Development of AFLP markers and genotyping of elite maize inbred lines

(Received: 10.11.2003; Accepted: 1.1.2004)

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ABSTRACT

The AFLP technology was used to develop AFLP markers characterizing 10 maize inbred lines and generating unique fingerprint for each inbred line. Selective amplification of the genomic DNA from the ten inbred lines, using eighteen primer combinations, revealed a total number of 1143 amplicons. The number of polymorphic amplicons across the 10 inbred lines was 902, representing a level of polymorphism of 78.9%. Genetic distances between lines were calculated from AFLP data using the Dice coefficient. UPGMA analysis and dendrogram clustered the ten inbred lines into two main clusters, each composed of different groups revealing relationships which were basically consistent with the pedigree of the inbred lines. Moreover, unique AFLP markers characterizing each of the ten inbred lines were identified. The total number of unique markers was 122 positive markers and 73 negative markers. The number of unique markers per genotype ranged from 4 to 45. Inbred line Sd 7 was characterized by the highest number of unique markers (39 positive and 6 negative markers). While, line A188 revealed the lowest number of unique markers (4 markers). The results assess the potentiality of the AFLP technology for characterizing at the molecular level and for generating unique fingerprint for each inbred line. This could have great impact in plant improving programs, particularly, of important crops such as maize.

Keywords: *Molecular markers, AFLP, maize, fingerprinting, genotyping, structural genomics and genetic similarity.*

INTRODUCTION

The emerging technologies have revolutionized plant genetics and breeding in the last few decades. Plant genomic research has produced molecular tools for scientist to improve breeding efficiency and accuracy. One of these important tools is the development of molecular markers. The usefulness of DNA markers for germplasm characterization and estimation of genetic relationships has been well demonstrated for different crops (Ahmed, 1999; Cao *et al.*, 1999; Moeller and Schaal, 1999; Hussein *et al.*, 2000, 2002 a & b and 2003, Adawy *et al.*, 2002, Fernandez *et al.*, 2002; Coulibaly *et al.*, 2002 and El-Khishin *et al.*, 2003).

In addition, the use of DNA markers for marker assisted selection brings extraordinary promise for streamlining many crop improvement efforts (Tanksley *et al.*, 1988 and Ribant and Hoisington, 1998).

Moreover, DNA markers proved to be useful in predicting genotypes that may be used in new crosses, particularly in certain crops such as maize where the challenge to maize breeders is to identify inbred lines that

produce highly heterotic hybrids (Ajmone Marsan *et al.*, 1998).

Different molecular markers have been applied for germplasm identification and characterization such as RFLP (restriction fragment length polymorphism, Botstein *et al.*, 1980), RAPD (random amplified polymorphic DNA, Williams *et al.*, 1990) and SSRs (simple sequence repeats, Litt and Lutty, 1989).

More recently, a DNA fingerprinting technique, called amplified fragment length polymorphism (AFLP) has been developed by Zabeau and Vos (1993) and Vos *et al.* (1995). AFLP markers are genomic restriction fragments detected after selective amplification using the polymerase chain reaction (Saiki *et al.*, 1985). AFLPs are Mendelian markers with a number of appealing features relative to other molecular markers including (i) a high multiplex ratio, thus, revealing a 10-fold increase in the number of informative markers per analysis, (ii) its ability to give highly reproducible banding patterns, and (iii) no priori sequence information of the DNA is necessary. Therefore, they provide a very powerful tool for genomic DNA fingerprinting of genomes of any origin or complexity, including those of maize (Vos *et al.*, 1995 and Ajmone Marsan *et al.*, 1998).

The present study has been conducted with the main objectives to develop AFLP markers characterizing 10 maize inbred lines, to develop a fingerprint identifying each inbred line and to establish the genetic relationships among these lines as detected by AFLP analysis.

MATERIALS AND METHODS

Plant material

The plant material used in this study consisted of 10 maize inbred lines. Seeds were kindly provided by the Maize Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. The genetic backgrounds of the 10 inbred lines are described in Table (1).

Genomic DNA isolation

The total genomic DNA was isolated from young leaves of the 10 inbred lines as described by Aitchitt *et al.* (1993). Leaf tissues (2 g) were harvested and frozen in liquid nitrogen; the frozen tissue was then ground to a fine powder. The powdered tissue was homogenized in 14 ml of DNA extraction buffer (1.4 M NaCl, 20 mM EDTA, 100 mM tris-HCl pH 8.0, 3% w/v CTAB, 1% v/v 2-mercaptoethanol) and incubated at 65 C for 30 min. This mixture was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1), and the DNA in aqueous phase was precipitated with an equal volume of isopropanol. The DNA was pelleted by centrifugation, washed with 70% (v/v) ethanol and dissolved in 3ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA solution was then treated with RNASE (10 µg/ml at 37°C for 30 min), re-precipitated with 7.5 ml ethanol, 0.3 M sodium acetate and dissolved in 1 ml of TE buffer. Agarose gel electrophoresis confirmed that the DNA was of high molecular weight with no degradation or contaminating RNA.

AFLP analysis

The AFLP procedure was performed, with minor modifications according to the protocol of Vos *et al.* (1995) that is supplied with the AFLP Analysis System I kit (Gibco, BRL). Approximately 500 ng DNA of each DNA sample was digested simultaneously with EcoRI and MseI at 37°C for 2 hr. The digested samples were incubated at 70°C for 15 min to inactivate the restriction endonucleases. *EcoRI* and *MseI* adapters were ligated to the digested samples at 20°C for 4 hr. This was done to generate template DNA

for amplification. Preamplification was carried out with +1 - primers each carrying one selective nucleotide (*EcoRI* + A and *MseI* +C) in a thermocycler for 20 cycles (94°C/30s, 56°C/60s and 72°C/60s). The amplification products were diluted 50 fold in TE buffer and stored at -20°C. Selective amplification was carried out with *EcoRI* + 3 - primers and *MseI* +3 – primers and 5 µl of the diluted PCR products from the preamplification. Eighteen primer pair combinations were employed in this study (Table 2). The PCR amplification was performed as follows: one cycle at 94°C for 30s, 65°C for 30s and 72°C for 60s, followed by 13 cycles of touchdown PCR in which the annealing temperature was lowered by 0.7°C every cycle. This was followed by 23 cycles at 94°C for 30s, 56°C for 30s and 72°C for 60s.

Gel analysis

The reaction products were mixed with equal volumes of formamide loading buffer (98%formamide, 10mM EDTA, bromophenol blue and xylene cyanol), denaturated by incubating at 90°C for 3 min and quickly cooled on ice. The products were analyzed on 8% denaturing polyacrylamide gels. The gel was run at constant power (50-55 W) until the dye was about 2/3 down the length of the gel. The gel was silver stained using a DNA silver staining system from Promega, according to the manufacturer instructions.

Data analysis

The amplified fragments were scored visually as present (1) or absent (0). The Dice coefficient of similarity (Sneath and Sokal, 1973) was used to obtain estimates of genetic similarity for phenetic analysis. A dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

Molecular markers have become, over the last few decades, a promising method for maize fingerprinting, which are based not only on restriction fragment length polymorphisms (RFLPs), but particularly on polymerase chain reaction (PCR) generated markers, such as random amplified polymorphic DNAs (RAPDs) (Williams *et al.*, 1990), microsatellites (SSRs) (Litt and Lutty, 1989) and more recently, amplified fragment length polymorphisms (AFLPs) (Zabeau and Vos, 1993).

In the present study, ten maize inbred lines were assayed for AFLPs using 18 selective primer combinations. Fingerprinting revealed a total number of 1143 unambiguous amplified fragments, ranging in size from 50 to 2700 nucleotides. The number of amplified fragments detected after selective amplification with the different primer combinations varied from 33 to 128 (Fig. 1 and Table 2). The number of polymorphic amplicons across the 10 inbred lines was 902 representing a level of polymorphism of 78.9%. The different primers revealed different number of polymorphic amplicons ranging from 29 to 102 with an average of 50.1 polymorphic amplicon per primer combination. In this respect, Ajmone Marsan *et al.* (1998) analyzed the genetic diversity among 13 maize inbred lines and reported that the number of AFLP polymorphic bands ranged from 19 to 52 with an average of 34.8. While El-Khishin *et al.* (2003) detected 47-118 polymorphic bands with an average of 85.3 among 20 maize inbred lines representing a level of polymorphism of 80.1%. The level of polymorphism revealed by the different primer combinations ranged from 53% in primer combination 1/3 to 100% in primer combination 3/4. Hussein *et al.* (2002a) reported that the level of polymorphism

revealed by six AFLP primer combinations in cotton ranged from 38% to 65%. While, in maize El-Khishin *et al.* (2003) revealed a level of polymorphism ranging from 64.4% to 86.3%. In addition, Adawy *et al.* (2003) claimed that the level of polymorphism in date palm ranged from 17% to 81% as revealed by 28 AFLPs primer combinations.

Genetic distances among inbreds

The scoring data obtained from the eighteen primer combinations were used to determine the genetic similarity among the ten maize inbred lines using the Dice coefficient. These similarity matrices were then used in the cluster analysis to generate a dendrogram using the UPGMA method. A summary of the genetic similarity among lines is presented in Table (3).

The genetic similarity estimates ranged from 65.1% to 80.9%. The highest genetic similarity (80.9) was between inbreds Sd 7 and Gz 649 and between Sd 7 and Gz 603. While, the lowest genetic similarity (65.1%) was exhibited between Sd 62 and Sd 7. These results are in good agreement with the pedigree of these lines. As shown in Table (1) Sd 7 is one of the progenators of Gz 603 only, therefore it contributed to its genetic make-up which was reflected as the closest genetic similarity. While the low similarity value between Sd 7 and Sd 62 reflects their highly divergent origin, where Sd62 is derived from Tipalcinco No.5, while Sd 7 is derived from a cross between American early dent x composite A4.

The genetic relationships were expressed as shown in the dendrogram (Fig. 2) which illustrates the graphical representation of the genetic distances among the ten maize inbred lines. The dendrogram clustered the ten inbred lines into 2 main clusters. One cluster was composed of two groups where Sd 63 and Sd 62 formed one group which reflects their close relationship since both lines were derived from Tipalcinco No.5. The second group comprised Gz 643 and Gz 624. This is also in close correspondence with their pedigree, as they are both resulting from crossing B73 with Sd 62. In the second cluster, Gz 649 and Gz 639 grouped together and Sd 7 grouped with Gz 603, thus, reflecting their pedigree relationships. In this respect, Goulao *et al.* (2001) in apple and El-Khishin *et al.* (2003) in maize reported that the hybrid cultivars sharing common parents tend to group together in cluster analysis, suggesting that the genetic similarity values obtained by molecular markers appear to correspond to the known pedigree information. Moreover, Assem (2001) and El-Itriby *et al.* (2003) studied the regeneration ability of the same inbred lines and showed that Gz 643 and Gz 624 revealed the highest regeneration ability, while Sd 7 and Gz 603 revealed the lowest regeneration ability. Lines Sd 62 and Sd 63 showed moderate response on the callus induction and regeneration media.

Characterization of inbred lines by AFLP unique markers

In the present study, genotype specific AFLP markers were successful in characterizing all the tested inbred lines. As shown in Table (4), the AFLP assay permitted the identification of the different lines by a total of 122 unique positive markers (UPM) and / or 73 unique negative markers (UNM). The total number of UPM per genotype ranged from 3 to 39, while the number of UNM per genotype varied from 1 to 24. The highest number of unique markers (45) was exhibited by the inbred line Sd 7 which was characterized by 39 positive and 6 negative markers. While, the lowest number (4) was revealed by line A188 (3 positive and 1 negative markers). The different primer combinations revealed different numbers of unique positive and or negative markers across the ten inbred lines, thus revealing a unique

fingerprint for each inbred line.

The results obtained in this study point out the usefulness of the AFLP marker system for fingerprinting maize inbred lines with high accuracy. This could be attributed to the high multiplex ratio of the AFLP assay. Different authors reached to the same conclusion concerning the efficiency of the AFLP technology (Negi *et al.*, 2000 in pepper, Degani *et al.*, 2001 in strawberry; Hussein *et al.*, 2002a in cotton, and 2003 in citrus; Adawy *et al.*, 2003 in date palm and El-Khishin *et al.*, 2003 in date palm). Therefore, the use of AFLP's fingerprint in maize breeding programs is highly recommended to assess seed purity, selection of parental lines that could provide the highest heterotic effect, determination of marker assisted selection, prediction of heterotic response of hybrids and protection of breeders rights.

Table (1): Pedigrees of the ten maize inbred lines.

Maize line	Pedigree
[b]Sd 62	Tepalcinco No.5
[b]Sd 63	Tepalcinco No.5
[b]Sd 7	American early dent x composite A4
A188	American line
[a]Gz 643	B73 x Sd 62
[a]Gz 624	B73 x Sd 62
[a]Gz 639	B73 x Sd 62
[a]Gz 649	B73 x Sd 62
[a]Gz 650	B73 x Sd 62
[a]Gz 603	B37 x Sd 7 ([c]Bc1 Sd 7)

[a]Gz = Giza [b]Sd = Sids [c]Bc = backcross

Table (2): Summarized results obtained by AFLP data analysis showing AFLP primer combinations, total number of amplicons, number of polymorphic amplicons and level of polymorphism detected by different primer combinations among the ten maize inbred lines.

Code	Primer combination	Total no. of amplicons	Polymorphic amplicons	Polymorphism %
1/2	AAC/CAC	79	51	64.56
1/3	AAC/CAG	53	36	53.00
1/4	AAC/CAT	75	45	60.00
2/2	AAG/CAC	74	57	77.03
2/3	AAG/CAG	33	29	87.88
2/4	AAG/CAT	51	37	72.55
2/7	AAG/CTG	63	51	63.00
2/8	AAG/CTT	54	45	83.33
3/3	ACA/CAG	52	38	73.10
3/4	ACA/CAT	37	37	100.00
4/4	ACC/CAT	90	78	86.67
4/5	ACC/CTA	65	42	64.62
4/6	ACC/CTC	98	97	98.98
5/4	ACG/CAT	41	30	73.17
5/6	ACG/CTC	41	39	95.12
6/6	ACT/CTC	128	102	79.69
7/8	AGC/CTT	51	41	80.39
8/2	AGG/CAC	58	47	81.03
Total		1143	902	78.90

Table (3): Genetic similarity matrix computed according to Dice coefficient based on AFLP data.

		1	2	3	4	5	6	7	8	9	10
1	Sd 62	100.0									
2	Sd 63	79.4	100.0								
3	Gz 624	74.4	77.9	100.0							
4	Gz 643	74.1	79.8	80.3	100.0						
5	Gz 639	71.5	74.9	76.3	80.8	100.0					
6	Gz 649	69.8	72.0	74.8	78.3	87.3	100.0				
7	A188	68.1	71.3	72.9	73.9	78.1	78.5	100.0			
8	Gz 650	66.6	73.6	73.7	74.2	75.5	75.2	72.0	100.0		
9	Gz 603	65.7	66.0	69.0	72.2	77.2	77.1	75.9	68.4	100.0	
10	Sd 7	65.1	67.4	69.9	72.6	80.8	80.9	78.1	69.2	80.9	100.0

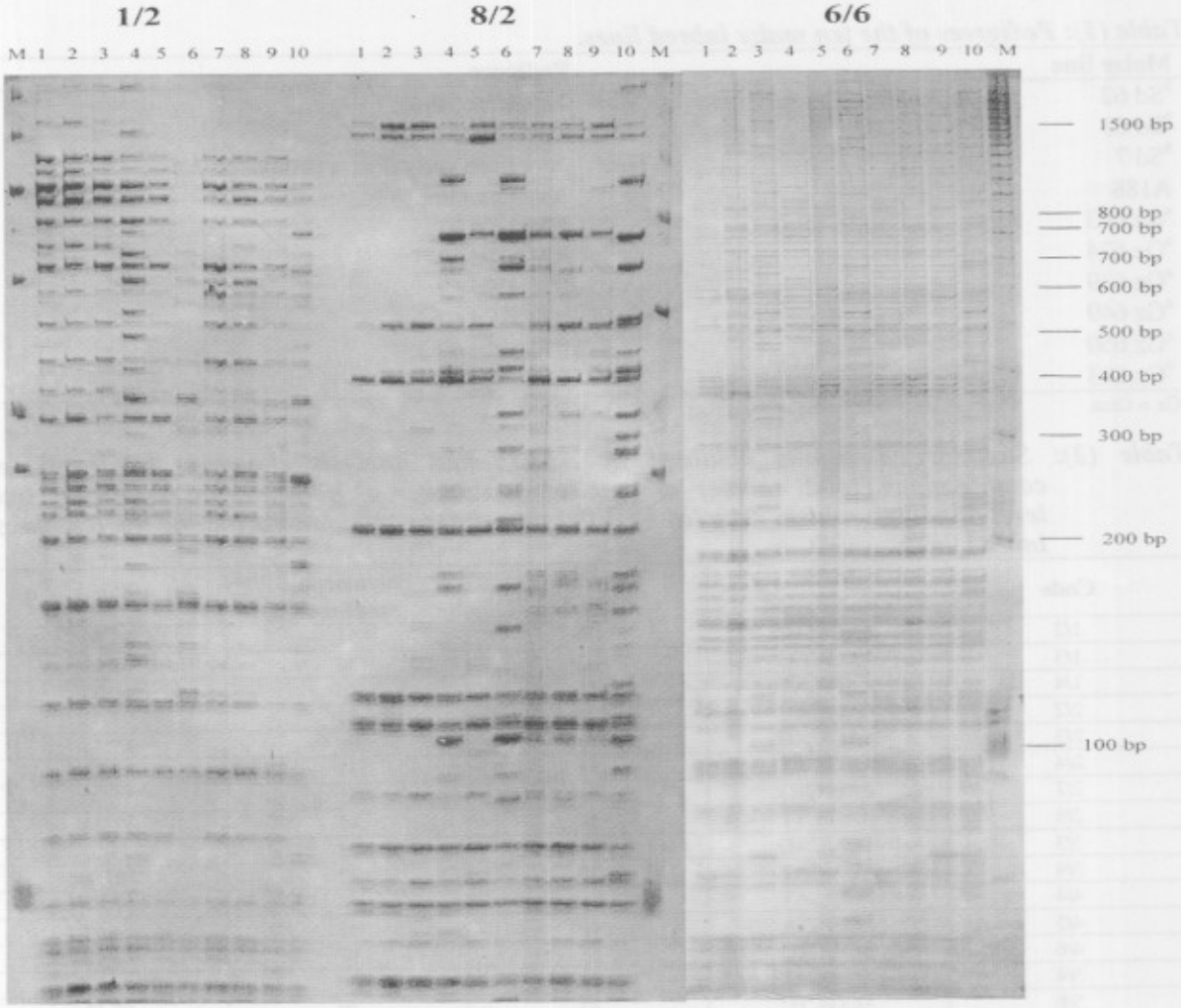

Fig. (1): AFLP profiles of the ten maize inbred lines as revealed by primers combinations 1/2, 8/2 and 6/6. Lanes 1 to 10 refer to inbred lines: Sd 62, Sd 63, G 624, G 643, G 639, G 649, A188, G 650, G 603, Sd 7. M: refers to 100 bp DNA ladder.

Table (4): Unique positive (UPM) and negative (UNM) AFLP markers, markers size and total number of markers characterizing each of the ten maize inbred lines.

Primer comb. code	Sd62		Sd63		Gz 624		Gz 643		Gz 639		Gz 649		A188		Gz 650		Gz 603		Sd 7		Total	
	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM
1/2	780 760		880 790				850 840 580 390 370					835 590 500 490 380							190 120		11	5
1/3															1980 1180 1160				1170 1150 900		6	
1/4							550	175		160								1400	1180		2	3
2/2					690 410			1300 1100	420		200	1350						700 370 360 340 180			4	8
2/7		110 130	80				70 65				135	60							120	220 120 80	5	6
2/3					180		120				300 180					2900 1700				800	4	3
2/4	400				93							80						350	80		3	2
2/8											100				310 290						3	
3/4			148								290 260							190	285 280 270		6	1
3/3		246			311 275 265		225		120		336 110	130							105		8	2
4/5	350 300					330		390 380 220 200 110			200	180					400		710 650 580 420		8	7
4/4	197 196	127			450 390 248		590 580	190							570		380 330	160 150 118 117 113	500 180	180 142	12	9

Cont. Table (4):

Primer comb. code	Sd62		Sd63		Gz 624		Gz 643		Gz 639		Gz 649		A188		Gz 650		Gz 603		Sd 7		Total	
	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM	UPM	UNM
4/6			180 95 60	320 100					320				600		245		130 102 125 122 90 120 50	400 100 170 160 130 90 80 60 50 40	400 340 250 195		17	12
5/4	285								290									240	175		3	1
5/6			200							2150 400 165 100											1	4
6/6		2400 2220 460	200 165 145 130 115 77	695 610 76	710 355 320	230													400 360 290 125 110		14	7
7/8								400			270		490 180	200					400 360 290 120 110		8	2
8/2		320			350														700 220 680 210 400 140		7	1
Total	6	8	16	5	14	2	12	10	4	5	11	10	3	1	7	2	10	42	39	6	122	73

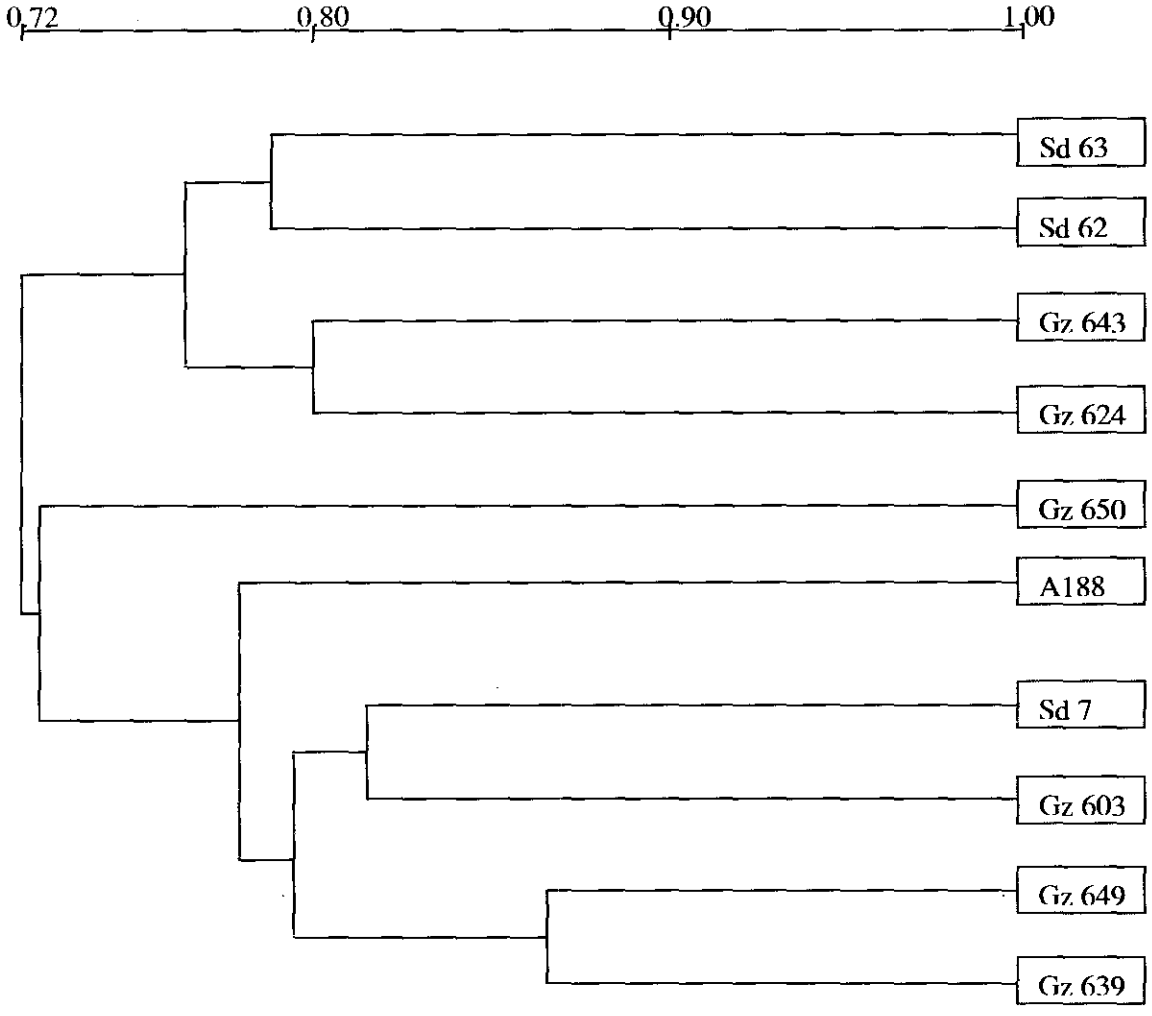

Fig. (2): Dendrogram of 10 maize inbred lines revealed by UPGMA cluster analysis.

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الملخص العربي

تحديد واسمات جزيئية من نوع AFLP و البصمة الوراثية لبعض سلالات من الذرة الشامية

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أجريت هذه الدراسة بهدف تحديد واسمات جزيئية من نوع ال AFLP و تحديد البصمة الوراثية لعشر سلالات من الذرة الشامية حيث تم اجراء تحليل ال AFLP باستخدام ١٨ توليفة من البادئات (EcoRI and MseI) . أظهرت الدراسة ١١٤٣ شظية من ال د.ن.أ. على مستوى السلالات العشرة و كان عدد الشظايا التي أظهرت تبايناً هو ٩٠٢ شظية مما يشير إلى وجود مستوى من التباين يبلغ ٧٨،٩ % . وقد استخدمت المعلومات الناتجة من دراسة التباين الوراثي بهذه التقنية لتقدير درجة التشابه الوراثي بين السلالات المختلفة و ذلك باستخدام معامل دايس و رسم دندروجرام يوضح علاقات القرابة الوراثية بين السلالات المدروسة. و قد فصل الدندروجرام السلالات إلى مجموعتين أساسيتين كل منهما يتكون من مجموعات .