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Study of Genetic Diversity of Some Pomegranate Cultivars by Using ISSR Technique

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Abstract

The study aimed to investigate the genetic diversity of seven cultivars of pomegranate in one of the private orchards in the city of Heet, Anbar Governorate, during the 2021 growing season. The analysis of ISSR primers revealed variability in their ability to amplify DNA bands, influenced by factors such as nitrogenous base composition, binding efficiency, and base sequence diversity. Primer ISSR 8932809 produced the highest number of bands (13), while ISSR 8932804 generated the fewest (6). The highest polymorphism rate (91%) was observed with ISSR 8932799, compared to the lowest (66%) with ISSR 8932806, indicating sequence diversity impacts binding and polymorphism rates. Genetic similarity among cultivars varied, with Malisi and Hitawi showing the highest similarity (lowest genetic distance of 0.76). In contrast, Shahrban and Nab Al-Jamal were the most genetically distinct (highest genetic distance of 0.12). The dendrogram, based on ISSR UPGMA analysis, grouped the seven genotypes into two main clusters (A and B). Cluster A included Nab Al-Jamal, Wonderful, Malisi, and Hitawi, with distinct sub-branches. Nab Al-Jamal was closest to Wonderful (0.8 units apart) and furthest from Hettawi (2.4 units apart). Cluster B contained Seedless Rawa, Salimi, and Shahrban, with Salimi and Seedless Rawa being the most genetically similar (0.8 units apart).

Keywords: Pomegranate, *Punica granatum*, cultivars, genetic diversity, ISSR technique.
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Introduction

Pomegranate (Punica granatum) belongs to the Punicaceae family, the original homeland of pomegranate was believed to be at the Northern part of Iraq and Iran [1]. Pomegranate trees thrive in semi-tropical regions of the world between latitudes 41 degrees north and south of the equator. They can grow at high elevations (1050-1400 meters above sea level) as well as in coastal areas and near water bodies [2], [3]. In Iraq, pomegranate cultivation succeeds well due to the suitability of environmental conditions, with the necessity of protecting its fruits from sunburn in the summer. More than 23 cultivars of pomegranate are cultivated in Iraq, with the most common being the Salimi, Salta Khani, Sweet Pomegranate, Masabek, and Nab Al-Jamal cultivars [4]. The total production of pomegranate trees accounts for 28.16% of the total production of summer fruit trees in the country. Diyala Governorate leads in production, followed by Salah al-Din Governorate, then Karbala, with production percentages of 54.94%, 38.28%, and 3.50% respectively. The rest of the provinces accounted for a percentage of 3.28% [5]. Iraq is considered one of the Arab countries rich in plant genetic resources due to its great genetic and climatic diversity. It harbors numerous genetic resources of fruit trees, including pomegranates. Despite this, it is considered a neglected crop in terms of scarcity of studies and scientific research conducted on it. Most cultivars and cultivars are labeled with local names, and to date, there have been no studies to distinguish local pomegranate cultivars, characterize them, and highlight their economic value [6].

Apart from its relevance for the ecosystem, genetic diversity helps to meet human requirements for food, clothes, medicine, and energy. Because of this, plant breeders are now using genetic diversity indicators to obtain genetic data on crops, for use in indirect selection of traits associated with these indicators [7]. Since plant genetic resources are characterized by their high genetic diversity and tolerance to both biotic and abiotic stresses, the genetic diversity of plant species plays an important role in biodiversity [8]. Inter-simple sequence repeat (ISSR) is highly efficient, as it combines the advantages of both RAPD (Amplified Random Polymorphic DNA) and SSR markers. It is also characterized by high levels of polymorphism, and is more accurate, reliable, and stable than the RAPD technique. It has been widely used for the genetic evaluation of pomegranate cultivars in different countries [9], [10]. Additionally, this molecular marker has been extensively employed in research on the identification genetic resource, species evolution, plant classification, development, and genetic diversity [11], [12]. A study was conducted by Amar and El-Zayat [13] that used ISSR technology to determine the genetic relationships between 10 pomegranate cultivars using 10 primers. The genetic similarity between these cultivars ranged from 64 - 97%, and the number of resulting bands was 88, of which 72 bands were polymorphic bands with an average of 8 for all cultivars studied. The percentage of polymorphism was 80.66%. Based on the aforementioned, the idea of this study came to try to describe the variation between 7 selected pomegranate cultivars grown in an orchard in the city of Heet - Anbar Governorate, and to identify their genetic diversity as a basic requirement for the conservation of biodiversity due to its importance in pomegranate genetic improvement processes.

Materials and Methods

This investigation was carried out at the University of Baghdad's - College of Agricultural Engineering Sciences laboratories., Palm Research Unit, using the ISSR amplified DNA technique. This was done by taking samples of young pomegranate leaves. Seven pomegranate cultivars were selected that are widely cultivated in the city of Heet - Anbar Governorate, namely (Salimi, Malisi, Rawa Seedless, Hitawi, Shahrban, Nab Al-Jamal, and Wonderful). The main characteristics of the cultivars studied are presented in Table 1 [14].

Table (1): Characteristics of the studied pomegranate cultivars

Cultivar	Characteristics
Salimi	Elliptic leaves with a pointed tip, green-reddish blade edge color, calyx flower shape with 7-5 sepals of orange-red color. Very large fruits, slightly yellow-red skin color, many juicy seeds, sweet taste, sometimes sour.
Al-Meleisi	Elliptic leaves with a pointed tip, green blade edge color, ribbed fruits of medium size, small and soft red seeds, smooth and thin skin with some redness, sweet taste.
Rawa Seedless	Elliptic leaves with a pointed tip, green blade edge color, calyx flower shape with 6 sepals, medium-sized fruits without seeds, slightly reddish-green skin color, sweet taste and high juice content.
Heetawi	Elliptic leaves with a pointed tip, green blade edge color, white seed color that turns pink at full maturity, light green skin.
Shahrban	Elliptic leaves with a pointed tip, green blade edge color, calyx flower shape with 6 red sepals, large ribbed fruits with a yellow-red color.
Nab Al-Jamal	Elliptic leaves with a pointed tip, green blade edge color, calyx flower shape with 6 red sepals, spherical large fruits, smooth and thin skin, juicy seeds, sweet with medium acidity.
Wonderful	Elliptic leaves with a pointed tip, green blade edge color, calyx flower shape with 6 red sepals, dark red large fruits, red seeds, very juicy and sour taste.

DNA isolation

From the young leaves (4 months) of the cultivars under study, the DNA was extracted efficiently by using EZ-10 Spin Column Fungal Genomic DNA Mini –Preps Kit . Purity and concentration measured using the standard method [15]. The yield of the extracted DNA was in range of (490-570) µg per ml of fungal growth with purity of (1.7-1.9). The amount of DNA obtained was estimated to be between 50 and 150 ug per 1.5 gm of leaves for each pomegranate cultivar, with a purity of between 1.7 and 2, as measured by the Nano drop device made in Japan. The DNA samples were diluted to a concentration of 50 nanograms microliter-1, which is proper for PCR reactions, is the proper concentration. The technique of [16] was applied to separate plant DNA. This is one of the efficient methods for isolating DNA from plants such as pomegranate [17].

Application of ISSR Technique

The employed was Inter Simple Sequence Repeats (ISSR), used 7 primers that are appropriate for all plants and PCR technology, were used in the study, which were obtained by importing them from Bloneer Company in South Korea. The nucleotide sequence and annealing temperature of the study's primers are displayed in Table 2

The PCR reaction was performed according to [18] with a few changes. Using Master mix X2 from Bloneer Company, final reaction volume of 25 µL in the end. In the experiment, a 10 mM concentration of primer in 2 µL was used, 12.5 µL of Master Mix, 9 µL of purified water and DNA at a level where 40 ng μL^{-1}. This reaction was performed in a thermal cycler made in USA in the circumstances listed below:

1. Denaturation: at 94 °C for 5 minutes to separate the two DNA strands.
2. 40 cycles each including the following steps:
   a. Denaturation 94 °C for 30 seconds. Annealing: For one minute, each primer from (Table 2) was annealed at a different temperature.
   b. Extension at 72°C for one minute.

Table (2): Nucleotide Sequence and annealing Temperature of ISSR Primers

Primer	Nucleotide Sequence 3-5	Annealing temperature
ISSR 8932798	(AG) 4AGA	52
ISSR 8932799	(AG) 6GC	55.4
ISSR 8932804	(CA) 6GT	42
ISSR 8932805	(CA) 6GC	44
ISSR 8932806	(CA) 6GA	44
ISSR 8932807	(CA) 6AA	42
ISSR 8932809	(GT) 6TG	42

3. The reaction is completed –PCR at 72°C for one minute.

Then the samples were stored at 4°C, after which we perform electrophoresis on agarose gel.

Electrophoresis, Staining, and Imaging

Electrophoresis was performed on 2% agarose gel in 1X TBE buffer

(TBE buffer = 55g Boric acid+ 108 g Tris borate + 9.2 EDTA, pH 0.810) =TBE 1X to which 5 µL of ethidium bromide dye (10 mg mL^{-1}) was added. The DNA samples were loaded onto the agarose gel by adding 5 µL of the special loading buffer (Bromophenol blue 1X Loading buffer). A 1 Kbp DNA marker from Geneaid Company was also injected in order to measure the size and molecular weight of the formed bands. The next step in the process was electrophoresis, which involved separating the amplified DNA bands using an electric field of 100 volts, until the transfer of DNA bands stops. The gel was then photographed using an agarose gel imager (Agle Eye II Staratagene, Digi Cam 120 camera model, 115 V, made in USA).

Statistical analysis

A table was created based on the amplification procedure results and the existence or lack of DNA bands in the samples under investigation, where the number 0 denotes the band's absence and the number 1 denotes the existence of the clear DNA band exclusively (Figure 1). Using the Past statistical program, the tables were arranged for each primer individually, and the Unweighted Pair Group approach with Arithmetic (UPGMA) Averaging approach was used to create the genetic association tree.

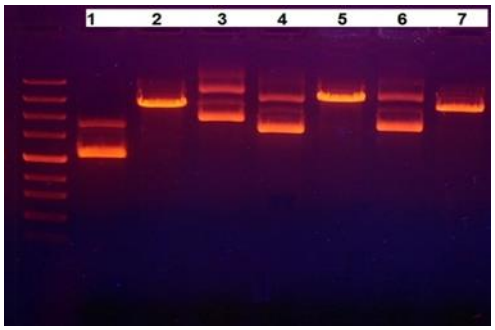

Figure (1): Electrophoresis results of PCR amplification on 1.5% agarose gel

Results and discussion

The ISSR indices showed significant variation in the replication patterns depending on the primer used, due to differences in the number of complementary binding sites on the genome of each plant studied. These differences influenced the presence and quantity of amplification bands. Analysis of genetic relationships relied on identifying the presence or absence of these bands, which resulted from duplications of specific gene segments.

Polymorphism

Ten primers were used, 7 of which were distinguished by their ability to detect genetic variations between different pure cultivars. Table 3 shows that the primers utilized

provided 68 bands, of which 53 were polymorphic, with 77% polymorphic. This indicates the presence of genetic variation between the pomegranates cultivars utilized. The table also shows that primer no. (ISSR 8932809) provided the highest number of bands (13 bands) in contrast to the other primers used, while the primer ISSR 8932804) showed the lowest number of bands (6 bands) during the electrophoresis of the samples on polyacrylamide gel. The degree to which each primer binds to the plant genome, the nitrogenous base composition of each primer, and the variance in base sequence among the primers all affect how many bands each pair of primers produces, this results in variations in the primers' binding sites within the plant genome being studied, which in turn causes variations in the quantity of bands that the primers generate [19]. The highest percentage of polymorphism was 91% in the primers (ISSR 8932799), while the primer (ISSR 8932806) generate only 66% of the total polymorphism during the electrophoresis of the samples on polyacrylamide gel. This change in percentages, which affects the binding positions of the primers, is caused by the diversity of base sequences in the plant genome. Among other things, genetic crossing over, linkage, and rearrangement are the causes of this discrepancy. We observe that in particular regions of the gel, the polymorphic bands either clearly show up or do not [20].

Table (3): Number of bands and polymorphism of the primers used

Primers	Number of bands	Polymorphism	polymorphism %
ISSR 8932798	10	7	70%
ISSR 8932799	12	11	91%
ISSR 8932804	6	5	83%
ISSR 8932805	7	5	71%
ISSR 8932806	9	6	66%
ISSR 8932807	11	10	90%
ISSR 8932809	13	9	69%
Total	68	53	
Average	9.71	7.57	%77

Genetic relationship between the studied cultivars

The genetic distance between the studied cultivars was calculated based on the equation [21]. According to the data, which displayed the degree of similarity and difference between the pure lines, the cultivars Malisi and Hitawi had the highest percentage of similarity, which is equivalent to the lowest genetic distance (0.76), while the lowest percentage of genetic similarity, which corresponds to the highest genetic distance (0.12), was between the cultivars (Shahrban and Nab Al-Jamal). From the results of the table (4), we can see that the two cultivars (Shahrban and Nab Al-Jamal) were the most genetically dis

Table (4): Genetic similarity values between the studied pomegranate cultivars

	Salimi	Malisi	Rawa Seedless	Hitawi	Shahrban	Nab Al-Jamal	Wonderful
Salimi	1						
Malisi	0.33	1					
Rawa Seedless	0.72	0.38	1				
Hitawi	0.41	0.76	0.38	1			
Shahrban	0.50	0.16	0.60	0.31	1		
Nab Al-Jamal	0.17	0.65	0.16	0.52	0.12	1	
Wonderful	0.33	0.62	0.31	0.63	0.21	0.46	1

Discussion

Cluster Analysis

Cluster analysis allows the studied genotypes to be divided into multiple groups that reflect the degree of genetic relationship between them. Samples may cluster together based on their original habitat or based on their origin and pedigree which constantly generate genetic variation. The dendrogram of the genetic relationship, which is based on genetic distance values using the ISSR UPGMA method (Figure 2) shows that the seven genotypes that divided into A and B, the two main categories. Cluster A includes four genotypes (Nab Al-Jamal, Wonderful, Malisi and Hitawi). We notice group A to be divided into two main branches, the first of which includes Nab Al-Jamal, while the second group is also divided into two branches, the first of which includes the Wonderful pomegranate cultivar and the second branch includes both the Hettawi and Malisi cultivars. We notice in group A that the Nab Al-Jamal and Hettawi varieties are 2.4 units apart, between Nab Al-Jamal and Malisi 1.6 units apart, and between Nab Al-Jamal and Wonderful 0.8 units apart. As for cluster (B), It was split into two primary branches: Seedless Rawa and Salimi were in the second branch, and Shahrban was in the first. Find that the Salimi of 0.8 units away from Seedless Rawa, while Shahban is 1.6 units away from Seedless Rawa. The results of the above study agree with [22], [23], [24]

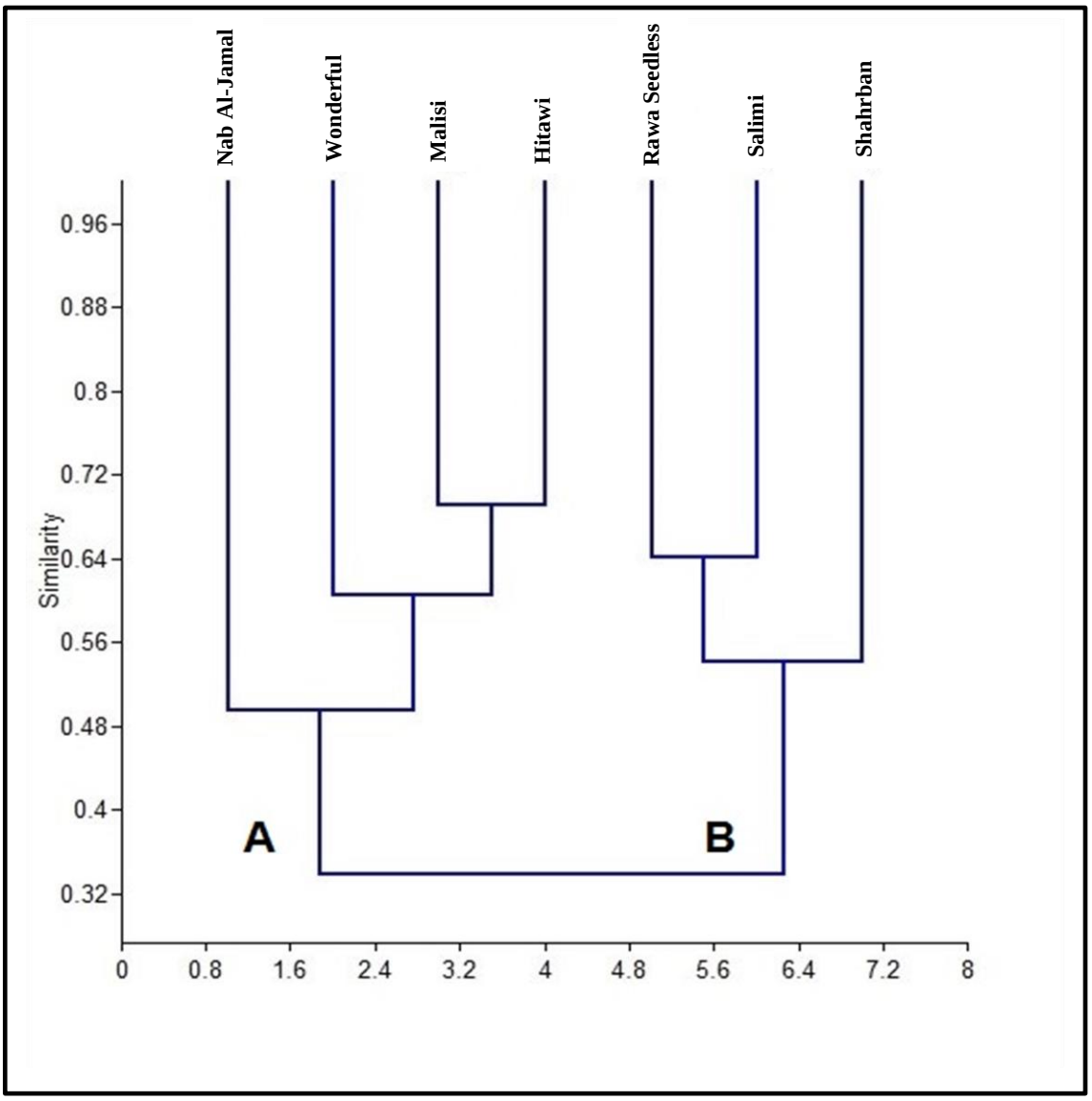

Figure (2): Dendrogram of the studied pomegranate cultivars

Conclusion

The application of the ISSR technique helped to separate, distinguish, and determine the degree of genetic relationship between the studied cultivars, which makes it useful for adoption in breeding and improvement programs, studying these cultivars, and creating genetic maps for each one.

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

References

[1] Mahmoud, H.I. (2019). Production of Deciduous Fruits. Dar Al Fajr for Publishing and Distribution. The Egyptian Arabic Republic, p. 266.

[2] Madadi, M., Zamani Z. and Fatahi, R. (2017). Assessment of Genetic Variation within Commercial Iranian Pomegranate (*Punica granatum* L.) Cultivars, Using ISSR and SSR Markers. Turk. J. Agric. Food Sci. Technol., 5, 22.

[3] Alani, O.M.T. and Al-Dulaimy, A.F.Z. (2024). Response of Rawa seedless pomegranate fruits to different types of packaging and spraying with calcium. Kirkuk University Journal For Agricultural Sciences,15(3), 310-323.

[4] Mohammad, A.A.R. and Abu Al-Saad, M.A.W.A. (1990). Deciduous Fruits, Authority of Technical Institutes, Ministry of Higher Education and Scientific Research. Iraq.

[5] Central Statistical Organization, Summer Fruit Tree Production Report (2021). Agricultural Statistics Directorate. Ministry of Planning - Central Statistical Organization, Republic of Iraq.

[6] Almiahy, F.H. and Farouk, F.J. (2017). Evaluation of the genetic diversity of pomegranate accessionssome Iraqi Pomegranate (*Punica granatum* L.) genotypes using ISSR marker. Journal of Agriculture and Veterinary Science, 10(8), 44-49.

[7] Hamdalla, M.S. (2009). The role of molecular techniques in genetic maps, genetic diversity and fingerprinting. Iraqi J. of Agric. Sci., 40(3), 50-62.

[8] Shaherli, M. and Al-Oubry K. (2004). Conservation of Genetic Resources of Plant Species in Syria, Project on the Conservation and Sustainable Use of Agricultural Biodiversity in Dry Areas GEF, General Authority for Scientific Agricultural Research, Ministry of Agriculture and Agrarian Reform, Syria.

[9] Abdullah, G.and Ahmed M. M. (2010). Variation of morphological and pomological characters of some pomegranate genotypes in northwestern regions of syria. Mesopotamia Journal of Agriculture, 38(1), 1-12.

[10] Al Shawish, F., Hamed, F. and Al–Aisa. I. Evaluation of some qualitive and chemical characteristics for the most important pomegranate (*Punica granatum*) accessions in Yemen. Damascus University Journal of Agricultural Sciences, 22(2), 227-241.

[11] Amar, M.H. and El-Zayat, M.A.S. (2017). Utilization of ISTR, ISSR and SRAP Molecular Markers to Reveal and Classify Egyptian Pomegranates (*Punica granatum* L.). Plant Omics, 10(5), 237–246.

[12] Ansari, S.A., Narayanan, C., Syed, A.W., Randhir, K., Nidhi, S., and Suresh, S.K. (2012). ISSR markers for analysis of molecular diversity and genetic structure of Indian teak (*Tectona grandis* L.) populations. Annals of forest research,

[13] Farsani, T.M., Etemadi, N., Badraldin, E.S. and Majid, T. (2011). Assessment of genetic diversity of bermudagrass (*Cynodon dactylon*) using ISSR markers. International Journal of Molecular Sciences, 13(1), 383-392.

[14] Al-Jumaili, A.A., and Al-Dujaili, J.A.H. (1989). Fruit production. House of Wisdom, Ministry of Higher Education and Scientific Research, Baghdad University, Iraq.

[15] Al-Mayahi, F.H.R. (2018). A study of genetic variation and the effect of some physiological treatments on the growth and yield of pomegranate and its content of medically active compounds. PhD thesis. faculty of Agriculture. Baghdad University.

[16] Weigand, F., Baum, M. and Udupa, S. (1993). DNA molecular marker techniques, technical manual No.20. International Center for Agricultural Research in the Dry Area (ICARDA). Aleppo, Syria pp:135.

[17] Fazio, G., Staub, J.E. and Stevens, M.R. (2003). Genetic mapping and QTL analysis of horticultural traits in cucumber (*Cucumis sativus* L.) using recombinant cucumber (*Cucumis sativus* L.) using recombinant inbred lines. Theor. Appl., Genet. 107, 864-874.

[18] Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research, 18(22), 6531-6535.

[19] Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J. Kuiper, M. and Zabeau, M. (1995). AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research, 23(21), 4407– 4414.

[20] Bradeen, J.M., Staub, J.E., Wyse, C., Antonise, R. and Peleman, J. (2001). Towards an expanded and Integrated linkage map of cucumber (*Cucumis sativus* L.). Genome, 44,111-119.

[21] Powell,W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol.Breed. 2, 225-238.

[22] Al Shawish, F., Hamed, F. and Al–Aisa, I. (2006). Evaluation of some qualitive and chemical characteristics for the most important pomegranate (*Punica granatum*) accessions in Yemen. Damascus University Journal of agriculture sciences, 22(2), 227-241.

[23] Al-Jaf, I.H.M., Mubarak, A.K., Al-Dulaimy, A.F.Z. and ALTaey, D.K.A. (2024).Variance Estimation and Genotypic, Phenotypic Correlation and Broad Heritability Percentage in Cucumber (*Cucumis sativus* L.). IOP Conf. Series: Earth and Environmental Science 1371(042020, 1-8.

[24] Jbir, R., Melgarejo P., Hernández F., Haddioui A., and Hannachi, A.S. (2014). Efficiency of Inter Simple Sequence Repeat (ISSR) markers for the assessment of genetic diversity of Moroccan Pmegranate (*Punica granatum* L.) cultivars. Biochemical Systematics and Ecology, 56, 24-31.

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دراسة التنوع الوراثي لبعض طرز الرمان باستخدام تقنية ISSR

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الخلاصة

هدفت الدراسة إلى التحقق من التنوع الوراثي لسبعة أصناف من الرمان في أحد البساتين الخاصة في مدينة هيت، محافظة الأنبار، العراق، خلال الموسم الزراعي 2021. استخدمت تقنية ISSR لتحديد العلاقات الوراثية بين أصناف الرمان المدروسة باستخدام عشرة بادئات، سبعة منها قادرة على الكشف عن الاختلافات الوراثية. أظهر التحليل الإحصائي إمكانية تقسيم الأصناف المدروسة إلى مجموعتين: المجموعة الأولى تتكون من أربعة أصناف (ناب الجمل ، رائع ، مليسي ، وهيتاوي). أما المجموعة الثانية فتضم ثلاثة أصناف (شهربان ، راوة بدون بذور ، وسليمي). تراوحت نسبة التشابه الوراثي بين هذه الأصناف من 66% إلى 91%، وتم إنتاج 68 حزمة، منها 53 حزمة متعددة الأشكال، بمتوسط 7.57 حزمة لكل صنف مدروس. كانت نسبة تعدد الأشكال 77%. وجد أن البادئ ISSR 8932809 أنتج أكبر عدد من الحزم بواقع 13 نطاقا، بينما أنتج البادئ ISSR 8932804 أقل عدد من الحزم (6) ، بمتوسط 9.71 نطاقا لكل بادئ.

الكلمات المفتاحية: الرمان، الأصناف، التنوع الوراثي، تقانةISSR