice dissimilarity coefficient and a cutoff point of 0.25 (point of abrupt change in group formation), based on the generated dendrogram, 4 major groups were identified. Groups I, II, and III were formed by variety UNB-2, the Argentina

genotype, and variety PA-091, respectively, while group IV was composed of the remaining genetic samples analyzed in this study. Upon subdividing group IV, a group was formed that included the genotypes with the smallest genetic distances together, including C. Fracaro, SE-013, BRS-Ângela, C. V1, C. Matheus, Zélia, UEM-M2, Jade, Iguatemi-2, P1 sintético, PR-023, IAC-125, Iguatemi-1, and Viçosa. Another subdivision of group IV was composed of a group of 8 genotypes with intermediate genetic distances (C. V2, IAC-112, Laranjeiras do Sul, C. Aelton, C. Barreto, Chile, RS-20, and CMS-42) and the IAC-112-F2 population, which was the most divergent cultivar in this group (Figure 2).

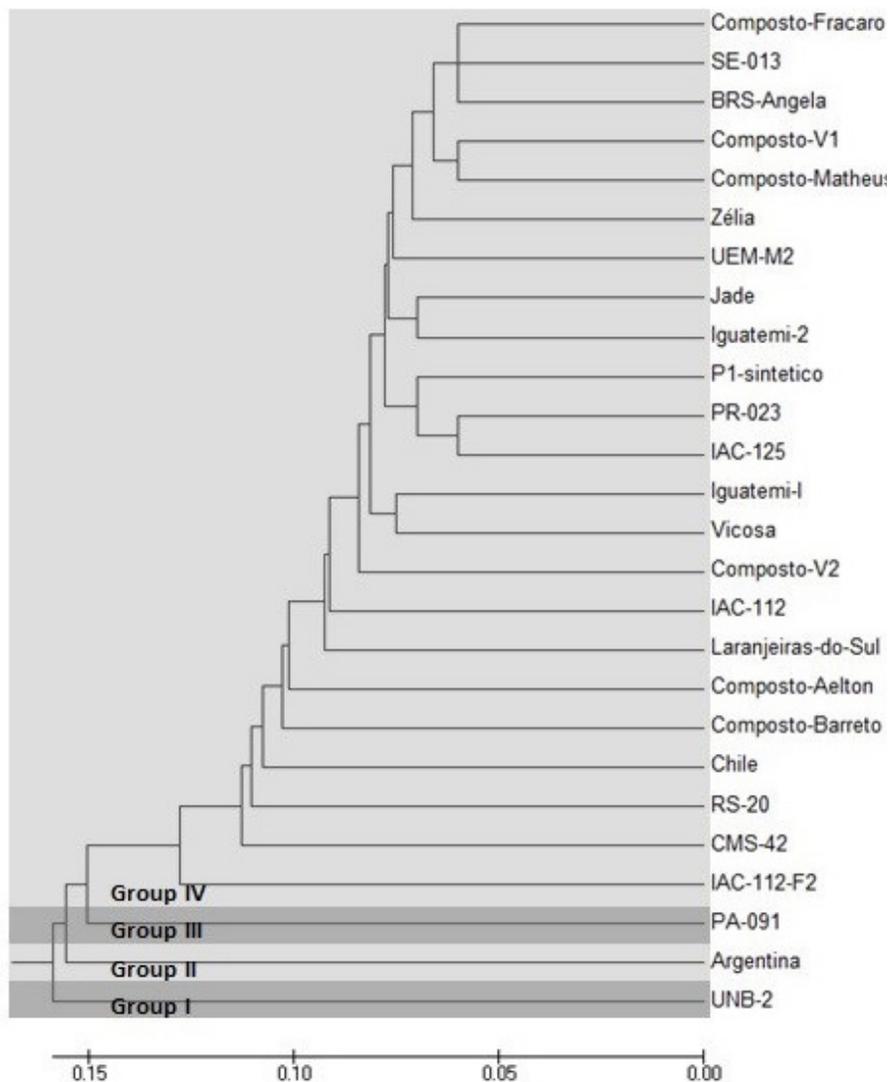


Figure 2. Dendrogram of genetic dissimilarity to 26 popcorn genotypes analyzed, based on the Dice coefficient determined by clustering UPGMA method (cophenetic coefficient of correlation = 0.90).

Upon analyzing the cluster-generated using the matrix obtained using the Jaccard dissimilarity coefficient, the grouping pattern was very similar to that obtained using the Dice coefficient, with the formation of 4 major groups and 4 subgroups within group IV (Figure 3). Differences were observed in the composition of the group with the smallest genetic distances, with the inclusion of genotype IAC-112, which, using this method, grouped more closely to genotype IAC-125. With the inclusion of IAC-112, the genotypes with the smallest genetic distances were separated into 2 groups, of which group IV-D-1 was formed by the genotypes C. Matheus, C. Fracaro, C. V1, Zélia, UEM-M2, SE-013, BRS-Angela, and IAC-112, while group IV-D-2 was formed by the genotypes PR-023, IAC-125, Jade, Iguatemi-2, and P1 sintético. Within group IV, the genotype IAC-112-F2 was also the most dissimilar, indicating that segregation of the F2 generation generated individuals that were genetically distinct from the F1 plants from which they originated. The order of the most divergent cultivars was the same as that described using the Dice coefficient (Figure 3).

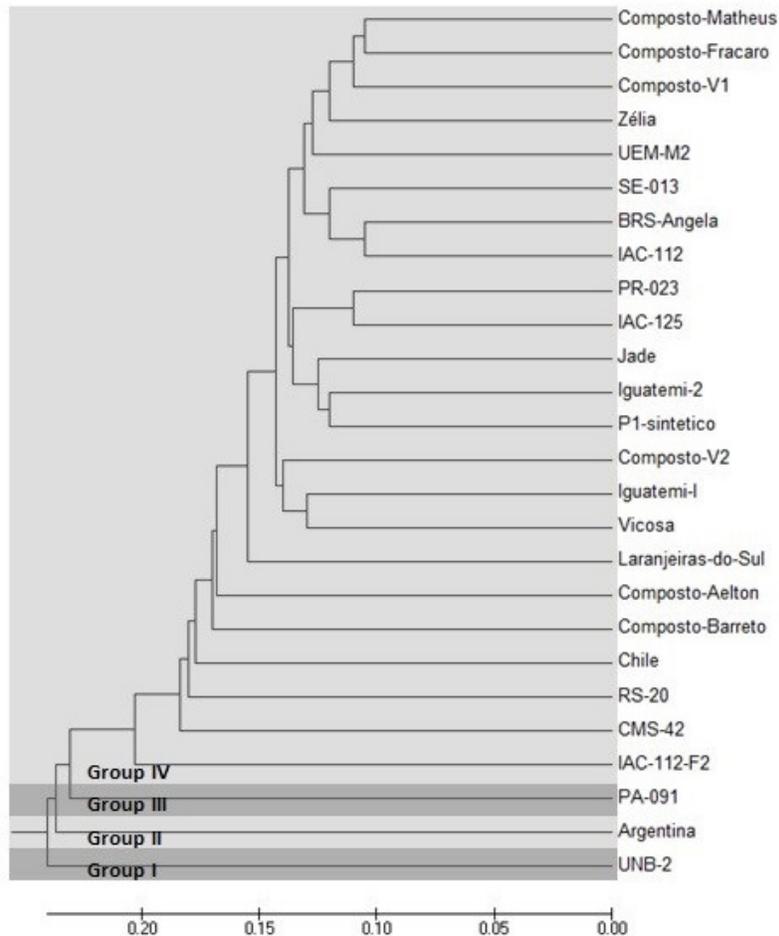


Figure 3. Dendrogram of genetic dissimilarity for the 26 popcorn genotypes analyzed based on Jaccard coefficient, determined by the UPGMA clustering method (coefficient of cophenetic correlation = 0.91).

Using the Rogers and Tanimoto coefficient to estimate genetic distance, there was a slightly different grouping pattern compared to the patterns observed for the Dice and Jaccard coefficients. Using a cutoff point in the abrupt change region of a mean distance of 0.28, 3 main groups were formed, of which group I was formed by genotype UNB-2, group II was formed by the Argentina and PA-091 genotypes, and group III consisted of the other genotypes analyzed in this study, which was equivalent to group IV in the groupings described above. Similar to the results observed using the Jaccard and Dice coefficients, genotype IAC-112-F2 was the most divergent within group III. Compared to the results for the other coefficients, there was also no difference in the formation of subgroups with smaller distances within group III. In this case, the subgroup with the smallest distance included 15 genotypes, with the inclusion of genotypes C. V2, Iguatemi-1, and Viçosa. Within this subgroup, these 15 genotypes were clustered into 3 smaller groups. The first subgroup was formed by the genotypes C. Matheus, C. Fracaro, C. V1, Zélia, SE-013, BRS-Angela, and C. V2; the second subgroup was formed by genotypes Iguatemi 1 and Viçosa; and the third subgroup was formed by the genotypes PR-023, IAC-125, UEM-M2, Jade, Iguatemi 2, and P1 sintético. The remaining genotypes had intermediate genetic distances within group III (Figure 4).

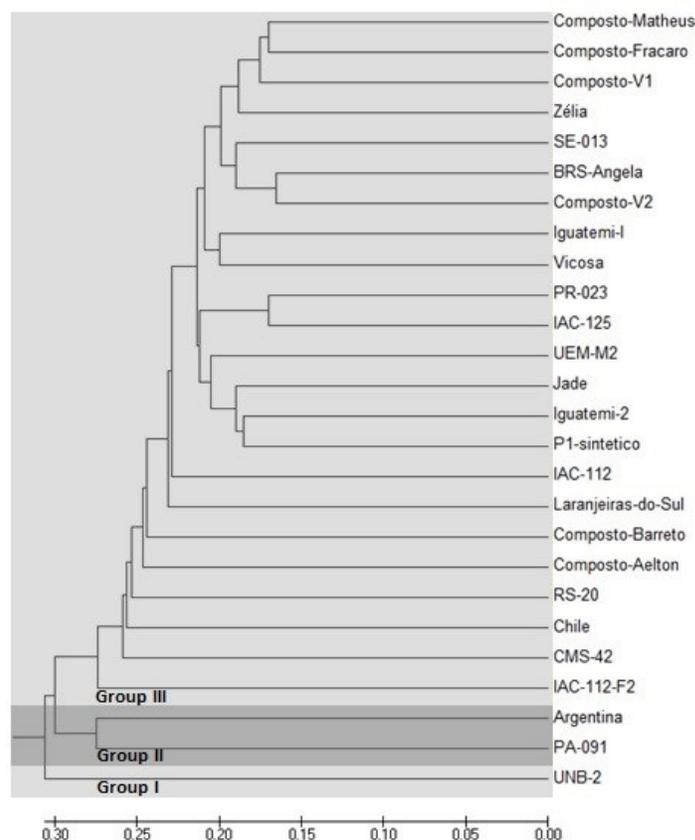


Figure 4. Dendrogram of genetic dissimilarity for the 26 popcorn genotypes analyzed, based on the coefficient of Rogers and Tanimotto determined by UPGMA clustering method (cophenetic correlation coefficient = 0.89).

The comparison of the 3 dissimilarity coefficients analyzed in this study revealed no major differences in their grouping patterns because there was a high level of agreement in the determination of genotypes with large, small, and intermediate genetic distances. The largest genetic distances were observed when the Rogers and Tanimoto dissimilarity coefficient (0.74) was analyzed, followed by the Jaccard coefficient (0.65) and the Dice coefficient (0.48). In regards to the intermediate distances, values of 0.51, 0.36, and 0.22 were observed for the Rogers and Tanimoto, Jaccard, and Dice coefficients, respectively. The 3 coefficients also generated similar estimates for the cophenetic correlation coefficient, a parameter that measures the consistency of the grouping pattern in relation to the genetic distance matrix. The cophenetic correlation coefficient estimates showed high magnitudes, with values of 0.91, 90.0, and 0.89 for the Dice, Jaccard, and Rogers and Tanimoto coefficients, respectively (Figures 2 to 4). Similarly to the observations by da Silva Meyer et al. (2004), correlations between the matrices obtained using the 3 coefficients in the present study also had high magnitudes and were positive and highly significant based on the Mantel test at the 1% probability level (Table 3), demonstrating a strong association between the results.

Table 3. Correlation between genetic distance matrices obtained by the coefficients of Jaccard, Dice, and Rogers and Tanimotto.

Matrices	Jaccard	Dice	Rogers and Tanimotto
Jaccard	-		
Dice	0.9969**	-	
Rogers and Tanimotto	0.9844**	0.9792**	-

**Significant at 1% probability by the Mantel test with 1000 permutations.

Our results showed that the 3 coefficients generated consistent and concordant results in the analysis of genetic distances among popcorn genotypes based on dominant molecular markers, but the Jaccard coefficient may be slightly better in estimating genetic distance values. Leal et al. (2010) compared the efficiency of RAPD and simple sequence repeat markers in studying the genetic diversity of popcorn and found that both markers produced identical genetic divergence values, indicating that RAPD markers were as effective as simple sequence repeat markers for analyzing genetic diversity, while lowering costs and simplifying application of the technique. The authors also noted that RAPD markers are an appropriate strategy for monitoring and guiding improvement programs for popcorn.

The observed genetic distances for popcorn in this study indicate which crossings will allow for the generation segregating populations with a high degree of genetic variability, allowing for the selection of superior genotypes. Furthermore, the results of molecular analyses using RAPD markers will enable definition and allocation of available popcorn germplasms within groups with different heterotic patterns, thus increasing the chances of achieving significant genetic progress in crop improvement programs. In this context, Lanza et al. (1997) reported that RAPD markers are efficient for establishing consistent heterotic groups among corn genotypes. Other studies focusing on corn cultivation have also reported success in defining heterotic groups based on RAPD markers (Parentoni et al., 2001; Bruel et al., 2006; Souza et al., 2012). However, for popcorn, studies in this area remain limited, and additional studies are needed for a better understanding of the pedigree of the germplasm used in breeding programs, as well as knowledge of the genetic and genomic relationships for the consistent establishment of heterotic groups.

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