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Selection index and molecular markers in reciprocal recurrent selection in maize

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ABSTRACT - *Reciprocal recurrent selection among full-sib families is one of the outstanding methodologies of maize improvement, since gains are possible in the per se and/or in cross populations. Here the selection index proposed by Smith and Hazel was used in the cited methodology; besides, an additional phase involving RAPD markers was introduced to preserve the genetic variability of the selected genotypes and identify contaminants before their recombination. Multivariate techniques of grouping and discriminant analyses were used for this purpose, to assure the continuance of the improvement program and amplify the genetic distance between the populations Cimmyt and Piranão, which were used for an intervarietal hybrid. The molecular technique proved useful to identify contaminants and helpful in the choice of the genotypes to be recombined to maximize heterosis among populations. The technique can be included in recurrent selection programs, mainly those that target the development of hybrids.*

Key words: *Zea mays*, recurrent selection, selection index, molecular markers, discriminant analysis.

INTRODUCTION

The demand for food is on the rise due to the growth of the human population. The global maize stocks that have been shrinking uninterruptedly over the last 5 years already reflect the increased demand (Dias 2005). The development of improved varieties with high yield potential can be seen as a possibility to increase production. Such varieties with qualitative and/or quantitative superior traits over previously recommended varieties are developed by genetic improvement, which represents one of the most successful modern technologies in agriculture, and accounts for approximately 50 % of the yield increments of most crops (Fehr 1987).

To obtain genetic gains in different traits there are some methodologies of simultaneous selection (Cruz and Carneiro 2003). Of these, the selection index proposed by Smith (1936) and Hazel (1943) has been well-accepted in maize improvement programs. This index associates the information of different traits of agronomic interest, based on economic weights, genotypic and phenotypic variances of each trait and the respective covariances. This methodology alongside with improvement strategies makes gains for

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diverse traits in a same program and selection cycle possible.

Of the improvement procedures with maize, reciprocal recurrent selection among full-sib families presents salient features, (Hallauer and Eberhart 1970) as it allows gains in the populations *per se* and in crosses. It targets a continuous and systematical increase of the mean of two populations, whilst preserving the genetic variability. On the other hand, the methodology is labor-intensive and requires skilled labor and considerable resources.

In the search for more efficient selection methods, the DNA markers were discovered as auxiliary tool. They are a support to the selection procedure, by saving time and optimizing the efficiency (Frish and Melchinger 1999), and warrant the existence of the genetic diversity required for the continuity of the program (Labate et al. 1997 and Popi et al. 2000).

This work aimed to improve the yield as well as several other morpho-agronomic traits of the maize populations Cimmyt and Piranão, from which the intervarietal hybrid of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) was derived. The objective is the development of an intervarietal maize hybrid with superior traits that outmatches hybrids generated in the past by the institution.

In this study, a phase based on RAPD markers was included in the classic procedure of reciprocal recurrent selection among full-sib families, to prioritize the maintenance of variability within and among populations to avoid genetic contaminations, which would result in the loss of the populations' identity. The objective of this procedure was to amplify the genetic distance between the populations for a clearer expression of heterosis in the target intervarietal hybrid and to allow the development of superior lines, which could be used to develop single-cross hybrids in the future.

MATERIAL AND METHODS

Plant Material

Two brachytic populations, belonging to distinct heterotic groups, Cimmyt (flint-type) and Piranão (dent-type) were chosen. Both populations had already been improved at the Universidade Federal de Viçosa and the Universidade Estadual do Norte Fluminense Darcy Ribeiro in Campos dos Goytacazes. Subsequently to the recurrent selection program conducted at the UENF, the ninth reciprocal recurrent selection cycle among full-sib families was performed here.

Experimental and improvement procedures

Each reciprocal recurrent selection cycle consists basically of three phases: 1 - generation of progenies or full-sib families and development of S_1 seed; 2 - evaluation of the full-sib families, identification of the best; and 3 - recombination of S_1 seed, corresponding to parents of the best families, to obtain populations in a more advanced cycle.

The methodology applied was as described by Hallauer and Miranda Filho (1988), with an additional phase using RAPD markers, to maintain the variability of the populations and preserve or even extend the genetic distance between them. Before the recombination phase of the superior parents, a process of molecular analysis of the RAPD type was therefore included. The results were used in multivariate grouping and discriminant analyses. The four stages of the process are described below, in chronological order:

Generation of full-sib families and S_1 seed

In March 2002, the populations Cimmyt and Piranão were sown to establish the full-sib families. Seeds of the two populations were sown every 0.4 m separately and alternately in 6 m long rows spaced 1 m apart, at a depth of 0.05 m.

Thirty days after emergence the plants were thinned to one plant per hill. In this as well as in all other phases the cultural practices were applied whenever necessary, as recommended for the crop (Fancelli and Dourado Neto 2000).

The families of full-sibs as well as S_1 seed, derived from selfing, were obtained by the following procedures: the ears were covered with transparent plastic bags before releasing the stigma. Consecutively, the pollen-releasing tassels were covered with paper bags for approximately 24 hours, to be used for the pollination of the ears. The pollen taken from the paper bag of each plant was used to pollinate a viable ear of one plant of the adjacent row as well as its own ear. Each plant used in a cross was therefore selfed to generate S_1 progenies, which implies that the plants used were prolific.

All S_1 ears were stored in a cold chamber, to warrant that those representing superior families,

identified in the test of progeny evaluation and considered viable for recombination in the RAPD marker analysis, would later be available.

Evaluation and selection of superior full-sib progenies

The 116 full-sib families were evaluated in the growing season 2002/2003, in a complete randomized block design with two replications, in two environments (Campos dos Goytacazes and Itaocara in Rio de Janeiro State). Every experimental unit consisted of one 5 m long row, rows spaced 1 m and planting spots 0.2 m apart. Thirty days after sowing plants were thinned to 25 plants per plot (one plant per planting spot) which would be equal to a population of 50.000 plants ha^{-1}.

After flowering, the following traits were evaluated:

a) Number of Days to Flowering (NDF): number of days from sowing until the exteriorization of the style/stigma on the ears of 50% of the plants of the experimental unit;

b) Plant Height (PH): mean height, in meters, of six competitive plants;

c) Ear Height (EH): mean height, in meters, of the same six competitive plants, measured from the soil level up to the insertion node of the highest ear on the stem;

d) Total Number of Plants (TNP): total number of plants at harvest;

e) Broken Plants (BP): number of plants broken below the highest ear at harvest;

f) Lodged Plants (LP): number of plants with an inclination angle of over 45º to the vertical, at harvest;

g) Husk leaves (PHE): number of partially unhusked ears at harvest;

The followings traits were measured after harvest:

h) Number of Ears (TNE): total number of harvested ears;

i) Number of Diseased Ears (NDE): number of ears with disease symptoms;

k) Ear Weight (EW): weight, in kg ha^{-1}, of the unhusked ears;

l) Grain Weight (GW): weight, in kg ha^{-1}, of the threshed grains; and

m) Weight of 100 grains (WHUN): weight, in kg, of a sample of 100 healthy grains.

The analysis of variance with the data was based on the following model:

$$Y_{ijk} = \mu + G_i + E_j + GE_{ij} + B / E_{jk} + e_{ijk} ;$$

where:

Y_{ijk}=value observed in genotype i, belonging to block k within environment j;

μ = overall mean;

G_i = effect of the i^{-th} genotype, considered random;

E_j = effect of the j^{-th} environment, considered fixed;

GE_{ij} = effect of the interaction between genotype i and environment j, considered random;

B/E_{jk} = effect of the k^{-th} block within the j^{-th} environment, considered random; and

e_{jk} = effect of the experimental error, considered random.

To select the progenies with best morpho-agronomic attributes, the classic selection index proposed by Smith (1936) and Hazel (1943) was used. A total of 43 families, of the 116 evaluated in each population, were selected to compose the set to be used in the genetic divergence study based on RAPD.

For this purpose, economic weights were randomly attributed to each trait with significant differences in the F test, in the analysis of variance, to optimize gains for each trait, emphasizing grain weight.

Evaluation of genetic diversity by RAPD technique

For an evaluation of genetic diversity within and among the populations Cimmyt and Piranão and identification of contaminants, we used plants derived from part of the S_1s seed of the parents that originated the 43 families selected as superior by the

selection index. For this purpose, these seeds were sown in 5 kg pots with soil, to obtain plant material enabling DNA extraction. Leaves of 20 seedlings per genotype were collected 21 days after planting. Then the samples were ground in liquid nitrogen and deep-frozen (-70 ºC) in 15 ml tubes.

For DNA extraction, an extraction kit with the plant DNAzol Reagent (Life Technologies) was used according to the manufacturer's protocol and the DNA of the samples quantified in a spectrophotometer. The samples were prepared as follows: 5 µl of the gross DNA were diluted in 245 µl TE buffer and homogenized by vortex mixing. The device was zeroed at 250 µl TE for a standard sample, and readings of the genotype samples performed at 260 and 280 nm. After quantification, DNA work samples were prepared by dilution to 10 $ng.ul^{-1}$.

The amplification reactions were carried out as described by Williams et al. (1990) with modifications, in a final volume of 25 µl containing the reagents in the

followings concentrations: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.4 mM MgCl2; 100 µM of each deoxyribonucleotide (dATP, dCTP, dGTP and dTTP); 0.3 µM of oligonucleotide primer; 20 ng genomic DNA and 0.5 units of Taq DNA polymerase (Pharmacia Biotech). The thermocycler (Perkin Elmer GeneAmp PCR System 9600) was programmed at 95 ºC for 1 minute followed by 45 cycles of 1 minute at 94 ºC, 1 minute at 36 ºC and 2 minutes at 72 ºC and a final extension step of 7 minutes at 72 ºC, at a transition temperature of 1 ºC s^{-1}.

A total of 60 primers were tested in eight of the 86 genotypes, four of each population to identify the polymorphic primers, in a procedure similar to the above described.

The amplification products were obtained by electrophoresis in 1.2 % agarose gel and 1X TAE buffer, at 70 volts for 3 hours and 5 µl loading buffer (blue juice) added to each sample. At the end of electrophoresis, the gel was stained with ethidium bromide and photo-documented under ultraviolet light for posterior band readings.

The polymorphic RAPD bands were used to construct a binary data matrix, by scoring the presence or absence of a particular band with 1 and 0, respectively. The distance between the pairs of accesses was calculated based on the arithmetic complement of the Jaccard index, expressed by $C_{ij} = 1 - \frac{a}{a+b+c}$, where **a** represents the number of DNA fragments, codified with 1, common to both plants; and **b** and **c** indicate the number of DNA fragments in which the two plants disagree, represented by 1-0 and 0-1, respectively.

This distance matrix was used to group the genotypes by Tocher's optimization method and the hierarchical method of Ward's modified minimum variance (instead of the Euclidean distance, the square of the genotype distance was used, obtained by the arithmetic complement of the Jaccard index) and the discriminant analysis by the technique of the mean distance of a plant to each population.

This phase was thought as a support for taking the decision on which genotypes should be recombined in each population, to conclude another selection cycle. Thus, 25 genotypes of each population were selected for the follow-up recombination phase.

Recombination of the selected progenies with S_1 seed of its parents

The 43 superior parents of each population identified in the field evaluation were planted in separate 6 m long rows spaced 1 m apart, with 15 plants per row, spaced 0.4 m apart. This phase was simultaneous with the end of the phase of establishing the molecular markers.

Of the 43 genotypes planted of each population, 18 were eliminated and the other 25 were used for recombination and formation of the improved populations Cimmyt and Piranão in cycle 9. The selection focused on a maximization of the genetic variability in the study populations, to maintain or amplify the genetic distance amongst them.

Different factors were taken into consideration to choose the genotypes that should be maintained and recombined and those that should be eliminated, cited below in decreasing order of importance:

1º – Elimination of the genotypes identified by discriminant analysis that were considered to be contaminant, that is, belonging to one population, but genetically closer to another;

2º – Elimination of genotypes of a population which, by Tocher's optimization and Ward's hierarchical clustering methods, were grouped together with plants that belong to another population;

3º – Elimination of genotypes with early or late flowering compared to the set, inhibiting their participation in the crosses required for the ninth selection cycle;

4º – Elimination of genotypes with problems of germination, generating few plants to be used for recombination;

5º – Elimination of genetically close genotypes, keeping the ones with highest mean grain yield.

Seventy days after sowing, the recombination process of the selected families began. Analogously as for the establishment of the progenies, the ears were covered with transparent plastic bags before the emission of the stigmas.

When the ears were ready for pollination, the tassels in the initial phase of pollen grain release were covered, when approximately the third part of the anthers had opened. On the following day the pollen grains of all tassels, previously prepared, were collected and blended into a single sample. This sample was used

to pollinate all receptive ears, except the ones whose plants contributed with pollen grains to compose the cited sample, warranting that there would be no selfing. The procedure was repeated for several days, while there were tassels releasing pollen and ears ready for fecundation.

RESULTS AND DISCUSSION

Evaluation of full-sib progenies

Due to heavy rains in the week before harvesting the trial in Itaocara, the number of broken plants and number of lodged plants were added and transformed into a single variable, denominated sum of the number of broken and lodged plants (BLP).

Apart from the traits NDF, PH, EH, and WHUN, the others expressed the existence of significant genotype x environment interaction, which made the partitioning of the genotypes in each environment necessary. In the case of the traits NDF, PH, EH, and WHUN the ANOVA identified significant ($P<0.05$) differences for environment, as well as for genotypes, revealing sufficient genetic variability in full-sib families for the continuity of the breeding program.

Of the other traits with significant genotype x environment interaction, TNP, TNE, EW, GW, and BLP performed similarly in both environments, that is, presented significant ($P<0.01$) differences between genotypes in each environment. PHE and NDE however performed differently in the environments under study. In Campos dos Goytacazes, these traits presented significant differences between genotypes, while no differences were observed in Itaocara.

Of the 116 evaluated families, a total of 43 were selected by the classic index proposed by Smith (1936) and Hazel (1943). After several attempts, the economic weights that obtained best results for the program concerned were: -1, -1, 5, -13, -1, 10, 400, 10 and -13, respectively for the traits PH, EH, TNP, PHE, NDE, EW, GW, WHUN, and BLP.

For the trait grain weight, considered the most important, the estimated gain was 4.68 %. (Table 1). This value is low compared with the 14.58 % gain estimated in cycle 8 of this same improvement program. The difference could be result of the different selection intensities applied in these two cycles. While the selection intensity in the present study was 37.1 %, it was 23.1 % in cycle 8. Souza Jr. and Pinto (2000) obtained a mean direct gain of 7.2% with reciprocal recurrent selection of half-sib families, using a methodology proposed by Souza Jr. (1987). The number of progenies used by the authors was somewhat higher than here - 200 half-sib families of each population. Consequently, they used a higher selection pressure. For the other traits NDF, PH, EH, TNP, PHE, TNE, NDE, EW, WHUN, and BLP the gains were -0,34, 2,05, 2,21, 1,09, 1,05, 2,40, -8,55, 3,92, 0,84 and –0,07%, respectively (Table 1). The negative values for estimated gains are of interest for the improvement of the concerned traits.

RAPD marker analysis

The DNA extraction by the Plant DNAzol kit (Life Technologies) proved efficient, making the extraction of a large DNA quantity of good quality possible. The resulting DNA quantity varied from 114.25 to 238.5 $ng.ml^{-1}$. The purity of the samples was estimated by the ratio between the spectrophotometric readings at 260 and 280 nm. These values varied from 1.616 to 1.758, indicating the high purity degree of the obtained DNA, free of chloroform and protein contaminations. The DNA quality was evaluated by electrophoresis in 0.8% agarose gel. The DNA samples were neither degraded nor problematical in the amplification reactions.

After screening several primers, 14 were chosen for the amplification process of the 86 genotypes (43 Cimmyt parents and 43 Piranão), generating 86 bands, 10 monomorphic and 76 polymorphic, which were converted into a binary data matrix, used to compose the distance matrix based on the Arithmetic Complement of the Jaccard Index. The most dissimilar genotypes were number 14 and 60, with a distance of 0.5439. In turn, genotypes 4 and 24 were the most similar, at a distance of 0.1111. It is noteworthy that the shortest distances (<0.2111) were found coincidently between genotypes belonging to the same group and the longest distances (>0.4439) mostly between genotypes of different groups; this had already been expected since these groups constitute distinct heterotic groups.

The Tocher clustering method formed 36 groups (Table 2). The largest one, group II, contained 16 genotypes, while the 17 other groups contained 1 genotype each. Genotypes 4 and 24, separated by the shortest genetic distance, were included in group I, together with other 6 genotypes. Genotypes 14 and 60 in turn, with the largest genetic distance, represented

Table 1. Estimates of the mean of the original population ($\bar{X}_0$),the selected population ($\bar{X}_S$),heritability (h^2 %), selection gains based on the mean (GS) and selection gains in percentage (GS %) by the statistical analysis of the classical selection index proposed by Smith (1936) and Hazel (1943) for 46 common superior maize full-sib families in the ninth reciprocal recurrent selection cycle

Trait[1/]	$\bar{X}_0$	$\bar{X}_S$	h^2 %	GS	GS %
NDF	65.06	64.66	54.13	-0.22	-0.34
PH	1.97	2.04	59.23	0.04	2.05
EH	1.27	1.32	60.19	0.03	2.21
TNP	22.3	23.26	25.54	0.24	1.09
PHE	2.25	2.30	50.96	0.02	1.05
TNE	31.94	33.49	49.46	0.77	2.40
NDE	5.13	4.02	39.41	-0.44	-8.55
EW	5640	6100	47.34	220	3.92
GW	4640	5080	49.82	220	4.68
WHUN	0.02527	0.02574	45.48	0.00021	0.84
BLP	11.16	11.14	38.88	-0.01	-0.07

[1] NDF = number of days to flowering; PH = plant height (in m); EH = ear height (in m); TNP = total number of plants; NBP = number of broken plants; NLP = number of lodged plants; PHE = number of partially husked ears; TNE = total number of ears; NDE = number of diseased ears; EW = ear weight (in kg ha^{-1}); GW = grain weight (in kg ha^{-1}); WHUN = weight of 100 grains (in kg); BLP = sum of broken and lodged plants.

Table 2. Grouping of 86 maize genotypes based on 76 polymorphic and 10 monomorphic RAPD bands by Tocher's method, using the Arithmetic Complement of the Jaccard Index

Group	Genotype	Group	Genotype
I	4, 24, 27, 28, 29, 3, 58, 37	XIX	31, 36
II	68, 77, 75, 78, 50, 51, 70, 63, 79, 80, 57, 74, 45, 48, 44, 47	XX	38
III	1, 42, 5, 33, 13	XXI	35
IV	65, 67, 60	XXII	6
V	8, 82	XXIII	39
VI	15, 32, 21	XXIV	86
VII	53, 54, 76, 62, 49	XXV	46
VIII	2, 43, 18	XXVI	22
IX	56, 64, 59	XXVII	10
X	69, 83, 23	XXVIII	52
XI	25, 26	XIX	72
XII	61, 71	XXX	11
XIII	17, 34	XXXI	7
XIV	73, 81	XXXII	66
XV	12, 20	XXXIII	14
XVI	30, 40	XXXIV	41
XVII	16, 19	XXXV	9
XVIII	55, 85	XXXVI	84

the groups XXXIII and IV, respectively, demonstrating that genotype 14 is one of the most divergent of the group.

Since the genotypes 1 to 43 belong to population Cimmyt and genotypes 44 to 86 to Piranão, the genotypes 8, 23, 58 and 82 appear as "contaminants" (Table 2), defined here as genotypes belonging to one population, but grouped together with genotypes of another. Several hypotheses could explain this, as for example: the plant originated from selfed seed could have already been derived from the other population through the seed mixture of the two populations; pollen

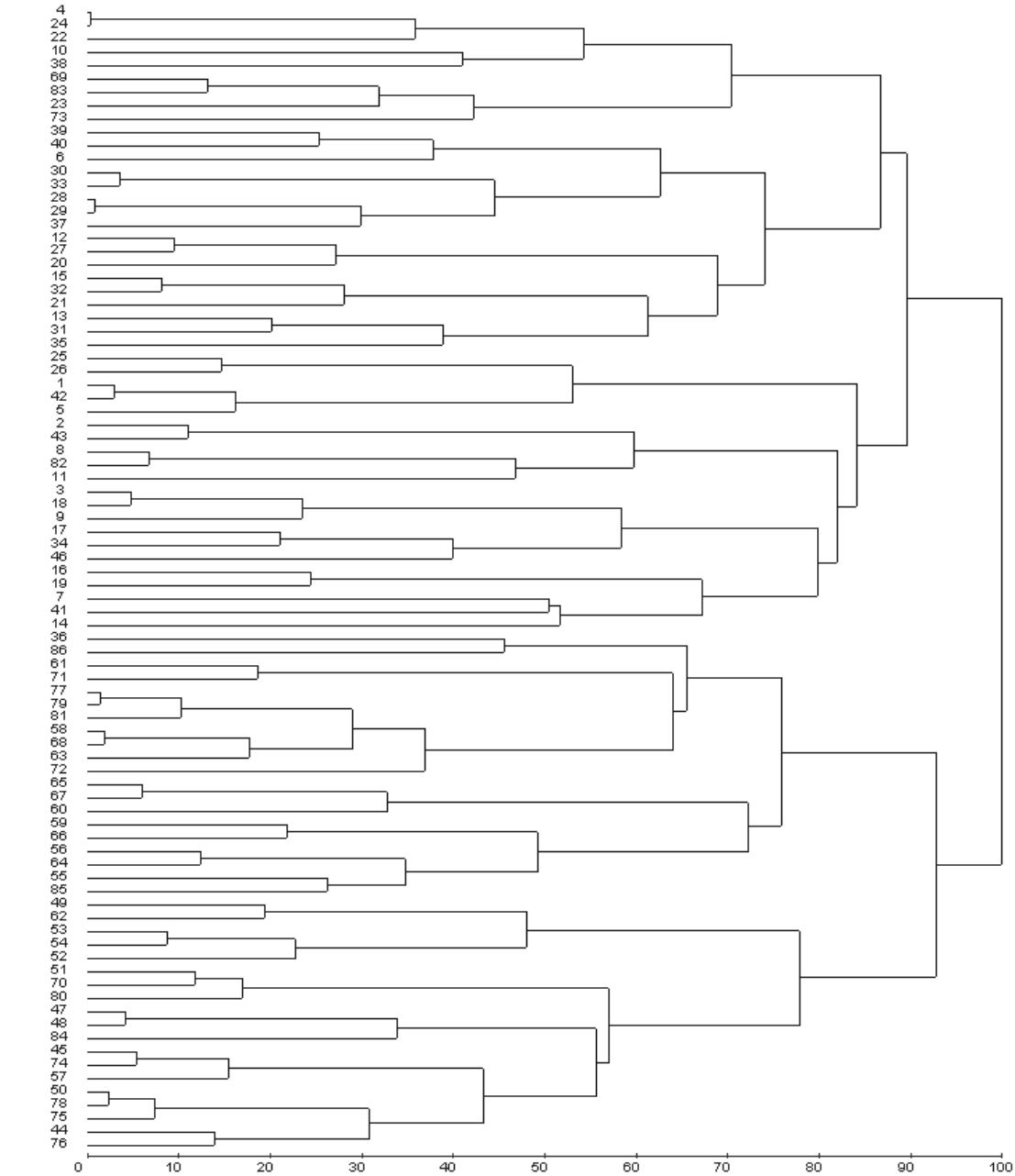

Figure 1. Dendrogram of 86 maize genotypes with 76 polymorphic and 10 monomorphic RAPD bands based on Ward's hierarchical method, using the dissimilarity matrix of the Arithmetic Complement of the Jaccard Index

of the other population could have pollinated the plant belonging to the population that originated the seed, whereas the molecular analysis identified the genetically closest to the paternal population; or even when considered to be from distinct heterotic groups, the populations could overlap, that is, have similar descents, as a point of intersection between two sets.

By Ward's hierarchical method (Figure 1), where the group formation is based on abrupt changes in the dendrogram, the formation of two large groups was verified very clearly: one formed by the genotypes belonging to Cimmyt and the other with those of Piranão. Similarly to grouping by Tocher's method, the presence of contaminants was verified as well. Genotype 36, derived from Cimmyt, appears in the large group of the Piranão population; and the genotypes 46, 69, 73, 82 and 83, belonging to this population, appear grouped with the Cimmyt genotypes.

The discriminant analysis in turn identified seven incorrect classifications, with an error rate of 8.14 %, where the genotypes 3, 7, 10, 16, 23, 29, and 36, classified as belonging to Piranão, have lower mean genetic distances to Cimmyt than to their own population, that is, they should be classified as belonging to Cimmyt and not to Piranão. It was further observed that there was no misclassification for the plants classified initially as belonging to Piranão, since all plants presented the lowest genetic distance to the population they had first been classified in.

This suggests that the selfed ears of the parents belonging to Piranão may have been contaminated by Cimmyt pollen, or that the parents already belonged to this population. Whatever the explanation, such genotypes must be discarded in the recombination process.

The results of the analysis of variance and the grouping techniques demonstrate the existence of sufficient variability for the continuity of the recurrent selection program and, associating these techniques with discriminant analysis and RAPD analysis of the genotypes, proved a useful and important tool for the identification of contaminants. These must be eliminated from recombination, to warrant the genetic distance between populations, which is an important factor when interpopulation heterosis is exploited, and to preserve the traits of each studied population.

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Aplicação de índice de seleção e marcadores moleculares na seleção recorrente recíproca em milho

RESUMO – *Das metodologias de melhoramento aplicáveis a cultura do milho, a seleção recorrente recíproca entre famílias de irmãos completos merece destaque pois permite ganhos nas populações per si e/ou em cruzamento. O presente trabalho além de utilizar o índice de seleção proposto por Smith e Hazel, nesta metodologia, introduziu, para os genótipos selecionados, uma etapa baseada em marcadores RAPD visando preservar a variabilidade genética e identificar contaminantes antes da recombinação dos mesmos, utilizando para tal, técnicas multivariadas de agrupamento e análise discriminante, no intento de dar longevidade ao programa de melhoramento e ampliar a distancia genética entre as populações 'Cimmyt' e 'Piranão', utilizadas para obtenção de um híbrido intervarietal. A técnica molecular mostrou-se útil para identificar contaminantes e auxiliar na escolha dos genótipos a serem recombinados de forma a maximizar a heterose entre as populações, podendo ser inserida em programas de seleção recorrente, principalmente aqueles que visam à obtenção de híbridos.*

Palavras-chave: *Zea mays,* seleção recorrente, índice de seleção, marcadores moleculares, análise discriminante.

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