ISOENZYMATIC VARIABILITY IN TROPICAL MAIZE POPULATIONS UNDER RECIPROCAL RECURRENT SELECTION

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ABSTRACT: Maize (*Zea mays* L.) is one of the crops in which the genetic variability has been extensively studied at isoenzymatic loci. The genetic variability of the maize populations BR-105 and BR-106, and the synthetics IG-3 and IG-4, obtained after one cycle of a high-intensity reciprocal recurrent selection (RRS), was investigated at seven isoenzymatic loci. A total of twenty alleles were identified, and most of the private alleles were found in the BR-106 population. One cycle of reciprocal recurrent selection (RRS) caused reductions of 12% in the number of alleles in both populations. Changes in allele frequencies were also observed between populations and synthetics, mainly for the *Est 2* locus. Populations presented similar values for the number of alleles per locus, percentage of polymorphic loci, and observed and expected heterozygosities. A decrease of the genetic variation values was observed for the synthetics as a consequence of genetic drift effects and reduction of the effective population sizes. The distribution of the genetic diversity within and between populations revealed that most of the diversity was maintained within them, *i.e.* BR-105 x BR-106 (G_{ST} = 3.5%) and IG-3 x IG-4 (G_{ST} = 4.0%). The genetic distances between populations and synthetics increased approximately 21%. An increase in the genetic divergence between the populations occurred without limiting new selection procedures.
Key words: *Zea mays,* genetic markers, genetic variability, isozymes, population improvement

VARIABILIDADE ISOENZIMÁTICA EM POPULAÇÕES TROPICAIS DE MILHO SOB SELEÇÃO RECORRENTE RECÍPROCA

RESUMO: O milho (*Zea mays* L.) é uma das culturas em que a variabilidade genética tem sido extensivamente estudada com base em locos isoenzimáticos. A variabilidade genética das populações de milho BR-105 e BR-106, e dos sintéticos IG-3 e IG-4, obtidos após um ciclo de seleção recorrente de elevada intensidade, foi investigada para sete locos isoenzimáticos. Foram identificados 20 alelos, sendo que a maioria dos alelos exclusivos foi detectada na população BR-106. Um ciclo de seleção recorrente recíproca (RRS) causou reduções de 12% no número de alelos em ambas populações. Mudanças nas freqüências alélicas foram observadas entre populações e sintéticos, principalmente para o loco *Est 2*. As populações mostraram similaridade para número de alelos por loco, porcentagem de locos polimórficos, e para heterozigosidade observada e esperada. Houve decréscimo nas estimativas da variabilidade dos sintéticos em conseqüência dos efeitos da deriva genética e redução do tamanho efetivo populacional. A distribuição da diversidade genética dentro e entre as populações revelou que a maior parte da diversidade permaneceu dentro delas, *i.e.* BR-105 x BR-106 (G_{ST} = 3,5%) e IG-3 x IG-4 (G_{ST} = 4,0%). A distância genética entre as populações e os sintéticos aumentou em torno de 21%. Houve aumento na divergência genética entre as populações, porém sem comprometer novos procedimentos de seleção.
Palavras-chave: *Zea mays,* marcadores genéticos, variabilidade genética, isoenzimas, melhoramento de populações

INTRODUCTION

Maize is one of the major crops in which the genetic variability has been extensively studied at the isoenzymatic level (Doebley et al., 1985; Kahler et al., 1986; Llauradó et al., 1993; Sanou et al., 1997; Gimenes & Lopes, 2000). The simplicity of the isoenzyme assays together with the lower cost, still guarantees its use today (Liu et al., 2001). In addition, it seems that abundant isoenzymatic variability exists in maize (Cardy & Kannenberg, 1982). Information about this variability has practical implications for germoplasm preservation and for trailing the variability behaviour during the selection cycles.

Improved sources of maize germplasm can be achieved by recurrent selection. This selection scheme was developed to increase the frequency of favourable alleles without reducing the genetic variability, which is essential for continued selection (Hallauer et al., 1988). Two populations are used in the case of reciprocal recurrent selection (RRS) and the progenies are selected on the basis of their performance in crosses with the reciprocal population (Schnicker & Lamkey, 1993). Several studies showed changes in allele frequencies at maize isoenzymatic loci under recurrent selection (Stuber & Moll, 1972; Stuber et al., 1980; 1982) and reciprocal recurrent selection (Brown & Allard, 1971). These changes were attributed to random genetic drift associated to decreasing population sizes. Recently, Labate et al. (1997; 1999) focused on the genetic diversity of two maize populations after 12 RRS assessed at restriction fragment length polymorphism (RFLP) loci. Significant changes occurred in allele frequencies and genetic diversity distribution between the improved populations.

Tropical maize populations are composed of a mixture of races, and present a considerable, unexploited genetic variability. BR-105 and BR-106 are important tropical maize populations from different origins and with distinct genetic structures, ensuring high levels of heterosis in their crosses (Naspolini Filho et al., 1981). Rezende & Souza Jr. (2000) showed that one cycle of a high-intensity RRS applied to BR-105 and BR-106 was effective, but the improvement of the populations *per se* was limited by the effects of genetic drift. However, the interpopulation genetic variances remained unchanged indicating that significant improvement could be achieved in the next cycle of selection.

The objectives of the present study were to evaluate, at the isoenzymatic level, the genetic variability of the populations BR-105 and BR-106 as well as the effects of one high-intensity RRS cycle over the genetic structure of these populations.

MATERIAL AND METHODS

Origin of the maize populations

Populations BR-105 and BR-106 were submitted to one cycle of high-intensity RRS (2.0 and 2.5%, respectively) using 400 S_3 lines. These lines were crossed with the opposite population, and superior interpopulation half-sib (HS) progenies were identified. Eight S_3 lines derived from BR-105, and 10 derived from BR-106, both related to the selected interpopulation HS progenies, were intercrossed in a diallel mating design within each population, giving rise to the IG-3 and IG-4 synthetics, respectively. Considering that the effective population size (*Ne*) of each S_3 is approximately 0.57, the *Ne* of IG-3 and IG-4 are 4.56 and 5.7, respectively (Rezende & Souza Jr., 2000). The inbreeding coefficients were 10.9 (IG-3) and 8.75% (IG-4).

Isoenzyme assays

Seeds randomly chosen from 100 individuals of each population and synthetic were grown in a greenhouse for 15 days. Approximately 200 mg of leaf tissue were collected from each plant, ground under liquid nitrogen and mixed with 2 mL of extraction buffer, as described by Alfenas (1999). Isoenzyme electrophoresis was carried out on polyacrylamide gels in a vertical apparatus according to Laemilli (1970). Eight enzymatic systems, *i.e.* Esterase (EST), Peroxidase (PO), Superoxide dismutase (SOD), Malate dehydrogenase (MDH), Phosphohexose isomerase (PHI), Acid phosphatase (ACP), Catalase (CAT) and Malic enzyme (ME) were revealed by staining procedures shown in Table 1. For details, see Stuber et al. (1988) and Pasteur et al. (1988).

In this study, a high quality of protein extraction procedure combined with electrophoresis conditions for selecting polymorphic loci led to reproducible band profiles, thus eliminating genotyping errors.

Table 1 - Staining solutions for isoenzymes detection on gels.

Isoenzyme	Staining solution
Esterase	α-naphthyl acetate; fast Garnett GBC salt; 0.1 M Phosphate buffer pH 6.5
Peroxidase	3-amine-9-ethilcarbazole; dymethilphormamyde; 0.1 M $CaCl_2$; 3% H_2O_2; 50 mM acetate buffer pH 5.0
Superoxide dismutase	0.2 M Tris-HCl buffer pH 8.0; 0.5 M $MgCl_2$; NAD 1%; PMS 1%; NBT 1%
Malate deehydrogenase	2 M D,L,-malic acid pH 7.0; 0.5 M $MgCl_2$; NAD 1%; NBT 1%; MTT 1%; 0.2 M Tris-HCl buffer pH 8.0
Phosphohexose isomerase	D-frutose-6-phosphate; 6-phosphogluconic acid; 0.5 M $MgCl_2$; NADP; MTT; PMS; NADP-dependent glucose-6-phosphate dehydrogenase; 0.05 M Tris-HCl buffer pH 8.0
Acid phosphatases	α-naphthyl acid phosphate; fast blue BB; 0.15 M acetate buffer pH 5.0
Catalase	Potassium ferricyanide; ferric chloride; 0.01% H_2O_2
Malic enzyme	2 M D,L,-Malic acid pH 7.0; 0.2 M Tris-HCl buffer pH 8.0; 0.5 M $MgCl_2$; NADP 1%; PMS 1%; NBT 1%; MTT 1%

NBT: nitro blue tetrazolium; MTT: tetrazolium thiazolyl blue; PMS: phenazine methosulfate; NAD: β-nicotinamide adenine dinucleotide; NADP: β-nicotinamide adenine dinucleotide phosphate.

Data analysis

Allele frequencies were calculated by using BIOSYS-1 (Swofford & Selander, 1989). The genetic diversity was measured based on the mean number of alleles per locus, percentage of polymorphic loci, mean observed heterozygosity ($\overline{Ho}$), and mean expected heterozygosity at Hardy-Weinberg proportions ($\overline{He}$). The standard error (s) of each allelic frequency as well as the 95% confidence intervals for $\overline{Ho}$ and $\overline{He}$ were determined based on the variances of the respective estimates, as described by Weir (1996). Wright's fixation index $\hat{f} = 1 - (Ho / He)$ was estimated to quantify the lack or excess of heterozygosity. The 95% confidence intervals for the fixation index were determined through the bootstrap method (10,000 replicates) using the GDA program (Lewis & Zaykin, 2001). The loss of heterozygosity for a particular locus (*Est 2*) was predicted by the commonly used equation for the loss of heterozygosity by random drift, *i.e.* $H_t = H_0\left(1 - 1/2Ne\right)^t]$, in which *Ne* is the effective population size, and *Ho* and *Ht* are heterozygosity values for generations *0* and *t*, respectively (Lacy, 1987). The distribution of gene diversity was carried out according to the model proposed by Nei (1973), in which the mean estimates of the total genetic diversity (H_r) were decomposed in one component attributed to the mean gene diversity within the population (H_s), and to a component attributed to the mean gene diversity between populations (D_{ST}). The proportion of the total gene diversity among populations (G_{ST}) or genetic differentiation was given by: $G_{ST} = D_{ST} / H_r$. Total gene diversity was defined for the populations BR-105 and BR-106 before selection (C_0) and for the synthetics IG-3 and IG-4 after one cycle of selection (C_1) using the FSTAT program (Goudet, 1995). Nei's genetic distance (Nei, 1972) was estimated by BIOSYS-1. A dendrogram was constructed using the unweighted paired group method with arithmetic averages (UPGMA) according to the NTSYS program (Rohlf & Slice, 1992). The bootstrap method with 1,000 replicates was performed to support the relative strength of each node of the dendrogram using the BOOD program (Coelho, 2000).

RESULTS AND DISCUSSION

Isoenzymatic polymorphism

Of the eight assayed enzymatic systems, the malic enzyme system revealed one locus that was monomorphic in both populations and their synthetics. Stuber et al. (1988) also found no variation in U.S. materials for the malic enzyme. For the other seven systems (Figure 1), the banding patterns were consistent with a Mendelian interpretation, giving rise to eight polymorphic loci (Table 2). A total of twenty alleles were identified. The number of alleles observed at each locus ranged from two (*Est 2, Po 1, Sod 1, Mdh 1* and *Acp 1*) to four (*Est 1*). According to Messmer et al. (1991), of the 22-isozyme loci evaluated in 21 U.S Corn Belt maize inbreds, 15 presented polymorphism with a maximum number of three alleles at a given locus.

Private alleles were found in the original populations for *Est 1*, *Sod 1*, *Mdh 1* and *Cat 1* loci, most of them were present in the BR-106 population. One cycle of RRS caused reductions around 12% in the number of alleles. Changes in allele frequencies were also observed between populations and their synthetics. The *Est 2* locus experienced a considerable change in allele frequency after one RRS over BR-105. As mentioned, the loss of heterozygosity for this locus was predicted by the equation described by Lacy (1987). At this locus the loss of heterozygosity was significant as the observed value for IG-3 (0.328 ± 0.047) did differ from the predicted value (0.292 ± 0.045) expected after one RRS (Figure 2). Raybould et al. (1998) suggested that "badly behaved loci" might be identified and excluded from the analysis. Thus, the subsequent analyses were completed without this locus.

Genetic variability within populations

The original populations BR-105 and BR-106 were similar regarding the number of alleles per locus, percentage of polymorphic loci, as well as observed and expected heterozygosities (Table 3). Because of the private alleles present in BR-106 (*Est-1*, *Mdh-3* and *Cat-1*), this population was typified by an allelic richness of 2.40 ± 0.40 per locus, which was slightly superior in relation to BR-105 values (2.10 ± 0.30). In terms of the mean expected heterozygosity over all loci, the original populations have the same quantity of potential variability (0.366 for BR-105 and 0.368 for BR-106).

These values of diversity are similar to those of two adapted exotic populations: Cateto Flint and Mexican Dent 0.300 ± 0.06 and 0.320 ± 0.06 characterized by 13 enzyme loci (Kahler et al., 1986). This diversity was considerably high in face of the estimative (0.248 ± 0.059) reported for 90 African landraces at 18 enzyme loci (Sanou et al., 1997).

A decrease in all genetic variation measures was observed for the synthetics as a consequence of genetic drift effects and reduced effective population sizes. Random genetic drift does predict disappearing of low frequency-alleles due to sampling errors (Reedy et al., 1995).

The values of the Wright's mean fixation index ($\hat{\hat{f}}_{IS}$) indicated that there was no inbreeding (Table 3). However, a particular bootstrapping, excluding IG-3, presented non significant values. According to Ritland (1989) the uncertainty of estimates arises from the sampling variances among loci. The number of loci and alleles are fundamental to provide a good accuracy for the estimation of the fixation index within populations (f) (Carlini-Garcia et al., 2001).

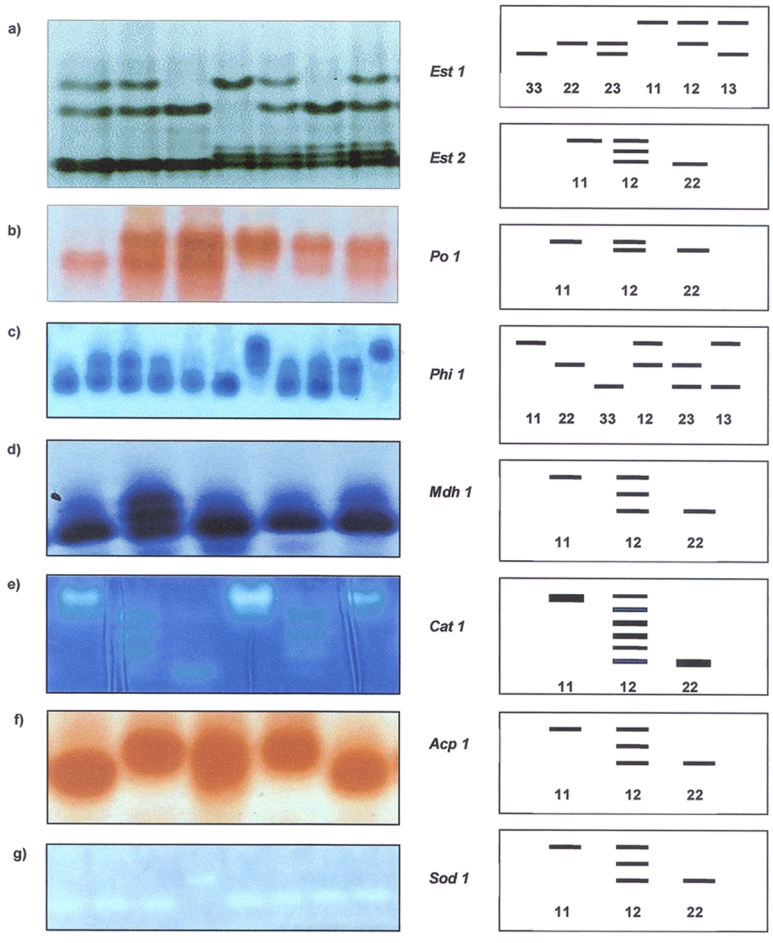

Figure 1 - Left side: Seven isoenzymatic systems revealed in polyacrylamide gels. a. Esterase (*Est 1*, *Est 2*); b. Peroxidase (*Po 1*); c. Phosphohexose isomerase (*Phi 1*); d. Malate dehydrogenase (*Mdh 1*); e. Catalase (*Cat 1*); f. Acid phosphatase (*Acp 1*); g. Superoxide dismutase (*Sod 1*). Right side: Zymograms of all genotypes detected in the maize populations BR-105 and BR-106, and/or their synthetics IG-3 and IG-4.

Distribution of genetic diversity

The distribution of the genetic diversity within and between populations showed that most of the diversity, either in BR-105 x BR-106 (G_{ST} = 3.5%) and in IG-3 x IG-4 (G_{ST} = 4.0%), was maintained within them (Table 4). Considering the 95% confidence intervals, the degree of gene diversity (G_{ST}) was significant. In maize germplasm collections assayed by isoenzymes, the gene diversity was primarily distributed within the collections than between them (Doebley et al., 1985). Gimenes & Lopes (2000) found that 84.4% of the alloenzymatic variability was distributed within the Brazilian indigenous races Caingang, Entrelaçado, Lenha and Moroti.

The G_{ST} estimative between the original populations (BR-105 x BR-106) was lower than the value of 30% reported for 65 maize collections from different origin (Europe, North America, Argentina and Chine) using RFLP markers (Rebourg et al., 1999). In terms of conservation, it can be suggested that efforts have to be directed at sampling a great number of individuals

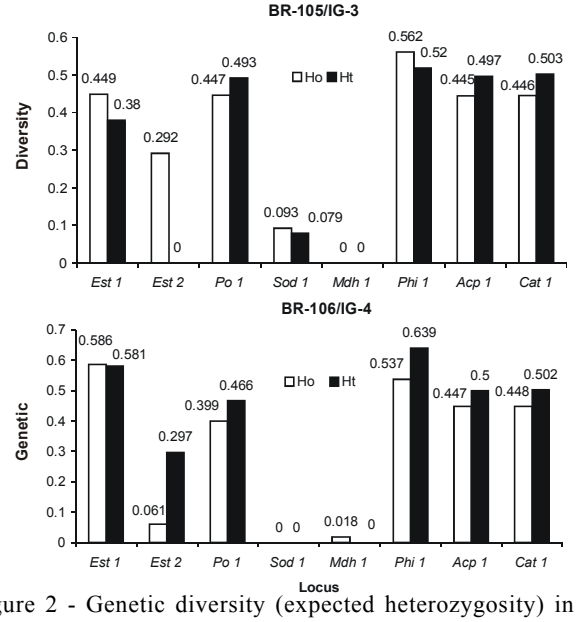

Figure 2 - Genetic diversity (expected heterozygosity) in the synthetics IG-3 (top) and IG-4 (bottom) after one cycle of RRS with the values predicted by random genetic drift model: $H_t = H_0 (1\text{-}½_{Ne})^t$

Table 2 - Allelic frequencies (p) and standard errors (σ) at 8 isoenzymatic loci in the maize populations BR-105 and BR-106, and their synthetics IG-3 and IG-4, respectively.

Locus	Allozyme	BR-105 $p \pm \sigma$	IG-3 $p \pm \sigma$	BR-106 $p \pm \sigma$	IG-4 $p \pm \sigma$
Est 1	1	0.000 ± 0.000	0.000 ± 0.000	0.364 ± 0.037	0.475 ± 0.076
	2	0.555 ± 0.035	0.747 ± 0.031	0.449 ± 0.038	0.435 ± 0.075
	3	0.435 ± 0.035	0.253 ± 0.031	0.157 ± 0.028	0.085 ± 0.042
	4	0.010 ± 0.007	0.000 ± 0.000	0.030 ± 0.013	0.005 ± 0.011
		*n=100	n=95	n=99	n=100
Est 2	1	0.205 ± 0.028	0.000 ± 0.000	0.030 ± 0.013	0.180 ± 0.058
	2	0.795 ± 0.028	1.000 ± 0.000	0.970 ± 0.013	0.820 ± 0.058
		n=100	n=100	n=100	n=100
Po 1	1	0.515 ± 0.035	0.570 ± 0.035	0.335 ± 0.036	0.365 ± 0.073
	2	0.485 ± 0.035	0.430 ± 0.035	0.665 ± 0.036	0.635 ± 0.073
		n=100	n=100	n=100	n=100
Sod 1	1	0.055 ± 0.016	0.041 ± 0.014	0.000 ± 0.000	0.000 ± 0.000
	2	0.945 ± 0.016	0.959 ± 0.014	1.000 ± 0.000	1.000 ± 0.000
		n=100	n=100	n=100	n=100
Mdh 1	1	1.000 ± 0.000	1.000 ± 0.000	0.990 ± 0.008	1.000 ± 0.000
	2	0.000 ± 0.000	0.000 ± 0.000	0.010 ± 0.008	0.000 ± 0.000
		n=100	n=100	n=100	n=100
Phi 1	1	0.215 ± 0.029	0.560 ± 0.035	0.155 ± 0.028	0.470 ± 0.076
	2	0.485 ± 0.035	0.410 ± 0.034	0.325 ± 0.036	0.305 ± 0.070
	3	0.300 ± 0.032	0.030 ± 0.012	0.520 ± 0.039	0.225 ± 0.063
		n=100	n=100	n=100	n=100
Acp 1	1	0.535 ± 0.035	0.550 ± 0.035	0.420 ± 0.039	0.535 ± 0.039
	2	0.465 ± 0.035	0.450 ± 0.035	0.480 ± 0.039	0.465 ± 0.039
		n=100	n=100	n=100	n=100
Cat 1	1	0.466 ± 0.039	0.500 ± 0.035	0.565 ± 0.038	0.483 ± 0.039
	2	0.534 ± 0.039	0.500 ± 0.035	0.425 ± 0.038	0.517 ± 0.039
	3	0.000 ± 0.000	0.000 ± 0.000	0.010 ± 0.008	0.000 ± 0.000
		n=87	n=100	n=100	n=88
**NA	20	17	15	19	17

*n: number of sampled individuals.**NA: total number of alleles.

Table 3 - Measures of genetic variation at 7 isoenzymatic loci in the maize populations BR-105 and BR-106, and their synthetics IG-3 and IG-4, respectively.

	Sample size[a]	Mean n° of alleles per locus	Percentage of polymorphic loci[b]	Observed heterozygosity ($\overline{Ho}$)	Expected heterozygosity ($\overline{He}$)[c]	Wright's fixation index ($\hat{f}_{IS}$)
BR-105	(98.1)	2.10 ± 0.30	85.70	0.366 ± 0.106	0.392 ± 0.090	0.067 (-0.120 ; 0.305)[d]
IG-3	(98.9)	2.00 ± 0.20	85.70	0.331 ± 0.081	0.353 ± 0.083	0.062 (0.005 ; 0.127)
BR-106	(99.9)	2.40 ± 0.40	85.70	0.368 ± 0.104	0.388 ± 0.101	0.053 (-0.140 ; 0.230)
IG-4	(98.3)	2.10 ± 0.40	71.40	0.359 ± 0.103	0.384 ± 0.101	0.066 (-0.035 ; 0.191)

[a]Mean sample size per locus. [b]A locus was considered polymorphic if more than one allele was detected. [c]Unbiased estimate. [d]90% confidence interval estimated with 10,000 replicates.

within the original populations, as the majority of the gene diversity is allocated within them. The mean gene diversity within the original populations (H_S) slightly diminished after selection, contributing for an increase in the mean gene diversity between the synthetics (D_{ST}). Consequently, the differentiation between them also increased. These values, however, did not differ since the 95% confidence intervals did overlap.

Table 4 - Mean values of gene diversity within and between the original populations (BR-105 and BR-106) and the synthetics (IG-3 and IG-4) assessed at 7 isoenzymatic loci.

	H_S	H_T	D_{ST}	G_{ST}(%) (*$CI_{95\%}$)
BR-105 vs BR-106	0.39	0.41	0.01	3.5% (0.019 ; 0.120)
IG-3 vs IG-4	0.36	0.38	0.05	4.0% (0.003 ; 0.180)

*CI; 95% confidence intervals obtained by bootstrap with 10,000 replicates.

Genetic distance and cluster analysis

The genetic distance between all pairwise comparisons ranged from 0.026 (Br-106 x IG-4) to 0.090 (BR-106 x IG-3) (Table 5). Comparing the distances between the original populations (BR-105 x BR-106) and the synthetics (IG-3 x IG-4) an increase of 21% was noted (contrast 0.047 to 0.057). This increase was very large considering that just one cycle of selection was performed, though somewhat less than the tenfold value reported by Labate et al. (1997) for the genetic distance, estimated for 82 RFLP loci. Results provided evidence that one cycle of a high-intensity RRS was able to increase the genetic divergence between the synthetics.

The heterosis between two particular populations depends on the difference of gene frequency between the populations (genetic divergence), and is greater when one allele is fixed in one population, and the other allele in the other (Falconer & Mackay, 1996). In this study, the cophenetic value was high (r=0.88; p=0.93) and significant.

The genetic relationships represented by the dendrogram (Figure 3) shows that each population was clustered with their respective synthetic. The selected progenies are intermated within each population, in the RRS process, in such away that each population can maintain their identity (Souza Jr., 2001). Moreover, the dendrogram grouped the materials into two clusters, which correspond to major maize heterotic groups or endosperm types. Thus, the dent type represented by BR-106 and IG-4 could be separated from the flint type composed by BR-105 and IG-3, even considering a reduced number of loci. Isozyme data of northern flints and southern dents have also showed that these two corn types are isozymically distinct at most loci (Smith, 1986).

Table 5 - Nei's genetic distances considering 7 isoenzymatic loci. BR-105 and BR-106 are the original maize populations and IG-3 and IG-4 are their synthetics, respectively.

Material pairs	D_{ist}
BR-105 x BR-106	0.047
BR-105 x IG-3	0.031
BR-105 x IG-4	0.062
BR-106 x IG-3	0.090
BR-106 x IG-4	0.026
IG-3 x IG-4	0.057

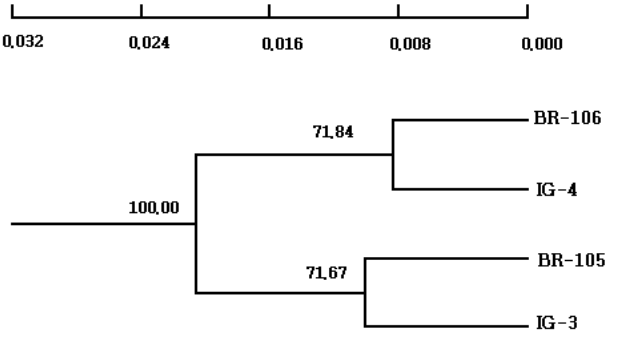

Figure 3 - Dendrogram constructed by using the Nei's genetic distances (*top*). The numbers at nodes represent the percentage of bootstrap values from 1,000 replications of re-sampled loci. BR-105 and BR-106 are tropical maize populations, and IG-3 and IG-4 are their synthetics, respectively.

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