GENETIC VARIABILITY OF ELITE BARLEY GENOTYPES FOR BRAZILIAN SAVANNA IRRIGATED SYSTEMS BASED ON RAPD MARKERS

VARIABILIDADE GENÉTICA DE ACESSOS ELITE DE CEVADA PARA SISTEMAS IRRIGADOS NO CERRADO COM BASE EM MARCADORES RAPD

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ABSTRACT: The objective of this work was to characterize and quantify the genetic variability of 39 barley elite genotypes from a Brazilian working collection belonging to Embrapa, using RAPD (Random Amplified Polymorphic DNA) molecular markers. Genomic DNA samples were extracted from leaves of each genotype and 15 decamer primers were used to obtain RAPD molecular markers. Molecular markers were converted in a binary data matrix utilized to estimate genetic dissimilarities between genotypes and to realize grouping and dispersion graphic analysis. A total of 160 RAPD markers were obtained, making 10.7 markers medium per primer. From all the markers, 141 (88.12%) were polymorphic. Genetic dissimilarities varied from 0.049 to 0.337 among the genotypes. PFC 2004033 and Prestige cultivar showed biggest genetic dissimilarities to others genetic materials. Grouping and dispersion graphic analysis showed a clustering tendency between the Mexican and American genotypes. Another clustering tendency was also found concerning the six-rowed materials. Accessions developed and used in Brazil and also in Germany, UK and Australia have shown the greatest genetic dissimilarity among themselves, being considered promising options to increase the genetic base of breeding programs.

KEYWORDS: Genetic diversity. *Hordeum vulgare* L. RAPD. Genetic resources.

RESUMO: O objetivo deste trabalho foi caracterizar e quantificar a variabilidade genética de 39 acessos de cevada elite da coleção de trabalho da Embrapa Cerrados, utilizando marcadores moleculares RAPD. Foram utilizados 15 iniciadores decâmeros para a obtenção dos marcadores RAPD, que foram convertidos em uma matriz de dados binários, a partir da qual foram estimadas as dissimilaridades genéticas entre os diferentes acessos e realizadas análises de agrupamento. Foram obtidos 160 marcadores RAPD, dos quais 141 (88,12%) foram polimórficos. As dissimilaridades genéticas variaram de 0,049 a 0,337, entre os acessos de cevada. A análise de agrupamento e de dispersão gráfica mostrou uma tendência de agrupamento entre os genótipos mexicanos e americanos. Outra tendência de agrupamento também foi encontrada entre os genótipos de seis fileiras de grãos. Acessos desenvolvidos e utilizados no Brasil e também os genótipos provenientes da Alemanha, Inglaterra e Austrália têm demonstrado a maior divergência genética entre si, sendo considerados opções interessantes para aumentar a base genética dos programas de melhoramento.

PALAVRAS-CHAVE: Diversidade genética. Cevada. *Hordeum vulgare* L. RAPD. Recursos genéticos.

INTRODUCTION

The barley (*Hordeum vulgare* sp. *vulgare*) - the fourth most important food crop in the world (FAOSTAT, 2012) – has shown a high potential to be integrated to the crop production in the savannas of Central Brazil. In general, barley is only grown in the coolest regions in the South of Brazil. However, Embrapa (Brazilian Agricultural Research Corporation) has developed researches on barley in the savannas using irrigated systems, showing the technical and economic feasibility of growing the cereal in this kind of environment (AMABILE et al., 2007a).

The characterization of genetic resources is critical in crop improvement programs because it allows significant quality and quantitative gains for the Brazilian agriculture. Therefore, a greater knowledge about the genetic resources of barley is fundamental for the selection and evaluation of crop accessions that meet the requirements of the irrigated productive system of the savannas. This will allow the barley crop to be considered an agronomic and economic alternative for this area.

The technological innovation used to carry out selection through genetic analysis of the targeted characteristics allows the improvement of species in the country, being essential to establish the crop in the savannas. One of the best strategies to obtain superior genotypes is through genetic recombination between the local and exotic adjusted germplasm of higher quality and the agronomic type. In order to do it, a genetic identification and separation of the accessions included in the working collection is needed, so the target crosses can be performed among the related parents, increasing the genetic variability for the barley improvement program (RASMUSSON; PHILLIPS, 1997; NASS, 2001).

Random Amplification of Polymorphic DNA (RAPD) has been successfully used to identify and quantify the genetic variability in several groups of plants, being therefore used as an additional tool for the characterization and use of genetic resources programs and also in improvement programs (FALEIRO, 2007; FERREIRA et al., 2007).

Molecular markers are often used to study the genetic variability of barley using the RAPD technique, since it has the great capacity of accessing information of its genome and because it is easy and fast to be performed, being effective and able to provide reliable results (SELBACH; CAVALLI-MOLINA, 2000; TODOROVSKA et al., 2003; KROTH et al., 2005; TANYOLAC, 2003; HOU et al., 2005; ABDELLAOUI et al., 2007; KARIM et al., 2009).

Therefore, the purpose of this research was to characterize and quantify the genetic variability of 39 elite genotypes from a Brazilian working collection belonging to Embrapa Cerrados, using RAPD (Random Amplified Polymorphic DNA) molecular markers.

MATERIAL AND METHODS

The present study was carried out at the Genetic and Molecular Biology Lab at Embrapa Cerrados, Brazil. Thirty-nine Mexican, American, Canadian, Australian, British and German elite, malting and hull-less barley accessions obtained from the working collection of Embrapa Cerrados, besides Brazilian lines obtained through the barley improvement program at Embrapa, were used (Table 1). Samples were cultivated in a green house at Embrapa Cerrados, and after 8-day germination, leaflet samples of two plants were obtained to extract the genomic DNA. CTAB method with some modifications was used for DNA extraction (BELLON et al., 2007). The amount of DNA was calculated using a spectrophotometer at 260 nm (A260) and a ratio of A260/A280 in order to evaluate its purity and quality (SAMBROOCK et al., 1989). Samples of each DNA accession were diluted to 5 ng/µL.

Table 1. Elite barley genotypes characterized in this study. Brasília, DF, 2011.

Number	Genotypes	Number of rowed	Origin
1	PFC 2001090 *	2	Brazil
2	CEV 96046 *	2	Brazil
3	PFC 213660 *	2	Brazil
4	PFC 99318 *	2	Brazil
5	PFC 213106	2	Brazil
6	PFC 2003122 *	2	Brazil
7	Alliot	2	UK
8	Foster *	6	USA
9	C-70	2	USA
10	Lacey *	6	USA
11	M 14	2	USA
12	CPAC 20011	6	Mexico
13	PFC 2005123	2	Brazil
14	CIMMYT 42	6	Mexico
15	CIMMYT 48	6	Mexico
16	CIMMYT 2	6	Mexico
17	CIMMYT 25	6	Mexico
18	PFC 2001049 *	2	Brazil
19	Danuta *	2	Germany
20	BRS 195 *	2	Brazil
21	BRS 180 *	6	USA
22	Cellar*	2	UK
23	CPAC 20020098 *	6	Mexico
24	BRS Deméter *	2	Brazil
25	Prestige *	2	UK
26	Scarlett*	2	Germany
27	PFC 2004345	2	Brazil
28	BRS Sampa *	2	Brazil
29	PFC 2004216	2	Brazil
30	BRS Elis *	2	Brazil
31	PFC 98252	6	Brazil
32	Vicente Morales	6	Mexico
33	BRS Greta *	2	Brazil
34	PFC 99324 *	6	Brazil
35	PFC 2004033 *	2	Brazil
36	PFC 214827-10	2	Brazil
37	Antartica-1	2	Brazil
38	Nandi	6	Australia
39	FM 404	2	Brazil

* Genotypes used in Brazilian improvement program.

The amplification reactions were carried out in a total volume of 13 uL, with 10µL of the buffer, containing 10 mM Tris-HCl (pH 8.3); 50 mM of KCl; 3 mM of $MgCl_2$; 100 µM of each

desoxyribonucleotide (dATP, dTTP, dGTP and dCTP); 0.4 µM of a primer (Operon Technologies Inc., Alameda, CA, USA); one unit of Taq DNA polymerase and 3 µL of DNA (15 ng).

First 48 decamer *primers* [OPD (02, 03, 04, 07, 08, 09, 10, 16 and 27), OPE (03, 04, 07, 15, 16, 17, 18, 19 and 20), OPF (01, 02, 03, 04, 05, 09, 10, 11, 14, 17 and 20), OPG (01, 05, 07, 08, 15, 17 and 20) and OPH (01, 04, 08, 09, 12, 13, 14, 16, 17, 18, 19 and 20)] were tested to be adjusted in the PCR (*Polimerase Chain Reaction*). To accomplish this, the DNAs of 4 genotypes (ICARDA/CIMMYT 48, Danuta, Prestige and PFC 99324) selected by their morphological divergence (two and six-rowed materials) and original geographical distance were chosen. Through these tests, 15 decamer primers which generated the greatest amount and quality of amplifications were selected: OPD (03, 07 and 08), OPF (05, 09, 14 and 20), OPG (05, 08, 15 and 17) and OPH (04, 12, 14 and 20).

The amplifications were performed in a thermocycler programmed for 40 cycles, following the sequence: 15 s at 94 ºC, 30 s at 35 ºC and 90 s at 72 ºC. After 40 cycles, a final extension that lasted 6 minutes at 72 ºC was made and then the temperature was reduced to 4 ºC. After the amplification, 3 µl of bromophenol blue (0.25%) and glycerol (60%) in water were added to each sample. Right after that, the samples were loaded in agarose gel (1.2%), stained with ethidium bromide and embedded in a TBE buffer (90 mM Tris-Borato, 1 mM EDTA). The electrophoretic separation was performed for about 4 hours at 90 volts. At the end of the run, the gels were photographed under ultra-violet light.

The generated RAPD markers were converted in a binary data matrix and the genetic distance were calculated among the different genotypes, based on Nei & Li's coefficient of similarity (NEI & LI, 1979), using Genes Program (CRUZ, 2007). The genetic similarity (GS) was calculated using $Sgij = 2Nij/(Ni + Nj)$; where: Nij is the number of bands present in both i and j genotypes and Ni and Nj is the number of bands in i and j genotypes, respectively. The genetic dissimilarity was calculated subtracting the GS of the unit (1 - GS).

The dissimilarity distance matrix was used to perform a cluster analysis through a dendrogram, with the UPGMA (*Unweighted pair-group method arithmetic average*) method (SNEATH; SOKAL 1973) as the cluster criteria and the graphic dispersion based on multidimensional scales with the principal coordinates methods and the aid of SAS Program (SAS Institute Inc., 2010) and STATISTICA (Statsoft Inc., 1999).

In order to define the number of groups, a stop in the clustering algorithm was performed based on the average genetic dissimilarity among the genotypes. The cophenetic correlation coefficient among the original genetic distances and those represented by the dendrogram between the accession pairs, according to SOKAL; ROHLF (1962) was calculated to estimate the adjustment between the dissimilarity matrix and the generated dendrogram using software NTSYS pc 2.1 (ROHLF, 2000). The clustering stability was estimated using the Bootstrap Analysis with 500 replications and the Genes program (CRUZ, 2007).

RESULTS AND DISCUSSION

The analysis of 39 barley genotypes using 15 primers generated a total of 160 RAPD markers, where 141 (88.12%) were polymorphic (Table 2), with an average of 10.7 bands per *primer*. OPD07, OPD08, OPF05 and OPH12 decamer primers showed the biggest number of polymorphic bands, while primer OPH14 presented the biggest number of monomorphic bands and an equal amount of monomorphic and polymorphic bands.

Table 2. Primers used to generate RAPD markers for the barley genotypes and respective numbers of polymorphic and monomorphic bands. Brasília, DF, 2011.

Primer	Sequence (5´→3´)	Number of polymorphic bands	Number of monomorphic bands
OPD03	GTCGCCGTCA	8	0
OPD07	TTGGCACGGG	12	0
OPD08	GTGTGCCCCA	14	0
OPF05	CCGAATTCCC	12	0
OPF09	CCAAGCTTCC	9	1
OPF14	TGCTGCAGGT	9	0
OPF20	GGTCTAGAGG	11	1
OPG05	CTGAGACGGA	10	2
OPG08	TCACGTCCAC	11	1
OPG15	ACTGGGACTC	4	0
OPG17	ACGACCGACA	11	2
OPH04	GGAAGTCGCC	6	3
OPH12	ACGCGCATGT	13	2
OPH14	ACCAGGTTGG	5	5
OPH20	GGGAGACATC	6	2
	Total	**141**	**19**

The high percentage of polymorphic markers and high average of markers by primer have shown the highest genetic variability among the barley elite genotypes from the working collection of Embrapa Cerrados. This behavior might be explained by its wide genetic base found at the germplasm bank in Brazil and by the efficiency of the RAPD technique when quantifying the variability for the species. A high genetic variability was also found in the collections analyzed by SELBACH; CAVALLI-MOLINA (2000), HOU et al. (2005), ABDELLAOUI et al. (2007) and KARIM et al. (2009). This high genetic variability found is important for the barley improvement program at Embrapa, since it allows the selection of different parents that will be included during the crossing blocks and hybridizations in an irrigated environment. Therefore, there will be a reduction in costs because fewer crosses per group will be performed, maximizing the chances of obtaining the desirable genetic combinations without losing or narrowing the genetic base.

Genetic dissimilarity between barley genotypes ranged from 0.049 to 0.337 (data not shown). This wide range shows the analysis of accessions with different degrees of dissimilarity, as found in other collections which were assessed with RAPD markers by TODOROVSKA et al. (2003); KROTH et al. (2005) and HOU et al. (2005); corroborating the results found by SELBACH; CAVALLI-MOLINA (2000); TANYOLAC (2003) and KARIM et al. (2009). American cultivars Foster and C-70 showed the smallest distances, while English barley Prestige and Brazilian FM 404 presented the highest dissimilarity. As for the average of genetic dissimilarities, C-70 and PFC 2003122 genotypes had the lowest absolute value - (0.136) and (0.138), respectively and PFC 2004033 and cultivar Prestige showed the highest average (0.265 and 0.259, respectively).

A good magnitude ($r = 0.81$) and significant ($P \leq 0.001$) cophenetic correlation coefficient for the dendrogram was found, being greater than the value of 0.70 proposed by ROHLF (2000). This result indicated the consistency in the adjustment between the graphical representation of genetic similarities and its original matrix, ensuring the performance of inferences through a visual evaluation of the dendrograma (Figure 1).

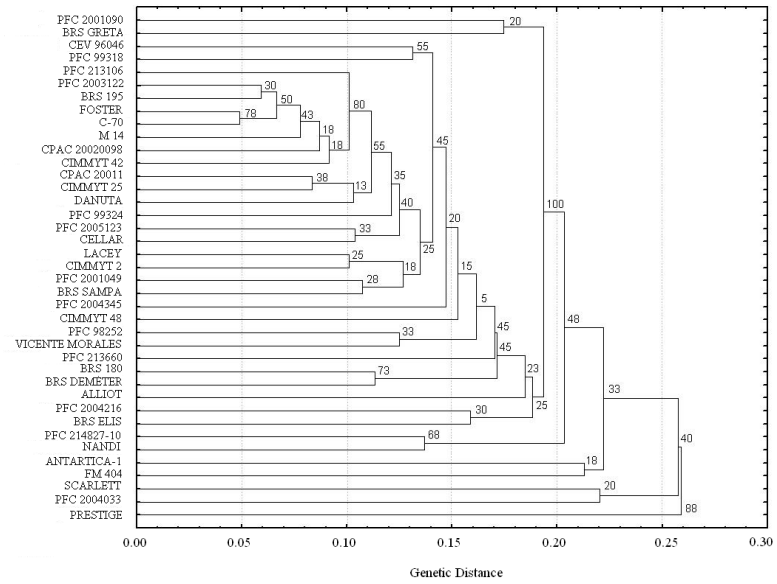

Figure 1. Cluster analysis of 39 barley genotypes based on the genetic distance matrix calculated using 160 RAPD markers. UPGMA method was used as a cluster criteria. All figures found in the groups show the percentage of times that the genotypes were clustered in a Bootstrap analysis with 500 replicates. The value for cophenetic correlation coefficient (r) was set at 0.81.

Through the clustering analysis, using the UPGMA method, a big group of similarity was observed, setting as the cut point (decision of when to stop a clustering algorithm) the mean genetic distance of 0.181. This particular group showed a high stability (100%) based on the Bootstrap Analysis (Figure 1). The present study has shown that all genotypes originated in Mexico and the USA were in this group, regardless of the fact that the samples had covered or hulless seeds. The explanation might be that the feature "presence/absence of hulls" is determined by a gene, while the RAPD markers are obtained in all the genome.

Inside this main group of similarity, two other groups could be observed. One of them includes BRS 195 cultivar and PFC 2003122 genotype, with dissimilarity genetic of 0.059. The dissimilarity genetic in this case is small because BRS 195 is PFC 2003122 genotype's parent. In this group, all elite six-rowed materials also generated from selections and hybridizations made in the USA and Mexico and from materials from Brazil that were introduced, selected or hybridized in the improvement programs of the mentioned countries, were clustered. This result indicates that the cluster is a consequence of the selection pressure produced by breeders from those countries who work in the improvement programs. For instance, the cluster including C-70 and Foster, both originated in the USA and six-rowed samples, showed the greatest genetic convergence (0.049) and stability (78%). Another sub-group of similarity was composed by CPAC 20011 genotypes – Brazilian material selected along with CIMMYT program – and CIMMYT 25, six-rowed materials and coming from the same geographic area, that is, the cooperative program established between Embrapa and ICARDA/CIMMYT.

The English cultivar Prestige known as a reference of international malting quality (EUROBARLEY, 2010; RATH, 2001) and for its excellent agronomical performance and quality under irrigation in the savannas (AMABILE et al., 2007b, 2009a e 2009b) showed to be the most isolated genotype, with the greatest genetic distance in comparison to all the other genotypes. The separation of this genotype compared to the others presented a high repeatability (88%). This fact proves the importance of this material when used in more divergent crossing block with the purpose of increasing the genetic base of the improvement programs, more specifically targeted to the development of cultivars in an irrigated environment of the savannas.

Also, by using the dendrogram analysis, cultivars FM 404, Antártica-1, Prestige, Scarlett and Nandi and PFC 2004033 and PFC 214827-10 genotypes showed the highest dispersions and were expressed in

relation to the only cluster considered stable with a repeatability of 100% (Figure 1). Except for Scarlett, Prestige and PFC 2004033, none of the other genotypes have been used in crossing blocks, even those who present good malting quality, as FM 404 and Antártica-1. The confirmation of the distances between those genotypes might be useful to the program when choosing new parents for the hybridizations to be made to get a malting quality.

Several clusters have shown low percentages of coincidence indicating that there is no tendency for accessions to cluster hierarchy, that is, high similarity within the groups and high dissimilarity among the groups (Figure 1). It is possible that the lack of cluster hierarchy occurred because the barley improvement program at Embrapa uses a wide genetic base to generate genetic constitutions for several Brazilian barley production systems: the irrigated and dry environment**.**

As for the genetic material used in the improvement programs in Brazil, they are widely distributed in the dispersion graph (Figure 2), showing the genetic variability of these materials. This variability is caused by the new approach given to the improving program at Embrapa in 2000 in order to increase the genetic base. The results clearly show the existence of a wide genetic variability in the accessions groups used, being a direct outcome of the analysis of genetic samples of origins and several improvement programs. The existence of a significant variability in Brazilian barley cultivars, even those resulting from a self-polinated crop, was also detected by ECHART (1996) and SELBACH; CAVALLI-MOLINA (2000). With the re-direction and re-modeling of the Brazilian barley program – adjusted to a new national reality, so that it becomes more competitive, produces a better malt quality and shows better agronomical performance, the goal here was to introduce and use wider genotype variability with similar and pre-defined morpho-agronomic characteristics to this system in hybridizations. This system was developed to meet the demand of the irrigated barley crop in the savannas located in the Southern and Mid-West region of Brazil.

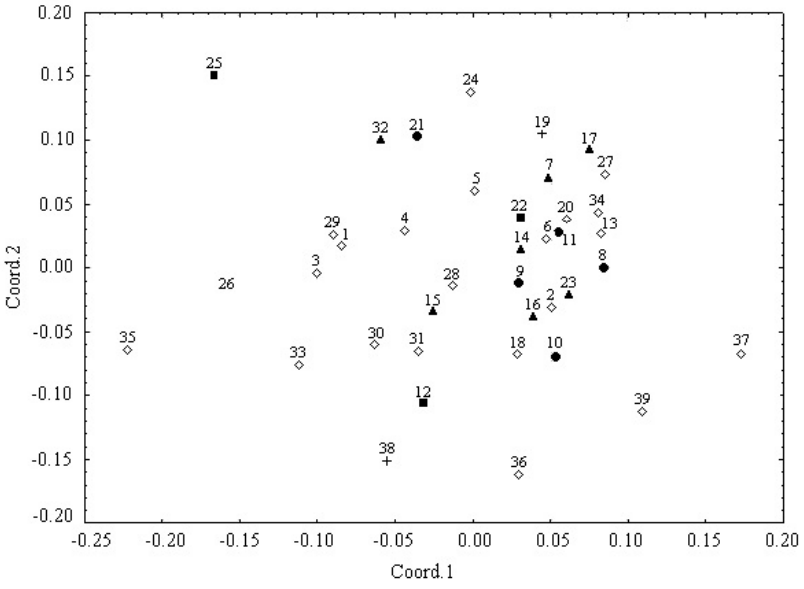

Figure 2. Cluster analysis of 39 barley genotypes based on the genetic distance matrix calculated using 160 RAPD markers. UPGMA method was used as a cluster criteria. All figures found in the groups show the percentage of times that the genotypes were clustered in a Bootstrap analysis with 500 replicates. The value for cophenetic correlation coefficient (r) was set at 0.81. Brasília, DF, 2011.

When analyzing the dispersion of barley genotypes in relation to the characteristic 'number of rows/ear" (six/two-rowed) in comparison to the two first principal coordinates (Figure 3) a cluster tendency in the six-rowed samples, except for Nandi genotype, was observed as shown in the dendrogram based on the UPGMA method.

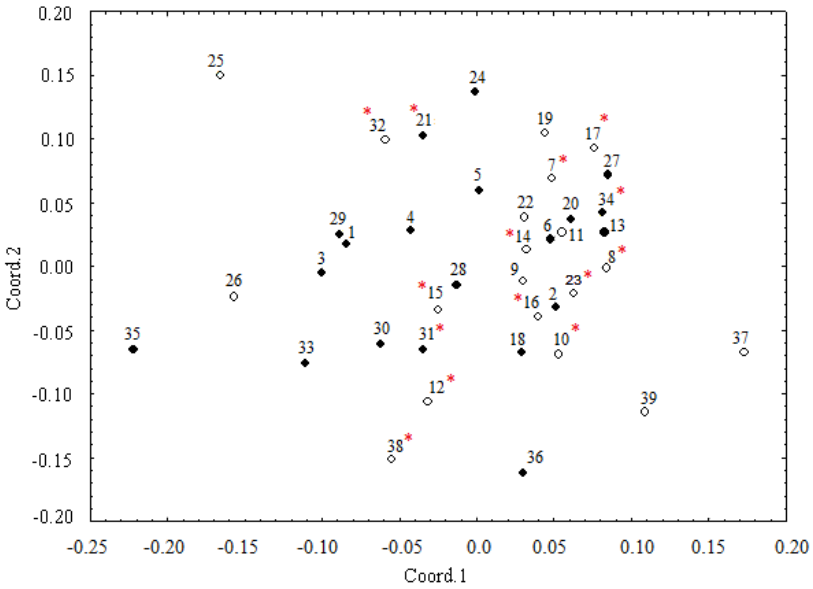

Figure 3. Graphic dispersion of 39 barley accessions of different geographical regions based on the genetic distance matrix using 160 RAPD markers. All figures correspond to accessions showed in Table 1. Origin of the genetic materials: (◊) Brazil; (▲) Mexico; (■) UK; (+) Germany; (●) United States and (✳) Australia. * Six-row barleys. Brasília, DF, 2011.

When considering all different geographical origins of the barley genetic materials studied, a concentration of Mexican genotypes was found, except for Vicente Morales cultivar (Figure 3). This kind of clustering tendency was also observed with materials originated from the North American improvement programs, where they were clustered, except for the BRS 180 cultivar. It is important to highlight that regardless of being hybridized in the USA, the final generations provided after the selection of cultivar BRS 180 were performed in an irrigated environment in the savanna, being influenced by the environment, in a way that probably resulted in a so different cultivar compared to the others in the group.

A cluster in the Mexican and American samples was observed because a lot of emphasis was put into the selection and collection of six-rowed samples and irrigated environments in a certain point in time of the improvement program made in these countries. On the other hand, Brazilian samples and samples from Germany, England and Australia have shown the greatest genetic dissimilarity, being interesting options to increase the genetic base of the improvement programs.

CONCLUSIONS

A high genetic variability was found in the present working collection proving to be a feasible material to be used for genetic improvement.

There is a genetic structure among the genotypes evaluated, with a clustering tendency around the Mexican and the American genotypes. Another clustering tendency was also found concerning the six-rowed materials.

Accessions developed and used in Brazil and also in Germany, UK and Australia have shown the greatest genetic dissimilarity among themselves, being considered interesting options to increase the genetic base of improvement programs.

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