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Evaluation of the genetic diversity of pomegranate accessionssome Iraqi Pomegranate (Punicagranatum L.) genotypes using ISSR marker

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Abstract: *This study has been performed in the biotechnology laboratory of Agriculture faculty- Baghdad University during 2016. Ten genotypes from local pomegranates from different geographical regions of Iraq were studied to estimate and evaluate the genetic diversity and the relationship among them. 10 starters of the IntersimpleSequence Repeat Markers (ISSR) are used to determine the diversity level among the studied genotypes. 9 of them showed an activity to give a polymorphism among the studied genotypes which give 72 alleles, the ratio of this polymorphism was 66 %. The higher bands (13) were obtained from starter ISSR8932809, while the starter ISSR8932804 gave the lower bands (6). The study showed that the genetic similarity of the genetic affinity ranged between 0.119-0.438 in which the higher genetic affinity (0.438) was for the genotypes of Zazli and Karbalaye, followed by (0.432) betweenZazli and Baladi genotypes, while the lowest (0.119) between the both genotypes of Taeif and Shahraban and for Khushi and Nab aljamal.*

Keywords: *Pomegranate, genetic diversity, molecular marker, ISSR.*

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I. Introduction

The Pomegranate (*Punicagranatum*L.) is belongs to the Punicaceae family and it is one of the oldestrecognized edible fruits [1]. Thepomegranate has garnered increasing interest over the world due to its high nutritional value[2], and the phytochemical and medicinal properties of its juice[3-5]. The pomegranate is a temperate species that is native to theregionsfrom Central Asia, especially Iran and perhaps some surrounding areas, from where it has spread to the to different parts of the world [6],such as the Mediterranean countries like India, Pakistan, Afghanistan, California, China, Japan, and Russia [7, 8]. It requires high summer temperatures in order to mature properly so commercial production is limited to coastal areas or those with mild summers [9]. It has been cultivated and naturalized overthe Mediterranean region, the Caucasus region of Asia and dry, hotareas of the United States and Latin America [10].

Distinctionamong pomegranate cultivars is mainly based on morphologicalcharacteristics, for example fruit size, husk andaril color, sweetness, ripening time, juiciness, and theproportions of seeds and flesh. Because morphologicalcharacteristics such as husk and aril color are affected byagro-ecosystems, for this ancient and widespread fruit it islikely that the same genotypes have different names indifferent regions (synonymy and/or homonymy)[10].Due to thelong historic cultivation of pomegranate, synonymiesand homonymies can be observed among genotypes cultivatedin different varities. Precise identification of genotypes and determination of the genetic relationships among them will be necessary for conserving its genetic diversity. Such data will also facilitate improved selection of genotypes with traits preferred by consumers[5].However, due to the effects of environmental factors onthese attributes, their use can be ambiguous. Therefore, markersindependent from the environment are necessary for reliableidentification and discrimination of genotypes and cultivars[11].

In recent years, use of molecular markers has been found to be a reliable means of systematically reconstructing phylogenetic relationships among plants[12].Different types of marker systems have been used for genetic analysis and genotyping, including morphological, cytological, biochemical and DNA markers. These markers including for example,Random Amplified Polymorphic DNA (RAPD)[11, 13, 14], Restriction Fragment LengthPolymorphisms (RFLP) [15], Amplified Fragment Length Polymorphism (AFLP) [16-18], Simple Sequence Repeat (SSR)[19, 20], Sequence-Related Amplified Polymorphism(SRAP) [12], and Inter Simple Sequence Repeats (ISSR) [19, 21]have been used to determine genetic diversityamong some pomegranate cultivars.These molecular markers are based ondifferent principles, are obtained by using procedures ofvarying complexity and generate different amounts of polymorphic data[11].Inter-Simple

sequence Repeats (ISSR) analysis isconsidered as efficient molecular marker, and could be used to show genetic variation in the wild pomegranate populations[22].

The ISSR combines the advantages of RAPD and SSR markers at the same level. Thus, it can produce more polymorphism than RAPD, and the reaction system is more sensitive, more stable, and has good repeatability [23, 24]. This molecular marker has been widely used in studies on germplasm resource identification, phylogeny of species, plant taxonomy, evolution, and genetic diversity [25, 26].

Iraq is one of the main pomegranate producers among the middle east countries, but the availableinformation on pomegranates from Iraq is very littles[1]. According to the information from the Central Organization of Statistics,The pomegranate production in Iraq was about 98,683 tons in 2015and production has rapidly increased from year toyear. To our knowledge, there is no proviousestudy concerning the use of molecular markers to study the genetic diversity of the differentvarieties of pomegranate. Thus, the objectives of this study were to assess the genetic relationships and population genetic structure of tenth pomegranategenotypes collected from defferent location in Iraq by using of ISSR markers.

Experimental work and material

This study was conducted in the Biotechnology Laboratories of the Faculty of Agriculture, University of Baghdad during 2016. Ten genotypes of local pomegranates were studied to estimate genetic diversity and determine the degree of genetic affinity between them, using 9 ISSR initiators.Sampling was based on different geographicalorigins (north, center, south, northeast, andsoutheast of Iraq). All samples of plant were cultivated and collected directly from the field, (Table 1; Fig. 1).

Pomegranatefruits were hand-harvested in the September of 2016 when fullyripened, to ensure their best flavor and color, and transportedunder ventilated conditions to the laboratory.

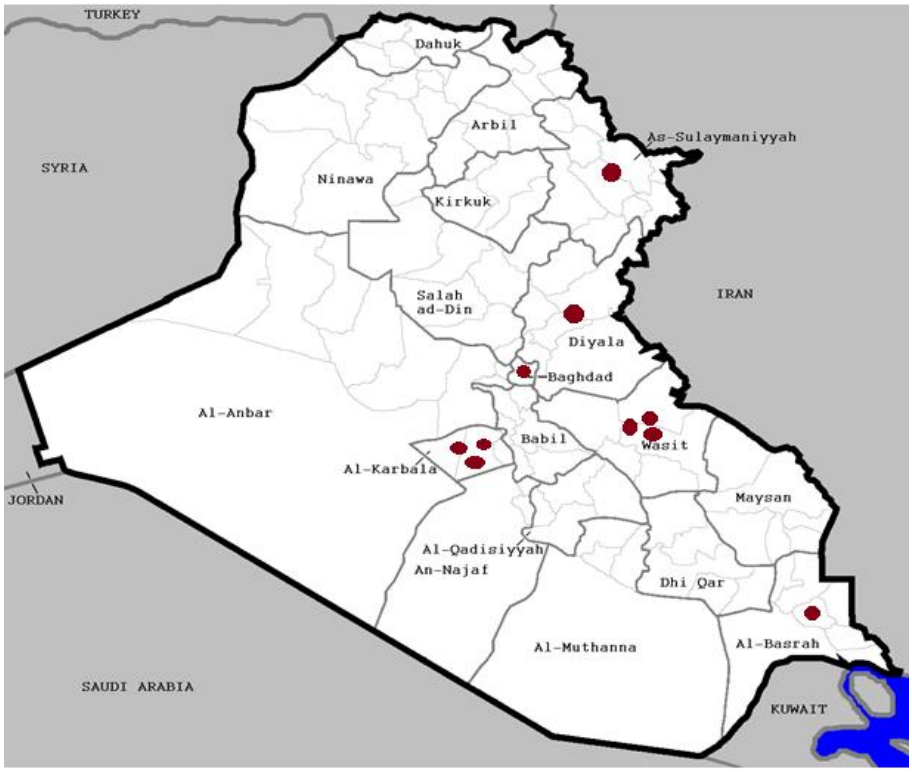

Fig. 1 The location of the pomegranate genotypes sample used in the study

Table 1 Pomegranate genotypes used in this study with their geographical origins and types

No.	Genotype name	Sampling Location	Type
1	Salimi	Karbala	culverted
2	Wonderful	Wassit	culverted
3	Shahraban	Diyala	culverted
4	Nab aljamal	Baghdad	culverted
5	Rasalbaghl	Wassit	culverted
6	Baladi	Karbala	culverted
7	Taief	Basrah	culverted
8	Karbalaye	Karbala	culverted
9	Khushi	Wassit	culverted
10	Zazli	Sulaymaniyyah	culverted

DNA isolation of the genome

DNA was isolated from the young leaves of the studied cultivars. A DNA quantity of 50-150 micrograms per 1.5 g of leaves was obtained for each pomegranate with a purity of 1.7-2 measured by Nanodrop. 50 ng. mL^{-1} which is the appropriate concentration for PCR reactions. There are several methods for isolating nucleic acids from plants because different plants contain different amounts of plant compounds, such as proteins and polysaccharides, as well as nucleic acids, technology was used between simple sequence sequers (ISRs) based on PCR technology and 10 of the prefixes Suitable for all plants (Table 2), the method mentioned by Weigandof isolating the DNA from the pomegranate plant was adopted[27]. It is one of the most effective methods of isolating DNA from plants such as pomegranates. Other organisms because of the thick wall surrounding the cell membrane as well, some plants contain a large amount of phenolic substances and polysaccharides, which are contaminants, sometimes deposited with DNA, giving high viscosity liquid and inhibiting PCR reactions. To remove these substances, the extracted DNA was reduced to reduce the percentage of inhibitory sugars.

Application of ISSR technology:

In this study, 11 starters were used after importing from Bloneer Company, I. Table2showed the Nucleotide Sequence and the coalescence temperature of the starters used in the study.

Table 2 : ISSR starters used to analyze pomegranate genotypes

Sr. No.	Starters	Sequence (5'-3')	Annealing temperature (ºC)
1	ISSR 8932798	(AG)4AGA	25
2	ISSR 8932799	(AG)6GC	55.4
3	ISSR 8932804	(CA)6GT	42
4	ISSR 8932805	(CA)6GC	44
5	ISSR 8932806	(CA)6GA	44
6	ISSR 8932807	(CA)6AA	42
7	ISSR 8932809	(GT)6TG	42
8	ISSR 8932811	(GT)6CT 44	44
9	ISSR 8932812	(GT)6AT	39

The PCR was conducted according to procedure listed byWilliams[13] with some modifications and the final reaction volume was 25ul using 2x Master mix obtained from Bloneer Company. The reaction consisted from 2ul of the starter with a concentration of 10m M, 12.5ul of Master mix, 9ul of distilled water, and DNA with concentration of 40 $ng.ul^{-1}$. This reaction occurred into thermo-rotation system according the following conditions:

1- Separation: carried out at 94˚C for 5 minutesduration, the two series of DNA to be separated.
2- 40 rotations, each includes the following stages:
2-1 Separation occurred at 94˚C for 30 sec.
2-2 Coalescence: according to the temperature of each starter, from table 2, for 1 minute.
2-3 Elongation at 72˚C for 1 minute.
3- Reaction completion at 72˚C for 1 minute.
The samples were kept under 4˚C, then deported on Acaruz gel.

The Electric Deportation, Coloring and Imaging

The deportation on Acaruz gel (2%) was done into the buffer solution TBE 1x.

TBE 1x = (10x TBE buffer = 108g Tris borate + 55g Boric acid + 9.2 EDTA, ph 0.8)

5ul of ethidium bromide stain (10 $mg.ml^{-1}$) where DNA samples were loaded on Acaruz gel by adding 5ul of the special loading liquid (Bromophenol blue 1x loading buffer). 1kpb DNA, from Geneaid Company was injected to determine the volume and molecular weight of the resulted bands, then deporting by passing through an electric field of 100 v to separate DNA bands resulted from the amplification, then imaging the gel by the image analyzer (Eagle Eye II Stratagene).

II. Statistical Analysis

The results of the amplification process were written in a table depending on the presence or absence of DNA bands in the studied samples, the number 1 indicates to the presence of a clear DNA band only, while the number 0 indicates the absence of the band. Individually the tables were organized for each starter and the dendrogram was drawn by applying Unweighted Pair Group Method with Arithmetic Averaging (UPGMA) using the Statistical Past.

III. Results and discussion

3.1 Polymorphism

Ten starters are used, 9 of them, had an ability to detect the genetic differences among different pure varieties. Table(3) showed that used starters gave 72 bands, 88 of them had a polymorphism with a ratio of 80.66%. That indicated to the genetic distance among the pomegranate varieties used in the study. The table showed that the starter ISSR 8932809 gave a greater band number (13 bands) compared to the other used starters, while the starter ISSR 8932804 showed least bands number (6 bands) through sample deportation on poly acryl amide gel. This variation in the number of bands, resulted from each pair of used starters, depends on the corresponding extent on the starter link with plant genome as well as the components of each starter of nitrogenous bases. The sequence difference of used starters bases led to the difference of their link sites with the plant genome and this led to difference in bands number resulted from the used starters[28]. In spite of the difference in the bands number, the starters succeeded to give polymorphism among the resulted bands which reached 100% in the starters ISSR 8932799, ISSR 8932807, while the starter ISSR8932798 had no more than 70% of polymorphism. This variation in the percentages attributed to the difference in the sequence of bases in the plant genome which affected the starters link sites, and this difference was caused by genetic rearrangement, link, passing and other. Different bands appeared clearly on the gel or do not appear in certain sites on the gel [29].

Table 3: Number of bands and polymorphism of the used starters

premier	Bands number	Polymorphism	Polymorphism %
ISSR 8932798	10	7	%70
ISSR 8932799	12	12	%100
ISSR 8932804	6	5	%83
ISSR 8932805	7	5	%71
ISSR 8932806	9	7	%77
ISSR 8932807	11	11	%100
ISSR 8932809	13	10	%76
ISSR 8932811	11	8	%72
ISSR 8932812	9	7	%77
Total	88	72	
Average	9.77	8	%80.66

3.2 Determination of Genetic Affinity among Studied Genotypes

Genetic distance, among studied genotypes, was determined according to the equation obtained from Powell [30]. The result showed the similarity and dissimilarity extent among the pure ancestries. The higher similarity percentage, which corresponded to the less genetic distance (0.438), was between Khushi and Zazli followed by Salimi and Zazli, Taief and Zazli, and Wonderful and Zazli which formed a small group, while less similarity percentage, which corresponded to a great genetic distance (0.175), was between Wondeful and Rasalbaghl followed by Shahraban and Baladi. From the results shown in the table 4, it can be observed that the Khushi and Zazli genotypes had greater genetic distance than others.These results agree with results obtained from Egyptian pomegranates [31, 32] and similar values were obtained from Iranian pomegranates cultivars [33].

Table 4: Values of the genetic similarity among 10 genotypes of pomegranate

Genotype name	Matrix File Input									
	Salimi	Wonderful	Shahraban	Nab aljamal	Rasalbaghl	Baladi	Taief	Karbalaye	Khushi	Zazli
Salimi	0.000									
Wonderful	0.200	0.000								
Shahraban	0.281	0.279	0.000							
Nab aljamal	.0.173	.0.232	0.190	0.000						
Rasalbaghl	0.188	0.119	0.236	0.186	0.000					
Baladi	0.290	0.304	0.147	0.193	0.259	0.000				
Taief	0.247	0.267	0.339	0.238	0.255	0.259	0.000			
Karbalaye	0.327	0.304	0.194	0.210	0.241	0.246	0.315	0.000		
Khushi	0.183	0.248	0.270	0.238	0.235	0.278	0.255	0.315	0.000	
Zazli	0.432	0.417	0.368	0.402	0.385	0.364	0.417	0.400	0.438	0.000

3.3Cluster Analysis:

The data obtained from ISSR analysis of 10 pomegranate genotypes were subjected to cluster analysis. Cluster analysis lets dividing the studied genetic structures into groups reflecting the genetic affinity among them according to their originality. The dendrogram, depended on the values of the genetic distance using the UPGMA method (Fig 2) which created according to the results of ISSR indicators, showed that the 10 genotypes distributed into two main groups, A and B. The cluster A included 9 genetic structures which divided of two sub-clusters A1 and A2. The first main sub cluster (A1) includes (Karbalaye, Baladi and Shahraban). The sub cluster A1 shown that the genotypes of Karbalaye andBaladihad a distance between each other of 10, and the same distance for genotypes of Baladi and Shahraban.

The second main sub cluster (A2) includes (Taief, Khushi, Nab aljamal, Salimi,Rasalbaghl and Wonderful. This sub cluster shows that the distance between Taiefand Khushiwas 9, while the distance was 10 for the genotypes of Khushiand Nab aljamal. Also, the distance between the genotypes of Nab aljamal and Salimi was 10, and the same distance for genotypes of Rasalbaghl and Salimi, and for genotypes of Rasalbaghl and Wonerful too.

The cluster B included the genotypes of Zazlifromthe north region was clustered separately.

The analysis of cluster analysis can be concluded that molecular study showed the studied cucumber genotypes contained high genetic variety and these results can be used as an essential material in the programs of breeding by hybridizing to improve the quantity and quality properties and produce cucumber individual hybrids. The results of the study found to be similar to to the results of previouse works done by .

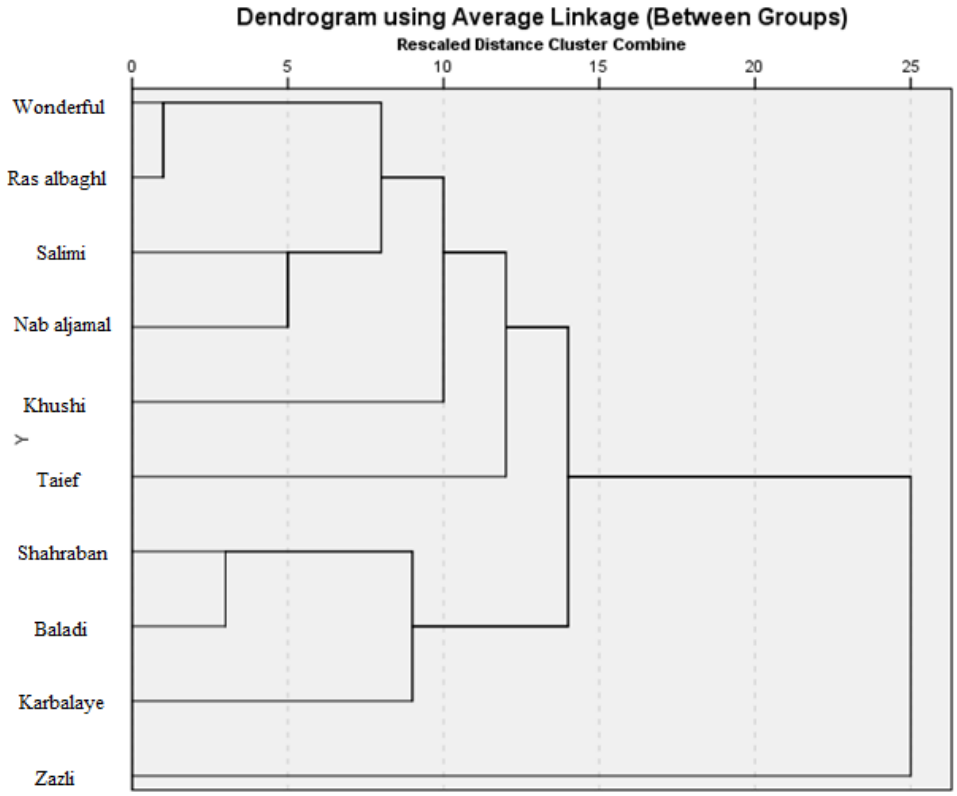

Fig. 2 Cluster layout of genotypes for 10 pomegranate samples using UPGMA based on the ISSR data.

IV. Conclusion

The high efficiency obtained from ISSR marker which used to study the genetic diversity among pomegranate genototypesmake it an effective technique could be used to show the genetic diversity in plant. It provides a quick screen forDNA polymorphism and at the same very small amounts of DNAare required. ISSR primers consist of 9 nucleotides and optimization of annealingtemperature is important. The choice of annealingtemperature for further ISSR analysis is based on thecomplexity and reproducibility of banding patterns. Inthis study, when optimal conditions for PCR had beendetermined, reproducible patterns were obtained forISSR assays. Among the 10 cultivars analyzed withISSR marker, two main groups were recognized byUPGMA. Thefirst group contained 9 cultivars; the second groupincluded 1 cultivar, respectively. In the presentstudy, ISSR provided good insist of genetic diversityavailable in gladiolus germplasm. Due tounique ISSR fingerprints, it can be useful fordetermining of cultivar purity and efficient use andmanagement of the genetic resources collection.

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