Egypt. J. Agron. **Vol. 46**, No. 1, pp: 75 - 83 (2024)

Egyptian Journal of Agronomy

http://agro.journals.ekb.eg/

Identification of Informative Simple Sequence Repeat (SSR) Markers for Testing of Hybridity in Maize

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Maize (*Zea mays* L.,) is the second-most significant cereal crop after wheat in Egypt and worldwide. The aim of this study was to evaluate the genetic diversity among six maize inbred lines and their 15 F1s single crosses using Simple sequence repeat (SSR) markers by five SSR primers. The current investigation were conducted during two successive summer seasons of 2020 and 2021 at National Research Centre, Agriculture Research Station in Nubaria, El-Beheira governorate to produce all possible hybrid combinations. A total number of amplified fragments were 75 bands. Forty-six out of 75 bands were monomorphic (61.33%) and 29 fragments were polymorphic bands. The number of amplicons per primer varied between 6 (p-dupssr 7) and 23 (p-umc1358). The Jaccard's similarity coefficient separated the six maize lines and 15 single crosses (Sc) into two major groups. The first group I: Included three sub-groups: Sub-group a: (similarity range 0.72 to 0.84) consisted of Sc (P1xP4) and sub-group b (similarity range 0.78 to 0.87): contained two Sc (P1xP9) and (P1xP12).Sub-group c (similarity range 0.75 to 0.91) such as P1; (P1xP15); (P1xP17); and (P4xP15). The second group (II) composed of three sub-groups: Sub-group d (similarity range 0.72 to 0.90) involved P15; (P15xP17). Sub-group e (similarity range 0.70 to 0.91) included P17; (P12xP17); and (P12xP15). Sub-group f (similarity range 0.75 to 0.93) contained P4; (P4xP9); (P4xP12); (P4xP17); P9; P12; (P9xP12); (P9xP15); and (P9xP17). The SSR markers can be used as test of hybridity and seed genetic purity in the breeding programs of maize.

Keywords: *Zea mays* L., genetic diversity, DNA, molecular marker, cluster analysis.

Introduction

Maize (*Zea mays* L.) is considered one of the most important strategic grain crops in Egypt and worldwide. It is used in different food industries and food for humans and animals (Ismail *et al*., 2024). It is a short-duration crop that is grown in the summer and spring in order to increase the economy and achieve the desired yield (Dogar 2023). Identifying the genetic relationship between the maize lines is considered an important method for the selection of parents in breeding programs. Genetic variability analysis discriminates between breeding groups and helps classify inbred lines into specific homogeneous groups (Begna 2021; Swarup *et al*., 2021). The molecular diversity of maize is highly significant and could play a major role in the breeding programs in the future. The study of genetic variability is necessary for comprehension of the genotype and aids breeders to choose parents, which have good traits for performing breeding in the future (Al-Badeiry *et al*., 2014). On principle, inbred genotypes are considered as a necessary source in breeding programs and are used in the production of new hybrid lines of maize (Troyer 2001; Kamal *et al*., 2023; Nadeem *et al*., 2023). Precise selection of inbred genotypes from heterotic groups is a base of efficient employment of new inbred lines. Molecular markers that could offer quick, effective, precisely defined, and repeatable genotypic Galović *et al*., (2004) and Sharma *et al*., (2014) provide descriptions. Single nucleotide polymorphisms (SNPs) are enduring patterns of naturally occurring genetic variation and provide variation suitable for differentiating between closely related lines. Simple sequence repeats (SSRs) distributed in genomic

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Received 24/3 /2024 ; Accepted 26/5 /2024
DOI: 10.21608/agro.2024.275958.1420

DNA and were considered sufficient tool for determination genetic variability in *Z. mays* L. (Rupp *et al*., 2009; Fernandes *et al*., 2015). Abdel Sattar *et al*., (2015) determined the genetic diversity among different genotypes of maize by SSR and Inter-simple sequence repeats (ISSR) assays. SSR and ISSR tools were detected to be adequately credible genetic markers for the estimation of genetic distances among the studied maize genotypes.

The objective of this study was to study the genetic diversity among six maize inbred lines and their 15 F1s single crosses by using Simple sequence repeats (SSR) markers with five primers. Detect the best inbred lines and F1crosses may be used in future maize breeding programs.

Materials and Methods

Plant materials

The plant materials used in this study included six inbred lines of white maize tolerant to drought, i.e., P1, P4, P9, P12, P15, and P17, supported by the International Maize and Wheat Improvement Center (CIMMYT) (Tables 1 and 2). In 2020 summer growing season half diallel cross among the six maize inbred were conducted at National Research Centre, Agriculture Research Station in Nubaria, El-Beheira Governorate to produce all possible hybrid combinations. The twenty-one genotypes (six parents and their 15 F1 hybrids) were grown under a pot experiment in the next growing season 2021.

Genomic DNA isolation

0.5 g of 15-day-old maize seedlings of six maize lines and 15 single crosses (SC) were collected and soaked in liquid nitrogen for DNA extraction using the 2% CTAB procedure as described by Saghai-Maroof *et al*., (1984).

SSR fingerprints

A total of five SSR primers were used to amplify the DNA of six maize lines and 15 single crosses (Sc) (Table 3) (Gemenet *et al*., 2010).

Amplification product analysis

The amplified DNA (15 µl) for all samples was electrophoresed on 1% agarose containing Ethidium Bromide. (0.5 µg/ml) in 1X TBE buffer at 75 constant volt, and determined with a UV transilluminator. The size of each fragment was estimated with reference to a size marker of 1 Kb DNA ladder (BioRoN, Germany). Gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

Data analysis

A matrix for SSR was generated by scoring reproducible bands as 1 for their presence and as 0 for their absence across six maize lines and 15 Sc. Genetic similarity coefficients were computed following Nei and Li (1979). The data was subsequently used to construct a dendrogram using the un-weighted pair group method of arithmetic averages (UPGMA) (Sneath and Sokal 1973) employing sequential, agglomerative hierarchic and non-overlapping clustering (SAHN). All the computations were carried out using the software NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1 (Rohlf 2000). Correlation coefficients were calculated using similarity coefficients obtained from SSR analysis

Principal component analysis (PCA)

PCA was also carried out to show multiple dimension of the distribution of the six maize lines and 15 single crosses in a scatter-pot by PAST software version 1.62 (Hammer *et al*., 2001)

Results and Discussion

SSR profiles

Five SSR primers were applied in this study to characterize the six maize lines and 15 single crosses (SC) as illustrated in Figure (1) and Table (3). The total number of amplified fragments was 75 bands. Forty-six out of 75 bands were monomorphic (61.33%) and 29 fragments were polymorphic fragments, resulting in a polymorphism of 38.67%. The number of amplicons per primer varied between 6 (p-dupssr 7) and 23 (p-umc1358). These results were in agreement with Barcaccia *et al*., (2003) found that polymorphisms result from variation in DNA sequence at primer binding sites and differences in DNA length between primer binding sites. The molecular size of the amplified fragments varied between 70 bp (p-dupssr7 and p-phio31) and 1560 bp (p-umc1358). The extent of polymorphism per primer ranged from 25 (p-mc1542) to 66.67% (p-dupssr 7) (Table 3). Table (4) showed polymorphic bands revealed in six maize lines and 15 Sc. These the results agree with those observed by Gerdes and Tracy (1994) mentioned that pedigree relationships can be applied as a benchmark to test the effectiveness of DNA markers in estimating relationships among maize genotypes. Reif *et al*., (2003) indicated that SSR is an efficient marker to classify closely related lines.

TABLE 1. **Pedigree of 15 maize accessions used in this study**.

Code	Name	Pedigree	Origen
1	DTMA-2	[SYN-USAB2/SYN-ELIB2]-12-1-1-2-BBB	AF1oA-607-2
4	DTMA-79	(A.T.Z.T.R.L.BA9o 5-3-3P-1P-4P-2P-1-1-1-B x G9B Co R.L.23-1P-2P-3-2P-3-2P-1P-B-B-B)-B-16TL-3-1-4-B	TL11A-1639-79
9	DTMA-210	CL-G1829=G18C23-61-3-1-1-B*7	TL11A-1639-210
12	DTMA-252	La Posta Seq C7-F71-1-2-1-2-B-B-B	TL11A-1639-252
15	DTMA-269	DTPWC9-F2-3-2-1-B-B-B	TL11A-1639-269
17	DTMA-261	La Posta Seq C7-F180-3-1-1-1-B-B-B	TL11A-1639-261

TABLE 2. Parents and 15 single crosses used in this the study.

No.	Parents and single cross	No.	Parents and single cross	No.	Parents and single cross
1	P1	8	Sc (P4xP9)	15	Sc (P9xP17)
2	Sc (P1xP4)	9	Sc (P4xP12)	16	P12
3	Sc (P1xP9)	10	Sc (P4xP15)	17	Sc (P12xP15)
4	Sc (P1xP12)	11	Sc (P4xP17)	18	Sc (P12xP17)
5	Sc (P1xP15)	12	P9	19	P15
6	Sc (P1xP17)	13	Sc (P9xP12)	20	Sc (P15xP17)
7	P4	14	Sc (P9xP15)	21	P17

TABLE 3. SSR-PCR amplified bands, polymorphic bands, and unique markers for six parents and 15 single crosses using five primers.

Primer Code No.	Sequence (5′ to 3′)	Size range of the scorable bands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	% Polymorphism
p-phio31	F: GCAACAGGTTACATGAGCTGACGA R:CCAGCGTGCTGTTCCAGTAGTT	70-1400	21	14	7	33.33
p-dupssr7	F:GAAGCTTAATCTGGAATCTGG R;TGTTGCTTCCTTGTAAAATCT	70-500	6	2	4	66.67
p-dupssr10	F:AGAAAATGGTGAGGCAGG R:TATGAAATCTGCATCTAGAGAAATTG	80-1050	17	8	9	52.94
p-umc1358	F:AGAACCTCCCGCTTGACGAC R:ACCTCAACCTCGACCTCTGCAT	100-1560	23	16	7	30.43
p-umc1542	F:TAAAGCTATGATGGCACTTGCAGA R:CATATTTGCCTTTGCCCTTTTGTA	90-1300	8	6	2	25
Total	**-**	**70-1560**	**75**	**46**	**29**	**38.67**

TABLE 4. Polymorphic bands and unique markers for six parents and 15 single crosses using five primers.

Primer Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
p-phio31																					
+1400	+1		+1										+1	+1				+1	+1		+1
+1250		+1	+1	+1		+1							+1	+1				+1	+1		+1
+850			+1	+1									+1	+1	+1				+1		+1
+650	+1		+1		+1	+1		+1		+1		+1	+1	+1	+1	+1	+1		+1	+1	
+310	+1		+1	+1		+1		+1	+1		+1						+1	+1			+1
+305	+1																+1				
+280	+1		+1	+1		+1	+1	+1	+1	+1								+1			
p-upssr7																					
+500	+1			+1	+1	+1				+1								+1	+1	+1	
+415	+1	+1		+1	+1	+1				+1											
+320	+1	+1		+1	+1	+1				+1								+1	+1	+1	
+240	+1	+1		+1	+1	+1															
p-dupssr1o																					
+1050																					+1
+1000	+1																+1	+1		+1	+1
-720		-1	-1	-1									-1	-1						-1	
+550						+1	+1	+1	+1	+1	+1	+1		+1	+1					+1	+1
+250		+1	+1	+1	+1		+1	+1												+1	+1
-200												-1	-1	-1							
+150		+1						+1	+1							+1	+1	+1	+1	+1	+1
+130		+1																		+1	+1

+1= Presence of band, -1= Absence of band, 1= P1, 2= Sc (P1xP4), 3= Sc (P1xP9), 4= Sc (P1xP12), 5= Sc (P1xP15), 6= Sc (P1xP17), 7= P4, 8= Sc (P4xP9), 9= Sc (P4xP12), 10= Sc (P4xP15), 11= Sc (P4xP17), 12= P9, 13= Sc (P9xP12), 14= Sc (P9xP15), 15= Sc (P9xP17), 16= P12, 17= Sc (P12xP15),18= Sc (P12xP17), 19= P15, 20= Sc (P15xP17), and 21= P17.

TABLE 4. Continued.

Primer Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
p-umc1358																					
+1460							+1	+1			+1			+1	+1			+1	+1	+1	+1
+1200						+					+1							+1	+1	+1	+1
-1000			-1	-1																	
+600	+1	+1	+1	+1	+1	+1										+1				+1	+1
+335			+1					+1			+1	+1	+1		+1	+1	+1	+1		+1	+1
+279				+1			+1	+1	+1	+1							+1	+1	+1	+1	+1
+211					+1	+1					+1	+1	+1			+1	+1	+1	+1	+1	+1
p-mc1542																					
+1300	+1		+1	+1	+1	+1															
+155			+1				+1	+1				+1	+1				+1	+1			+1

+1= Presence of band, -1= absence of band , 1= P1, 2= Sc (P1xP4), 3= Sc (P1xP9), 4= Sc (P1xP12), 5= Sc (P1xP15), 6= Sc (P1xP17), 7= P4, 8= Sc (P4xP9), 9= Sc (P4xP12), 10= Sc (P4xP15), 11= Sc (P4xP17), 12= P9, 13= Sc (P9xP12), 14= Sc (P9xP15), 15= Sc (P9xP17), 16= P12, 17= Sc (P12xP15), 18= Sc (P12xP17), 19= P15, 20= Sc (P15xP17), and 21= P17.

TABLE 5. Similarity indices between the six parents and 15 single crosses as estimated using SSR analysis.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1.00																				
2	0.78	1.00																			
3	0.80	0.78	1.00																		
4	0.83	0.84	0.87	1.00																	
5	0.89	0.84	0.78	0.84	1.00																
6	0.89	0.79	0.79	0.85	0.91	1.00															
7	0.75	0.81	0.78	0.78	0.78	0.76	1.00														
8	0.75	0.78	0.81	0.76	0.76	0.77	0.93	1.00													
9	0.78	0.79	0.79	0.79	0.77	0.80	0.91	0.92	1.00												
10	0.86	0.78	0.75	0.81	0.87	0.88	0.87	0.84	0.88	1.00											
11	0.76	0.74	0.77	0.72	0.80	0.83	0.85	0.85	0.86	0.82	1.00										
12	0.74	0.77	0.77	0.70	0.81	0.78	0.83	0.84	0.81	0.83	0.88	1.00									
13	0.73	0.79	0.85	0.74	0.77	0.75	0.79	0.79	0.77	0.76	0.80	0.91	1.00								
14	0.76	0.76	0.82	0.74	0.77	0.77	0.85	0.82	0.83	0.82	0.83	0.84	0.89	1.00							
15	0.77	0.75	0.81	0.73	0.81	0.79	0.86	0.87	0.85	0.87	0.91	0.89	0.85	0.91	1.00						
16	0.78	0.82	0.82	0.74	0.85	0.80	0.79	0.82	0.83	0.82	0.86	0.91	0.86	0.80	0.88	1.00					
17	0.85	0.72	0.80	0.75	0.80	0.81	0.80	0.86	0.87	0.83	0.84	0.82	0.78	0.75	0.83	0.87	1.00				
18	0.81	0.74	0.76	0.77	0.79	0.83	0.76	0.79	0.80	0.79	0.86	0.76	0.77	0.77	0.79	0.80	0.86	1.00			
19	0.76	0.82	0.79	0.79	0.79	0.80	0.76	0.76	0.77	0.79	0.77	0.75	0.83	0.83	0.78	0.83	0.75	0.85	1.00		
20	0.77	0.78	0.73	0.73	0.84	0.81	0.80	0.84	0.79	0.83	0.85	0.80	0.74	0.76	0.83	0.84	0.82	0.87	0.87	1.00	
21	0.75	0.73	0.81	0.76	0.76	0.79	0.78	0.81	0.82	0.75	0.85	0.77	0.79	0.79	0.80	0.79	0.85	0.90	0.79	0.83	1.00

1 = P1, 2= Sc (P1xP4), 3= Sc (P1xP9), 4= Sc (P1xP12), 5= Sc (P1xP15), 6= Sc (P1xP17), 7= P4, 8= Sc (P4xP9), 9= Sc (P4xP12), 10= Sc (P4xP15), 11= Sc (P4xP17), 12= P9, 13= Sc (P9xP12), 14= Sc (P9xP15), 15= Sc (P9xP17), 16= P12, 17= Sc (P12xP15), 18= Sc (P12xP17), 19= P15, 20= Sc (P15xP17), and 21= P17.

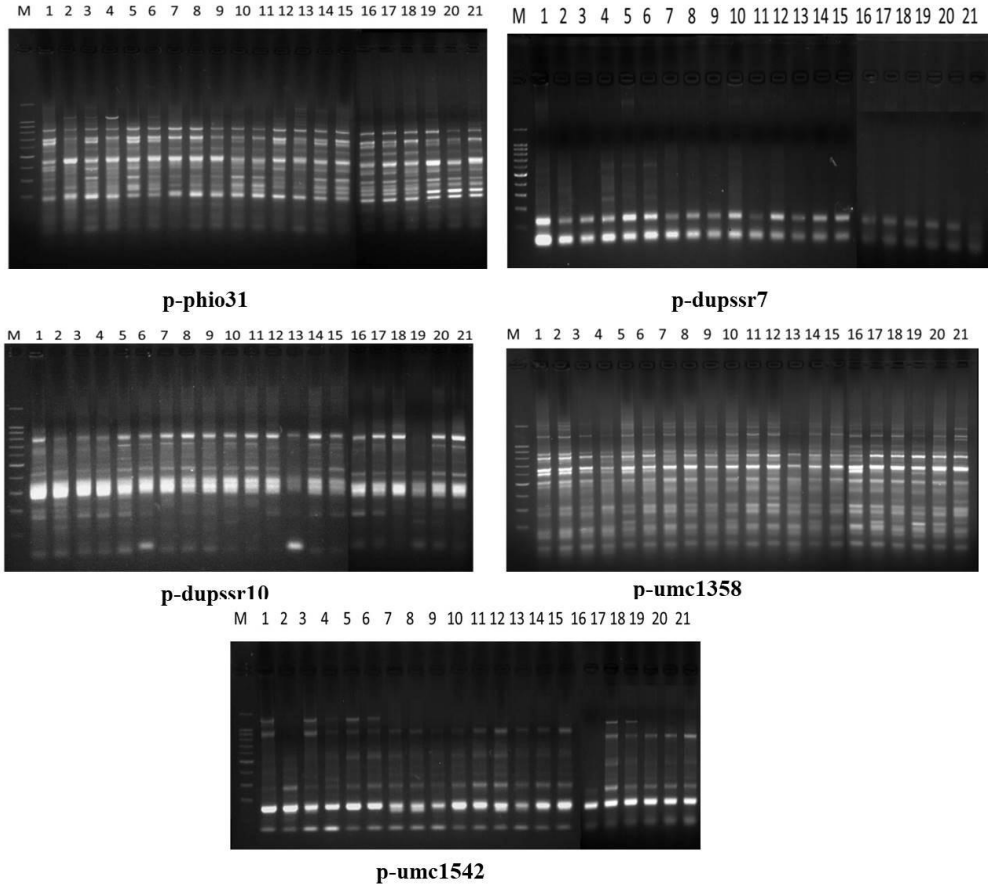

Figure 1. SSR-PCR analysis of six maize lines and 15 single crosses (SC) with five primers, lane M = 1 Kb DNA ladder. Lanes 1, 7, 12, 16, 19, and 21: parents No. 1, 4, 9, 12, 15, and 19, respectively. Lanes 2, 3, 4, 5, 6, 8, 10, 11, 13, 14, 15, 17, 18, and 20: Sc (P1xP4); Sc (P1xP9); Sc (P1xP12); Sc (P1xP15); Sc (P1xP17); Sc (P4xP9); Sc (P4xP12); Sc (P4xP15); Sc (P4xP17); Sc (P9xP12); Sc (P9xP15); Sc (P9xP17); Sc (P12xP15); Sc (P12xP17); and Sc (P15xP17); respectively.

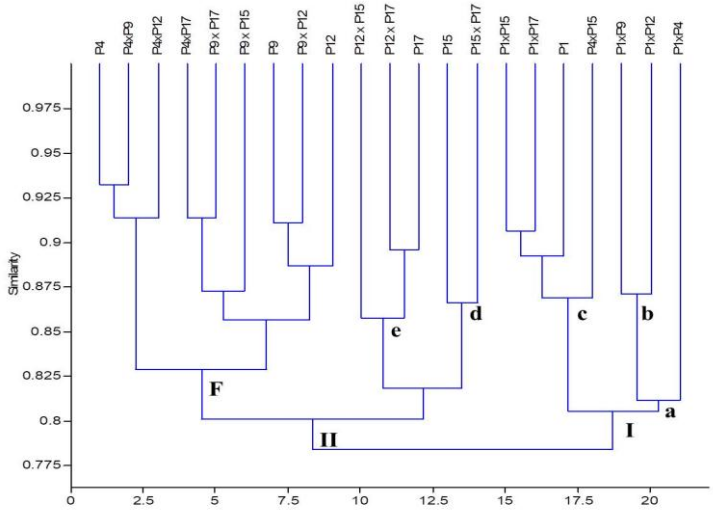

Figure 2. Dendrogram of six maize parents and their F1 single crosses based on SSR-PCR by UPGMA algorithm using Jaccard's similarity coefficient.

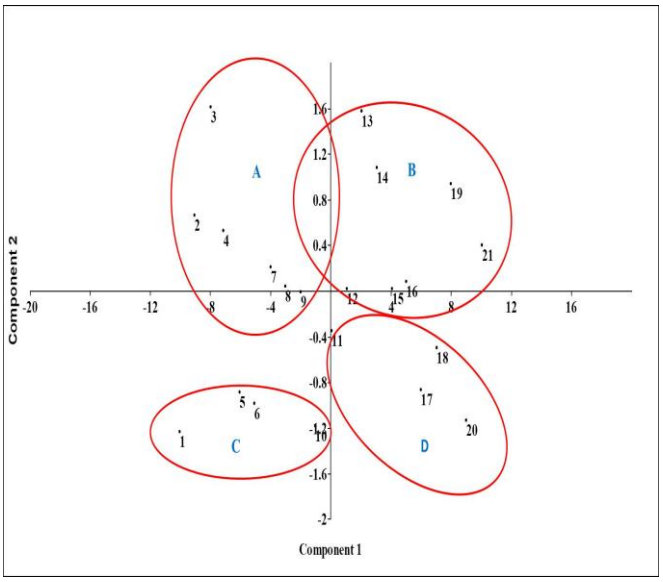

Figure 3. Principal component analysis (PCA) seperated six maize parents and their F1 single crosses to four groups (A, B, C, and D), using five SSR primers. 1= P1, 2= Sc (P1xP4), 3= Sc (P1xP9), 4= Sc (P1xP12), 5= Sc (P1xP15), 6= Sc (P1xP17), 7= P4, 8= Sc (P4xP9), 9= Sc (P4xP12), 10= Sc (P4xP15), 11= Sc (P4xP17), 12= P9, 13= Sc (P9xP12), 14= Sc (P9xP15), 15= Sc (P9xP17), 16= P12, 17= Sc (P12xP15), 18= Sc (P12xP17), 19= P15, 20= Sc (P15xP17), and 21= P17. Cluster analysis

The dendrogram was produced according to five SSR markers (Table 5 and Figure 2). Six maize lines and 15 single crosses were clustered in two major groups. The first group I: Included three sub-groups: Sub-group a: (similarity range 0.72 to 0.84) consisted of Sc (P1xP4) and sub-group b (similarity range 0.78 to 0.87): contained two Sc (P1xP9) and (P1xP12).Sub-group c (similarity range 0.75 to 0.91) such as P1; (P1xP15); (P1xP17); and (P4xP15). The second group (II) composed of three sub-groups: Sub-group d (similarity range 0.72 to 0.90) involved P15; (P15xP17). Sub-group e (similarity range 0.70 to 0.91) included P17; (P12xP17); and (P12xP15). Sub-group f (similarity range 0.75 to 0.93) contained P4; (P4xP9); (P4xP12); (P4xP17); P9; P12; (P9xP12); (P9xP15); and (P9xP17) (Table 5 and Figure 2). The dendrogram was confirmed by principal coordinate analysis (PCA) (Figure 3). The six maize lines and 15 single crosses in the PCA scatter plot were detected by ellipses numbered with A, B, C, and D. These lines are very close, so they were put in groups A, B, C, and D as presented in Figure (3). The lines and single crosses 2, 3, 4, 7, 8, and 9 were clustered into the group (A). However, 12, 13, 14, 15, 16, 19, and 21 were put together in group (B), while 1, 5, 6, and 10 were clustered in the group (C). Finally, lines and single crosses 11, 17, 18, and 20 were clustered in group (D). In this study, it was observed that the results from the

UPGMA agreed with the PCoA (Lopes *et al*. 2015). As it is easy to see, the six lines and 15 single crosses in four major groupings. These results were in agreement with Shiri (2011) who mentioned that most studies in maize were focused on the development of microsatellite markers for genetic mapping and germplasm analysis (Phelps *et al*., 1996). A complete genetic map of maize may be created using microsatellites because of their diversity, abundance, and wide microsatellites the genomes distribution. Due to their high polymorphism in DNA molecule between genotypes, so microsatellite markers are a useful tool for assessing genetic diversity in various plants, including maize (Barbosa-Neto *et al*., 1996). SSR and ISSR assays are recognized as repeating sequences of 2 to 6 bp, which create bands of 20 to 100 bp (Agrawal *et al*., 1999; Goldstein and Schlotterer, 1998). Heckenberger *et al*., (2002) indicated that genetic distances (GDs) depending on molecular markers are significant factors for characterizing essentially derived varieties (EDVs). Information regarding the genetic marker variability within maize inbred lines is crucial in this regard.

Conclusion

Maize hybrids were distinguished clearly from their parental lines using five SSR markers. In addition, SSR markers clearly grouping the six maize lines and 15 single crosses into two major groups, with a similarity coefficient ranging from 0.70 to 0.93. Therefore, SSR assays can be confidently used for the identification of maize seed purity tests without any genetic confusion, and the determination of diversity analysis of the progeny of maize hybrids. This marker is considered an important tool for

improving the breeding efficiency of new lines and exploring the genetic background of maize varieties.

Consent for publication

All authors declare their consent for publication.

Author contribution

The manuscript was edited and revised by all authors.

Conflicts of Interest

The author declares no conflict of interest.

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