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BIOCHEMICAL AND MOLECULAR IDENTIFICATION OF SIX MAIZE INBRED LINES

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ABSTRACT: The present investigation was done to define the identity of six maize inbred lines using SDS-PAGE protein analysis and some isozymes variability, DNA fingerprinting detected by inter simple sequence repeat (ISSR)-PCR molecular markers. Protein electrophoresis revealed that total number of bands ranged from 12 in the inbred line Sids 1108 to 4 in inbred line Sids 1157 and inbred line Sids 63. Three common bands were found in all tested inbred lines. Two inbred lines; Sids 7 and Sids 1108 showed specific bands which could be used to distinguish them from the others. In addition, bands of two isozymes systems, peroxidase (Prx) and esterase (EST) were determined for the six maize inbred lines based on polyacrylamide gel electrophoresis. The number of bands and R_f values could be used to identify these inbred lines. Eleveen anchored ISSR primers were used for fingerprinting of the six maize inbred lines and obtained 73 scorable bands, 42 of them were polymorphic (58 %). Sids 7 produced two bands with two primers (ISSR 1and ISSR 10). Sids 34 produced three bands with three primers (ISSR 1, ISSR 9 and ISSR 11). Sids 1159 produced three bands with three primers (ISSR 2, ISSR3 and ISSR 8). Sids 1108 produced two bands with two primers (ISSR 6 and ISSR 7). Sids 1157 have one band marker with primer ISSR 11. Also, Sids 63 has one band marker with primer ISSR 3. Existing genetic differences between inbred lines are important from the point of view of the breeder to take advantage in maize improvement programs.

Key words: Maize (*Zea mays* L.) SDS- protein electrophoresis, Isozymes, ISSR- PCR.

INTRODUCTION

Maize (*Zea mays* L.) ranks as one of the world's three most important cereal crops, it is cultivated in a wide range of environments more than wheat and rice because of its greater adaptability (Koutsika-Sotiriou, 1999). Maize is an important cereal crop for food and feed in many parts of the world. In Egypt, maize is grown for food, feed, fodder and industrial purposes. Egypt imports approximately 35% of its maize needs (Khalifa and Zein El-Abedeen, 2000). Genetic diversity plays a key role in crop improvement. In this respect, Atif Elsadig *et al.* (2012) showed that utilized the ISSR primers to access on specific DNA fingerprints will be of high value for maize breeders working in the improvement of the crop. Several analyses of the maize genetic variability have been performed using molecular markers to assess the crop evolutionary aspects (Wu, 2000; Srdic *et al*., 2007; Abuali *et al*., 2011) or to obtain genotype characterization (Gethi *et al*., 2002) or to estimate heterosis among inbred lines. The use of DNA markers, compared to phenotypic aspects, becomes even more important. There are many markers available, among them are the simple sequence repeats (SSR) markers (Tautz, 1989), randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990), and inter simple sequence repeats (ISSR) (Zietkiewicz *et al*., 1994). One of these techniques is inter

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simple sequence repeats (ISSR) technique is considered simple and fast like RAPD, but has more stringency than RAPD. Also, ISSR markers are highly polymorphic, which makes them useful for studies on genetic diversity, phylogeny, genetic coding, genomic mapping and evolutionary biology (Reddy *et al*., 2002). Genetic diversity plays a key role in crop improvement. Atif Elsadig *et al*. (2012) identified genetic diversity in nine maize genotypes using eight primer sets. The results showed that the estimation of genetic distance among maize genotypes by utilization of ISSR markers could be used as a reliable method for the characterization of maize genotypes. The DNA based markers represent a powerful tool in assessment of genetic diversity among maize genotypes. They can be used to identify diverse sources in maize germplasm collections or to select groups of genotypes with desirable characters and contrasting phenotypes, if large numbers are employed. Particularly, genetic distance estimates determined by ISSR markers may help to identify suitable maize germplasm for introgression into breeding stocks. It is recommended that there are 9 genotypes could be used in efficient hybridization breeding programes for the object of increasing levels of genetic polymorphism in maize genotypes. In the present study six inbred lines of maize were assessed for diversity by utilization of 11 ISSR markers, SDS-protein electrophoresis, some isozymes polymorphism variability and combined analysis for six maize inbred lines by useing dendrogram clusters which illustrated the relationship between six maize inbred lines under study that great important for breeding programs.

MATERIALS AND METHODS

Genotypes Seed Samples

Seed samples of six inbred lines of maize were germinated to isolate their DNA. Number, name and source of six maize inbred lines are presented in Table 1.

The genetic materials involved in the present investigation were obtained from Maize Research Dep., Field Crops Research Inst. Giza, Egypt.

Methods of Analysis

The present work was conducted in the Seed Technology Research Department, Field Crops Research Institute, ARC, Giza during November 2015 season. Seeds were germinated in covered, sterilized, disposable Petri dishes containing Whatman filter paper moistened with distilled water.

SDS- protein electrophoresis

Soluble proteins were extracted from seeds based on their molecular weight and Sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE) technique was conducted according to the protocol described by Laemmli (1970) and modified by Studier (1973).

Protein marker range from (205, 97, 66, 36 and 14) kbp. The bands were recorded as either present (+) or absent (-)

Isozymes electrophoresis

Native polyacrylamide gel electrophoresis (Native-PAGE) technique was used to characterize the isozymes fingerprint of six maize inbred lines such as esterase (Est) and peroxidase (Prx). Isozymes fractionation was performed on vertical slab (19.8 cm × 26.8 cm × 0.02 cm) using the gel laconic electrophoresis apparatus according to Jonathon and Wendel (1990). The ingredients of compounds used are shown in Table 2.

Molecular Markers

Plant material and DNA extraction

For genomic DNA isolation, seeds of each of the six maize inbred line were germinated and grown to the four-leaf stage. The seedlings were used for DNA extraction by DNeasy plant minikit (Quigen Inc., Cat. no. 69104 and USA). The DNA concentration of the final samples was measured by ultraviolet (UV) spectrophotometer at 260 nm. The integrity of the DNA was checked by electrophoresis in a 1.2% agarose gel in TAE buffer.

ISSR-PCR analyses

Eleven ISSR primers were evaluated for six maize inbred lines. Name and sequences of primers are shown in Table 3.

Table 1. Name and source of the studied maize inbred lines

Number	Inbred line	Source
1	Sids 7	A.E.DX A4
2	Sids 34	A.E.D
3	Sids 1159	KAH-1
4	Sids 1108	G2EV-8
5	Sids 1157	Var.4
6	Sids 63	Tep.5

Table 2. The ingredients of staining solutions

Enzymes	Compounds and references	Amounts
Prx	A-sodium acetate (1M, pH4.7) Methanol	50 ml
	3,3,5,5 tetramethyl benzidine (TMBZ)	50 ml
	B-0.30 % H_2O_2 (Graham *et al.,* 1964)	2 ml
EST	Sodium phosphate (100 mM, pH 6.0)	50 mg
	α -naphthyl acetate	25 mg
	Fast blue RR salt	50 mg
	Jonathon and Wendel (1990)	

Table 3. Name and sequences of the 11 primers used for ISSR-PCR analyses

Primer name	Sequence
ISSR 1	98b (CACACA)²GT
ISSR 2	44b (CTCTCT) ² CTCTGC
ISSR 3	14a (CTCTCT) ²CTCTTG
ISSR 4	49a (CACACA)²AG
ISSR 6	(GTGTGT)²GC hb9
ISSR 5	hb12 (CAC)³GC
ISSR 7	(GAGAGA)²GG hb8
ISSR 8	hb10(GAGAGA)²CC
ISSR 9	hb15(GTG)³GC
ISSR 10	hb13(GAG)³GC
ISSR 11	hb14(CTC)³GC

Polymerase chain reaction (PCR) conditions

ISSR-DNA amplification was carried out in PCR tubes containing 25 µl reaction mixtures, having 1 µl template DNA, 1 µl ISSR primer, 15 µl of dd H_2O and 7 µl PCR mix. Amplification was carried out in a PTC- 200 thermal cycle (MJ Research, Watertown, USA) programmed as follows: Denaturation, 94°C for 3 minutes, then for 40 cycles. Each cycle consisted of 30 second at 94°C, 1 minute at 40°C, 2 minutes and one minute at 72°C, followed by a final extension time of 12 minutes at 72°C and 4°C (infinitive). Domenyuk *et al*. (2002).

Gel electrophoresis

Gel electrophoresis was applied according to Sambrook *et al.* (1989). The run was performed for one hour at 80 volt in pharmacia submarine (20 × 20 cm). Bands were detected on UV-transilluminator and photographed by gel documentation 2000, Bio- Rad. Fragment sizes of ISSR were estimated from the gel by comparison with the DNA marker fragment of 100 to1.5 kbp ladder marker. The bands were recorded as either present (+) or absent (-)

RESULTS AND DISCUSSION

Protein Electrophoresis [SDS-PAGE]

The SDS-PAGE for the six maize inbred lines are shown in Fig. 1 and Table 4, bands were detected with different molecular weights (MW) (ranged from about 150 kda to 20 kda). The total number of bands between inbred lines ranged from 12 in inbred line Sids 1108 to 4 in inbred line Sids 1157 and inbred line Sids 63. While inbred line Sids 7 had 6 unique bands, inbred line Sids 34 had 5 unique bands and inbred line Sids 1159 had 7 unique bands.

Furthermore, the different tested inbred lines were also varied in molecular weight (MW) of their protein fractions. The molecular weight (MW) of the lightest protein band was 20 kilo dalton (kda.) in all tested inbred lines, whereas the heaviest protein band was about 150 kilo dalton (kda.) in the inbred lines. Based on SDS-PAGE of protein, the inbred line Sids 1157 and inbred line Sids 63 were identical, *i.e.,* they have the same banding patterns. There are three common bands that were found in all tested inbred lines, which have molecular weight (MW) of 65, 30 and 20 kda. Band which has MW about 90 kda was found in inbred line Sids 7 considered as positive specific marker for this inbred line, which was present only in this inbred line but absent in all other inbred lines under study, while bands of MW about 110 and 100 were considered as positive specific markers for inbred line Sids 1108. All the proteins from MW of the tissue are present in the gel, so that individual enzymes must be identified using an assay that links their function to a staining reaction. Stephan *et al*. (1992) found that a major percentage of protein variation can be explained by variation in neutral mutation rate and a minor percentage by strong selection. The recombination of few individuals, *e.g.* ten S1 lines, during the recurrent selection program carried out in EPS6 and EPS7, may have reduced variability and changed genetic distances due to random drift. Osman *et al*. (2013) studied genetic relationship between some species of *Zea mays* and sorghum was determined using SDS-PAGE of seed protein and RAPD-PCR marker.

Isozymes Electrophoresis

One of the aims of this study was to use two different isozymes systems, peroxides (Prx) and esterase (EST)) for identification of six maize inbred lines under investigation based on polyacrylamide gel electrophoresis profiles.

Peroxidase (Prx)

Results in Fig. 2 and Table 5 show that all of the six maize inbred lines contained six peroxidase bands. The peroxides produced different number of bands and different R_F values of the bands. Hence, these values can be adequately used for identification among these inbred lines. Peroxidase produced two monomorphic bands in R_F 0.223 and 0.351, Also produced three marker bands for three different inbred lines, R_F 0.242 considered as positive specific marker for inbred line Sids 1159 while R_F 0.441considered as positive specific marker for inbred line Sids 7, also R_F 0.691 considered as positive specific band marker for inbred line Sids 1108; all three different R_F bands were present for these inbred lines but absent in all other inbred lines under study.

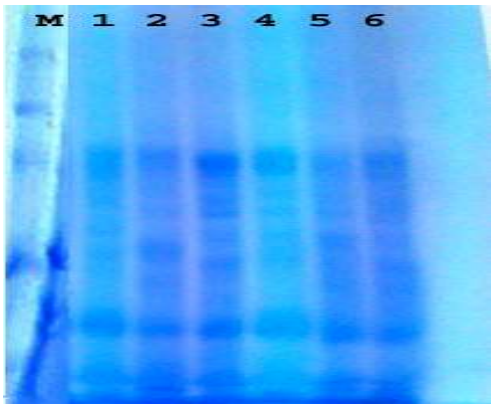
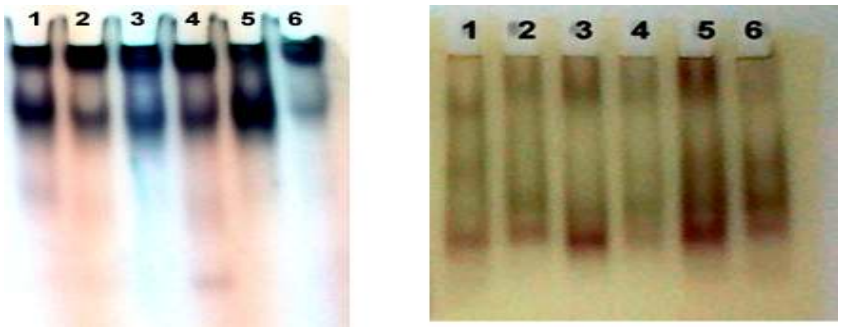


Fig. 1. SDS-PAGE for the six maize inbred lines

Table 4. Molecular weight (MW) of SDS-PAGE protein electrophoresis for six inbred lines

Band number	MW	1-Sids 7	2-Sids 34	3-Sids 1159	4-Sids 1108	5-Sids 1157	6-Sids 63
1	150	+	+	+	-	-	-
2	120	-	-	-	+	+	+
3	110	-	-	-	+	-	-
4	100	-	-	-	+	-	-
5	90	+	-	-	-	-	-
6	80	+	-	-	+	-	-
7	75	-	+	-	+	-	-
8	65	+	+	+	+	+	+
9	50	-	-	+	+	-	-
10	40	-	-	+	+	-	-
11	35	-	-	+	+	-	-
12	30	+	+	+	+	+	+
13	25	-	-	+	+	-	-
14	20	+	+	+	+	+	+
Total bands		6	5	7	12	4	4



(Prx) **(EST)**

Fig. 2. Peroxidase (Prx) and Esterase (EST) isozyme polyacrylamide gel electrophoresis profiles in six maize inbred lines

Table 5. R_F of peroxidase isozyme (Prx) bands in six maize inbred lines

R_F	1-Sids 7	2-Sids 34	3-Sids 1159	4-Sids 1108	5-Sids 1157	6-Sids 63
0.223	+	+	+	+	+	+
0.242	-	-	+	-	-	-
0.351	+	+	+	+	+	+
0.441	+	-	-	-	-	-
0.539	-	+	-	+	-	-
0.691	-	-	-	+	-	-

Esterase (EST)

Results in Fig. 2 and Table 6 show that all of the six maize inbred lines contained six esterase bands. The esterase produced six different number of bands and different R_F values of the bands and no monomorphic bands. Esterase produced two marker bands for one inbred line Sids 7, band R_F 0.241 and R_F 0.505 these two bands considered as negative specific marker for inbred line Sids 7, while band R_F 0.301 considered as positive specific marker for this inbred line. R_F 0.432 was present in two inbred lines *i.e.,* Sids 7 and Sids 63, also band with R_F 0.589 was present in two inbred lines *i.e.,* Sids 7 and Sids 1159, while band with R_F 0.573 was absent in two inbred lines, Sids 7 and Sids 1159, but was present in all other inbred lines under study. Isozymes are useful tools to study the genetic structure of populations (Hattemer, 1991; Richardson *et al*., 1990). This conclusion inferred from isozyme results confirms the lack of inbreeding depression revealed by agronomic data and it is in agreement with some reports where inbreeding depression was not detected after several cycles of selection using selfed lines (Helms *et al*., 1989; Holthaus and Lamkey, 1995; Lamkey, 1992).

Molecular Marker

In the present study, 11 primers of ISSR were selected to differentiate among six inbred lines. These primers produced multiple bands, which ranged from 10 bands for primer ISSR 8,9 and 11 to 3 bands for primers ISSR 4 and ISSR 7. The total number of bands were 73, 42 of them were polymorphic (58%). The highest level of polymorphism was observed in primer ISSR 4 which showed 100% polymorphism, while the lowest polymorphism was 33% in primer ISSR 10 as shown in Table 7 and Fig. 3.

Primer ISSR1 produced 5 bands, 2 of them were polymorphic (40% polymorphism). This analysis indicated the presence of two markers for two inbred lines Sids 7 and Sids 34. Primer ISSR2 showed 4 bands, 3 of them were polymorphic (75% polymorphism), this primer (ISSR2) indicated the presence of one marker for inbred line Sids 1159. Primer ISSR3 showed 6 bands, 4 of them were polymorphic (67% polymorphism). This analysis indicated the presence of two markers for two inbred lines Sids 1159 and Sids 63. Primer ISSR4 revealed 3 bands, 3 of them were polymorphic (100% polymorphism) with no marker for this primer. Primer ISSR5 revealed 9 bands, 7 of them were polymorphic (78% polymorphism), also no marker for this primer. Primer ISSR6 produced 4 bands, 2 of them were polymorphic (50% polymorphism) indicated the presence of one marker for inbred line Sids 1108. Primer ISSR7 produced 3 bands, 2 of them were polymorphic (67% polymorphism), this primer has one marker for inbred line Sids 1108. Primer ISSR8 produced 10 bands, 6 of them were polymorphic (60% polymorphism) indicated the presence of one marker for inbred line Sids 1159. Primer ISSR9 produced 10 bands, 5 of them were polymorphic (50% polymorphism) this primer has two markers for sids 34. Primer ISSR 10 revealed 9 bands, 3 of them were polymorphic (33% polymorphism) this primer has one marker for Sids 7. Primer ISSR 11 produced 10 bands, 5 of them were polymorphic (50% polymorphism) indicated three markers for two different inbred lines, Sids 34 and Sids 1157.

Table 6. R_F of esterase isozyme (EST) bands in six maize inbred lines

R_F	1-Sids 7	2-Sids 34	3-Sids 1159	4-Sids 1108	5-Sids 1157	6-Sids 63
0.241	-	+	+	+	+	+
0.301	+	-	-	-	-	-
0.432	+	-	-	-	-	+
0.505	-	+	+	+	+	+
0.573	-	+	-	+	+	+
0.589	+	-	+	-	-	-

Table 7. Levels of polymorphism and unique inbred lines specific bands for six maize inbred lines identified by 11 ISSR primers

Primer	Total band	Polymorphic band	Monomorphic band	Polymorphic (%)	Specific band	
					Inberd line	MW (bp)
ISSR 1	5	2	3	40	1-Sids 7	170
					2-Sids 34	380
ISSR 2	4	3	1	75	3-Sids 1159	980
ISSR 3	6	4	2	67	3-Sids 1159	320
					6-Sids 63	70
ISSR 4	3	3	0	100		
ISSR 5	9	7	2	78		
ISSR 6	4	2	2	50	4-Sids 1108	350
ISSR 7	3	2	1	67	4-Sids 1108	330
ISSR 8	10	6	4	60	3-Sids 1159	420
ISSR 9	10	5	5	50	2-Sids 34	80, 40
ISSR 10	9	3	6	33	1-Sids 7	550
ISSR 11	10	5	5	50	2-Sids 34	880, 220
					5-Sids 1157	100
Total	73	42	31	58		

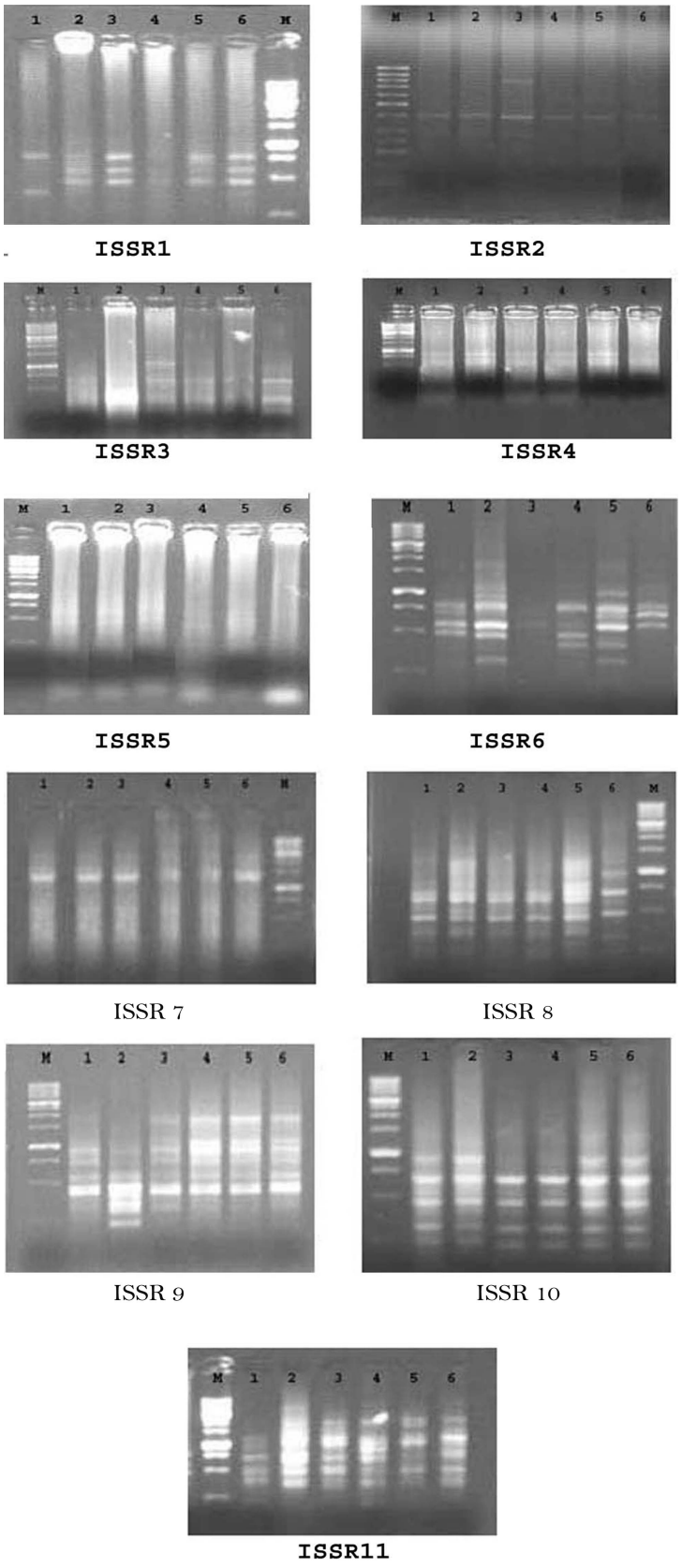


Fig. 3. ISSR banding patterns amplified with 11 primers

ISSR markers can produce three number of markers as observed in inbred line Sids 1159 which was identified by 3 primers at different molecular weights (MW), one of them in primer ISSR 2 (980 bp) and the other of primer ISSR 3 (320 bp) and the last one band in primer ISSR 8 (420 bp). Also inbred line Sids 34 showed three band markers, two of them in primer ISSR 9 (80 and 40 bp) and one in primer ISSR 1 (380 bp). Inbred line Sids 7 recorded two bands in two different primers, ISSR 1 (170 bp) and ISSR 10 (550 bp). Also inbred line Sids 1108 has two band markers, one of them in primer ISSR 6 (350 bp) and the other in primer ISSR 7 (330 bp). Inbred line Sids 63 produced one marker at primer ISSR 3 (70bp) as well as inbred line Sids 1157 has one marker at primer ISSR 11 (100 bp). The previous genetic variability among maize inbred lines is very important to plant breeder in hybrid maize breeding programs for producing F1 hybrid with heterotic effect. Osipova *et al*. (2003) used RAPD and ISSR markers to analyze the genetic divergence between the regenerated plants derived from callus cultures and the original maize line A188. Analysis of polymorphism by using 38 RAPD- and 10 ISSR-oligonucleotide primers showed that the differences between eight examined somaclones and the original line ranged from 6.5 to 23%. Atif Elsadig *et al*. (2012) used Inter-simple sequence repeat (ISSR) markers to assess genetic diversity in a selected group of maize inbred (*Zea mays* L.) genotypes. A high level of polymorphism of 69% was detected among these genotypes. The ISSR primers showed 10 fingerprints for six genotypes out of nine studied which are Frantic, Huediba-1, Balady, Huediba-2, Giza 2 and Mogtamaa 45-2. The maximum genetic distance of 0.48% was detected between Huediba-2 and Mogtama- 45-2. While, the minimum genetic distance of 0.16% was observed between Giza-2 and Var.113. The results indicated that variation can be attributed to the use of ISSR.

Combined Analysis for Six Maize Inbred Lines

Similarity index and dendrogram across the six maize inbred lines under investigation based on ISSR analyses are shown in Table 8 and Fig. 4, respectively. The comparison revealed that the highest closely related inbred lines were inbred line Sids 1159 and Sids 1108 (similarity matrix of 861), followed by inbred line Sids 1157 / inbred line Sids 63 (similarity matrix of 857). The lowest relationships were recorded for inbred line Sids 7/inbred line Sids 34 (similarity matrix of. 0.737). These inbred lines resulted in two main clusters. One of them involved the inbred line Sids 7, while the second cluster involved the rest of the inbred lines. The second cluster was divided into two subclusters, one included inbred line Sids 34, while the other subcluster involved inbred line Sids 1159, inbred line Sids 1108, inbred line Sids 1157and inbred line Sids 63. Ramakrishnan *et al*. (2014) used inter simple sequence repeats analysis to reveal that 17 Indian genotypes of maize were assessed for their regeneration potential. The similarity matrix pair-wise value was 1, the Mantel test value was p 1.0; analysis of molecular variance and genetic variances were 93% within the population and 7 % between populations.

Table 8. Similarity matrix among six maize inbred lines using ISSR analysis

Inbred lines	1-Sids 7	Sids 34	Sids 1159	Sids 1108	Sids 1157
2-Sids 34	0.737				
3-Sids 1159	0.791	0.766			
4-Sids 1108	0.805	0.841	0.861		
5-Sids 1157	0.766	0.842	0.817	0.848	
6-Sids 63	0.838	0.774	0.804	0.837	0.857

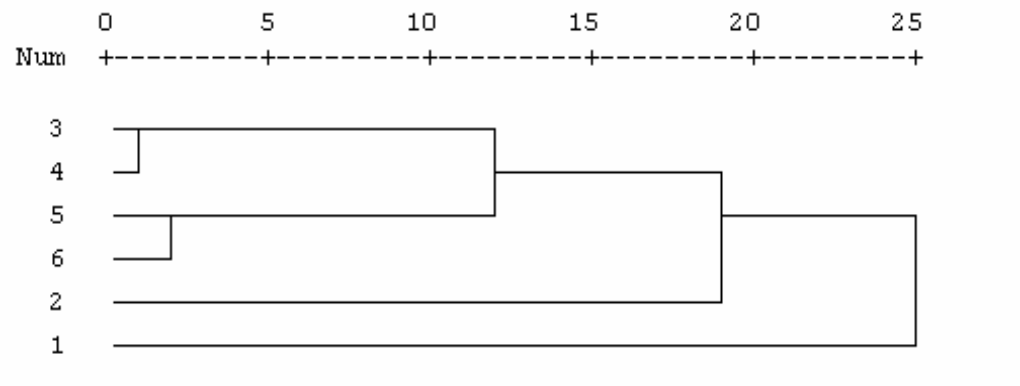


Fig. 4. Dendrogram resulting from the analysis of eleven ISSR primers showing the relationships among the six maize inbred lines

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التوصيف البيوكيميائى والجزيئى لست سلالات من الذرة الشامية

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أجريت هذه الدراسة فى المعمل للتمييز بين ستة تراكيب وراثية من الذرة الشامية باستخدام التفريد الكهربى للبروتين وأثنين من المشابهات الإنزيمة وهى البيروكسديز والاستيريز وتكنيك ISSR-PCR وكانت النتائج المتحصل عليها من الدراسة بأستخدام تكنيك التفريد الكهربى للبروتين تراوحت عدد الحزم البروتينه بين 12 فى سدس 1108 إلى 4 فى سدس 63 وسدس 1157 وهناك ثلاث علامات وراثية مشتركة بين السلالات تحت الدراسة وهناك اثنين من السلالات الوراثية بهم علامات جزيئية مميزة لهم و هم سدس 7و سدس 1108، باستخدام المشابهات الانزيمية البيروكسديز والاستيريز للتفرقة بين السلالات تحت الدراسة وقد أظهرت نتائج مختلفة فى R_F تميز بين هذه السلالات، بأستخدام 11 بادي من ISSR-PCR أظهرت النتائج 73 علامة جزيئية منهم 42 مختلفة بنسبة 58% كما وجد أن سدس 7 به علامتين جزيئيتين فى اثنين من البادئات (ISSR 1 and ISSR 10) ، سدس 34 به ثلاث علامات جزيئية فى ثلاث بادئات، (ISSR 1, ISSR 9 and ISSR 11) سدس 1159 به ثلاث علامات جزيئية فى ثلاث بادئات، (ISSR 2, ISSR 3 and ISSR 8) سدس 1108 به علامتين جزيئيتين في أثنين من البادئات، (ISSR 6 and ISSR 7)، سدس1157 به علامة جزيئية واحدة فى البادي (ISSR 11)، وسدس 63 به علامة جزيئية واحدة فى البادي (ISSR 3)، وتمثل هذه الأختلافات أهميه من وجهة نظر مربى النبات للاستفادة بها في برنامج تحسين الذرة الشامية.

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