Analysis of Genetic Diversity in Maize(*Zeamays* L.)Varieties Using Simple Sequence Repeat(SSR)Markers

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Abstract

A total of 41 alleles were detected among the tested varieties using 10 Simple Sequence Repeat (SSR) loci distributed on 9 chromosomes of maize.The molecular size of bands obtained from amplification of SSR products ranged from 91 to 288 bp. Alleles ranged from one in umc1653 to ten in bnlg1189 loci. The values of heterozygosity for each locus varied from 0.46 for umc1641 and umc1069 loci to 0.88 for bnlg1189 locus with a mean of 0.55. The polymorphic information content (PIC) values for the SSR loci ranged from 0.17 to 0.85, with an average of 0.44. The highest PIC values were observed in primers bnlg1017 (0.85) and umc1038 (0.79) and the lowest PIC values was observed in primer umc1946 (0.17).The study revealed that, the lowest genetic distance was (0.2410) between varieties IPA 5011 (lane 6) and Sarah (lane 8), while, the highest genetic distance was (0.7853) between varieties DKC 6120 (lane 15) and Manlcet (lane 19). The genetic similarity values ranging from (0.2147 to 0.7590) depending upon the genetic distance values that ranging from (0.2410 to 0.7853), which indicate the larger diversity with percentage of (24 to 78%) among the tested varieties. Analysis of the obtained results from genetic distances and Neighbor-joining dendrogram (unrooted tree) revealed that, the 20 tested maize varieties can be grouped into two major groups: one small cluster A containing 4 varieties and a large cluster B containing 16 varieties. The results obtained in this study may assist maize cultivation and in maize breeding programes.

Key words: Genetic diversity; *Zea mays*; SSR markers; genetic distance.

الخلاصة

تم الكشف عن 41 من الأليلات بين الأصناف باستعمال 10 من المواقع الوراثية ذات التتابع المتكرر البسيط (SSR) Simple Sequence Repeat والمنتشرة على تسعة من كروموسومات الذرة الصفراء. وتراوح الحجم الجزيئي للحزم الناتجة من تضاعف المواقع الوراثية للـ SSR من 91 إلى 288 زوج قاعدي. وتراوح عدد الأليلات في المواقع الوراثية من أليل واحد في umc1653 إلى عشرة أليلات في الموقع الوراثي bnlg1189. ووجد أن قيم التغاير الزايكوتي heterozygosity لكل موقع تراوح من 0.46 في (umc1641) إلى 0.88 في (bnlg1189) وبمعدل قدره 0.55. تراوحت قيم محتوى المعلومات متعددة الأشكال polymorphic information content (PIC) لكل موقع وراثي من 0.17 إلى 0.85، وبمعدل قدره 0.44. ولوحظ أن أعلى قيمة للـ PIC كانت للبادئين bnlg1017 (0.85) و umc1038 (0.79) في حين كانت أقل قيمة في البادئ umc1946 (0.17). توصلت الدراسة من خلال هذا المؤشر الجزيئي إلى أن أقل بعد وراثي هو (0.2410) والذي حصل بين الصنفين IPA 5011 (رقم 6) والصنف سارة (رقم 8)، بينما وجدت أكبر بعد وراثي هو (0.7853) بين الصنفين DKC 6120 (رقم 15) و Manlcet (رقم 19). وعلى أساس قيم البعد الوراثي تم إيجاد قيم التشابه الوراثي التي تراوحت من 0.2147 إلى 0.7590، والتي تشير الى التنوع الكبير الذي نسبته (24 إلى 78%) بين الأصناف المدروسة. كشف تحليل النتائج التي تم الحصول عليها من البعد الوراثي وشجرة العلاقة الوراثية Neighbor–joining dendrogram إلى أن كل الأصناف قيد الدراسة توزعت إلى مجموعتين رئيسيتين: مجموعة واحدة صغيرة تدعى A تحتوي على أربعة أصناف، ومجموعة كبيرة تدعى B تحتوي على ستة عشر صنفا. النتائج التي تم الحصول عليها في هذه الدراسة يمكن أن تساعد في زراعة الذرة الصفراء وبرامج تربيتها.

الكلمات الدالة: التنوع الوراثي، الذرة الصفراء، واسمات التتابع المتكرر البسيط، البعد الوراثي.

Introduction

Maize (*Zea mays*) is one of the world's most important crop plants after wheat and rice, which provides staple food to large number of human population in

the world. It is belonging to the family of grasses (Poaceae) (Farhad *et al*., 2009). It has greater nutritional value as it contains about 72% starch, 10% proteins, 8.5%fiber, 4.8% oil, 3% sugar and 1.7% ash. It is not only an important human nutrient, but also a basic element of animal feed and raw material for manufacture of many industrial products (Farhad *et al*., 2009 and Ministry of Environmental and Forests, 2010). Information concerning the genetic diversity of maize hybrids is very important for germplasm enhancement, hybrid breeding and preventing environmental damage that may occur due to the genetic uniformity of hybrids grown on large areas. Unless there is sufficient genetic diversity in the germ plasm, it is practically not possible to increase the yield and other desirable characters of a crop, because the selection of improved genotypes depends on the availability of genetic variability within the breeding material (Bauer *et al*., 2007). Among DNA marker methods, most commonly used is polymerase chain reaction (PCR). There are two kinds of PCR-based assays, for example, random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSRs), powerful DNA fingerprinting techniques (Cholastova *et al*., 2011). Microsatellites or (SSRs) composed of a DNA sequence motif of 2–6 bases in length that is repeated tandemly usually five or more times are markers that have become widely used in genetic studies in maize (Qi-Lun *et al*., 2008). Besides its high level of polymorphism, this technique is useful molecular markers because they are abundant, uniformly distributed, codominant, rapidly produced by PCR, relatively simple to interpret and easily accessed by other laboratories via published primer sequences. Besides, for measuring diversity, it is very useful tool for assigning lines to heterotic groups and for genetic fingerprinting (Park *et al*., 2008).Measuring genetic diversity in populations of sweet corn, e.g., is important for understanding of the genetic structure and subsequently improving the breeding process (Rupp *et al*., 2009). Nearly 1000 SSR markers are available in maize under public domain facilitating their utilization for diverse purposes in genetics and plant breeding, (Kumari *et al*., 2005) and also used as an important tool for purity identification of maize hybrid (Wu *et al*., 2010).The aim of this study was detection of polymorphism at DNA level among varieties of maize genetic deployed in Iraq by investigation and application of DNA marker such as, SSR and to find of DNA fingerprint distinctive for varieties and that serves as an identity that is used to diagnose these varieties; and assessment and evaluation of genetic distance and phylogenetic diversity of varieties so as to guide breeder an appropriate choice for parents to conduct breeding.

Materials and Methods

Samples Collection:

Collected grain maize varieties from different regions in the country and certified sources, such as: Buhooth 106, IPA 2052, IPA 5015, IPA 5012, IPA 5018, IPA 5011, IPA 5026, Sarah, Al-Maha, SNH 8605, SNH 5610, Kr 640, 3078, Pio 3751, DKC 6120, DKC 5783, 89 May 70, Biotech Bag, Manlcet and DKC 6418. Its grains were sown on 21/7/2011 for autumn cultivation and samples of maize leaves (2 weeks old) were collected for DNA isolation.

DNA Isolation:

The genomic DNA Mini Kit (Geneaid Biotech. Ltd; Taiwan Company) provides a quick and easy method for purifying total DNA (including genomic DNA,mitochondrial and chloroplast DNA) from plant tissue. DNA was isolated from leaves according to the method protocol.

DNA Quantification and Quality Determination:

To determine the concentration, 20µl of DNA from each sample was added to 1980 µl of TE buffer and mixed thoroughly then optical density (OD) was measured using a spectrophotometer at wavelengths of 260 nm and 280nm. The DNA concentration in the solutions was calculated according to the following formula: DNA conc. (µg /µl) = [OD260 *100 (dilution factor) * 50 µg/ml] /1000. Theoretically, OD260 of one corresponds to approximately (50µg/ml) for double strand DNA. The ratio between the reading at 260nm /280nm provides estimate of the purity of nucleic acid (Sambrook and Russell, 2001).

PCR Amplification of SSR-Primers:

According to the Experimental Protocol of AccuPower® TLA PCR PreMix, the PCR reaction mixture was prepared as follows: **1.** 5µl template DNA and 4 µl of primer (10 pmole/µl, 2µl forward and 2µl reverse), were added to each AccuPower® TLA PCR PreMix tube. **2.** Sterilized deionized distilled water was added to AccuPower® TLA PCR PreMix tubes to the final volume of 20 µl. **3.** The tubes were mixed with vortex to dissolve the lyophilized blue pellet, and briefly spine down (all these steps were done in ice). a sequence was amplified individually using SSR primer (listed in table 1).Amplification were performed in thermocycler programmed according to annealing temperatures as follows: **1.** one cycle of 1 min at 94C°, for 40 cycle of each 15 sec at 94C°, 15 sec at 55C° and 30 sec at 72C°, with a final extension for one cycle of 1 min at 72C° (umc2013, umc1038 and bnlg1017). **2.** one cycle of 5 min at 94C°, for 35 cycle of each 40 sec at 94C°, 35 sec at 60C° and 45 sec at 72C°, with a final extension for one cycle of 5 min at 72C° (umc1630, umc1946, umc1641 and bnlg1189). **3.** one cycle of 4 min at 94C°, for 35 cycle of each 1 min at 94C°, 1 min at 58C° and 2 min at 72C°, with a final extension for one cycle of 7 min at 72C° (umc1653, umc1069 and bnlg1810).

Table 1: Names and sequences of SSR primers used in this study.

Primer	Sequences (5' → 3')
umc1630	Forward: CAGACCTTCGAGGGCAAGAACT Reverse: AGTTTTGGCTTCTTCTCCCAAGTC
bnlg1017	Forward: ATTGGAAGGATCTGCGTGAC Reverse: CAGCTGGTGGACTGCATCTA
umc1946	Forward: GAAACGACCAGCACAGCACAT Reverse: GCACCACACCATCAGATCCAG
umc1641	Forward: CTCCCTTCGTCTCCCGACTC Reverse: CAGATCGGCTCAGCCACAAC
bnlg1189	Forward: CGTTACCCATTCCTGCTACG Reverse: CTTGCTCGTTTCCATTCCAT
umc2013	Forward: GGGACGAGAGTCTGTTGTTGTTG Reverse: GTTGATGCATGTGACTCTGGAAAC
umc1653	Forward: GAGACATGGCAGACTCACTGACA Reverse: GCCGCCCACGTACATCTATC
umc1069	Forward: AGAGAATCCCCAAGCAAACAAAC Reverse: CTTCATCGGAGCCATGGTGT
bnlg1810	Forward: ATGCTCCTCCTCTCCTCCAT Reverse: GCGATGATGAGCTGCAAGTA
umc1038	Forward: CGTCACACTCCTCTGCCACTT Reverse: GAGGATTCAGAACTCGACTCGG

Then amplified DNA were separated by electrophoresis in 2-3 % agarose gels (1.5-2 hr, 100V) to be sure that the PCR process succeeded (Weigand *et al*., 1993). PCR products were visualized by U.V transilluminator and then were imaged by gel documentation system (Hashemi *et al*., 2009), the size of SSR-PCR products estimated by comparing with the marker 100 bp DNA ladder 100-2,000 bp.

Data Analysis of SSR Products:

The resulting microsatellite data were analysed using Power Marker V.3 software (http://www.powermarker.net) to calculate the number of alleles, heterozygosity and polymorphic information content (PIC). Genetic distance was computed using Nei's (1972) standard genetic distance (Ds). The distance method of Nei (1972) was used with program Power Marker V.3 for the construction of phylogenetic tree. Neighbore─joining method was used to obtain the tree. The tree was then viewed using the TREEVIEW version 1.6 (http://taxonomy. zoology. gla. ac. uk/ rod/rod.html) programs.

Results and Discussion

The isolated DNA (concentration ranges from 100 ng/µl to 295 ng/µl) with purity of 1.8─1.9 determined by spectrophotometric ratio A260/A280.

The results obtained in these experiments revealed that 10 primers have amplified the specific regions and produced specific bands (SSR primers were umc1630, umc1946, umc1641, umc2013, umc1038, bnlg1017, bnlg1189, umc1653, umc1069 and bnlg1810). For scoring and data analysis, the amplified loci were run on agarose gel with high concentration.

When SSR─PCR products were separated by agarose gel electrophoresis (Figures 1, 2, 3 and 4) either one band (homozygous) or two bands (heterozygous) were noticed for each primer depending on the types of microsatellite loci (homozygous or heterozygous). Microsatellites as they are co─dominant marker thus heterozygote produces two bands revealing the amplification of the two loci and could be readily identified (Wu *et al*., 2010). Microsatellite co─dominancy increases the efficiency and accuracy of population genetic measures based on these markers compared with other markers (Wang *et al*., 2009). The molecular weight of these bands obtained from amplification of SSR products of twenty investigated maize variety ranged from 91 to 288 bp.

In similar studies, Senior *et al*., (1998) reported that the molecular weight of bands obtained from SSR products of maize ranged from 62 to 284 bp. Also, in a study by Li *et al*., (2002) on Chinese maize inbred lines, the molecular weight was ranged from 49 to 286 bp. Park *et al*., (2008) found that the molecular weight of bands obtained from amplification of microsatellite loci in 76 korean waxy corn ranged from 75 to 175 bp.

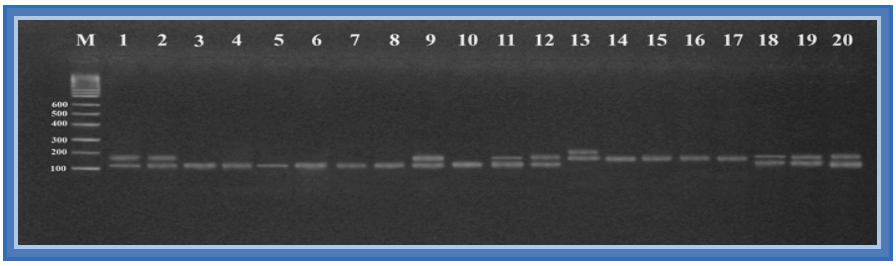

Figure 1: The amplification results of the SSR primer umc1630, lane M: DNA ladder and lanes 1-20: maize varieties.

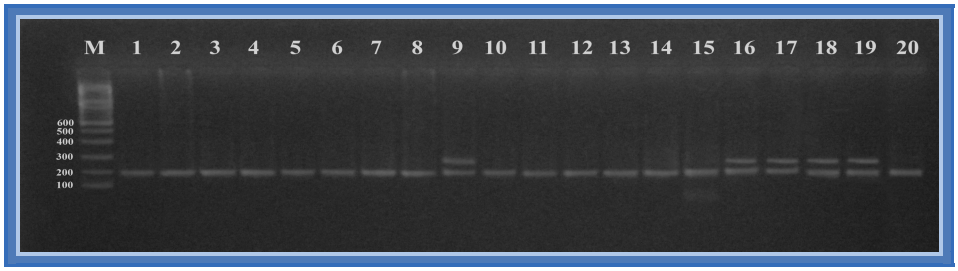

Figure 2: The amplification results of the SSR primer umc2013, lane M: DNA ladder and lanes 1-20: maize varieties.

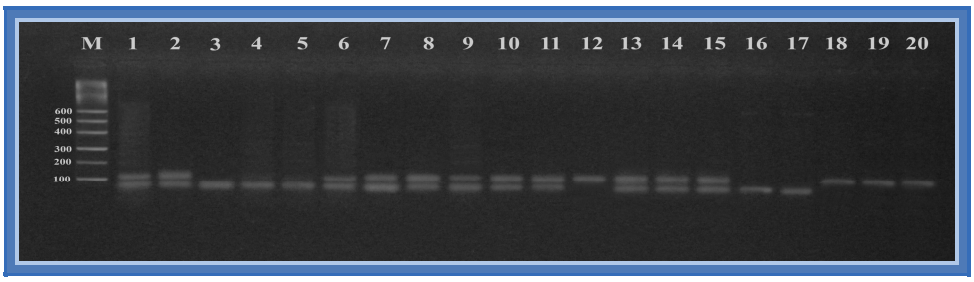

Figure 3: The amplification results of the SSR primer umc1069, lane M: DNA ladder and lanes 1-20: maize varieties.

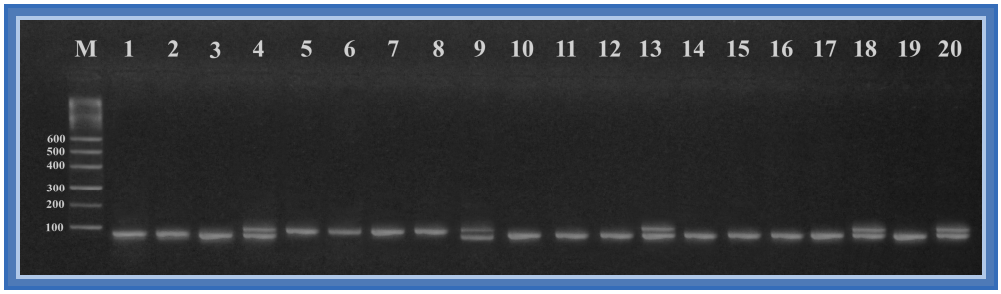

Figure 4: The amplification results of the SSR primer bnlg1810, lane M: DNA ladder and lanes 1-20: maize varieties.

The total number of alleles detected was 41 for ten SSR loci in twenty maize varieties (Table 2). The alleles ranged from one in umc1653 to ten in bnlg1189 loci. The average number of alleles per locus among the varieties was 4.1 and the mean number of alleles reported by many authors in previous studies was similar as those found in this study. Warburton *et al*., (2002) found an average of 3.8 alleles in 9 Asian inbred lines and 3.04 by Yen *et al*., (2002) in 13 Indian inbred lines. Similarly mean alleles detected by George *et al*., (2004) had higher (5 alleles) in 68 Asian maize inbred lines, 4.9 alleles in 64 tropical Asian lines.

Variation reported in the number of alleles of maize varieties may be related to variation in the loci studied as well as the number of genotypes and the locations or due to wild range of origin varieties used in the study (Gurung *et al*., 2010).

The values of heterozygosity for each locus varied from 0.46 for umc1641 and umc1069 loci to 0.88 for bnlg1189 locus with a mean of 0.55 (Table 2).

These results are similar to the results obtained in previous studies made in maize, for example: Kostova *et al*., (2006) found a mean heterozygosity of 0.51, and Morales *et al*., (2010) a value of 0.54 and which was higher than the findings by Yao *et al*., (2008), where they observed an average value of 0.39.

Heterozygosity gives an idea of the information available from the SSR loci and their potential to detect differences between lines based on their genetic relation. The differences among varieties may be attributed mainly to differences in the genetic base. We also considered the fact those microsatellites with show a higher number of allelic variants. The numbers of alleles as well as the diversity values confirm the wide genetic base of the maize varieties (Eyherabide *et al*., 2006 and Morales *et al*., 2010).

The PIC values for the SSR loci ranged from 0.17 to 0.85, with an average of 0.44 (Table 2). The highest PIC values was observed in primers umc1038 (0.79) and bnlg1017 (0.85) and the lowest PIC values was observed in primer umc1946 (0.17).

PIC demonstrates the informativeness of the SSR loci and their potential to detect differences among the varieties based on their genetic relationships. Legesse *et al*., (2007) also reported an average PIC value of 0.58, but their highest PIC value was 0.71 and also number of SSR loci having highest PIC values was very low. The high level of polymorphism is due to diverse varieties and more variation of SSR loci used in the present study.

SSRs comprised of dinucleotide repeats had the highest average PIC value of 0.85. These results were consistent with the results of Etten *et al*., (2008) who found an average PIC value for all SSR loci of 0.62 and an average PIC value for dinucleotide repeats of 0.70.

In contrast, trinucleotide repeats are more abundant in protein coding regions (Toth *et al*., 2000) with relatively small repeat numbers and total length (Odeny *et al*., 2007).

Table 2: Summary of genetic diversity at ten SSR loci in maize. Primer name, bin location, repeat type, fragment size range (pb), no. of amplified bands, number of alleles, heterozygosity and polymorphic information

No.	Primer	Bin location	Repeat type	Fragment size range (pb)	No. of amplified bands	No. of alleles	Heterozygosity	Polymorphic information content (PIC)
1	**umc1630**	1.11	(ATGGG)4	117-208	29	5	0.69	0.29
2	**bnlg1017**	2.02	(AG)18	210-254	30	3	0.54	**0.85**
3	**umc1946**	2.07	(GCTGCT)	91-141	27	4	0.63	**0.17**
4	**umc1641**	3.10	(TCGCC)4	200-264	25	2	**0.46**	0.22
5	**bnlg1189**	4.07	(AG)12	101-145	25	**10**	**0.88**	0.52
6	**umc2013**	5.07	(TGG)4	200-288	28	8	0.74	0.48
7	**umc1653**	6.07	(GAAA)24	94-94	**20**	**1**	0.00	0.00
8	**umc1069**	8.08	(GGAGA)6	124-143	27	2	**0.46**	0.31
9	**bnlg1810**	9.01	(AG)14	80-119	**31**	3	0.59	0.74
10	**umc1038**	10.07	(CT)15	88-103	25	3	0.51	0.79
			Mean			**4.1**	**0.55**	**0.44**

The genetic relationship between the varieties was determined using the genetic distances. This difference between the two varieties can provide a good estimate of how divergent they are genetically (Avise, 1994). The genetic

relationships between the genotypes were estimated with Nei, (1972) standard genetic distance (GD). Other authors also made use of (GD) estimates (Yu *et al*., 2012). The matrix for genetic distance estimates is shown in Table 3.

The lowest genetic distance was (0.2410) between varieties IPA 5011 (lane 6) and Sarah (lane 8) which means that the presence of similarity between these two varieties is high degree using SSR markers; may be because they were from local varieties and cultivated in the country, as well as morphological features and traits.

The highest genetic distance was (0.7853) between varieties DKC 6120 (lane 15) and Manlcet (lane 19) which means that the presence of similarity between them are very low and they were collected from different geographical origins (Baghdad cities and Kurdistan region respectively) but introduced to the country of the same place (USA) (Table 3).

The genetic similarity values ranging from (0.2147 to 0.7590) depending up on the genetic distance values ranging from (0.2410 to 0.7853), which indicate the substantial diversity (24% to 78%) among the varieties used for this study. The range of the genetic similarity obtained in this study were within the ranges reported by Sedaf, (2010) in a set of temperate in inbred lines of maize (0.17 to 0.92). In tropical and subtropical maize, Banisetti, (2012) reported a range of genetic similarities of (0.16 to 0.88). Genetic distance (or similarity) can reveal the genetic diversity of individuals (Sedaf, 2010).

Table 3: The genetic distance values between maize varieties studied in SSR analysis.

Varity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	0.0000																			
2	0.3645	0.0000																		
3	0.3859	0.4859	0.0000																	
4	0.5549	0.5338	0.3556	0.0000																
5	0.5816	0.5416	0.2456	0.3813	0.0000															
6	0.4734	0.5734	0.2842	0.2892	0.3527	0.0000														
7	0.3589	0.3589	0.4842	0.5137	0.3527	0.3572	0.0000													
8	0.5744	0.5214	0.3842	0.3892	0.2527	**0.2410**	0.3572	0.0000												
9	0.5335	0.6015	0.4505	0.4638	0.5116	0.3589	0.3487	0.3589	0.0000											
10	0.3859	0.5769	0.3541	0.4162	0.3256	0.3270	0.2670	0.3270	0.3859	0.0000										
11	0.2645	0.3632	0.4159	0.5488	0.5045	0.3906	0.4589	0.3906	0.2984	0.4459	0.0000									
12	0.4645	0.4616	0.3816	0.5047	0.5636	0.5434	0.5274	0.5244	0.6376	0.5343	0.5335	0.0000								
13	0.3935	0.3908	0.5162	0.5252	0.3813	0.2486	0.4286	0.5286	0.5303	0.4556	0.4621	0.4638	0.0000							
14	0.4602	0.5375	0.6705	0.4809	0.5116	0.4589	0.7175	0.5529	0.4735	0.5459	0.3984	0.4335	0.3303	0.0000						
15	0.4723	0.6638	0.5162	0.4606	0.4813	0.5286	0.4892	0.4286	0.4401	0.4556	0.5288	0.4663	0.4606	0.2503	0.0000					
16	0.5417	0.5531	0.6670	0.5292	0.2527	0.5278	0.5263	0.5620	0.5234	0.5842	0.5031	0.5336	0.7437	0.4924	0.4537	0.0000				
17	0.5093	0.4754	0.5182	0.2575	0.4438	0.4602	0.4602	0.4602	0.5013	0.5572	0.5376	0.5754	0.4316	0.5013	0.4316	0.0912	0.0000			
18	0.4905	0.4638	0.4556	0.2906	0.4813	0.4537	0.4537	0.4537	0.4638	0.6162	0.4638	0.4949	0.5442	0.4638	0.5452	0.2486	0.4626	0.0000		
19	0.6325	0.3145	0.3859	0.4638	0.5316	0.5324	0.5734	0.4924	0.5035	0.4561	0.6165	0.6315	0.5638	0.5076	**0.7853**	0.6589	0.6513	0.6952	0.0000	
20	0.5234	0.2689	0.4842	0.4537	0.4527	0.5172	0.3572	0.5108	0.5234	0.4842	0.5304	0.5274	0.4892	0.5264	0.4537	0.5178	0.5602	0.5537	0.3589	0.000

Yu *et al*., (2012) examined the genetic diversity of 80 inbred waxy maize lines using 22 SSR molecular markers that could be used to achieve heterosis in waxy maize and found the values genetic distance from (0.233 to 0.505). An SSR marker used for diversity studies has recorded genetic distance range of 16-53% among 14 genotypes of maize using four SSR markers (Sedaf *et al*., 2010). These results agreement with results of the present study.