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Assessment of genetic diversity in maize inbred lines using RAPD markers

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ABSTRACT - *RAPD molecular markers were used to analyze genetic diversity between 16 corn lines. Twenty-two primers were used resulting in the amplification of 265 fragments, of which 237 (84.44%) were polymorphic. Using the UPGMA method the genetic associations obtained showed 5 distinct heterotic groups. A principal coordinates analysis also showed an association of lines in 5 groups, in agreement with the results observed in the dendrogram. A bootstrap procedure was applied to verify whether the amount of markers used was sufficient to ensure reliability of the results, the procedure showed a coefficient of variation of 8.3%, suggesting that the markers were sufficient to assess genetic diversity between the analyzed lines. The high rate of polymorphism between lines revealed by RAPD markers indicated that the method is efficient to analyze genetic diversity in corn lines and that the genetic divergence can be used to establish consistent heterotic groups between corn lines.*

Key words: corn, distance genetic, molecular markers, polymorphism.

INTRODUCTION

The foundation of hybrid development in a corn breeding program consists in obtaining lines and evaluating their combination ability. Choosing suitable lines is essential for the success of the breeding program. In addition, breeding programs demand considerable time and resources, and the pressure for quick development of commercial hybrids encourages the search for alternatives that could be efficient in the short term.

Genetic divergence evaluation is frequently used by corn breeders as an alternative method for germoplasm selection. The aim of this method is to select more promising materials and to decrease expenditures and the time required to implement several hybrid combinations, which are sometimes unnecessary. Efforts are therefore focused on more promising combinations, that is, those between more divergent materials (Fuzzato et al. 2002).

The development of molecular biology techniques allowed genetic divergence to be evaluated by means of molecular markers. The advantage of this procedure is that a large number of markers can be obtained, which allows genetic variability to be sampled in a more efficient way, and consequently to obtain a more precise estimate of genetic divergence (Williams et al. 1990).

Such estimate can be obtained based on a large amount of information on the genetic diversity of the

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genotypes available for the breeder. In general, these data are obtained in the form of a matrix consisting of a certain number of genotypes, which could be either cultivars or lines, genotyped by a few tens or hundreds of markers (Pejic et al. 1998).

Among the markers used today, RAPD (Random Amplified Polymorphic DNA) have been the most utilized, since these are the most technically accessible markers and because of their lower cost. Another essential characteristic of RAPD markers is the fact that they behave as dominant genetic markers, even though dominance in this case does not refer to the classic concept of interaction between alleles of the same locus, but merely to a standpoint of the relative interpretation between the genotype and phenotype of an individual (Ferreira and Grattapaglia 1998).

One aspect that should be taken into account is that, from a genetic breeding standpoint, the greater the capacity of molecular markers in predicting the behavior of crosses, when evaluated in field experiments, the more useful they are. This will occur whenever an association is verified between loci that control molecular traits and controllers of morpho-agronomic traits.

Lanza et al. (1997) verified, from their research results, that RAPD can be used as an alternative to determine genetic divergence between corn lines, separating them into different heterotic groups, and to aid in selecting superior crosses, thus reducing the number of crosses required in field evaluations.

Among the associations between different estimates–general and specific combining ability, productivity, heterosis, and genetic divergence measures– the correlation between different methods for evaluating genetic divergence has been studied by some researchers, Ferreira et al. (1995), stated that heterosis and the combining ability of parents depend directly on the genetic divergence that exists between them, and that the chance of finding promising combinations is better when more divergent materials are used. The same work shows that when compared with diallelic cross results, the techniques for evaluating genetic divergence demonstrate that most crosses between cultivars that would be used as parents might be unnecessary, due to the low divergence that exists between some of these crosses, resulting in not very productive hybrids.

Therefore, the objective of this study was to analyze genetic diversity between 16 corn lines by means of RAPD markers.

MATERIAL AND METHODS

Sixteen corn lines from the genetic breeding program of Instituto Agronômico do Paraná - IAPAR were used, belonging to five heterotic groups (Table 1), according to personal information provided by the researcher Antônio Carlos Gerage:

a) Group 1: L69 - line obtained by self-fertilization from the IPTT36 population introduced by Cimmyt - Center for the Improvement of Maize and Wheat (Mexico);

b) Group 2: L72 - line obtained by self-fertilization from a commercial triple hybrid;

c) Group 3: L89, L90, L91, L92, L93, and L94 – all lines were obtained by self-fertilization from a commercial single hybrid;

d) Group 4: L95, L96, L97, L98, L99, L100, and L101 – all lines were obtained by self-fertilization from a IAPAR's experimental single hybrid;

e) Group 5: L102 - line obtained by self-fertilization within the amarillo cristalino variety from Cimmyt.

In order to extract the DNA, seeds of the 16 lines were placed to germinate in paper towel. After approximately 15 days, each line was represented by a bulk consisting of young leaves emitted by three individuals. The leaves were removed and macerated together after being frozen in liquid nitrogen, and then

Table 1. List of lines used to obtain hybrids and their corresponding codes in this study, with an indication of their segregation generations and their corresponding heterotic groups

Code	Line	Generation	Heterotic Group
1	L69	S12	A
2	L72	S12	B
3	L89	S8	C
4	L90	S8	C
5	L91	S8	C
6	L92	S8	C
7	L93	S8	C
8	L94	S8	C
9	L95	S8	D
10	L96	S8	D
11	L97	S8	D
12	L98	S8	D
13	L99	S8	D
14	L100	S8	D
15	L101	S8	D
16	L102	S8	E

transferred to a 1.5 ml vial at the proportion of 1/3 of the vial's capacity. The extraction method used was based on the protocol described by Ferreira and Grattapaglia (1998), with slight alterations. For each extraction, 700 ml of extraction buffer were used, consisting of 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1%PVP (polyvinylpyrrolidone), in addition to 0.2% b-mercaptoethanol added to the buffer in the beginning of extraction. The samples were incubated at 65 ºC for 60 minutes, with later centrifugation at 6,000 rpm for 10 minutes; the aqueous phase was transferred to another vial and the first extraction was performed with organic solvent by adding 700 ml of chloroform-isoamyl alcohol (24:1) and then agitating the vials (by inversion) for 5 minutes in order to obtain a homogeneous emulsion. These vials were centrifuged at 6,000 rpm for 15 minutes and again the aqueous phase was transferred to another vial, and extraction was again performed with organic solvent. The DNA was precipitated by adding 360 ml of ice-cold isopropanol (-20 ºC) to the aqueous phase from the second extraction. The vials were gently inverted to precipitate the nucleic acids and a new centrifugation followed (12,000 rpm for 10 minutes). The supernatant was discarded and the *pellet* was washed twice with 500 ml of 70% ethanol. The DNA (pellet) was then resuspended in 50 ml TE (10 mM Tris-HCl pH8.0 and 1mM EDTA) containing 10 mg/ml RNAse, and left overnight in the refrigerator for complete DNA dissolution. After DNA extraction, quantification was performed in a fluorometer and the samples were diluted to a final concentration of 10 ng/ml for amplification.

The amplification reactions were conducted in a final volume of 15ml, containing 1× buffer (75 mM Tris-HCl pH 9.0, 50 mM KCl, and 2.0 mM $(NH_4)_2SO_4$), 0.1 mM of each dNTP, 0.5 mM of primer, 0.7 unit of *Taq* DNA polymerase (Biotools), and 20 ng DNA, and ultra-pure water to complete the volume. The amplifications were performed in a model PT-100 thermocycler, programmed to an initial stage of 3 minutes at 94 ºC, 47 1-minute cycles at 94 ºC, 1.45 minutes at 38 ºC, 2 minutes at 72 ºC, and a final stage of 6 minutes at 72 ºC.

After amplification, the total volume was inserted into agarose gel (1.2%), stained with ethidium bromide (0.5 mg mL^{-1}). The amplified fragments were separated by electrophoresis in TAE buffer (0.04M tris-acetate and 0.01M EDTA pH 7.5) at 100 volts for 3 hours. After the run, the amplification products were visualized in ultraviolet light and the gel images were transferred to a microcomputer for analyses.

Twenty-two pre-selected decanucleotide primers acquired from "Operon Technologies" - California, USA, were used in the RAPD reactions.

During gel evaluation, a similarity matrix was constructed where each band was treated as a single character and its presence in an individual was designated as 1 (one) and its absence in another individual was designated by 0 (zero). This matrix was used to produce genetic distances between the pairs of cultivars; these distances were estimated between all possible pairs. The NTSYS-pc software (Numerical Taxonomy and Multivariate Analysis for Personal Computers), version 2.1 (Rohlf 2000) was used to evaluate the genetic associations between samples. Pairwise comparisons were made between genotypes based on Jaccard similarity coefficient. The genetic similarity estimate (GS) between each pair of genotypes was calculated using the expression GS=$a/(n-d)$, where a=number of positive coincidences, n=total number of fragments and d=number of negative coincidences. The genetic distances (GD) between the pairs of lines were estimated by GD=1 - GS.

The simplified representation of distances was done using a dendrogram obtained by the agglomerative hierarchical unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal 1973) and by the scattering of corn lines in a bidimensional graph using the first two principal coordinates. The bootstrap procedure was applied to calculate variance of the genetic similarities obtained from the markers and thus to verify the consistency of the obtained dendrogram. The variance coefficient was obtained from 1,000 bootstrap random draws using the DBOOT software program, version 1.1 (Coelho 2001).

RESULTS AND DISCUSSION

In this study, 22 primers were used, producing a total of 265 amplified fragments - bands (Table 2), with an average of 12.05 bands per primer. Of these, 237 were polymorphic (10.77 bands per primer) and 28 were monomorphic (1.27 bands per primer). The number of polymorphic bands varied from 3 for the OPAR-11 primer up to 18 for the OPAR-05 primer. An example of the electrophoretic pattern of RAPD fragments, amplified from the OPP-14 and OPAV-19 primers is presented in Figure 1.

The level of polymorphism (84.44%) obtained was higher than in some reports found in the literature, such as Melo et al. (2001), who obtained 61.46% of polymorphic bands working with corn hybrids. Lanza et al. (1997) obtained

Table 2. Primers used and their respective base sequences, number of amplified fragments, and number of polymorphic bands for the 16 lines analyzed

Primer	Sequence	Nr. of Fragments	Nr. of polymorphic bands
OPAD-06	AAGTGCACGG	15	15
OPAK-15	ACCTGCCGTT	13	11
OPAM-01	TCACGTACGG	17	17
OPAR-05	CATACCTGCC	18	18
OPAR-10	TGGGGCTGTC	14	11
OPAR-11	GGGAAGACGG	5	3
OPAR-15	ACACTCTGCC	5	4
OPAR-16	CCTTGCGCCT	6	5
OPAT-08	TCCTCGTGGG	16	14
OPAU-12	CCACTCGTCT	15	14
OPAV-13	CTGACTTCCC	13	12
OPAV-19	CTCGATCACC	13	12
OPAW-10	GTTGTTTGCC	14	12
OPAW-11	CTGCCACGAG	19	17
OPAW-19	GGACACAGAG	12	10
OPAX-10	CCAGGCTGAC	9	8
OPP-05	CCCCGGTAAC	11	10
OPP-14	CCAGCCGAAC	8	7
OPW-03	GTCCGGAGTG	12	12
OPW-08	GACTGCCTCT	7	5
OPW-09	GTGACCGAGT	9	7
OPW-13	CACAGCGACA	14	13

80.6% of polymorphism studying genetic divergence between corn lines using RAPD markers. It is important to consider that the level of polymorphism to be obtained depends on the degree of divergence between the genotypes under study. Another aspect to be considered is that, in this study, the primers used were rigorously pre-selected, taking into account the number and quality of the amplification products, and this may have contributed to increase polymorphism.

The 237 polymorphic bands used can be considered more than sufficient for an adequate evaluation of genetic divergence. According to Figure 2, and based on the bootstrap method, it can be observed that starting at approximately 130 bands, the coefficient of variation for the genetic distances (8.3%) between genotypes becomes stabilized, indicating that 130 randomly-sampled bands would produce the same cluster obtained with the 265 bands used in this study.

Based on the 237 polymorphic bands obtained, a genetic distance matrix was constructed using the complement of Jaccard's similarity coefficient, listing all corn line pairs. The mean genetic distance for the 120 distances obtained was 0.507, with an amplitude from 0.303 to 0.640. The smallest genetic distance obtained was observed between lines L89 and L90, while the greatest distance was between lines L97 and L91. Souza Sobrinho et al. (2001), obtained a mean genetic divergence of 0.65 between seven commercial corn hybrids.

Figure 3 presents the cluster for cultivars, according to the UPGMA clustering method, which groups cultivars by means of a dendrogram. The 16 lines were separated into five distinct groups, which were in agreement with the heterotic patterns described by Iapar, based on the genealogy of the lines (Table 1).

Figure 4 shows the projection of genetic distances between the 16 corn lines in a bi-dimensional plane, which also showed an association of the 16 lines in five groups, in agreement with the results observed in the dendrogram.

Lanza et al. (1997) described that RAPD markers are useful to establish consistent heterotic groups between corn lines. This statement agrees with the results obtained in this study, where the same markers were efficient to separate the 16 lines into 5 heterotic groups, confirming the previous separation of those lines according to their genealogy.

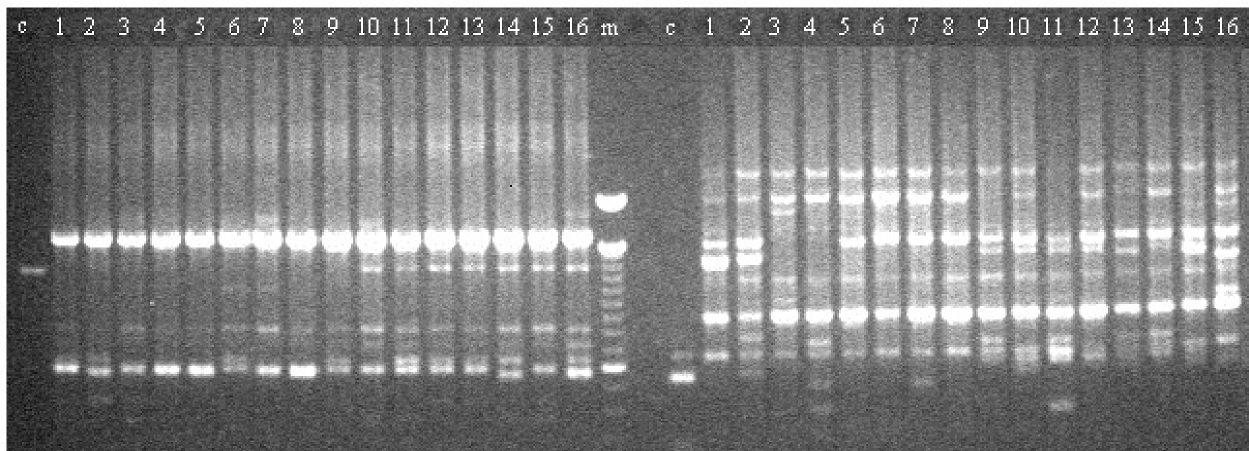

Figure 1. Photo of agarose gel (1.2%) showing amplified DNA fragments in the 16 lines analyzed with primers OPP-14 and OPAV-19, respectively

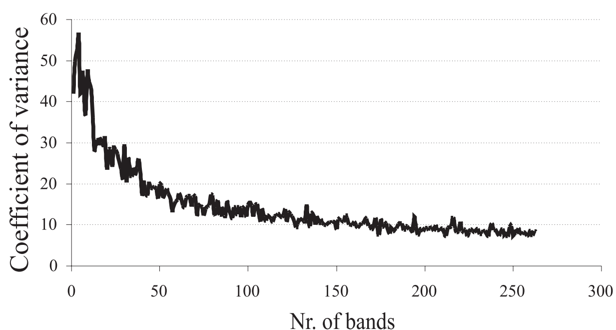

Figure 2. Coefficient of variation for the number of markers, estimated from 1,000 bootstrap random draws. The coefficient of variation for the 265 bands was 8.3%

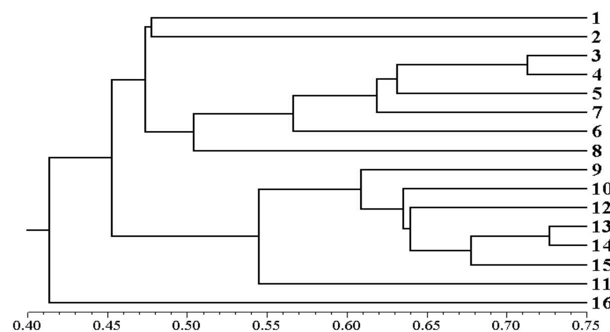

Figure 3. UPGMA clustering for the 16 lines based on Jaccard genetic similarities from RAPD data

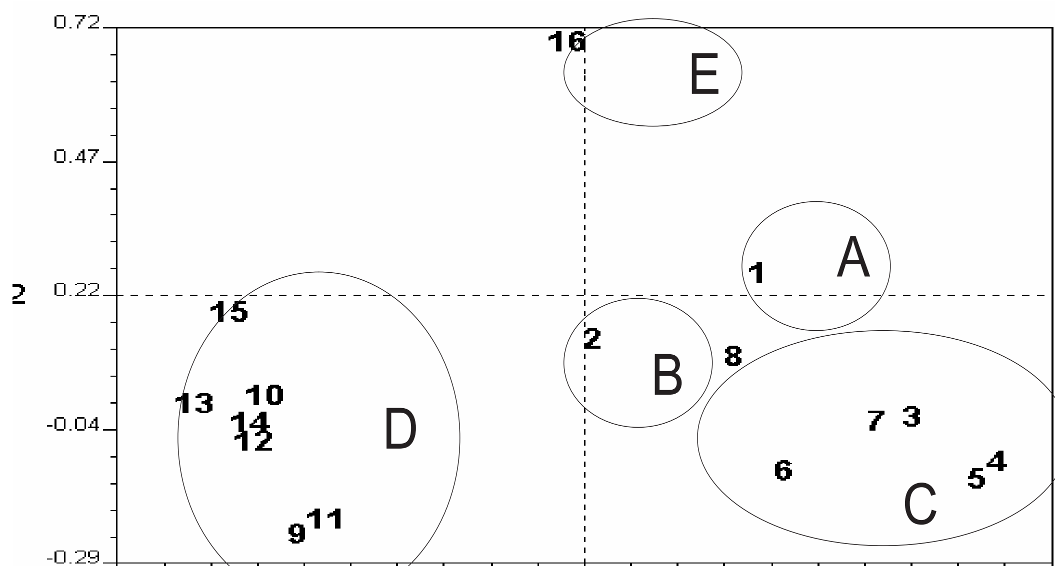

Figure 4. Bidimensional graph showing the distribution of lines according to coordinates 1 and 2. The numbers and letters inserted within circles correspond, respectively, to the lines and their heterotic groups

The high rate of polymorphism between lines revealed by RAPD markers indicated that the method is efficient to analyze genetic diversity in corn lines. This work's results showed that the genetic divergence obtained by RAPD can be used to establish consistent heterotic groups between corn lines.

Análise da diversidade genética em linhagens de milho usando marcadores RAPD

RESUMO - *Marcadores moleculares RAPD foram utilizados para analisar a diversidade genética entre 16 linhagens de milho. Foram utilizados 22 primers que resultaram na amplificação de 265 fragmentos, dos quais 237 (84,44%) foram polimórficos. Usando o método de agrupamento UPGMA, as linhagens foram agrupadas em 5 grupos heteróticos distintos. Análise de coordenadas principais mostrou igualmente uma associação em 5 grupos, concordando com os resultados observados no dendrograma. O procedimento de bootstrap foi aplicado para verificar se a quantidade de marcadores utilizada foi suficiente para garantir a confiabilidade dos resultados, apresentando um coeficiente de variação de 8,3%, sugerindo que os marcadores foram suficientes para acessar a diversidade genética entre as linhagens analisadas. O alto polimorfismo revelado com os marcadores RAPD mostrou que o método é eficiente para a análise de diversidade genética em linhagens de milho e a divergência genética pode ser usada para estabelecer grupos heteróticos consistentes entre linhagens de milho.*

Palavras-chaves: milho, distância genética, marcadores moleculares, polimorfismo.

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