Mansoura university

Mansoura journal of Biology

Genetic diversity of maize inbred lines as indicated by molecular markers

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Received 9 March 2014; accepted 2 June 2014

Keywords
Maize;
Molecular markers;
Cluster analysis;
SSR;
ISSR;
RAPD

Abstract Genetic diversity of seven inbred lines of maize (***Zea maize* L.**) was studied using different molecular markers (SSR, ISSR and RAPD markers). All used markers were able to clearly separate the inbred lines into two clusters. The inbred line Inb 2 was separated in one cluster along with the inbred line Inb 56 according to both ISSR and RAPD molecular markers while it was separated apart of all clusters according to SSR markers. The inbred lines Inb 66, Inb 72, Inb 76, Inb 82 and Inb 102 were clustered in one group according to all types of molecular markers. The results of both ISSR and RAPD markers are more or less the same but differ from the results obtained from the SSR data. Because of its specificity, SSR markers are more reliable than both ISSR and RAPD markers in addition to their co-dominance nature, so that their results can be considered for genetic relationships studies. Thus, it can be said that Inb 2 could be used for hybridization with all other inbred lines except of the inbred line Inb 56 to give single cross with the highest growth vigorous from the inbred lines used in this study.

Introduction

Maize (*Zea mays* L.) belongs to family *Poaceae*. It is the third important cereal crop of the world as well as of Egypt (after wheat and rice). The harvested area of maize in Egypt was increased from 750,000 ha to 825,000 ha in the years of 2012 and 2013, respectively. On the other hand, the yield productivity was 7.3 MMT in 2011 while increased to 7.6 MMT in 2012 (FAO., 2013). Thus, producing new maize hybrids with high potential of yield production could improve maize productivity in Egypt.

Maize breeding programs depend on knowledge of genetic diversity and relationship among inbred lines and breeding material. This is especially fundamental in assigning inbreds to heterotic groups and planning outstanding hybrid crosses. There are several approaches in assessing genetic similarity between breeding material (i.e. inbred lines, hybrids, populations, landraces and races), which include analysis of pedigree and heterotic data, morphological data and/or molecular data, such as DNA markers (Li *et al.,* 2002). There are several molecular markers used for plant genetic analysis. Each one of these marker systems offers a unique combination of advantages and disadvantages

(Kumar *et al.,* 2009). They differ in the type of sequence polymorphism detected, the information content, the dominance relationships between alleles, the amount of DNA required, the need for DNA sequence information in the species under analysis, the development costs, the ease of use and the extent to which they can be automated (Kumar *et al.,* 2009).

The choice of marker system is to significant extent dictated by the specific application and there is probably not single class of markers that can satisfy all the needs encountered by plant geneticists and breeders. The RAPD has been useful technique in studying polymorphism, identifying genes of interest and characterizing genetic resources. The RAPD consists in the random amplification of the DNA fragments with a single primer with an arbitrary sequence, resulting in the final synthesis of several DNA fragments, with different sizes. From this set of fragments, it is possible to determine the polymorphism in the DNA sequence, which can be used as a genetic marker (Williams *et al.,* 1990). The RAPD markers have been a powerful tool in the assessment of the genetic diversity between Maize inbred lines (Srdic *et al.,* 2007). ISSR markers are widely used for study of genetic fingerprinting and genetic diversity analysis of many species of plants. ISSR markers detect polymorphism in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Zietkiewicz *et al.,* 1994; Kumar *et al.,* 2009).The ISSR data are useful in the selection of inbred lines to be tested for general and specific combining abilities and further development of synthetics (Mbuya *et al.,* 2012).

Microsatellites, also known as simple sequence repeat (SSRs), Short Tandem Repeats (STRs) or Sequence-tagged Microsatellite Sites (STMS), are tandemly repeated units of short nucleotide motifs that are 1 to 6 bp long. Di-, tri- and tetra-nucleotide repeats are widely distributed throughout the genomes of plants and animals (Tautz and Renz, 1984). One of the most important attributes of microsatellite loci is their high level of allelic variation, making them valuable and informative genetic markers (Xu, 2010). SSR markers have been the most widely used DNA marker type to characterize germplasm collections of crops because of their easy use and detect, relatively low price, co-dominant and the high degree of polymorphism provided by the large number of alleles per locus (Vignal *et al.,* 2002). Currently, thousands of SSR primer pairs have been developed and mapped on corn genome, the list of which is available in the Maize GDB database (http://www.maizegdb.org). To date, SSRs have been used on corn for mapping (Taramino and Tingey, 1996), genetic fingerprinting (Smith *et al.,* 1997; Senior *et al.,* 1998) and to assess genetic diversity among inbred lines (Lu and Bernardo, 2001; Enoki *et al.,* 2002; Liu *et al.,* 2003).

In maize, one of the main tasks of breeders involve developing improved inbred lines and identifying the best parental combinations for creating hybrids that are phenotypically superior and with significantly higher yield compared to their parents therefore the objectives of this study are to (a) determine of genetic diversity of seven white inbred lines of maize using molecular markers (SSR, ISSR and RAPD markers), (b) compare the results from all of those markers.

Materials and methods

Plant material and DNA extraction

Seven white inbred lines of maize (*Zea maize* L.) were kindly provided by the Agricultural Research Center, Giza, Egypt and used in this study (Table 1). Maize grains were grown in the green house and leaves of seedlingswere collected after ten days of sowing and were directly ground in liquid nitrogen using pestle and mortar. DNA isolation and purification was carried out using modified Cetyl-tetramethyl ammonium bromide (CTAB) method (Saghai-Maroof *et al.,* 1984).

Table 1: Maize inbred lines used in the study and their pedigree.

No.	Genotype	Endosperm color	Pedigree
1	Inb. 2	White	G4 A. E.
2	Inb. 56	White	Rg – 11 g.s. (Bedia*Ci64) (S.C. 14)
3	Inb. 66	White	Rg – 21 g.s. (Bedia*303) (G216*Mo2Rt)
4	Inb. 72	White	Rg – 29 g.s. (Syn. Laposta*303) (G216*Mo2Rt)
5	Inb. 76	White	Rg – 33 g.s. (PI221866*307A) (S.C.14)
6	Inb. 82	White	Rg – 39 g.s. (Sanjuan*307) (S.C. 14)
7	Inb. 102	White	Rg – 59 g.s. (Syn. Laposta*307) (S.C.14)

SSR markers:

Nine maize specific primer pairs were used to carry out the SSR analysis (Hoxha *et al.,* 2004, Table 2). The PCR amplification reactions were performed in a 25 µl volume using 50 ng DNA containing 0.5 µmoles of each primer pair, 100 µM of dNTPs, 2.5 µl (1X) of Taq DNA polymerase buffer, 1.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (GoTaq Flexi DNA polymerase, Promega Inc.). The SSR reactions were carried out using Touchdown PCR program. The main program was 7 cycles at 94°C for 1 min, 59°C for 1 min, decreasing 1°C in every cycle, and 72°C for 1 min, followed by 28 cycles at 94°C for 1 min, 52°C for 1 min. and 72°C for 1 min. A denaturation step at 94°C for 2 min. and an extension step at 72°C for 5 min. were carried out before and after the previous programs, respectively. PCR products were separated on 1.5 % agarose gel electrophoresis.

Table 2: SSR primers sequences, total and polymorphic bands generated by each and their polymorphic information content (PIC).

No.	Primer name	Primer sequence	Total # bands	Polymorphic bands	PIC
1	phi015	F:5'-GCAACGTACCGTACCTTTCCGA-3' R:5'-ACGCTGCATTCAATTACCGGGAAG-3'	3	2	0.67
2	Phi021	F:5'-TTCCATTCTCGTGTTCTTGGAGTGGTCCA-3' R:5'-CTTGATCACCTTTCCTGCTGTCGCCA-3'	4	3	0.75
3	Phi022	F:5'-TGCGCACCAGCGACTGACC-3' R:5'-GCGGGCGACGCTTCCAAAC-3'	6	5	0.86
4	Phi034	F:5'-TAGCGACAGGATGGCCTCTTCT-3' R:5'-GGCGAGCACGCCTTGGTTCT-3'	9	8	0.89
5	Phi084	F:5'-AGCAGAACGGCAAGGGCTACT-3' R:5'-TTTGGCACACCACGACGA-3'	4	1	0.75
6	Phi127	F:5'-ATATGCATTGCCTGGAACTGGAAGGA-3' R:5'-AATTCAAACACGCCTCCCGAGTGT-3'	4	3	0.75
7	Umc1014	F:5'-GAAAGTCGATCGAGAGAGACCCTG-3' R:5'-CCCTCTCTTCACCCCTTCCTT-3'	8	6	0.72
8	Umc1152	F:5'-CCGAAGATAACCAAACAATAATAGTAGG-3' R:5'-ACTGTACGCCTCCCCTTCTC-3'	2	1	0.50
9	Umc2050	F:5'-CTCCTGCTGTGATTCTAGGACGA-3' R:5'-CTGGATCTCGGCATGGTCTT-3'	2	1	0.50

ISSR markers:

Eleven ISSR primers were selected in order to carry out the ISSR analysis (Zietkiewicz *et al.,* 1994, Table 3). The 25µl reaction volume contained 2.5 µl of reaction buffer (1X), 150 µM dNTPs, 1.5 mM $MgCl_2$, 1 µM of primer, 0.75 U Taq DNA polymerase (GoTaq Flexi DNA polymerase, Promega Inc.) and 50 ng of genomic DNA. The PCR program included a denaturation step at 94 ^{o}C for 4 minutes, followed by 40 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, and a final extension step at 72 oC for 4 minutes. The PCR products were separated on 1.5 % agarose gel electrophoresis

Table 3: ISSR primers sequences, total and polymorphic bands generated by each and their polymorphic information content (PIC).

No.	Primer name	Primer sequence	Total # bands	Polymorphic bands	PIC
1	844A	5'- $(CT)_8$GC-3'	7	7	0.86
2	17889A	5'- $(CT)_6$AC-3'	8	7	0.88
3	HB1	5'- $(CT)_8$TG-3'	8	8	0.75
4	HB9	5'- $(GT)_6$GG-3'	10	9	0.90
5	HB12	5'- $(CAG)_3$GC-3'	8	5	0.69
6	UBC815	5'- $(CT)_8$G-3'	7	5	0.82
7	UBC835	5'- $(AG)_8$YC-3'	6	3	0.67
8	UBC842	5'- $(GA)_8$YG-3'	5	4	0.72
9	UBC847	5'- $(CA)_8$RC-3'	7	6	0.82
10	UBC848	5'- $(CA)_8$RG-3'	6	6	0.83
11	UBC850	5'- $(GT)_8$YC-3'	8	8	0.81

RAPD markers:

Nine random 10-mer primers were used for RAPD analysis depending upon the literature (Carvalho *et al.,* 2004, Table 4). A total volume of 25 µl PCR reaction contained 75 ng template DNA, 150 µM dNTPs, 1.5 mM $MgCl_2$, 2.5 µl of reaction buffer (1X), 1 µM of primer and 0.75 U Taq DNA polymerase (GoTaq Flexi DNA polymerase, Promega Inc.). The volume was adjusted up to 25 µl by sterialized double distilled H_20. The PCR cycling condition involved initial denaturation at 94°C for 3 min. followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 38 ^{o}C for 35 seconds and extension at 72°C for 40 seconds. A final extension step at 72°C for 4 min. was conducted to extend all the annealed primers, followed by storage at 4°C. The PCR products were separated on 1.5 % agarose gel electrophoresis.

Table 4: RAPD primers sequences, total and polymorphic bands generated by each and their polymorphic information content (PIC).

No.	Primer name	Primer sequence	Total # bands	Polymorphic bands	PIC
1	OPA R4	5'-CCAGGAGAAG-3'	7	6	0.77
2	OPBI2	5'-CCTTGACGCA-3'	10	9	0.78
3	OPC07	5'-GTCCCGACGA-3'	4	2	0.63
4	OPR09	5'-TGAGCACGAG-3'	8	8	0.88
5	OPW05	5'-GGCGGATAAG 3'	4	3	0.75
6	OPV03	5'-CTCCCTGCAA-3'	10	7	0.82
7	OPAI7	5'-GACCGCTTGT-3'	10	10	0.87
8	UBC 228	5'-GCTGGGCCGA-3'	4	4	0.63
9	UBC 300	5'-GGCTAGGGCG-3'	4	3	0.75

Statistical analysis:

SSR, ISSR and RAPD gel bands were scored as 0/1 for absence/presence of bands, codominant marker. Dendrograms were constructed using the UPGMA method (Unweighted Pair-Group Method with arithmetical algorithms Averages; Sneath and Sokal, 1973) and the correlation cophenetic coefficients were calculated. Correlations among all obtained similarity matrices and the cophenetic of goodness of fit of each molecular marker analysis were performed using the Mantel's test (Mantel, 1967) of the NTSYS PC2.1 software (Rohlf, 2000). Polymorphism information contents (PIC) were calculated according to Anderson *et al.* (1993) using the following simplified formula: $PICi = 1 - \Sigma p^2 ij$ Where pi is the frequency of the jth allele for marker ith summed across all alleles for the locus.

respectively. Similarity coefficient matrices were calculated using the Jaccard similarity algorithm (Jaccard., 1908) for RAPD and ISSR markers, which are dominant markers, and the simple matching algorithm for the SSR

Results and Discussion:

Molecular markers patterns

The SSR dendrogram cophenetic goodness of fit was very high (r = 0.89, Table 5). The total number of bands generated from SSR primers ranged from two for the primer pairs Umc1152 and Umc2050 to nine for the primer pair Phi034 (Table 2). The number of polymorphic bands ranged from one band for the primer pairs Phi084, Umc1152 and Umc2050 to eight for the primer pair Phi034 (Table 2). The polymorphic information content for the SSR primers was generally moderate to high and ranged from 0.50 for the primer pairs Umc1152 and Umc2050 to 0.89 for the primer pair Phi034 (Table 2).

Table 5: Mantel’s test of the different types of molecular markers used in the study

	SSR	ISSR	RAPD
SSR	0.89		
ISSR	0.66	0.92	
RAPD	0.57	0.60	0.69

The goodness of fit of the ISSR analysis was very high (0.92, Table 5). A total of ten bands were generated from the primer HB9 and it was the highest total bands number generated from the ISSR primers. The smallest bands number (five bands) was obtained from the primer UBC842 (Table 3). The number of polymorphic bands ranged from three bands for the primer UBC835 to nine bands for the primer HB9 (Table 3). The polymorphic information content for the ISSR primers was generally very high and ranged from 0.67 for the primer UBC835 to 0.90 for the primer HB12 (Table 3).

The cophenetic of RAPD dendrogram showed high goodness of fit of cluster analysis (r = 0.69, Table 5). The number of total bands produced from the RAPD primers ranged from four bands for the primers OPC07, OPW05, OPC228 and UBC300 to 10 for the primers OPB12, OPV03 and OPA17 (Table 4). The number of polymorphic bands for RAPD analysis ranged from two bands for the primer OPC07 to ten for the primer OPA17 (Table 4). The polymorphic information content for the RAPD primers ranged from 0.63 for the primers OPC07 and OPC228 to 0.88 for the primer OPR09 (Table 4).

It seems that the goodness of fit of the analyses are very high (r= 0.89, 0.92 and 0.69 for SSR, ISSR and RAPD respectively, Table 2) which reflect the reliability of the analyses. High polymorphic percentages and high polymorphic information contents have been obtained from the present results indicating the efficiency of the analyses to detect the genetic diversity among the inbred lines in maize. The present results are in contiguous with those found by other researchers such as Abdellatif and Khidr., (2010). They found high polymorphic information contents and polymorphisms among the maize inbred lines

and the hybrids which they produced using those inbred lines.

Cluster analysis of molecular markers

The SSR analysis was able to separate maize inbred lines into two main clusters (Fig. 1). The first cluster contained the inbred lines Inb56, Inb66 and Inb72, while the second cluster included the inbred lines Inb76, Inb82 and Inb102. The inbred line Inb2 was separated away from the other two clusters (Fig. 1). The most related inbred lines according to SSR markers were inbred lines Inb76 and Inb82 followed by Inb66 and Inb72.

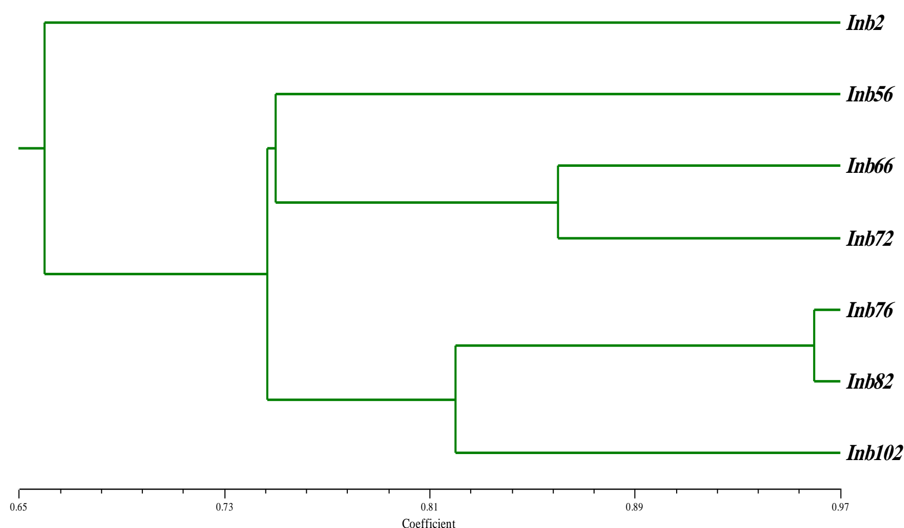

Fig. 1: Cluster dendrogram of maize inbred lines depending upon SSR markers using simple matching similarity coefficient and UPGMA method.

According to ISSR cluster analysis, maize inbred lines were separated into two main clusters (Fig. 2). The first cluster included both inbred lines Inb2 and Inb56. The second cluster divided into two branches, the first branch contained the inbred line Inb102 while the second branch included the inbred lines Inb66, Inb72, Inb76 and Inb82 (Fig. 2). The inbred lines Inb66 and Inb72 were the most related inbred lines comparing to the other inbred lines followed by Inb76 and Inb82.

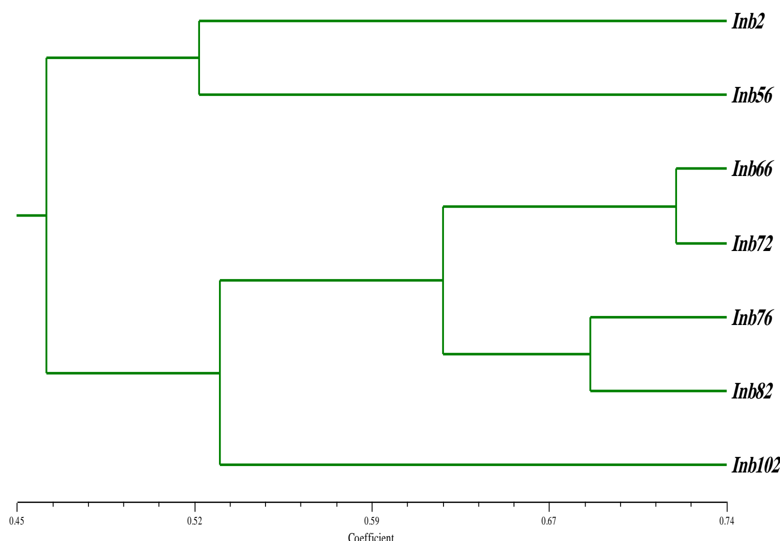

Fig. 2: Cluster dendrogram of maize inbred lines depending upon ISSR markers using Jaccard's similarity coefficient and UPGMA method**.**

According to RAPD cluster analysis, the maize inbred lines were separated into two main clusters (Fig. 3). The first cluster contained both inbred lines Inb2 and Inb56 likely to ISSR cluster analysis. The second cluster included the inbred lines Inb66, Inb102, Inb76 and Inb82. The inbred line Inb72 was separated from the second cluster (Fig. 3). The most related inbred lines according to RAPD data were the inbred lines Inb76 and Inb82 followed by the inbred lines Inb66 and Inb102 (Fig.3).

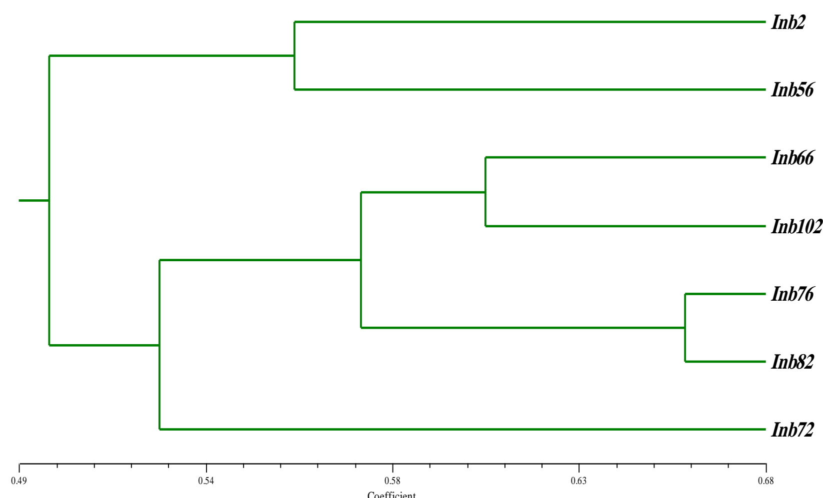

Fig. 3: Cluster dendrogram of maize inbred lines depending upon RAPD markers using Jaccard's similarity coefficient and UPGMA method.

It seems that both ISSR and RAPD results are similarly hence both markers are dominant markers and their results are differed from the SSR results. It is known that SSR markers are the best markers for genetic diversity determination (Holton ., 2001). From its results, the inbred line Inb2 seems to be differed from the other inbred lines and that is why it was used to create most of the Egyptian maize hybrids like single cross 10 and the triple cross 310.

Abdellatif and Khidr., (2010) studied the genetic diversity of new Egyptian maize hybrids based on SSR markers as compared with other molecular and biochemical markers. They found almost the same results; hence the SSR markers gave better resolution of the genetic diversity among the hybrids and their parents. They reported that the genetic relationships among the different inbred lines can be estimated efficiently using SSR markers. Morales *et al*., (2010) also found better performance of SSR markers when compared to the clustering based on genetic distance (UPGMA-Modified Roger's Distance). In another study, Hoxha *et al*., (2004) reported that SSR markers are powerful tool for detection of genetic diversity in maize population.

Molin *et al*., (2013), on the other hand, studied the genetic diversity of different Brazilian maize landraces derived from different tropical sites using RAPD, SSR and AFLP markers. They revealed high levels of variability across landraces within and between collection sites and they obtained comparable results from the different types of molecular markers. Yu *et al*., (2012) examined the genetic diversity of 80 inbred waxy maize lines using 22 SSR molecular markers and they obtained nine groups of those inbred lines. They concluded that it was more accurate to determine the difference between the populations using the highly stable DNA genetic markers.

Mantel's test

Mantel's test was used to determine the correlation between different types of molecular similarity matrices. There is a moderately correlation between the molecular similarity matrices (Table 5). The correlation between SSR and ISSR markers was 0.66 while it was 0.57 between SSR and RAPD markers. The correlation between ISSR and RAPD markers was 0.60 (Table 5).

These results are in agreement with those found by some researchers. Budak *et al.*, (2004) reported that correlation coefficient between between ISSRs and SSRs was r=0.66 while Leal *et al.,* (2010) compare the groups formed using SSR and RAPD markers and found relatively high correlation (0.55) between RAPD and SSR markers. Abdellatif and Khidr., (2010) found also comparable results in their study.

It can be concluded that all types of molecular markers are efficient to study the genetic diversity in maize. Among the different types of molecular markers, SSR is more accurate marker because of its co-dominance and stability of the results. It can be said also that the inbred line Inb2 is different from the other inbred lines and could be used in different combinations with other inbred lines to create new efficient maize hybrids. It can be said also that SSR markers are more reliable than both ISSR and RAPD markers and their results can be considered for genetic relationships studies. Thus, it can be said that Inb 2 could be used for hybridization with all other inbred lines used in this study except of the inbred line Inb 56 to give single cross with the highest growth vigorous.

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التنوع الوراثي لسلالات الذرة الشامية مدروسا بإستخدام الدلائل الجزيئية

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تمت دراسة التنوع الوراثي في سبعة سلالات من الذرة الشامية باستخدام ثلاثة أنواع من الدلائل الجزيئية المختلفة هي SSR, ISSR , RAPD وقد كانت هذه الدلائل (مجتمعة) قادرة على فصل سلالات الذرة الشامية بوضوح في مجموعتين. ووقد إنفصلت السلالة النقية Inb2 في مجموعة منفصلة مع السلالة النقية Inb56 وفقا للدلائل الجزيئية ISSRو RAPD في حين إنفصلت السلالة النقية Inb2 بعيدا من كل السلالات الأخري وفقا لدلائل الSSR . أما السلالات Inb66، Inb72، Inb76، Inb82، Inb102 فقد إنفصلت في مجموعة واحدة وفقا لجميع أنواع الدلائل الجزيئية .وقد كانت نتائج كل من ال ISSRوال RAPD متقاربة إلي حد كبير ولكنها مختلفة عن النتائج التي تم الحصول عليها من دلائل الSSR . ويمكن القول أن دلائل الSSR هي أكثر موثوقية من دلائل الISSR وRAPD ذوي طبيعة السيادة المشتركه كدلائل جزيئية، وهكذا يمكن القول أن نتائجها يمكن الوثوق فيها بشكل أكبر لدراشة العلاقات الوراثية بين أنواع الذرة الشامية. وأيضا يمكن القول أن السلالة النقية Inb56 يمكن استخدامها في التهجين مع جميع السلالات النقية الأخرى إلا مع السلالة النقية Inb56 وذلك لإعطاء أعلي قيمة لقوة الهجين بين السلالات المستخدمة في هذه الدراسة .