

DETERMINATION GENETIC DIVERSITY OF INBRED LINES AND HYBRIDS OF MAIZE USING ISSR TECHNIC

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ABSTRACT

This study was carried out in the biotechnology laboratories of the Seed Inspection and Certification Department - Ministry of Agriculture during 2019, to study the relation among molecular five inbred lines with hybrid crosses of maize. to estimate the genetic diversity and determine the degree of genetic similarity among them using the ISSR based on PCR technic with 6 primers. All primers produced 79 bands at a rate of 13.2 bands per primer. The number of amplified bands ranged from 10 to the primer UBC 835 and 16 in the UBC 897, with a molecular length ranged between 100 - 1700 bp. The total number and percentage of polymorphic bands was 71 and 90% respectively. The primers UBC 856 and UBC 866 the highest percentage of had polymorphic bands of 100%. While the UBC 835 showed the lowest (70%). The highest efficiency and specificity were 12.7% and 9.9% respectively in UBC835. Based on molecular marker data and genetic similarity using the UPGMA method in establishing the dendrogram, the study showed that genetic similarity between the inbred lines ranged from 0.105 to 0.40, the highest (0.40) between the two strains BK121 and Zm6 , While the lowest degree of similarity (0.105) was between the two strains BK121 and ART-817 .

Keywords: PCR Technology, polymorphism, primers, bands

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تحديد التباعد الوراثي لسلاسل وهجن من الذرة الصفراء بتقنية ISSR

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باحث

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المستخلص

نفذت الدراسة في مختبرات التقنيات الحيوية التابعة الى دائرة فحص وتصديق البذور - وزارة الزراعة خلال عام 2019 . بهدف دراسة التغيرات الجزيئية بين 5 سلالات مع هجنها التبادلية لنبات الذرة الصفراء . لتقدير علاقات التباعد الوراثي وتحديد درجة القرابة الوراثية فيما بينهم ، أستعملت تقنية ISSR المعتمدة على تقنية ال PCR و 6 من البادئات, أنتجت جميع البادئات الستة 79 حزمة بمعدل 13.2 حزمة للباديء الواحد تراوح عدد القطع المكوثة بين 10 للباديء UBC 835 و 16 في الباديء UBC 897 و بوزن جزيئي تراوح بين 100 – 1700 bp . بلغ العدد الكلي والنسبة المئوية للقطع المتباينة 71 و 90% بالتتابع. اعطى البادئان UBC 856 و UBC 866 أعلى نسبة مئوية للقطع المتباينة بلغت 100% بينما أظهر الباديء UBC 835 أوطأ نسبة مئوية للقطع المتباينة بلغت 70%, بلغت أعلى كفاءة وأعلى مقدرة تمييزية 12.7% و 9.9% بالتتابع في الباديء UBC835. اعتماداً على بيانات المؤشر الجزيئي والتشابه الوراثي بطريقة UPGMA في مخطط الصلة بينت الدراسة ان التشابه الوراثي ودرجة القرابة الوراثية بين السلالات تراوحت ما بين 0.105 – 0.40 كان اعلاها (0.40) بين السلالتين BK121 و Zm6 , في حين كان اقل درجة قرابة وراثية 0.105 بين السلالتين BK121 و ART-817 .

كلمات مفتاحية: التغيرات الجزيئية، PCR، البادئات، أحزمة

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INTRODUCTION

Maize (*Zea mays* L.) is considered as an important source of food for humans and livestock, as well as an important source of income for millions of people in the world (19), as well as the contents of the grains to starch, protein, oil, vitamins and minerals, in addition to biofuels (1, 2 and 4). Hybrid production was described as the agricultural revolution and the greatest event in plant breeding, and the vigor of the hybrid depends mainly on genetic diversity among parents. Specialists seek to find the best hybrids through the screening of the best parents to achieve the highest hybrid vigor. Most plant breeders' decisions are based on data of phenotypic traits in the case of traditional breeding programs, which makes it liable to many challenges and the impact of environmental conditions on those traits, which may lead to inaccurate conclusions sometimes, and thus still. There is a great opportunity to promote traditional genetic applications which have some sort of environmental independence (3,9,15,18). DNA indicators are a great importance for biochemical and morphological indicators because they are not affected by the environment and depend on the sequence of genes or genetic material, the difference in the genetic material carried by the individual can be detected. Biotechnologies are currently one of the most important tools for improving crop production and food production. This technique has become known and was applied in many developed countries. The use of biotechnology at the molecular level of DNA was accelerated crop improvement. As it helps in the processes of selection and breeding shorter than that of the conventional breeding processes (12,13,14). Molecular indicators, which are highly independent of environmental influences, have been proposed based on the individual genome to determine genetic diversity; Molecular markers have made significant contributions to the discovery of genetic diversity, including ISSR (Inter Simple Sequence Repeats), which is one of the most important technologies that depend on polymerase chain reaction (PCR). It has ability to detect a large number of multi-template strands for each primer with ease of

use with high efficiency and does not require prior knowledge with the DNA sequence of primers (15, 17, 20). This study was aimed to identify the genetic diversity among five inbred line fingerprint of 5 maize pure lines, i.e., the molecular characterization of the resulting strains and hybrids using the ISSR index and the genetic similarities between the strains and hybrids.

MATERIALS AND METHODS

In this study, five inbred lines of maize, genetically stable and locally derived were used and numbered from 1 to 5 as follows : 1. BK121, 2. ABS6, 3. BK116, 4. ART-817, 5. Zm6. These inbred lines were used in a half-diallel crossing program for single cross hybrids breeding. Samples were kindly supplied by the Department of Agricultural Research Inspection and Certification of Seeds, Abu Ghraib. The five inbred line and the ten crosses were planted at the fields of the Research Center (For the purpose of obtaining samples) Leaves were taken from the 15 strains and crosses. Samples were analyzed in the Inspection and Certification of Seeds laboratory.

DNA isolation of the genome

DNA was isolated from the young leaves of the studied hybrids and strains. with quantity of 50-150 micrograms per 1.5 g of leaves was obtained for each genotype of maize with a purity of 1.7-2 measured by Nanodrop. The dilution of the DNA samples was adjusted to obtain 50 ng.microliter⁻¹ concentrations, which is the appropriate for conducting PCR reactions. There are several methods for isolating nucleic acids from plants because plants with their diversity contain different amounts of plant compounds such as proteins and multiple polysaccharides, in addition to nucleic acids. Inter Simple Sequence Repeats (ISSR) which technique was used, based on PCR technology, and 6 primers were suitable for all plants (Table 1). The method of Weigand and Udupa, (21) was adopted to isolate the DNA from maize plant. The process of isolating DNA from plants is relatively more difficult than other organisms because of the thick wall surrounding cell membrane, as well as some plants containing a large amount of phenolic substances and polysaccharides, which are considered as contaminants,

sometimes deposited with DNA, giving high viscosity liquid and inhibiting PCR reactions. In order to get rid of these substances, DNA dilution was performed to reduce the percentage of inhibitory sugars.

ISSR Application

In this study, 6 primers were used, obtained through their importation from Biobasic-Canada, Table 1 shows the Nucleotide Sequence and their codes of the primers used in the study.

Table 1. Nucleotide Sequence of the primers used in ISSR

Primer code	Nucleotide Sequence 3 – 5	No.
UBC834	AGAGAGAGAGAGAGAGYT	1
UBC835	AGAGAGAGAGAGAGAGYC	2
UBC841	GAGAGAGAGAGAGAGAYC	3
UBC856	ACACACACACACACACG	4
UBC866	AGCAGCAGCAGCAGCAGC	5
UBC897	ACACACAGAGAGAGAGAC	6

The PCR reaction was performed according to Williams and Tingey, (22) with some modifications. The final reaction volume (25ul) using X2 Master Mix was obtained from Biobasic Co. Canada. The reaction consisted of 2ul of the primer at a concentration of 10m μ , 12.5 ul of Master mix, 9ul of distilled water, and DNA at the concentration of 40 ng.ul-1. This reaction was done using thermo-rotation system according the following conditions:

1. Partition: At a temperature of 94 °C for 5 minutes to separate the two DNA sequences
2. 40 cycle, each includes the following stages:
 - 2-1 Separation at 94°C for 30 sec.
 - 2-2 Coalescence: according to the temperature of each primer, from Table 1, for 1 minute.
 - 2-3 Elongation at 72°C for 1 minute.
- 3- Reaction completion at 72°C for 1 minute
- 4- The samples were stored at 4°C, and then deported on Acaruz gel.

Electrophoresis, pigmentation and imaging

Electrophoresis was done on Acaruz gel (2%) into the buffer solution TBE 1x.

TBE 1x = (10x TBE buffer = 108g Tris borate +55g Boric acid + 9.2 EDTA, ph 0.8)

Plus ul 5 of the ethidium bromide dye (10 mg. M l⁻¹), where DNA sample were loaded on Acaruz gel by adding 5ul of the special loading liquid (Bromophenol blue 1x) loading buffer). 1kpb DNA, from Geneaid Co., was

injected to determine the volume and molecular weight of the resulted bands, then deporting by passing through electric field of 100 v to separate DNA bands resulted from the amplification, then imaging the gel by the image analyzer (Eagle Eye II Stratagene).

Molecular and statistical analysis

The results of the multiplication processes of the primers used in the ISSR indicators were taken by comparing the presence and absence of DNA bands in the different samples, when the DNA is present, it represented by number 1 and absence by the number 0. The coefficient of genetic distance as well was calculated as the coefficient of similarity between the inbred lines and crosses using the coefficient of 72 s' Nei and lie's (17). Cluster analysis was used and dendrogram between the inputs using the UPMA method (17).

$$GD = 1 - \left[2x \left(\frac{N_{ij}}{N_i + N_j} \right) \right]$$

N_{ij}: - represents number of bands shared between i and j

N_i: represents number of packets in model i

N_j: - represents number of bands in model j

The calculation of the ratio of the different bands in the primers is based on the equation:

$$\text{Polymorphism\%} = \left(\frac{N_p}{N_t} \right) \times 100$$

N_p: - Number of different bands in the primers

N_t: - Total number of bands in the primers

Calculate the percentage of efficiency of primers used were:

$$= \frac{\text{Total number of primer bands}}{\text{Total number of bands of all primers}} \times 100$$

Calculation percentage of the specificity of each primer:

$$= \frac{\text{Number of Polymorphism bands for the primer}}{\text{Number of Polymorphism bands for All primers}} \times 100 \dots \text{by (2)}$$

RESULTS AND DISCUSSION

The results of five primers in ISSR reactions showed a difference in the number of amplified bands and molecular size depending on the primer used, resulting from the difference in the number of sites complementing this primer in the genome of each genotype of maize included in this study. Having achieved a remarkable success, it is worthy to detect genetic diversity. This is consistent with other researchers (6, 7, 8, and 11) differences.

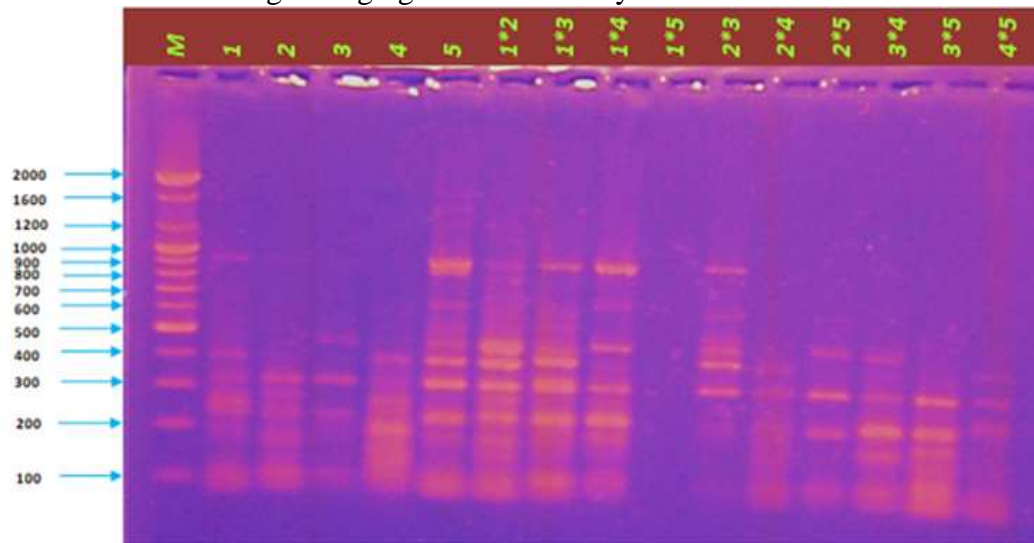
Table 2. Primers and the number of bands produced and polymorphism and their ratios with the efficiency and specificity and molecular weight for each primer

Primer	Bands number	Polymorphism	Polymorphism %	efficiency of primers %	Specificity %	Molecular weight bp
UBC 834	13	11	%84.6	%16.5	%15.5	1200-100
UBC 835	10	7	%70	%12.7	%9.9	600-100
UBC 841	11	9	%81.8	%13.9	%12.6	1600-200
UBC 856	15	15	%100	%18	%21.1	1100-100
UBC 866	14	14	%100	%17.7	%19.7	1200-100
UBC 897	16	15	%93.7	%20.2	%21.1	1700-100
total	79	71	%90			1700-100

Primers used**1. Primer UBC 834**

Thirteen bands were obtained, so the efficiency was 16.5% and the number of polymorphism bands was 11, recording a percentage of polymorphism 84.6%. This polymorphism bands was reflected in the specificity and reached 15.5%, which enabled the primer to identify the complementary sequences in the genomic DNA of the inbred lines and hybrids, showing a marked variation in location and molecular weight ranging from

100-1200 bp. Bands with a molecular weight of 300 bp appeared in all genotypes except 1x5 hybrid, where the primer had no complement and thus no bands were appeared. It is possible to invest in the absence of any bands on the Agarose gel using this primer to determine the classification of maize after an unknown classification after introduced to ISSR reactions, and then the appearance of any bands is evidence that the outstanding pure lines or the hybrids is not a hybrids under study.

**Figure 1. Bands produced by UBC 834 characterizing genetic diversity between five inbred lines and hybrid crosses in maize using ISSR indicators on Agarose****2. Primer UBC 835**

Ten bands were obtained, the efficiency decreased to 12.7% and the number of Polymorphism bands was 7, where the percentage of Polymorphism bands was 70%. This polymorphism bands was reflected in the specificity reached 9.9%, the primer was able to identify the complementary sequences in DNA genomes for inbred lines and hybrids

showing a clear variation in location and molecular weight ranging from 100-600 bp. In Figure 2 it is clear that the bands with a molecular weight of 500 bp appeared in most inbred lines and hybrids, with the smallest number of bands was in hybrids (2x4) and (2x5). Inbred lines 3 and 4 were characterized the absence of amplified bands.

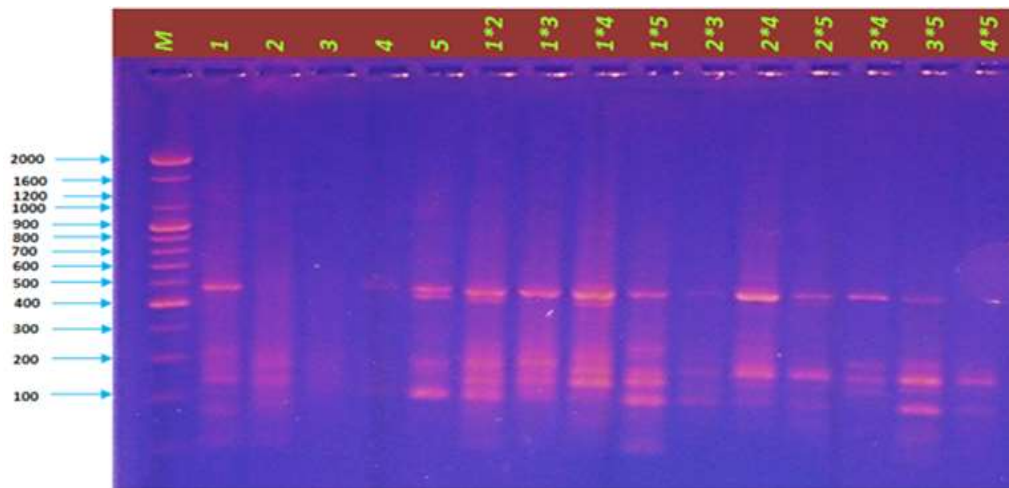


Figure 2. Bands produced by UBC 835 characterizing genetic diversity between five inbred lines and hybrid crosses in maize using ISSR indicators on Agarose

3. Primer UBC 841

This primer showed 11 bands, with from efficiency 13.9%. among these nine were Polymorphism bands among the inbred lines and the studied hybrids, achieving 81.8% of the bands that appeared in the primer, with 12.6% specificity. The primer UBC841 identified the complementary sequences of

DNA in the genome. It is clear from Figure (3) that the lowest number of bands was obtained in inbreds 1 and 2 and hybrids 2x5 while the highest number of bands appeared in the 1x5 hybrids Inbred lines 2 and 4 were characterized by the absence of amplified bands. The hybrids 1x4 and 2x4 share one band at 250 bp.

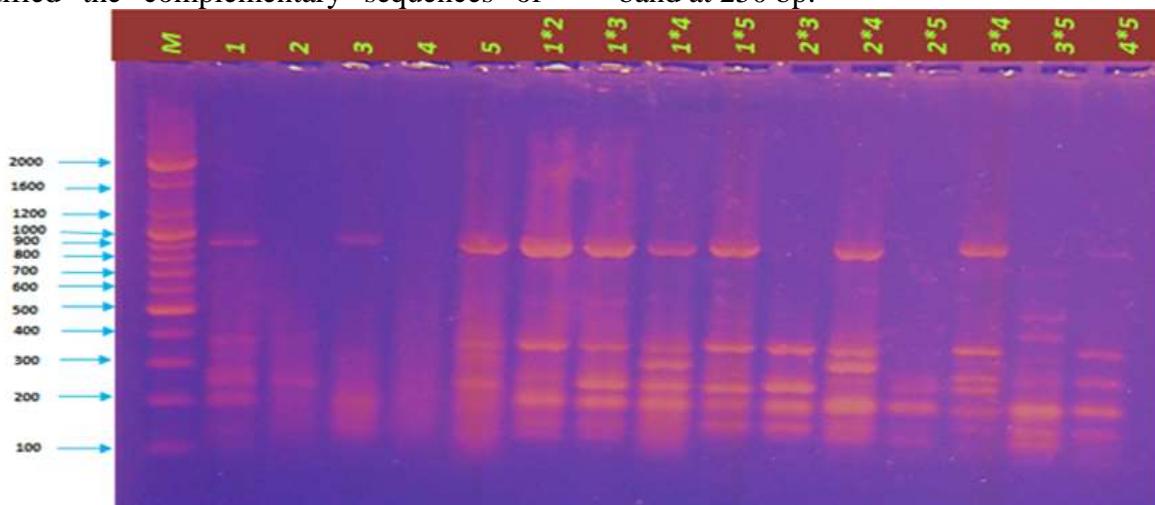


Figure 3. Bands produced by UBC 841 characterizing genetic diversity between five inbred lines and hybrid crosses in maize using ISSR indicators on Agarose

4. Primer UBC 856

The total number of bands reached 15 bands, all primer were Polymorphism with 100% Figure 4 shows that the molecular size of the bands exhibited in this primer was between 100-1000 bp. The hybrid 1x3 showed the highest number of bands (5), while two bands

appeared in the 4x5 hybrid while the hybrid with out any bands. This means that the primer did not find the sequence complementary and thus can be considered a fingerprint for this hybrid. The specificity of this primer achieved 21.1%.

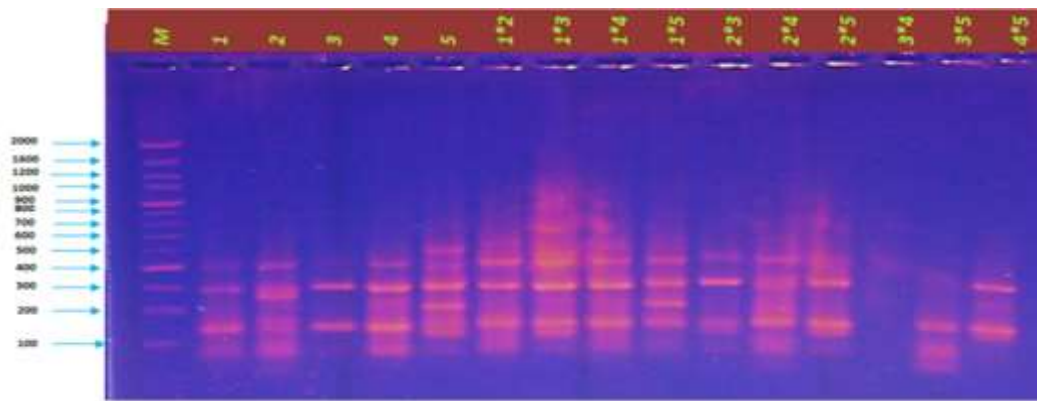


Figure 4. Bands produced by UBC 856 characterizing genetic diversity between five inbred lines and hybrid crosses in maize using ISSR indicators on Agarose

5. Primer UBC 866

The results of was primer multiplication showed 14 bands Table 2 all were Polymorphism, thus the percentage of Polymorphism bands was 100%. The molecular size ranged between 100-1200 bp. The total Specificity was 19.7%. The primer

UBC866 identified the complementary sequences of DNA in the genome. It is clear from Figure (5) that the lowest number of bands was obtained in hybrids 1x3 and 3x5 while the highest number of bands appeared in the 2x4 and 4x5 hybrids

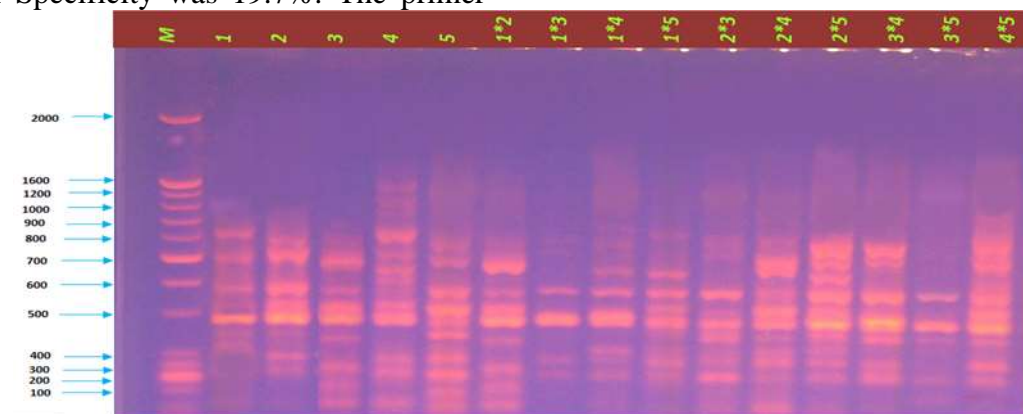


Figure 5. Bands produced by UBC 866 characterizing genetic diversity between five inbred lines and hybrid crosses in maize using ISSR indicators on Agarose

6. Primer UBC 897

DNA was synthesized for the genotypes involved in the study, producing approximately 16 bands , 15 were Polymorphism, thus the percentage of Polymorphism bands was 93.7%. The molecular size of the bands identified in this

primer was 100-1700 bp. Figure (6) shows the possession of a number of hybrids 1x4, 2x4 and 4x5 as well as inbred 1 number of link sites up to 6 sites. While the lowest number of link sites was one in strain 3, while no special band appeared. The total Specificity was 21.1%.

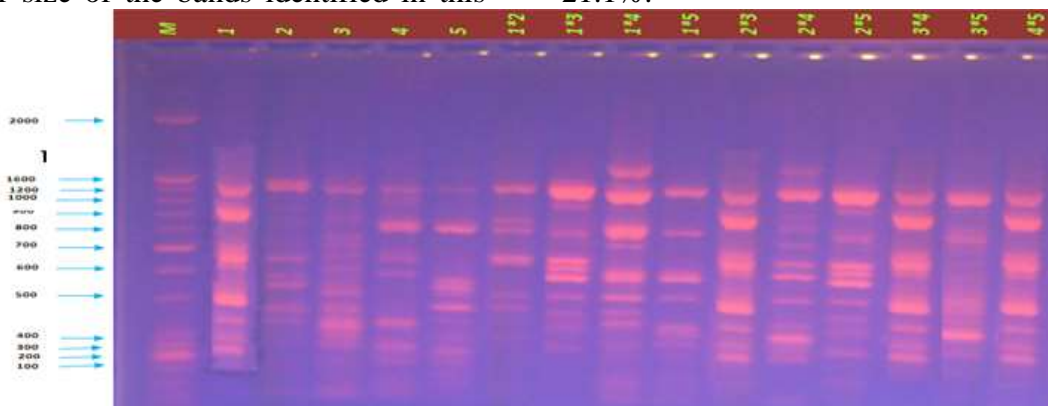


Figure 6. Bands produced by UBC 897 characterizing genetic diversity between five inbred lines and hybrid crosses in maize using ISSR indicators on Agarose

Genetic distance between inbred lines and hybrids using ISSR technique

After calculating the genetic distance between inbred lines that hybrids, the results showed 79 multi-shaped bands using PAST program, based on the Hamming Similarity Index, as shown in Table 3. The highest proportion of genetic similarity (less genetic distance) 0.40 was between inbred line 1 and 5. While the lowest genetic similarity (highest genetic

distance) was 0.105 between the inbred lines 2 and 5. It is noted that from the highest genetic similarity between hybrids is 0.631 between the hybrid 4x1 and inbred line 5, while the lowest genetic similarity was 0.06 between hybrid 1x5 and inbred line 2. The highest genetic similarity ratio 0.625 (less genetic distance) was between hybrid 4x1 and 5x1. The lowest genetic distance 0.19 was between the hybrid 4x1 and 5x3.

Table 3. The values of the genetic distance of the inbred lines and hybrids of maize using ISSR technique

	1	2	3	4	5	1x2	1x3	1x4	1x5	2x3	2x4	2x5	3x4	3x5	4x5
1	1														
2	0.234	1													
3	0.333	0.125	1												
4	0.333	0.125	0.2	1											
5	0.4	0.105	0.263	0.263	1										
1x2	0.444	0.117	0.375	0.375	0.545	1									
1x3	0.5	0.133	0.333	0.333	0.523	0.764	1								
1x4	0.437	0.142	0.461	0.187	0.631	0.526	0.588	1							
1x5	0.437	0.060	0.266	0.266	0.476	0.526	0.588	0.444	1						
2x3	0.352	0.142	0.357	0.266	0.55	0.611	0.588	0.625	0.444	1					
2x4	0.538	0.083	0.333	0.333	0.4	0.529	0.6	0.533	0.437	0.352	1				
2x5	0.333	0.181	0.454	0.333	0.4	0.529	0.6	0.533	0.437	0.437	0.428	1			
3x4	0.333	0.083	0.333	0.066	0.333	0.444	0.5	0.437	0.352	0.277	0.333	0.538	1		
3x5	0.166	0.076	0.214	0.133	0.208	0.421	0.388	0.19	0.333	0.263	0.235	0.4	0.4	1	
4x5	0.25	0.083	0.333	0.230	0.272	0.444	0.5	0.277	0.437	0.352	0.333	0.428	0.428	0.5	1

Cluster analysis of the studied genotypes based on ISSR indicators

According to the results of cluster analysis following UPGMA method with the nearest neighbor, the studied genotypes separated into three main groups reflecting their genetic background and draw guidelines for the suitable breeding program. There was a clear tendency to the parental genotypes to occupied the main clusters representing the higher ancestors, such as the S2 and S4 genotypes as they approved their unique and distinct genetic composition via occupying a single cluster for each. Meanwhile, the rest of genotypes grouped in one main cluster. Figure 7 indicated that the S6 and S7 genotypes gained the highest level of genetic similarity against each other compared to S2 parental line that revealed the higher genetic diversity against the entire tested genotypes. Traditionally, the individuals that are belonged to the same cluster expected to share a similar genetic

material (10). However, this procedure is to study the relationship and the historical development of the studied genotypes. In general, organisms that are to each other are likely to be closer to one another than individuals with different structures or sequences. The importance of determining the genetic relationship via molecular tools is to select the best parental inbreds necessary to create genotypes with new genes pool and predicting the best hybrid. The phenotypic assessment is environmental-dependent, thus, the detected differences may be due to environmental effects, which are not represents a true evaluation for a genotype in question and will not taken into consideration in determining the degree of genetic similarity between individuals. Furthermore, genotypes with higher genetic diversity like S6 and S7 have a big opportunity to produce super hybrids via hybridization process. (20). were the most divergent at the DNA level, by

joining the two most heterogeneous groups on the hierarchical path suggesting an increase in the total heterogeneity between species. This may contribute to the hybrid vigor, the rest of the inbreds were merged with all the crosses in another major group. The five inbred and the ten hybrids were divided into two groups. Within the first group inbred 2, and the second group, included the inbreds and hybrids. The

second group also included two main groups, inbred line 4 and other inbred and hybrids. The rest of the inbreds were merged with all the hybrids in another major group and the latter tended to form two secondary groups which included the first two diallel hybrids 1x5 and 1x4, while the second included the rest of the hybrids and the inbreds (11 genotype).

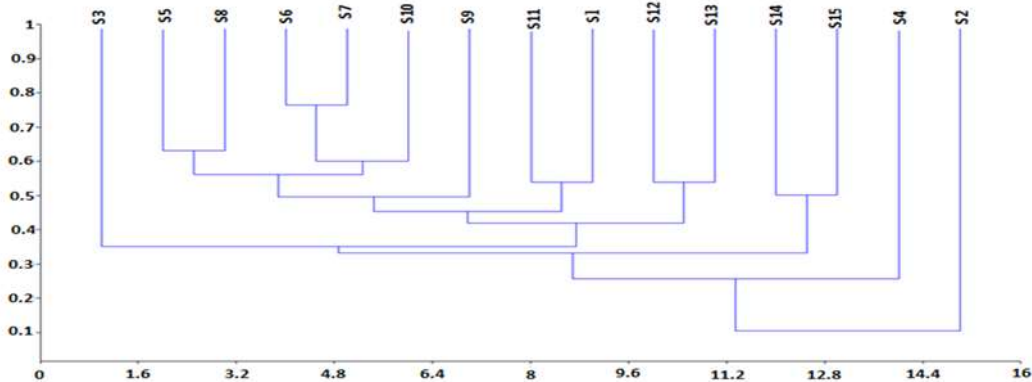


Figure 7. Dendrogram based on UPGMA method and nearest neighbor according to the results of 79 used ISSR markers of five inbreds and their ten hybrids of maize

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