FORMATION OF POTENTIAL HETEROTIC GROUPS OF MAIZE INBRED LINES USING VARIATION AT SIMPLE SEQUENCE REPEAT LOCI

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ABSTRACT

This study was aimed to identification of certain crosses to produce hybrids with higher performance *per se* can be aided by the determination of simple sequence repeats (SSR), which can improve our understanding of the genetic divergence of maize lines and their classification into different heterotic groups. Variability for each locus was measured using the polymorphism information content (PIC), with an average of 0.55, suggesting that the markers were highly informative. Analysis of the molecular variance (AMOVA) indicated higher divergence among the maize lines, suggesting the existence of different groups. The unweighted pair group method with arithmetic mean analysis (UPGMA) and the three-dimensional principal coordinate analysis (PCoA) revealed seven heterotic groups. Therefore, knowledge on the genetic diversity distribution in these maize inbred lines is essential to determine strategies to exploit heterosis in breeding programs in future studies.

Key words: *Zea mays* L., genetic diversity, polymorphism, AMOVA

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احتمالية تشكيل مجموعات غير متجانسة وراثيا من سلالات الذرة الصفراء باستخدام طريقة التكرار التسلسلي البسيط

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المستخلص

ان تحديد بعض السلالات من الذرة الصفراء يساعد في انتاج هجين ذات كفاءة عالية وذلك من خلال طريقة تكرار التسلسل البسيط (SSR)، والتي من الممكن ان ترقي من فهمنا للاختلافات الجينية بين السلالات وتصنيفها الى مجاميع غير متجانسة. وتم قياس التباين لكل موقع باستخدام محتوى معلومات تعدد الأشكال (PIC) والذي وصل الى متوسط 0.55 ، مما يشير ذلك إلى أن البرايمرات كانت مفيدة. كما وتشير نتائج تحليل التباين الجزيئي (AMOVA) إلى وجود تباين عالي بين السلالات الداخلة ضمن الدراسة، مما يشير إلى وجود مجاميع تختلف عن بعضها. كما وكشفت طريقة المجاميع الزوجية غير الموزونة مع تحليل المتوسط الحسابي (UPGMA) وتحليل الإحداثيات الرئيسية الثلاثية الأبعاد (PCoA) عن وجود سبع مجموعات غير متجانسة. لذا من الضروري تحديد التنوع الجيني في السلالات لتحديد استراتيجية التربية.

الكلمات المفتاحية: *Zea mays* L., الاختلاف الجيني, تعدد الاشكال, تحليل التباين الجزيئي

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INTRODUCTION

The limiting factors of maize researchers at the North of Iraq are the development, improvement, maintenance, and uncontrolled quality of inbred lines. Hence, because of the absence of locally produced hybrid seeds, maize farmers must pay a high price for imported seeds, which raises the price of production (1). The *Zea mays* L. information of the genetic variation and relationships among the maize inbred lines will significantly impact the identification of hybrid combinations (4). In conventional plant breeding, genetic diversity is usually identified through observational selection (5, 30). However, the methods utilizing the phenotypic traits are greatly affected by environmental factors, laborious and slow (23). Therefore, evaluations at the molecular level by breeders are essential as an additional tool to investigate genetic diversity in the selection process for superior parental lines. Polymerase chain reaction (PCR) has recently been used by researchers in the North of Iraq in a variety of applications, including the plant breeding program (1). The PCR has a key role in detecting genetic variation utilizing different molecular markers (12). Variety of DNA-based markers have been employed to analyze the genetic variation of plants, including restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), inter simple sequence repeats (ISSRs), microsatellite or simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs) (14, 32, 29). SSRs are one of the best markers for evaluating genetic variation among maize genotypes. This is because they have the advantages of reliability, a high degree of allelic variation, repeatability, and discrimination. Due to their highly variable nucleotide sequence and genome coverage, SSR DNA markers have attracted attention for a variety of plant applications (16). In SSR DNA analysis, locus-specific PCR primer pairs are made using sequence information of the regions flanking the repeats. Diversity in band size is due to the diversity in the numbers of repeated units, which are co-dominantly inherited, highly polymorphic, reveal allele size variations, locus-specific, and can be accessed by sharing primer sequences (6, 34). SSR DNA markers have been extensively used for the formation of heterotic groups in maize worldwide (3). While heterotic groupings of maize inbred lines in use in the North of Iraq are not yet clearly defined. (1) conducted a study to group seven maize inbred lines and three testers, using 10 SSR DNA markers to investigate their genetic diversity. They assigned these inbred lines into three main heterotic groups and two main heterotic groups for testers using the UPGMA algorithm. The present study was aimed to assess genetic variation among 29 introduced maize inbred lines using 10 SSR DNA markers, to determine the most informative among those markers, and to generate a dendrogram based on molecular characteristics.

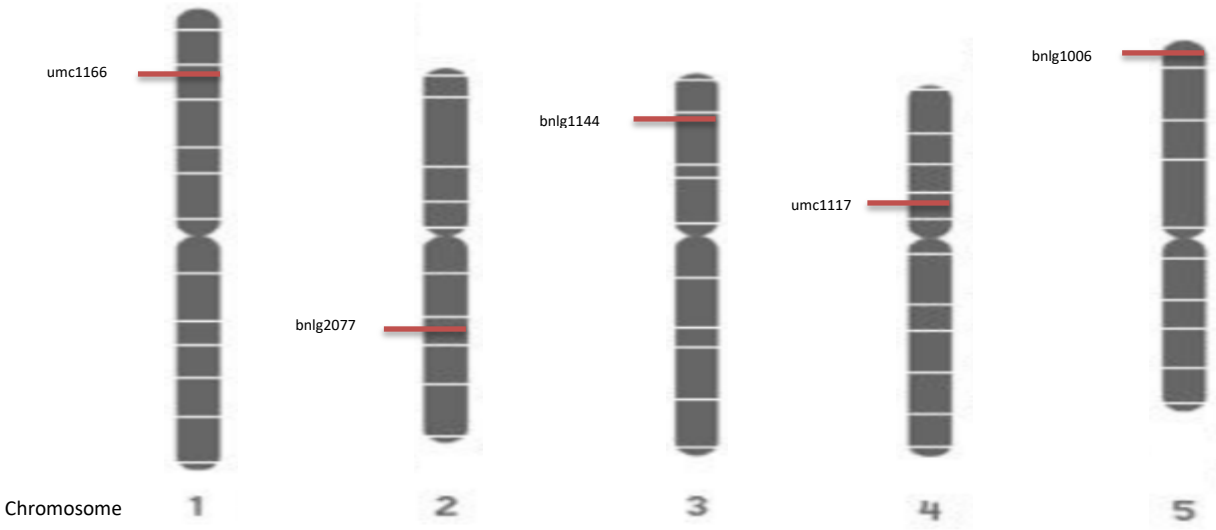
MATERIALS AND METHODS

Twenty-nine maize inbred lines from various sources and maintained through selfing undergoing at the College of Agricultural Engineering Sciences, Salahaddin University, Erbil, were taken for this study. Twenty-four inbred lines were introduced from Poland and the other five from International Maize and Wheat Improvement Centre (CIMMYT) (Table 1). Most of these lines were phenotypically evaluated and well-adapted to the environment in different studies that have taken on top-cross and performance *per se* evaluation to produce a single cross hybrid. Maize seeds were planted in plastic seed trays for around 3 weeks in the greenhouse. Sufficient amounts of fresh leaf were collected from each line, bulked, and powdered to a fine powder utilizing a mortar and pestle. Genomic DNA was isolated from the fresh leaf samples using the Beta Bayern tissue DNA Extraction Kit (Beta Bayern GmbH.90453 Bayern, Germany) following the manufacturer's instructions. DNA quality was checked by DNA quantification using a Nanodrop Spectrophotometer (Thermo Scientific, ND 1000 Spectrophotometer). Ten SSR DNA primers dispersed throughout the maize chromosome were selected from the maize genomic database (20) according to their PIC. The 10 SSR DNA markers used are listed in Figure 1. PCR (Bioresearch PTC-200 Gradient thermocycler) was performed in a total

reaction volume of 50 µl that comprised of 3 µl template DNA, 2 µl each of the forward and reverse primers, 18 µl DNase free water and 25 µl Taq Mastermix (AMPLIQON A/S Stenhuggervej (22).

Table 1. Germplasm sources, pedigrees, and origin of maize lines used in the study

No.	Lines	Source/Pedigree	Origin
1	SNZ	QW15	Poland
2	SSZ	SSS DeKalb	Poland
3	TTS	SSS Dekalb	Poland
4	STE	PM9out/80	Poland
5	TZE	CREOL	Poland
6	ETF	DK.435	Poland
7	ENZ	S5340	Poland
8	EFF	DK.505	Poland
9	PIO	Idt	Poland
10	LHF	sss	Poland
11	HCT	B73 rel Monsanta	Poland
12	FRF	sss-111.Found	Poland
13	DEO	B73Dekalb	Poland
14	SSS	B73Dekalb	Poland
15	LHS	SSS	Poland
16	KRZ	Idt	Poland
17	TZS	ERLEVO	Poland
18	SFN	unknown	Poland
19	SFT	unknown	Poland
20	STS	unknown	Poland
21	SOZ	unknown	Poland
22	BST	unknown	Poland
23	SZO	unknown	Poland
24	MSZ	unknown	Poland
25	ZPF	Monsanta	CIMMYT
26	MSN	CIMMYT	CIMMYT
27	MSE	CIMMYT	CIMMYT
28	MOS	CIMMYT	CIMMYT
29	FZT	CIMMYT	CIMMYT



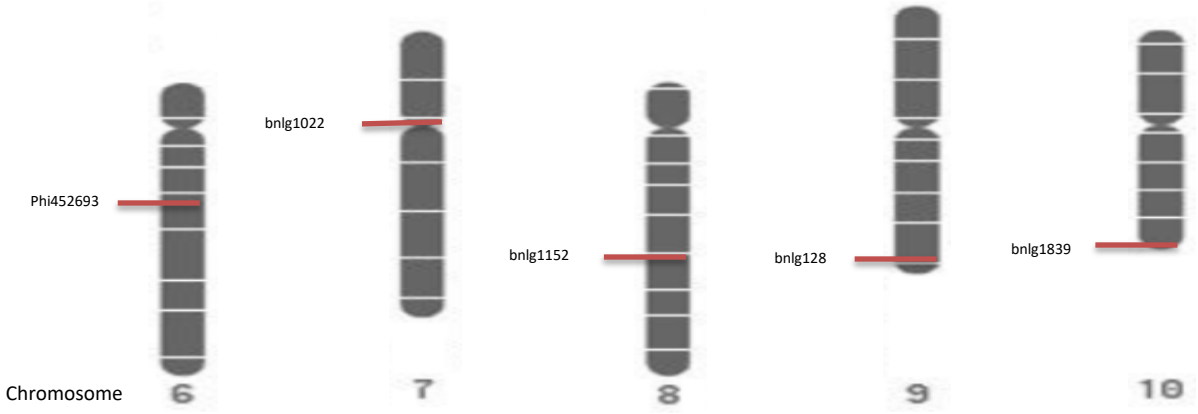


Figure 1. SSR DNA markers (10) were selected based on their PIC value and location throughout the maize chromosomes

For each primer pair, touch-down PCR amplifications were conducted individually. The protocol was 95°C for 3 minutes, followed by 30 cycles of 94˚C for 1 minute. Then, the 67°C annealing temperature was decreased by 0.5°C per cycle for 1 minute with 1 minute extension at 72°C. This was followed by one final extension cycle at 72°C for 10 minutes and an indefinite hold at 4°C. The PCR product was electrophoresed on 1.5% (w/v) agarose gel with 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0), after staining with ethidium bromide for 80 minutes until the loading dye was moved forward for 10 cm. After the completion of electrophoresis, the gels were observed under U.V. light. Photographed and documented utilizing a UV trans-illuminator. The allele bands were manually coded and repeated twice to reduce coding errors. Data were categorized as present (1) or absent (0) for all the genotypes, and bands that were diffused or too hard to code were categorized as missing data (9). Different statistical programs were utilized for each SSR marker to determine the average number of alleles per locus (n_a), effective number of alleles (n_e), observed homozygosity (H_o), expected heterozygosity (H_e) (17), Nei's expected heterozygosity (Nei) (24) and Shannon's information index (I) (18) were measured using the Population Genetic Analysis Software (POPGENE) Version 1.3.1 (36). Furthermore, the polymorphism information content (PIC) was estimated for each SSR marker using the PowerMarker Software Version 3.25 (19). $PIC = 1 - \sum P_{ij}$, where P_i is the frequency of j^{th} alleles for the i^{th} locus. The analysis of molecular variance (AMOVA) was computed utilizing Arlequin software version 3.5 (12). In addition, Dice coefficients (9) were used to measure genetic similarities (GS) among the inbred lines. Then, the dendrogram was constructed using UPGMA following the sequentia1 Agglomerative Hierarchica1 and Nested (SAHN) method by software NTSYS-pc Version 2.1 (27), to observe genetic diversity based on molecular distance coefficients. Consequently, three-dimensional graphs were constructed using the first three principal coordinate analyses (PCoA), using the software NTSYS-pc. The Mantel matrix correspondence test (19) was computed to construct the matrix of correlation for comparison between genetic distance and genetic similarity among the inbred lines.

RESULTS AND DISCUSSION

Genetic diversity is essential for successfully adapting to certain agro-climatic conditions and improving any crop species in any breeding program (23). Little is known about maize germplasm inbred lines adapted to the North of Iraq agro-ecologies. In plant evolution, most of the changes are due to changes in DNA nucleotide sequence (11). The 10 SSR markers with different repeats were assayed to select suitable polymorphic markers (Table 2). The investigation showed

that the AG and CA SSR DNA repeats and their adjacent regions were highly polymorphic in the genome of the 29 maize inbred lines. The overall percentage of polymorphic loci estimated among the 29 maize inbred lines using 10 SSR DNA markers was 100%. This indicates the existence of high diversity of the SSR loci among the maize inbred lines. (28) who found genetic diversity among 187 corn inbred lines using three microsatellite markers also found high polymorphism, with an overall level of polymorphic loci of 100%. However, (10) who found that molecular diversity between population and sub-population of maize ranged between (50-94)%. A total of 40 alleles were found in the 29 inbred lines. In this study, the number of alleles per SSR marker ranged from 2 (bnlg1006, bnlg1022, and bnlg1152) to 9 (umc1166), and the average number per marker was 4 (Table 2). This was less than what (26) reported, who detected a total of 145 alle1es and an average of 7.3 SSR alleles per locus among 20 loci over 500 Ghanaian maize landraces; (33) studied 35 inbred lines and found a total of 217 alleles with an average of 5.43 alleles across 40 loci. The variations in the number of alleles identified in this investigation compared to other investigations might be because of the

Table 2. Genetic information revealed by 10 SSR DNA markers amplified on 29 maize inbred lines

Marker	Bin	Repeat Motif	na	ne	Ho	He	Nei	I	PIC
umc1166	**1.02**	**(CT)10**	**9**	**7.07**	**1.00**	**0.87**	**0.86**	**2.07**	**0.84**
bnlg2077	**2.07**	**AG(33)**	**4**	**3.01**	**1.00**	**0.68**	**0.67**	**1.23**	**0.62**
bnlg1144	**3.02**	**Unspecified**	**5**	**3.22**	**1.00**	**0.70**	**0.69**	**1.37**	**0.65**
umc1117	**4.04**	**(TCGCA)4**	**3**	**2.32**	**1.00**	**0.58**	**0.57**	**0.96**	**0.50**
bnlg1006	**5.00**	**AG(20)**	**2**	**2.00**	**1.00**	**0.51**	**0.50**	**0.69**	**0.37**
phi452693	**6.04**	**AGCC**	**6**	**4.70**	**1.00**	**0.80**	**0.79**	**1.64**	**0.76**
bnlg1022	**7.02**	**AG(12)**	**2**	**1.82**	**1.00**	**0.46**	**0.45**	**0.64**	**0.35**
bnlg1152	**8.06**	**AG(18)**	**2**	**1.67**	**1.00**	**0.41**	**0.40**	**0.59**	**0.32**
bnlg128	**9.07**	**AG(15)**	**3**	**2.87**	**1.00**	**0.66**	**0.65**	**1.08**	**0.58**
bnlg1839	**10.07**	**AG(24)**	**4**	**2.69**	**1.00**	**0.64**	**0.63**	**1.11**	**0.56**
Mean			**4**	**3.14**	**1.00**	**0.63**	**0.62**	**1.14**	**0.55**
SD			**2.21**	**1.63**	**0.00**	**0.15**	**0.14**	**0.46**	**0.16**

Bin: a sub-region on corn chromosome, n_a: average number of alleles, n_e: number of effective alleles, Ho: observed homozygosity, He: expected heterozygosity, Nei: Nei's expected heterozygosity, I: Shannon's information index, PIC: polymorphism information content, SD: standard deviation

sample size and probably the fewer SSR markers analyzed.

The results of the present investigation also indicated that the SSR DNA markers were able to reveal genetic diversity across the 29 maize inbred lines. This was strongly supported by the high I and Nei estimated, which were indicators of gene diversity. High values for I (1.14) and Nei (0.62) were reported among the inbred lines studied, revealing that the inbred lines were differentiated at the DNA level (Table 2). A similar observation was also recorded by (1) in their study on maize, who found high values of I (1.03) and Nei (0.57). The extent of informativeness of the SSR DNA marker depends on its degree of polymorphism, which is reflected in the genetic variation among the studied genotypes (7, 25). In the current study, PIC ranged from 0.32 (bnlg1152) to 0.84 (umc1166), with an average 0.55 (Table 2), indicating that the 10 SSR DNA markers used across the genome of 29 inbred lines were highly informative. (31) stated that the high level of polymorphism was also affected by the degree of polymorphism of the SSR

markers. The highest results of the PIC value in this study was higher than that previously reported by Desai et al (8), which involved 22 maize inbred lines utilizing 17 SSR markers (PIC = 0.384). While lower than that were registered by Abu sin et al (2) from a study on 30 maize inbred lines utilizing 100 SSR markers (PIC = 0.624). The high informativeness of the SSR markers in this investigation suggests that diversity among the lines was high, making it possible to exploit heterosis in hybrid crosses in any breeding program. The results of I, Nei, and PIC were strongly supported by the results of AMOVA (partitioning of genetic variation), which indicated the same tendency of higher variation among (94.58%) than within (5.42%) of inbred lines (Table 3).

Table 3. Results of analysis of molecular variance (AMOVA) among 29 maize inbred lines using 10 SSR DNA markers

Source of variation	df	Sum of squares	Variance component	variation %
Among inbred lines	**28**	**242.29**	**4.31**	**94.58**
Within inbred lines	**29**	**7.00**	**0.25**	**5.42**
Total	**57**	**249.29**	**4.56**	**100**

df : degree of freedom

Kashiani et al (15), in their study among tropical sweet corn inbred lines, recorded that 92.9% of the total genetic diversity was because of diversity among the inbred lines. This is indicating the high degree of homozygosity at all the loci. Due to different pedigrees, origins, and breeding programs of these inbred lines, giving a reflection that these inbred lines belong to different genetic groups. This variation could be used to develop hybrid varieties with a high level of heterosis in the country by using parental lines from different groups. Genetic similarity (GS) coefficients across the maize inbred lines obtained from the amplification of 10 SSR DNA markers equally distributed throughout the genome are shows in Table 4. Among the inbred lines studied, MSN and MSE, DEO and SSS, and SSZ and TTS had the highest genetic similarity (all 0.80). However, the lowest genetic dissimilarity was found between SFT and KRZ, SNZ and SZO, ENZ and PIO, LHF and BST, DEO and KRZ, and MSZ and KRZ (all 0.00). Results of the Mantel test (21) revealed that genetic distance and genetic similarity among the inbred lines obtained from the amplifications of the 10 SSR markers were highly correlated, with positive magnitude (r = 0.625, t = 9.061, at $p \leq 0.0001$). This indicates that they could both be utilized for investigating relationships among the maize inbred lines examined using dendrogram analysis. The dendrogram was generated based on SAHN clustering (Figure 2), to represent genetic divergence across the 29 inbred lines. The inbred lines were grouped into seven different clusters as follows: Group I is represented by three Poland inbred lines, SFN, EFF, and Pio; Group II is represented by three other Poland inbred lines SOZ, SNZ, and TZS; Group III consisted of one CIMMYT inbred line, FZT and the line from Poland, KRZ. Group IV comprised of nine Poland inbred lines, SSS, DEO, SZO, MSZ, SFT, STS, HCT, FRF, and LHF, and two lines from CIMMYT, MSN, and MSE. Group V is represented by six Poland inbred lines, SSZ, TTS, STE, TZE, ENZ, and ETF. Group VI consisted of two Poland inbred lines, BST and LHS, and Group VII comprised of two CIMMYT inbred lines,

Table 4. Molecular genetic similarities among 29 maize inbred lines obtained from amplifications of 10 SSR DNA markers

Inbred Line	Genetic Similarity																											
	SFN	SSS	SFT	STS	SOZ	SNZ	SSZ	STE	TZE	ETF	ENZ	FZT	EFF	PIO	LHF	HCT	FRF	DEO	MSZ	MSN	MSE	MOS	BST	KRZ	TZS	LHS	TTS	ZPF
SSS	0.50																											
SFT	0.40	0.60																										
STS	0.30	0.50	0.70																									
SOZ	0.20	0.30	0.30	0.40																								
SNZ	0.20	0.10	0.10	0.10	0.60																							
SSZ	0.40	0.30	0.40	0.40	0.60	0.40																						
STE	0.30	0.60	0.60	0.60	0.50	0.20	0.70																					
TZE	0.40	0.30	0.40	0.30	0.30	0.60	0.50	0.50																				
ETF	0.20	0.40	0.40	0.30	0.30	0.30	0.50	0.60	0.50																			
ENZ	0.30	0.30	0.40	0.40	0.40	0.40	0.70	0.60	0.70	0.60																		
FZT	0.30	0.20	0.10	0.10	0.50	0.50	0.20	0.20	0.40	0.20	0.30																	
EFF	0.50	0.40	0.20	0.20	0.30	0.30	0.10	0.20	0.20	0.40	0.10	0.50																
PIO	0.50	0.30	0.30	0.20	0.20	0.30	0.10	0.10	0.10	0.10	0.00	0.30	0.60															
LHF	0.40	0.50	0.40	0.60	0.20	0.00	0.10	0.30	0.10	0.20	0.10	0.20	0.30	0.30														
HCT	0.40	0.40	0.60	0.60	0.50	0.30	0.40	0.40	0.20	0.30	0.20	0.20	0.30	0.30	0.50													
FRF	0.30	0.50	0.60	0.60	0.30	0.20	0.40	0.40	0.30	0.50	0.40	0.10	0.30	0.30	0.40	0.70												
DEO	0.50	0.80	0.70	0.60	0.50	0.30	0.50	0.60	0.50	0.40	0.50	0.20	0.20	0.20	0.40	0.50	0.60											
MSZ	0.50	0.40	0.60	0.50	0.30	0.20	0.50	0.40	0.40	0.40	0.50	0.10	0.20	0.20	0.30	0.30	0.50	0.60										
MSN	0.40	0.30	0.30	0.30	0.50	0.50	0.40	0.40	0.50	0.40	0.40	0.30	0.30	0.30	0.20	0.30	0.50	0.50	0.60									
MSE	0.40	0.50	0.50	0.50	0.50	0.30	0.50	0.60	0.50	0.40	0.50	0.30	0.10	0.10	0.30	0.30	0.40	0.70	0.70	0.80								
MOS	0.40	0.40	0.40	0.30	0.20	0.30	0.40	0.30	0.50	0.50	0.60	0.20	0.10	0.10	0.20	0.30	0.60	0.60	0.60	0.50	0.50							
BST	0.20	0.30	0.20	0.20	0.20	0.30	0.50	0.50	0.40	0.40	0.50	0.30	0.20	0.10	0.00	0.10	0.30	0.30	0.40	0.40	0.50	0.40						
KRZ	0.30	0.20	0.00	0.10	0.20	0.40	0.10	0.20	0.30	0.20	0.20	0.60	0.50	0.50	0.20	0.10	0.10	0.00	0.00	0.30	0.10	0.10	0.40					
TZS	0.00	0.30	0.30	0.40	0.60	0.40	0.30	0.60	0.40	0.40	0.50	0.50	0.30	0.10	0.30	0.30	0.30	0.30	0.20	0.40	0.40	0.30	0.40	0.40				
LHS	0.20	0.00	0.20	0.10	0.30	0.40	0.40	0.20	0.50	0.40	0.50	0.40	0.10	0.10	0.00	0.10	0.20	0.20	0.50	0.40	0.40	0.50	0.60	0.30	0.30			
TTS	0.40	0.50	0.50	0.40	0.40	0.30	0.80	0.70	0.60	0.60	0.70	0.20	0.10	0.10	0.20	0.30	0.60	0.70	0.60	0.60	0.70	0.60	0.60	0.10	0.30	0.40		
ZPF	0.40	0.20	0.30	0.30	0.20	0.30	0.40	0.20	0.30	0.40	0.40	0.10	0.10	0.10	0.30	0.60	0.50	0.30	0.40	0.20	0.20	0.70	0.30	0.10	0.20	0.40	0.30	
SZO	0.50	0.70	0.60	0.50	0.20	0.00	0.20	0.40	0.20	0.20	0.20	0.20	0.30	0.40	0.50	0.30	0.40	0.60	0.60	0.50	0.70	0.40	0.30	0.10	0.20	0.10	0.40	0.20

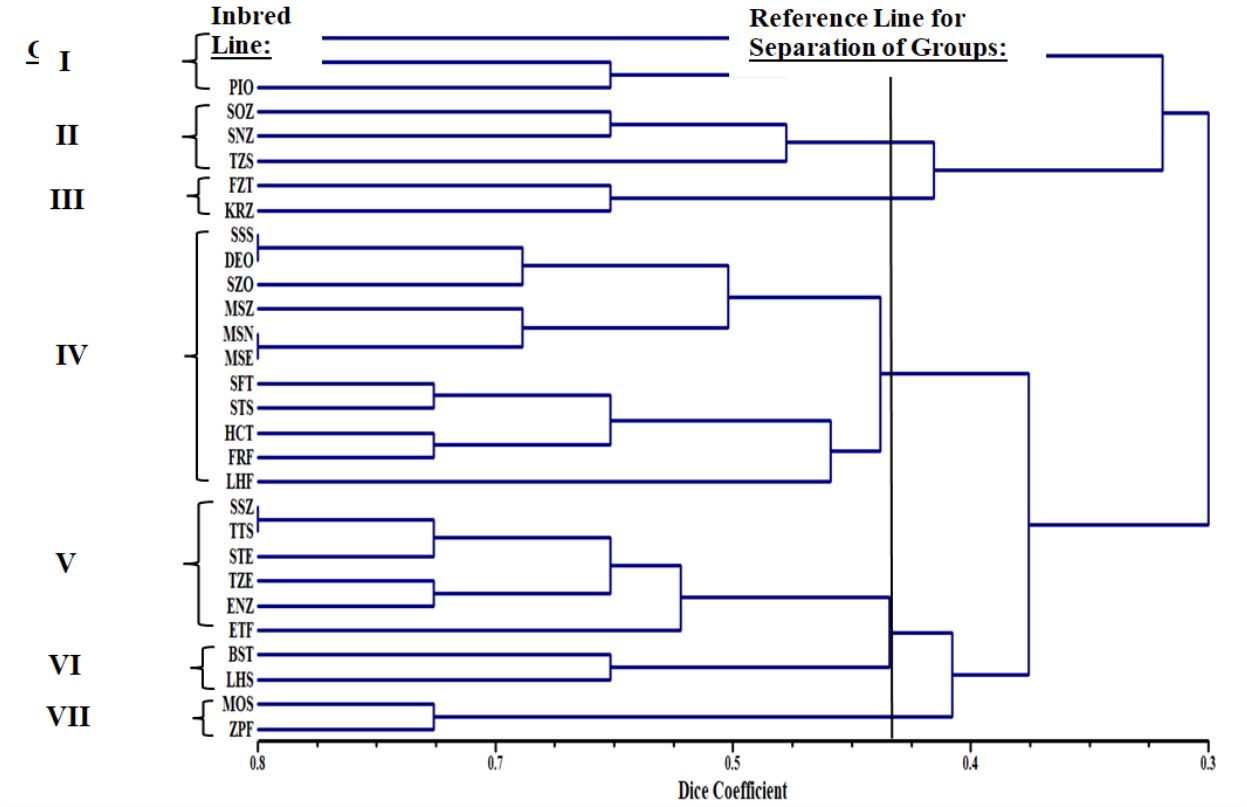


Figure 2. A dendrogram using an unweighted paired group method of arithmetic means (UPGMA) tree showing the grouping of 29 maize inbred lines conducted, using 10 SSR DNA markers

MOS and ZPF. Inbred lines originating from each country tended not to cluster together. This was expected since each inbred line was developed and selected from different sources and breeding programs. (13) reported that cluster analysis based on the UPGMA method for estimation of genetic similarity using SSR markers on 12 sweet corn inbred lines showed three clusters. In the present study, lines from Poland being the most diverse, grouped in seven, out of seven clusters. The CIMMYT lines were grouped into three groups of the seven clusters. The inbred lines from the same source populations were found to cluster together. Inbred lines SSZ and TTS which were descended from source SSS Dekalb were grouped in Cluster five. Inbred lines DEO and SSS which were descended from source B73Dekalb were grouped in Cluster four. Inbred lines MSN and MSE which were descended from source CIMMYT were also grouped in Cluster four. Although the last four lines were genetically related, they were descended from various sources (Table 1). Small variation among the lines was probably due to earlier mixing (intermating) among the source populations (35). The result of the PCoA (Figure 3) revealed a similar grouping of the inbred lines as shown by the UPGMA-rooted tree constructed from molecular dissimilarities among the inbred lines studied. Hence, the 29 maize inbred lines were grouped to seven heterotic groups. This was in agreement with previous reports using SSR DNA markers (2, 22). This indicates that crosses among the lines from various clusters could reveal high heterosis for yield and its components.

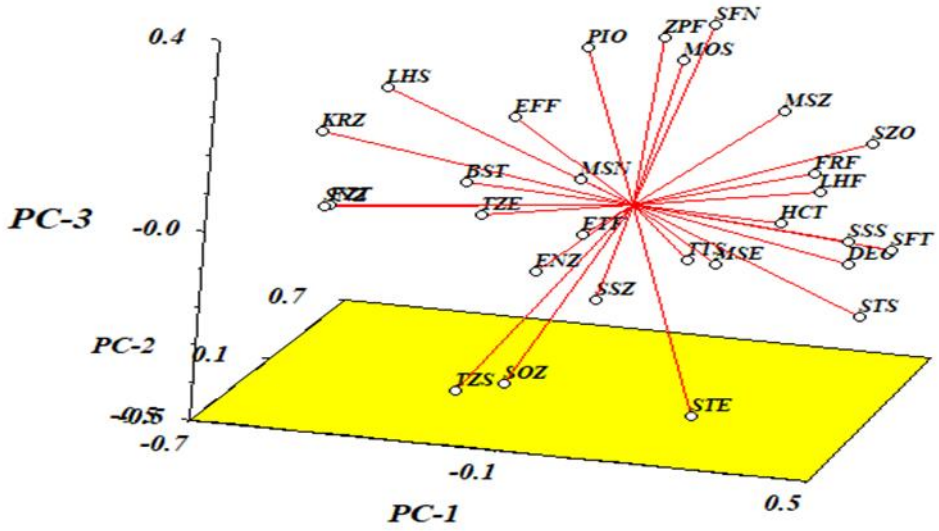


Figure 3. Three-dimensional graph showing relationships among 29 maize inbred lines based on the first three principal coordinates obtained from the amplification of 10 SSR DNA markers

The results of the present study suggested that clustering methods based on genetic variation measurements using SSR DNA markers offer a clear grouping of the 29 maize inbred lines into seven heterotic groups, supported by the three-dimensional principal coordinate analysis (PCoA). The total genetic differences across the maize inbred lines were mainly due to variations among the inbred lines rather than within inbred lines. This indicates that the SSR DNA markers were efficient in investigating genetic variations among the 29 maize inbred lines studied. This information helps select appropriate inbred lines as genetic materials in future plant breeding programs in the North of Iraq.

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