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Original Article

DNA Molecular Markers Depicted Genetic Variability and Heterotic Pattern Among Maize Inbred Lines

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Abstract

Crosses between lines with suitable genetic distance could lead to excellent hybrids, so the identification of genetic diversity between *Zea mays* L. inbred lines has a high priority in maize hybrid breeding programs. Here, fingerprinting of 27 maize inbred lines accompanied with lines B73 and Mo17 (as parents of commercial hybrid Sc704) were evaluated via15 Inter-Simple Sequence Repeat (ISSR) primers. A total of 127 bands were amplified which 107 (85.5%) out of them were polymorphic with an average PIC of 33% (between 0.44 for UBC-820 to 0.22 for ISSR-11). Classification of genotypes based on simple matching similarity coefficients and furthest neighbor (complete linkage) clustering algorithm located them into three distinguished groups. In spite of narrow information about pedigree of studied lines, results of present study expressed the existence of at least two heterotic groups in this germplasm. These findings could be applied in management and utilization of these studied lines for developing new maize hybrid.

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Introduction

Corn (*Zea mays* L.) ranks as one of the third most important cereal crops growth in the world. The constant increase of its yield requires more precision in breeding programs over the time. The strength of a breeding program depends on the genetic variability in breeding populations and success in the production of superior inbreds. The methodologies have been used to characterize genetic diversity in plant germplasm include morphological characters, pedigree analysis, and molecular markers. The evaluation of genetic diversity through DNA molecular markers presents some advantages over other methods because in addition to identifying high polymorphism, they do not interact with the environment and can be evaluated at any stage of plant development [1].

Combining ability is another key factor in maize breeding and plays a significant role in crop improvement, especially for cross-pollinated plants as it helps the breeder to study and compare the performance of the new lines in hybrid combinations [2]. In maize, the recognition of heterotic patterns among genetically divergent groups of germplasm is fundamental in hybrid breeding in order to achieve maximum exploitation of heterosis [3]. A heterotic group is a group of related or unrelated genotypes displaying similar combining ability and giving a heterotic response when crossed with an opposite or other geneticall-y distinct germplasm group, and heterotic pattern refers to a specific pair of 2 heterotic groups that express high heterosis and high hybrid performance in their crosses [2].

Classification of elite germplasm and assignment of new inbreds to established heterotic groups are major decisions in any hybridization breeding program for maize [4]. Assignment of inbred lines to the heterotic groups by means of molecular markers allows for the characterization of a greater number of lines, thus potentially increase the efficiency of maize breeding programs [5]. For germplasm with no pedigree information or ambiguous information, this could be the first step of establishing an estimation of likely heterotic groups in existence and designing a breeding strategy based on the information thus created. Inter-Simple Sequence Repeat (ISSR) is an easy-to-use multi-locus marker; its repeatability makes it a good choice among tools such as SSR, RAPD, and AFLP for less developed laboratories with limited financial resources. The ISSR technique involves anchoring of the designated primers to a subset of SSRs and amplifying the region between two closely spaced SSRs of opposing orientation. The primers used can be unanchored or, more usually, anchored at the 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences [6].

The advantage of this technique is its multiplexed banding profiles, high frequency of polymorphism, high throughput, and relatively low cost [7]. The ISSR method has proven especially useful in the Gramineae family [8], inbred lines of maize [7], sorghum [9], and fingerprinting in rice [10].

Because of the importance of heterosis in maize hybrid production, the present study has been done to identify heterotic groups in a germplasm from Agriculture and Natural Resources Research Center of Khuzestan (Safi-Abad) using DNA molecular markers. This study is the first report of genetic diversity and identification of potential heterotic groupings within the germplasm of Khuzestan maize inbred lines.

Materials and Methods

Plant material and genomic DNA extraction

A total of 27 maize genotypes (inbred lines) obtained from the Agriculture and Natural Resources Research Center of Khuzestan (Safi-Abad) and parents of commercial hybrid Sc704 (Mo17 and B73) were used in this study. The selection of inbred lines was based on available pedigree information, morphological characterization, and physiological traits (Table 1). The pedigree information of these lines is very limited; investigation of the germplasm confirmed the origin oflines in just two groups (of synthetic and CIMMYT origin). Studied lines originated from CIMMYT were achieved by selection from introduced CIMMYT open-pollinated populations, and lines of synthetic origin were created by intercrossing among miscellaneous genetic materials, but there is no clear information about these genetic materials. DNA was extracted from fresh leaf tissue of inbred lines using the CTAB method [11] with some modifications. Leaf sample of three plants was used for DNA extraction.

Table 1. Genotypes with their origin.

No. Inbred	Origin	No. Inbred	Origin
1	*B73	15	CIMMYT
2	*Mo17	16	CIMMYT
3	Synthetic	17	CIMMYT
4	CIMMYT	18	CIMMYT
5	CIMMYT	19	CIMMYT
6	Synthetic	20	Synthetic
7	CIMMYT	21	CIMMYT
8	CIMMYT	22	CIMMYT
9	CIMMYT	23	CIMMYT
10	Synthetic	24	CIMMYT
11	CIMMYT	25	CIMMYT
12	Synthetic	26	CIMMYT
13	CIMMYT	27	CIMMYT
14	CIMMYT	28 & 29	CIMMYT

* Famous parents of SC704

ISSR assay

A total of 15 ISSR primers were used based on di-nucleotide, tri-nucleotide, and tetra-nucleotide repeats (Table 2). The primers were anchored at 3ˈ-ends by one or two partially degenerate nucleotides. The final volume of polymerase chain reaction (PCR) was 25 µl, containing 2.0 µl (100 ng) diluted template DNA, 0.2 µl (1 U) Taq polymerase, 2.5 µl PCR 10X buffer, 0.5µl (2 mM/µl) dNTPs, 0.75 µl (50 mM) $MgCl_2$, 2.0 µl (10 pmol/µl) ISSR primer, and 17.5 µl distilled deionized water. The amplifications were performed in a thermal cycler using the following program: 94°C for 5 min, 35 cycles (1 min at 94°C, 1 min at annealing temperature and 1 min at 72°C), and final elongation of 5 min at 72°C. Gel electrophoresis with 1.5% agarose gels containing DNA Safe Stain was conducted with 1X TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) at 90 V and 100 mA for 1 h. Also a 100 bp DNA ladder (100 bp DNA Ladder, MBI Fermentas) was run for sizing of the bands. The gel was visualized under UV light using a gel documentation system.

Statistical analysis

ISSR bands were behaved as dominant markers and scored for the presence (1) or absence (0) of homologous bands for each primer. Polymorphic Information Content (PIC) of each ISSR marker was determined as described by Roldán-Ruiz *et al.,* [12]: PIC = 2×Pi (1-Pi), where P_i is the frequency of amplified allele (band present), and ($1-P_i$) is the frequency of the null allele (band absent). Also marker index (MI) was obtained by formula: MI = PIC×N (number of polymorphic bands) [13]. Shannon's information index [14] and Nei's gene diversity [15] were calculated by Popgene32 [16]. The classification dendrograms was constructed by means of furthest neighbor (complete linkage) method and using the simple matching similarity coefficients in NTSYS 2.02 [17]. To ensure the statistically correct number of clusters, the AMOVA (Analysis of Molecular Variance) was done by using GenAlEx, version 6.5 [18]. To ensure the statistically correct

number of clusters, the AMOVA (Analysis of Molecular Variance) was done by using GenAlEx, version 6.5 [18].

Table 2. Information of 15 ISSR primers that was used in this study.

Primer	Sequence 5'-3'	Annealing temperature
UBC-112	(GACA)4	50
UBC-808	(AG)8C	50
UBC-809	(AG)8G	51
UBC-811	(GA)8C	53.4
UBC-818	(CA)8G	49.2
UBC-820	(GT)8C	49.9
UBC-835	(AG)8YC	53.2
UBC-836	(AG)8YA	52
UBC-841	(GA)8YC	49
UBC-857	(AC)8T	55.2
UBC-864	(ACTG)4	55.2
UBC-866	(CTC)6	60.6
UBC-873	(ATG)6	53
ISSR-7	(GA)8T	50
ISSR-11	(AG)8CT	53.3

Results

A total of 107 polymorphic bands (85.5%) were detected from 127 bands with an average PIC of 33%, which shows low genetic diversity in the genetic material (Table 3). ISSR-11 revealed the minimum amount of polymorphic (40%) and PIC (0.22) content. Also UBC-820 revealed the highest polymorphic content (44%). MI varies between 1.32 for UBC-820 and 4.29 for UBC-118.

Dendrograms were produced based on simple matching similarity coefficients (Table 4) and furthest neighbor (complete linkage) clustering algorithm. In the dendrograms created, with 0.56 genetic similarity value as a threshold, 29 maize inbred lines could be divided into three groups (A, B, and C) (Fig.1).

The highest genetic distance (0.44 similarity) was seen between inbred line 1 (B73) and 19 from group C, and the least genetic distance (0.85 similarity) was between inbred lines 22 and 23 from group B, and inbred lines 3 and 4 from group C (Table 4).Analysis of molecular variance (AMOVA) revealed that 93% the genetic diversity existed within B and C group.

However, differences among groups were significant (Table 5). Shannon's information index, Nei's gene diversity and effective number of alleles were calculated for two groups (B and C) of created dendrogram (Table 6). Gene diversity in both groups was smaller than those investigated by chukan *et al.,* [19]. Also Nei's genetic identity and genetic distance between dendrogram clusters (A, B and C) was calculated, the lowest similarity was found between groups A with B and C (Table 7), as well as the highest distance.

In a principal coordinates analysis (Fig. 2) the first two principal coordinates accounted for 11.5 and 9.5 of the total molecular variation, respectively. Principal Coordinate 1 separates B73 (inbred line 1) from the other inbred lines, but could not properly distinguish two detected groups (C and B) in dendrogram from each other. When the original data do not highly correlate; usually, the first few PCs do not explain much of the original variation. In such cases, cluster analysis proved to be more sensitive and reliable for detecting pedigree relationships among genotypes rather than PCoA [20].

Table 3. Number of polymorphic and monomorphic bands, polymorphic information content (PIC) and marker index (MI).

Marker	No. Monomorphic Bands	No. Polymorphic Bands	No. Total Bands	%Polymorphic	PIC	MI
UBC-112	1	11	12	91.6	0.29	3. 48
UBC-808	1	6	7	66.6	0.37	2.59
UBC-809	1	8	9	88.8	0.37	3.33
UBC-811	0	11	11	100	0.38	4.18
UBC-118	2	11	13	84.6	0.33	4.29
UBC-820	1	2	3	40	0.44	1.32
UBC-835	0	5	5	100	0.34	1.70
UBC-836	0	6	6	100	0.39	2.34
UBC-841	0	7	7	100	0.36	2.52
UBC-857	0	9	9	100	0.34	3.06
UBC-864	0	8	8	100	0.34	2.72
UBC-866	1	6	7	85.7	0.31	2.17
UBC-873	1	9	10	90	0.31	3.06
ISSR-7	1	11	12	91.6	0.28	3.36
ISSR-11	4	4	8	40	0.22	1.76
Average	0.87	7.60	8.47	85.5	0.33	2.80

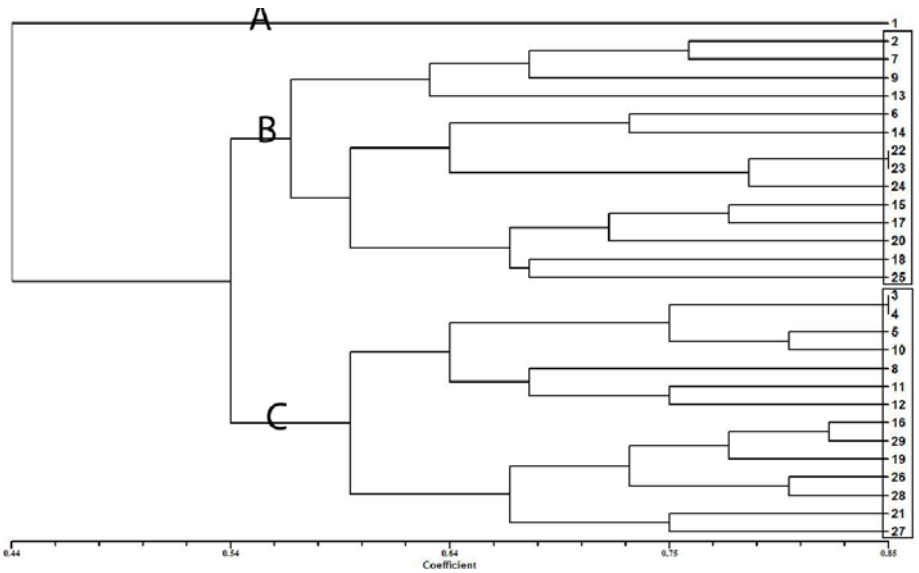

Figure 1. Dendrogram of the genetic relationships among inbred lines that is drawn based on simple matching similarity coefficients and furthest neighbor (complete linkage) clustering algorithm. Inbred lines 1 and 2 are B73 and MO17, respectively.

Table 4. Matrix of simple matching similarity coefficients.

Lines	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	1.00																												
2	0.75	1.00																											
3	0.60	0.64	1.00																										
4	0.65	0.70	0.85	1.00																									
5	0.53	0.62	0.75	0.77	1.00																								
6	0.52	0.70	0.66	0.63	0.64	1.00																							
7	0.60	0.76	0.63	0.64	0.71	0.70	1.00																						
8	0.67	0.61	0.66	0.64	0.69	0.61	0.74	1.00																					
9	0.56	0.72	0.68	0.68	0.67	0.72	0.68	0.63	1.00																				
10	0.58	0.64	0.76	0.76	0.80	0.63	0.70	0.68	0.78	1.00																			
11	0.57	0.67	0.69	0.67	0.64	0.71	0.71	0.69	0.71	0.75	1.00																		
12	0.54	0.66	0.64	0.66	0.69	0.63	0.70	0.68	0.68	0.76	0.75	1.00																	
13	0.50	0.67	0.62	0.64	0.64	0.64	0.67	0.54	0.64	0.67	0.68	0.67	1.00																
14	0.51	0.60	0.65	0.64	0.63	0.73	0.60	0.60	0.67	0.65	0.63	0.56	0.59	1.00															
15	0.55	0.65	0.62	0.60	0.64	0.71	0.77	0.65	0.65	0.65	0.64	0.77	0.63	0.68	1.00														
16	0.53	0.65	0.71	0.69	0.70	0.71	0.73	0.65	0.69	0.79	0.70	0.75	0.72	0.72	0.79	1.00													
17	0.55	0.65	0.69	0.67	0.68	0.64	0.69	0.64	0.64	0.71	0.66	0.69	0.63	0.63	0.78	0.81	1.00												
18	0.58	0.63	0.61	0.64	0.69	0.68	0.74	0.63	0.64	0.70	0.67	0.63	0.60	0.62	0.73	0.71	0.69	1.00											
19	0.44	0.62	0.67	0.71	0.72	0.71	0.67	0.62	0.67	0.69	0.68	0.71	0.64	0.70	0.72	0.78	0.68	0.69	1.00										
20	0.59	0.65	0.64	0.65	0.63	0.67	0.69	0.65	0.62	0.64	0.64	0.65	0.63	0.64	0.72	0.74	0.74	0.67	0.63	1.00									
21	0.54	0.61	0.63	0.68	0.65	0.63	0.72	0.66	0.63	0.68	0.69	0.66	0.67	0.62	0.69	0.73	0.71	0.68	0.75	0.62	1.00								
22	0.58	0.66	0.59	0.66	0.62	0.64	0.68	0.66	0.57	0.61	0.69	0.66	0.65	0.65	0.67	0.73	0.67	0.70	0.67	0.62	0.74	1.00							
23	0.62	0.70	0.63	0.66	0.64	0.64	0.68	0.68	0.57	0.63	0.67	0.64	0.64	0.65	0.73	0.73	0.71	0.66	0.67	0.65	0.74	0.85	1.00						
24	0.59	0.65	0.60	0.60	0.59	0.67	0.65	0.67	0.60	0.62	0.70	0.64	0.66	0.64	0.70	0.72	0.64	0.64	0.61	0.68	0.64	0.84	0.79	1.00					
25	0.52	0.64	0.57	0.61	0.71	0.68	0.68	0.64	0.66	0.64	0.65	0.70	0.64	0.60	0.71	0.75	0.71	0.68	0.65	0.67	0.66	0.70	0.66	0.75	1.00				
26	0.51	0.67	0.62	0.60	0.63	0.73	0.71	0.69	0.67	0.65	0.72	0.75	0.66	0.66	0.76	0.79	0.72	0.65	0.74	0.70	0.71	0.75	0.79	0.78	0.73	1.00			
27	0.53	0.67	0.62	0.64	0.66	0.69	0.65	0.65	0.60	0.62	0.68	0.62	0.59	0.59	0.63	0.70	0.68	0.60	0.72	0.59	0.75	0.71	0.73	0.72	0.71	0.76	1.00		
28	0.60	0.74	0.63	0.68	0.65	0.63	0.78	0.64	0.63	0.68	0.71	0.72	0.65	0.58	0.71	0.75	0.71	0.66	0.73	0.71	0.79	0.72	0.79	0.69	0.68	0.80	0.69	1.00	
29	0.58	0.66	0.66	0.68	0.71	0.68	0.72	0.64	0.70	0.72	0.71	0.74	0.67	0.64	0.71	0.82	0.69	0.68	0.79	0.64	0.78	0.66	0.68	0.62	0.72	0.75	0.67	0.81	1.00

Table 5. AMOVA analysis for the partitioning of variation of inbred lines among and within dendrogram groups (B and C).

Source	df	SS	MS	Est. Var.	%	PhiPT	P
Among Groups (AG)	1	46.429	46.429	1.704	7%	0.070	0.001
Within Groups (WG)	26	586.714	22.566	22.566	93%		
Total	27	633.143		24.270	100%		

Df, degrees of freedom; SS, sum of squares; MS, mean of squared; Var, variance of component; %, total-percentage of variation; P, the significance of the variance components after 999 random permutations, PhiPT, $^{V_{AG}}/_{(V_{AG} + V_{WG})}$; P, Probability of type I error.

Table 6. Shannon's information index and Nei's gene diversity for clusters (B and C) identified in this study.

Groups	Shannon's information index	Nei's gene diversity	Effective number of alleles
B	0.39	0.25	1.41
C	0.38	0.24	1.39
Overall	0.55	0.35	1.64

Table 7. Mean Nei's genetic identity between clusters (above diagonal) and genetic distance between clusters (below diagonal).

	A	B	C
A		0.62	0.60
B	0.47		0.98
C	0.51	0.02	

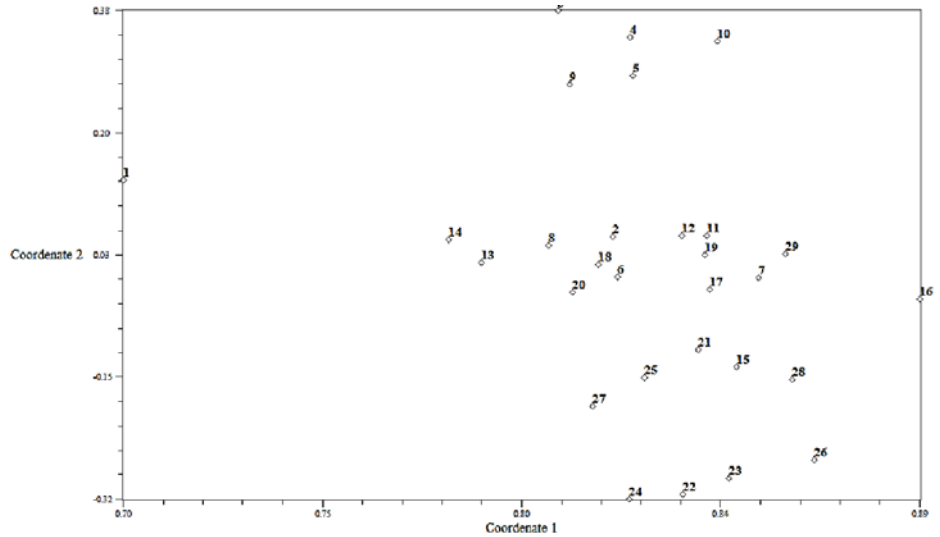

Figure 2. Inbred lines projection onto the plane defined by the Principal Coordinates 1 and 2.

Discussion

Due to the dendrogram (Fig. 1) group A included only the B73 inbred line, which belongs to the Red Yellow Dent heterotic group. So the maize germplasm of Agriculture and Natural Resources Research Center of Khuzestan (Safi-Abad) does not have this heterotic group. Hence, B73 is

a candidate for crossing with inbred lines from this center to produce promising hybrids. Also group B consisted of Mo17 with 13 other lines. Mo17 belongs to the Lancaster Sure Crop (LSC) heterotic pattern, and this group is probably heterotic pattern LSC. Cluster C included 14 lines in which the heterotic group was not identified.

A new and promising variety, SC Karoun, was produced by crossing Mo17 with inbred line number 10 from group C. Heterotic groups allow breeders to make fewer crosses, as they can maximize heterosis by crossing based on heterotic pattern only.

Choukan *et al.,* [21] used 46 SSR markers to assess genetic diversity and heterotic group prediction among 56 Iranian maize inbred lines. Four heterotic groups were predicted among 56 Iranian inbred lines by SSR markers. To assess the ISSR marker in the detection of polymorphism and heterosis, Kantety *et al.,* [7] evaluated the genetic diversity of 19 popcorn and 8 dent inbreds. The studied inbreds belonged to three (South American, Supergold, and Amber Pearl) and two (Reid Yellow Dent and Lancaster Sure Crop) heterotic groups for popcorn and dent inbreds, respectively. Finally, the existence of five heterotic groups was successfully predicted by ISSR results. The molecular genetic diversity of 58 Chinese maize inbred lines from significant heterotic groups (Lancaster Sure Crop, Reid Yellow Dent, Tangsipingtou, and Ludahonggu) and from miscellaneous origins was assessed by 40 SSR markers. SSR markers could separate inbred lines in accordance with their pedigree information, but there were some discrepancies between heterotic groups detected by SSRs and heterotic groups identified by conventional methods [22].

Conclusion

To sum up, findings of this research revealed the existence of a genetic variation among studied lines. It also could cover the narrow pedigree information of the studied lines and improve the management and utilization of this center's lines in further maize breeding programs. After supplementary confirmation such as classical or molecular breeding schedules, the detected heterotic groups could be applied efficiently by maize breeders.

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