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MOLECULAR ASSESSMENT OF GENETIC DIVERGENCE AMONG MAIZE GENOTYPES

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SUMMARY

Research carried out in 2021 at the Jeser Al-Musayyab Company, Baghdad, Iraq, determined the molecular variations among 10 inbred lines of maize (*Zea mays* L.), for use in the future production of promising F_1 hybrids. DNA sequencing random polymorphism technology using Inter Simple Sequence Repeats (ISSR) with five primers (UBC 834, UBC 835, UBC 856, UBC 866, and UBC 897) assessed the genetic variations among the 10 inbred lines of maize. All five primers produced 29 bands, averaging 5.8 strips each primer. The four primers, UBC 834, UBC 835, UBC 866, and UBC 897, had produced the same and highest number of proliferators with molecular weights ranging from 100 to 2000 bp. The primer UBC 856 showed the lowest number, with a molecular weight of 100–500 bp. The total number of polymorphs was 24, with a percentage of 82.4%, with the lowest (80%) polymorphs recorded in the primer UBC 856. The least discriminatory efficiency and ability values were 17.24% and 16.66%, respectively. Relying on binary data and genetic divergence using the UPGMA method to create a genetic kinship tree, the maize lines were separated into two groups, i.e., A, B, secondary, and sub-secondary groups. The highest genetic similarity (with the lowest genetic divergence) was estimated according to the Nei and Li coefficient of 0.23 between the two lines HS and S-10. The presented findings can provide further guidance and help analyze the genetic diversity among the maize populations.

Keywords: maize, ISSR, molecular, DNA, primer, cluster analysis

Key findings: The presented results authenticated the efficiency of ISSR markers in diagnosing genetic divergence among the inbred lines of maize (*Zea mays* L.).

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INTRODUCTION

Maize (*Zea mays* L.), in addition to its direct consumption by humans and livestock, also represents an important source and share of income for millions of people in several countries (Salami *et al.,* 2016; Ramadan *et al.,* 2021). Maize is also an indispensable source of raw materials for many industries worldwide, starting with the production of ethanol (biofuel) as an alternative to conventional car fuel and other industrial uses as an alternative to plastics and dyes (Yang *et al., 2017*).

Producing hybrids can denote an agricultural revolution and the most significant development in plant breeding. Maize hybrids' introduction also came from many researchers, according to Ramadan *et al.* (2020, 2022) and Mukhlif *et al.* (2021a, b). Heterosis depends primarily on the genetic divergence among the parental genotypes; therefore, the breeders always try to identify the best parent cultivars to acquire promising F_1 hybrids to reach the maximum hybrid vigor. The accuracy of selecting parental lines is the main pillar in successfully producing hybrids with commercial and economic heterosis versus other standard hybrid cultivars (Boonlertnirun *et al*., 2012; Al-Tamimi, 2020; Guevarra *et al*., 2022).

In traditional plant breeding programs, the breeder's decisions will mostly rely on phenotypic field data, which makes it vulnerable to many encounters, especially the environmental conditions' influences upon these traits. Consequently, it misguides the breeders and may, in turn, lead to erroneous decisions about the breeding material. However, at present, an extraordinary opportunity is at hand to enhance conventional genetic applications with molecular technologies that are mainly and ecologically independent (Alizadeh *et al.,* 2016).

Molecular markers have greater independence from environmental impacts, built on an individual's genome to determine the genetic diversity, and molecular markers have had tremendous influences in exploring genetic diversity, including ISSR markers. These markers can spot many polymorphic bands for each primer with high efficiency, ease of use and require no prior knowledge of DNA sequencing to design the primers (Muhammad *et al.,* 2017).

In previous studies, cluster analysis also helped determine genetic diversity among the genotypes and focused on identifying and studying genotypes' similarities and differences by placing them in relatively homogeneous groups that are different from the rest depending on a set of variables (Meena *et al*., 2017). In the above circumstances, the presented study sought to determine the genetic variations based on the molecular level in maize lines to identify the best ones for use in developing superior maize hybrids.

MATERIALS AND METHODS

Ten Inbred lines of maize obtained from the Agricultural Research Department, Baghdad, Iraq, had their symbols and origin indicated in Table 1. All the maize lines' seeds were planted in the fields in the Al-Bodiab area, north of Ramadi City, Iraq. At the maize plant's age of 7–10 days, the collected leaves from the ten lines served as samples for analysis in the Laboratory at the Jisr Al-Musayyab Company, Baghdad, Iraq.

Table 1. Maize inbred lines used in the study.

No.	Line symbol	Origin
1	Ast-B	Local
2	S-10	Local
3	Zm-17	Local
4	Syn-9	Local
5	Inb-6	Local
6	HS	America
7	Zm43R	Yugoslavia
8	Zm7R	Yugoslavia
9	Zm19R	Yugoslavia
10	ZP607	Yugoslavia

DNA extraction

The maize genotypes' young leaves underwent DNA extraction according to the CTAB method described by Weigand *et al*. (1993). About 50–150 µg of leaves were obtained for each maize's inbred line, then placed in a tube of 1.5 ml with a purity range of 1.7–2 as measured using nanodrop. DNA samples were diluted and adjusted to obtain a 50 ng/mıcroliter dilution, just right to perform PCR reactions. There are many ways for nucleic acid extraction from plants, varying with the variation of crop plants and the diversity in their compounds, such as, proteins, multiple and complex sugars, and nucleic acids. The best methods for extracting DNA from maize and wheat plants were described by Alfalahi (2011) and Altekriti (2013), respectively.

ISSR parameters

Given the accuracy of ISSR, the five primers were used to determine the genetic variations at the molecular level among the maize lines (Table 2).

DNA amplification

Each test tube received 5 µl of the master mix, then added with 2 µl of the used ISSR primer. Afterward, the mixture gained 5 µl of the DNA sample, then completed with distilled water to have a total volume of 20 µl. The mixture was centrifuged for five seconds and placed in a PCR machine for counting, as shown in the program (Table 3).

Table 2. ISSR primers' symbols and sequences used in this study.

No.	Primer sequence	Primer symbol
1	ACACACACACACACACG	UBC 834
2	AGAGAGAGAGAGAGAGYT	UBC 835
3	AGAGAGAGAGAGAGAGYC	UBC 856
4	GAGAGAGAGAGAGAGAYC	UBC 866
5	GACAGACAGACAGACA	UBC 897

Table 3. The thermal program of PCR reaction of ISSR primers.

Steps	Temperature °C	Time	Cycles number
Initial Denaturation	95	5 min	1
Denaturation	95	90 s	42
Annealing	55	90 s	42
Extension	72	30 s	42
Final Extension	72	70 min	1
Hold	4	――	――

Gel electrophoresis

Preparing acerose gel consisted of 1.5 g of agarose dissolved in 100 ml of TBE (Tris/Borate/EDTA) solution. The agarose was heated to boiling point and left to cool at about 45 °C temperature, afterward, adding five microliters of ethidium bromide dye. Leaving the liquid to solidify at room temperature, the comb quietly elevated. Then, transferring the gel to the electric relay basin holding a proper size of TBE, the electric relay was conducted at 50 volts for 10 min, then 90 volts for 3 h. The gel underwent an ultraviolet ray source (Transilluminator UV) exposure, with the migration results photographically documented.

Molecular statistical analysis

Based on the results from the electrical migration of the ISSR markers, a matrix was made for the results of the PCR reaction, with the number '1' indicating a band's appearance and '0' for non-appearance. The genetic diversity calculation among the maize genotypes used the MVSP program (Multi-Variate Statistical Package), with the cluster analyzed by calculating Euclidean Distance using the Nearest Neighbor method. The genetic diversity (GD) was estimated based on Nei and Li (1979):

$$GD = 1-(2N_{ij}/[N_i + N_j])$$

Where
GD = Genetic Distance
Nij = the number of bands found in both landraces i and j
N_i and N_j = the numbers of bands found in landrace i and j

Calculating the percentage of bands' diversity (polymorphism) in the starter was according to the following equation:

$$\text{Polymorphism } \% = (N_P/N_t) \times 100$$

Where
N_p = number of polymorphic loci
Nt = total number of loci

The percentage of primers efficiency and the percentage of the polymorphism of each primer was calculated as follows:

Efficiency percentage of used primers = The total amount of primer bands/total amount of bands for all primers × 100

Polymorphism percentage of each primer = Number of dissimilar bands of the primer/ Number of dissimilar bands of all primers × 100

Conducting cluster analysis helped draw the genetic diversity diagram, according to the Unweighted Pair Group Method Arithmetic mean (UPGMA) (Sokal and Michener, 1958).

RESULTS AND DISCUSSION

The molecular analysis through ISSR markers indicated that the five primers succeeded in detecting 29 bands in the genomes of maize genotypes and disclosed a significant variance in the quantity of replicated bands and their molecular weights, depending on the used primer. The analyzed results of the genetic relationship related to the presence and absence of the bands produced from the duplication of certain pieces of the maize genome and the molecular size of those bands linked to the complementary sites of the primer sequences on the DNA template (Table 4).

Table 4. The number of bands obtained using five ISSR primers with the percentage of polyforms in them, the percentage of each of the primer's efficiency, and the primer's polymorphism.

Primers	Total amplified fragments	Total polyform fragments	polyform fragments percentage %	Efficiency percentage of used primers %	Percentage of polymorphism %	Fragments size (bp)
UBC 834	6	5	83	20.68	20.83	100 -1200
UBC 835	6	5	83	20.68	20.83	100 1000
UBC 856	5	4	80	17.24	16.66	100 -500
UBC 866	6	5	83	20.68	20.83	100 -1000
UBC 897	6	5	83	20.68	20.83	100 -2000
Sum	29	24	82.4			100 -2000

Primer UBC 834

The primer UBC 834 showed six bands, five of which were varying, and therefore, the percentage of phenotypic shapes reached 83% of the total quantity of bands (Table 4). The efficiency and the polymorphism percentages of the primer were 20.68% and 20.83%, respectively, and the primer recognized the sequences that were supplemented with it in the DNA template of the maize genotypes used. It also presented a strong variation in the position and molecular size, ranging from 100 to 1200 bp. The results of genetic diagnosis showed an increase in the number of total bands, and it reached five bands in exotic maize genotype HS (6), with the lowest number of bands reached one band in local maize genotype S-10 (2), as shown in Figure 1.

Primer UBC 835

The primer UBC 835 had several bands that amounted to six, with five bands appearing different. The percentage of phenotypic shape was 83%, while the percent primer's efficiency and the polymorphism of the primer were 20.68% and 20.83%, respectively. The said primer successfully identified the matching sequences in the DNA of the genotype genome as it showed a variation ranging from 100 to 1000 bp in molecular size (Table 4). Through the molecular diagnosis, a band appeared with a molecular size of 100 bp in the genotypes under study (Figure 2). The genetic diagnosis also displayed an increase in the total number of bands in some genotypes, reaching six in foreign maize genotype HS, with some genotypes giving the lowest number of one, as observed in local maize genotype S-10.

Primer UBC 856

In primer UBC 856, the five bands were polymorphous, of which the phenotype's percentage reached 80% (Table 4). The primer recognized the genome sequence in the DNA with molecular weight ranging from 100 to 500 bp. The primer efficiency was 17.24%, while the percentage of its polymorphism was 16.66%. The outcomes of the genetic diagnosis revealed an increase in the total number of bands in some maize genotypes, reaching five in the exotic maize line HS (6) (Figure 3).

Primer UBC 866

The UBC 866 primer showed six bands, of which five were different; hence, the percentage of phenotypic shapes reached 83% in the total number of bands (Table 4). The efficiency and polymorphism percentages of the primer UBC 866 were 20.68% and 20.83%, respectively. The primer found its matching sequences in the DNA template of the maize genotypes, which varied in location and molecular size between 100–1000 bp. Also noted in Figure 4, the local maize genotype S-10 (2) showed the highest number of bands (5), ranging from 100 to 500 bp molecular weight, whereas the exotic maize line HS (6) exhibited the lowest number of bands (1), with a molecular weight of 100 bp.

Primer UBC 897

This primer showed six bands (Table 4), of which five displayed different forms, as reflected in the phenotype's percentage (83%) of the total number of generated bands. The complementary sequences of the primer in the DNA template occurred for the maize genotypes used in the study, which varied within the molecular weight of 100–2000 bp. The primer efficiency percentage was 20.68%, while the polymorphism percentage was 20.83%. The results also showed that exotic maize genotype HS (6) outperformed in the number of given bands, reaching five with a molecular weight of 100–1200 bp (Figure 5). However, all other maize genotypes showed a band appearance with a molecular weight of 100 bp.

In this context, several past primer studies also recorded the presence and absence of bands in some genotypes, which explains the discrepancy among the various populations concerning genetic diversity in maize (Berilli *et al.,* 2011; Junior *et al.,* 2011; Muhammad *et al.,* 2017; Abdul-Hamed and Amoon, 2020) and in germplasm of *Dactylis glomerata* L. (Costa *et al.,* 2016).

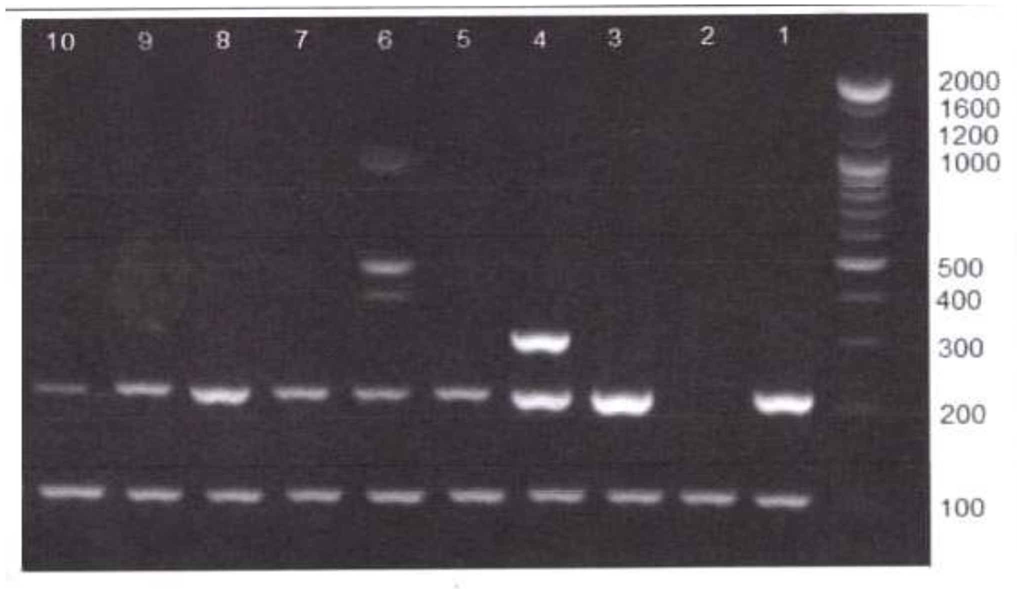

Figure 1. The bands produced by the UBC 834 primer in characterizing the genetic diversity among 10 inbred lines of maize using ISSR markers on the agarose gel.

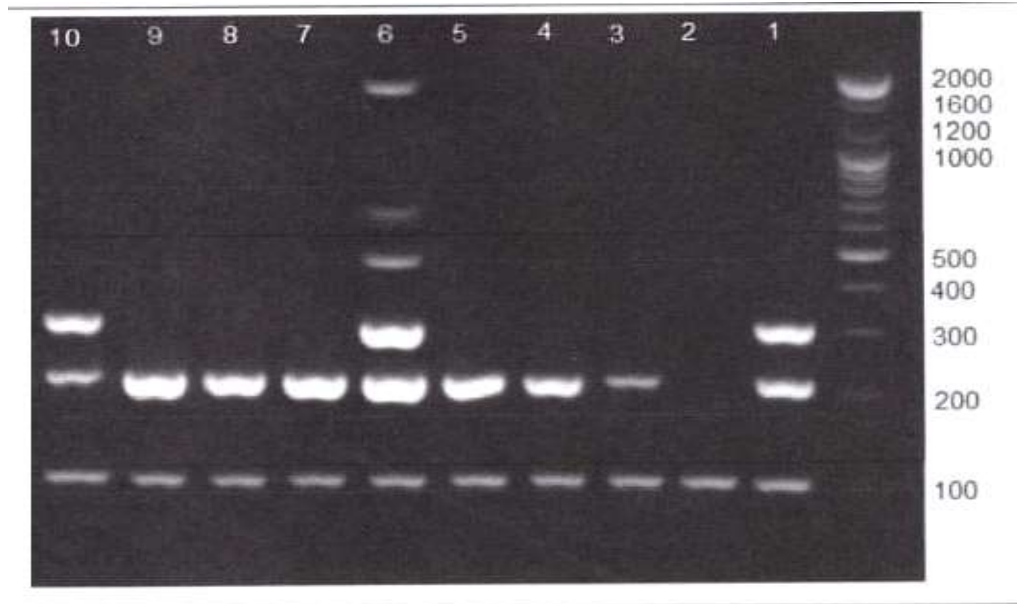

Figure 2. The produced bands using the UBC 835 primer in characterizing the genetic diversity among 10 inbred lines of maize by ISSR markers on the agarose gel.

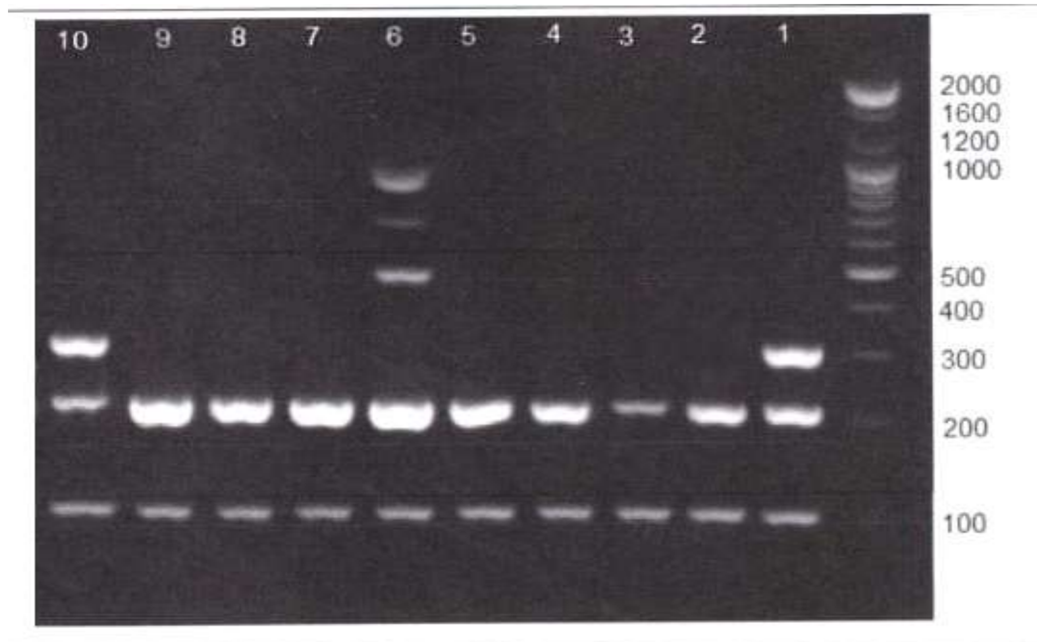

Figure 3. The produced bands using the UBC 856 primer in characterizing the genetic diversity among 10 inbred lines of maize by using ISSR markers on the agarose gel.

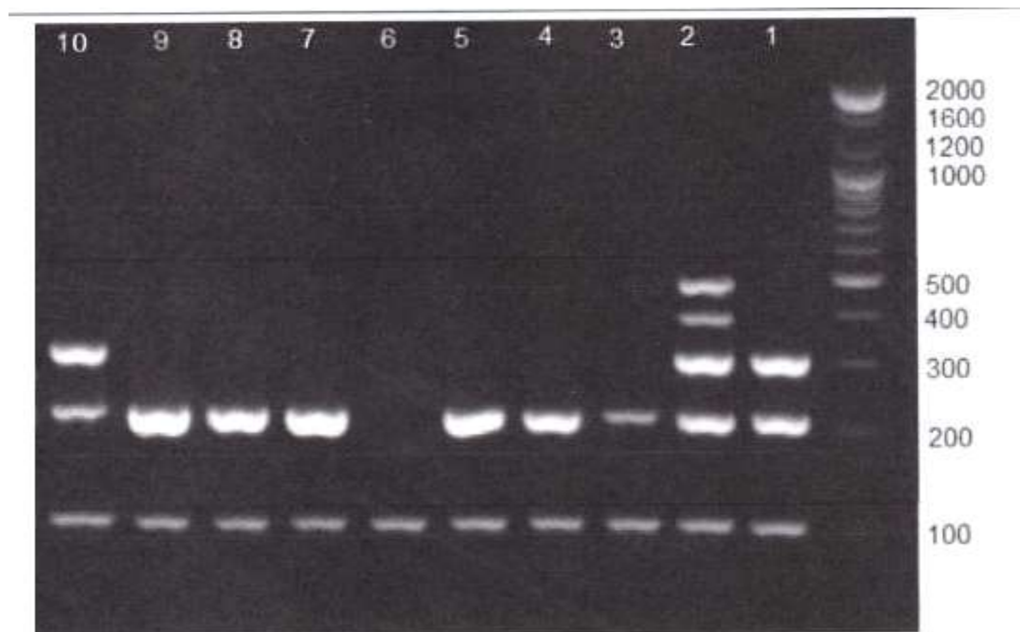

Figure 4. The bands produced by the UBC 866 primer in characterizing the genetic diversity among 10 inbred lines of maize using ISSR markers on the agarose gel.

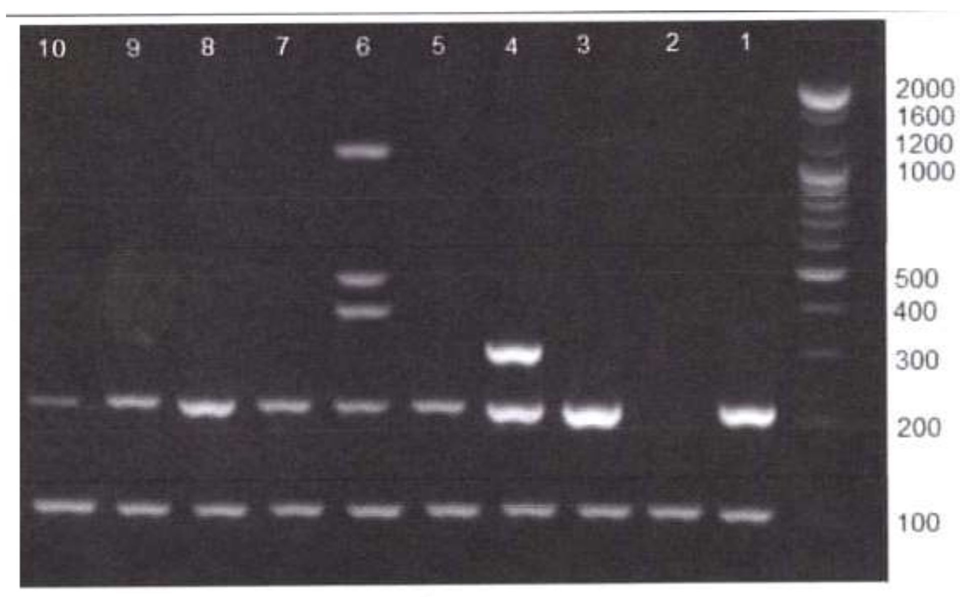

Figure 5. Bands resulting from the UBC 897 primer in characterizing the genetic diversity among 10 inbred lines of maize using ISSR markers on the agarose gel.

Table 5. Values of genetic diversity of maize lines using ISSR technique.

Maize lines	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1									
S2	0.53333	1								
S3	0.76923	0.53846	1							
S4	0.66667	0.46667	0.84336	1						
S5	0.76923	0.53846	1	0.83333	1					
S6	0.4	0.23077	0.3913	0.36	0.3913	1				
S7	0.76923	0.53846	1	0.83333	1	0.3913	1			
S8	0.76923	0.53846	1	0.83333	1	0.3913	1	1		
S9	0.76923	0.53846	1	0.83333	1	0.3913	1	1	1	
S10	1	0.53333	0.76923	0.66667	0.76923	0.4	0.76923	0.76923	0.76923	1

Genetic diversity among maize genotypes using ISSR

The calculation of the coefficient of the genetic diversity among the local and exotic maize lines under study showed 29 bands with polymorphism grounded on the 'Hamming Similarity Index' (Table 5). The results indicated that the lowest ratio of genetic similarity (with the highest genetic diversity) was 0.23 between the exotic maize line HS (6) and local maize genotype S-10 (2). The uppermost percentage of genetic similarity (with the lowest genetic diversity) was 0.84% between the two local maize lines, i.e., Syn-9 (4) and Zm-17 (3) (Figures 3 and 4).

Genetic kinship tree for maize lines based on ISSRs

The genetic kinship tree shows the evolutionary relationship of a group of organisms that arose from the same ancestor, a "common ancestor." The common ancestor was in the trunk of the diagram, and the organisms that arose from it were placed at the end of the tree branches. Distance between any couple of groups indicates the degree of kinship between them.

The maize genotypes underwent cluster analysis using ISSR markers and UPGMA methods, showing parents distributing the 10 local and exotic maize lines into two main groups (Figure 6). However, the first group included one maize line HS (No. 6), with the second group comprising two secondary groups, i.e., local maize line Ast-B (No. 1) and exotic maize genotype ZP607 (No. 10), and two sub-secondary groups, i.e., local maize line Syn-9 (No. 4) and exotic maize line Zm7R (No. 8). Past studies on genetic diversity through cluster and principal component analysis also revealed similar results in maize (*Zea mays* L.) (Kumar *et al.,* 2016; Matin *et al.,* 2017; Pahadi *et al.,* 2017; Suryanarayana *et al.,* 2017; Soliman *et al.,* 2021).

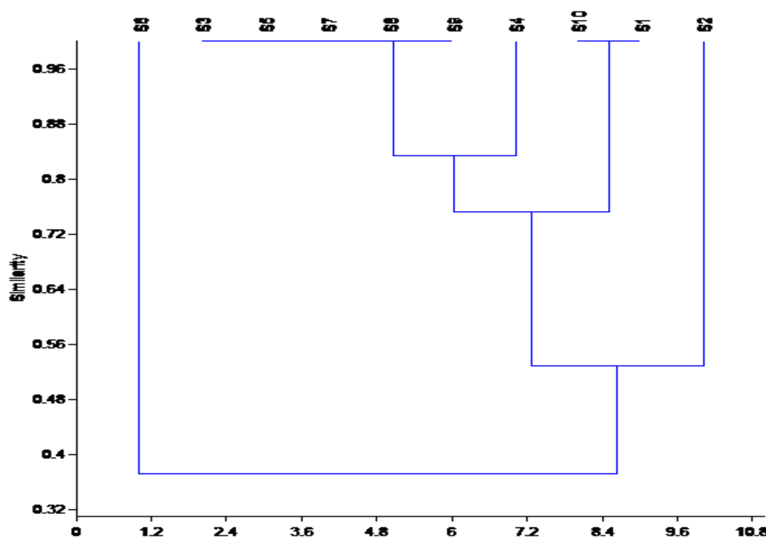

Figure 6. Cluster analysis of parental genotypes based on five ISSR primers.

CONCLUSIONS

The ISSR indicators were characterized by accuracy and high efficiency in detecting genetic variations among the studied maize genotypes. Cluster analysis also contributed to lining up similar individuals next to each other in a cluster, thus identifying the genotypes farther from each other to produce the best hybrids with high yield and quality. The inbred line S6 was the most distant genetically from the rest of the inbred lines; therefore, it is recommended to include it in the crossbreeding programs to obtain the best hybrids.

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