P-ISSN 1991-8941
E-ISSN 2706-6703
Vol. 20, No. 1 (2026)

Journal of University of Anbar for Pure Science (JUAPS)

journal homepage: juaps.uoanbar.edu.iq

Open Access

Original Paper

Genetic diversity and molecular characterization of olive cultivars (*Olea europaea* L.) in Iraq using ISSR markers

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ARTICLE INFO

Received: 13/07/2025
Accepted: 03/09/2025
Available online: 08/03/2026
April Issue
10.37652/juaps.2025.162802.1527

CITE @ JUAPS

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ABSTRACT

This study used ten different Olea europaea L. genotypes, including Ashrasi, Bashiqa, Manzanilla, Dakel, Al-Khudairi, Darmalali, Sorani, Qaisi, Nabali, and Ascolano. The genotypes were selected from seven sites representing the major areas of olive production and cultivation in Iraq. Genetic relationships among the 10 examined olive cultivars were determined using ISSR analysis, which revealed that 10 primers were highly efficient, generating 98 bands, 64 of which were polymorphic, yielding a mean polymorphism rate of 64.7%. These findings reveal significant genetic differentiation among the olive cultivars under study. Genetic similarity coefficients ranged from 0.11 to 0.83, indicating a broad spectrum of diversity. The Ashrasi cultivar was the most genetically distinct, suggesting it may be useful for enriching genetic diversity. In contrast, high genetic similarity was observed between Qaisi and Dakel, and between Darmalali and Al-Khudairi. These results were also supported by the dendrogram, which separated the cultivars into two major groups: one with higher similarity and the other with greater evolutionary distance, with Ascolano forming a separate branch, indicating its unique genetic identity.

Keywords: *Genetic diversity, Genetic similarity, ISSR markers, Molecular primers, Olive, Phylogenetic tree*

1 INTRODUCTION

The olive (*Olea europaea* L.), a member of the Oleaceae family, is considered a prominent and auspicious tree. The olive tree is medium-sized, evergreen, and typically grows to 5–8 m in height. It has an exceptionally long lifespan. Because its fruits are used to manufacture oil or as green or black table olives, olives have various nutritional and economic benefits. Furthermore, it also offers significant medical benefits for treating various ailments [1, 2].

The olive tree originated in the Mediterranean Basin, which can be visualized as an imaginary line reaching southward from northwest Iraq to Turkey, Syria, Lebanon, Palestine, and Israel. This is supported by the presence of wild and organic olive groves in the hilly region of northwest Iraq [3]. Given the availability of all economic, agronomic, and environmental conditions required for contemporary olive farming, olives may be among Iraq's most important fruits. Iraq has more than 40 types of olives out of the 2,629 varieties thought to exist [4]. The study, characterization, and conservation of olive genetic resources outside their natural habitats in genetic collections for olive breeding programs have gained prominence in recent decades [5, 6].

Genetic fingerprinting is now the most advanced and precise approach for identifying similarities among kinds or physical forms of various plant species, such as the molecular characterization of olive trees, palm

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trees, cucumber plants, and other plants [7–9]. Genetic studies, particularly those relevant to wild olives, have attracted researchers worldwide [10]. These studies have revealed genetic differences between cultivated varieties and wild forms of olive trees, as well as between eastern and western Mediterranean populations [5, 11, 12]. The research aims to employ the ISSR technique to determine the degree of genetic relatedness across farmed olive varieties and to characterize them molecularly.

Iraqi olive varieties demonstrate significant differences in their physical characteristics. The Ashrasi cultivar produces large, oblong fruits with robust flesh, while the Bashiqa cultivar features smaller, rounder fruits and wider leaves. High oil content is characteristic of both Manzanilla and Dakel, whereas Al-Khudairi and Darmalali are noted for their elongated pits and darker skin. The Sorani variety yields medium-sized fruits with a high pulp-to-pit ratio, and Qaisi is recognized for its distinctly pointed fruit tip. The Nabali variety produces oval fruits rich in phenolic compounds, while Ascolano offers larger fruits with a mild flavor profile.

In recent decades, efforts to study, characterize, and preserve olive genetic resources in collections outside their native environments for use in olive breeding programs have increased significantly [1, 12, 13]. Genetic fingerprints have emerged as the most advanced and accurate technique for determining parallels between diverse varieties, even in physical appearance. Genetic studies, particularly those on wild olive populations, have attracted the attention of scientists worldwide [9]. Such research has revealed genetic differences between domesticated olives and their wild counterparts, such as those found in both eastern and western parts of the Mediterranean [5, 11–13]. The study will therefore use the ISSR method to assess the genetic correlation among cultivated olive varieties and to perform molecular characterization of a few of them.

This study aimed to assess genetic diversity and linkage among 10 olive cultivars growing in Iraq using ISSR markers and to determine morphological attributes that can assist in breeding and conserving them.

2 MATERIALS AND METHODS

2.1 Plant material, research location, and time

This research was conducted at the Biotechnology Unit of the College of Agriculture, University of Anbar. Leaf samples were collected from olive cultivars (Ashrasi, Bashiqa, Manzanilla, Dakel, Al-Khudairi, Darmalali, Sorani, Qaisi, Nabali, and Ascolano) growing in different governorates across Iraq. All leaves were healthy and free from insect damage or disease symptoms. Genomic DNA was extracted from these samples using the CTAB method, as described by [14].

2.2 Dna extraction protocol

Young, healthy, fully developed leaves are collected from the current season's growth (preferably the youngest fully expanded leaf located near the shoot tip, typically the 2nd to 4th leaf from the top). Refrain from using very young (meristematic) leaves, as well as older, hardened, or diseased leaves. Gather samples during the cooler parts of the day, ensuring they remain clean and are quickly preserved (using liquid nitrogen or silica gel) prior to DNA extraction.

These leaves were used for total genomic DNA extraction, with slight modifications to the CTAB (hexadecyltrimethylammonium bromide) protocol, as described in [15]. One gram of leaf tissue was frozen in liquid nitrogen and ground into a fine powder. The powder was suspended in 3 mL of extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 2% CTAB), with 2% β-mercaptoethanol added. The mixture was incubated at 65 °C for 30 minutes. DNA was then extracted using a chloroform: octanol solution (24:1), and the resulting DNA was washed with 70% ethanol. The purified DNA was dissolved in 100–400 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing RNase (0.2–1 mg/mL). DNA quantity and purity were determined using a spectrophotometer by measuring absorbance at A260/A280. Stock DNA samples were diluted to a final concentration of 20 ng/µL and stored at -20 °C until use [16]. The quality of the isolated DNA was evaluated using a Nanodrop spectrophotometer, specifically by measuring the A260/A280 ratio, which ranged from 1.78 to 1.89, suggesting that the purity was adequate for PCR amplification.

2.3 Technology application - issr

ISSR (Inter-Simple Sequence Repeat) markers are used to detect genetic differences in microsatellite regions with the aid of single primers anchored to repeating units of 2 or 3 nucleotides. This method is used for its high reproducibility, similarity, and ability to generate multilocus profiles without prior sequence information, making it cost-efficient. This study resulted in the creation of a set of ten primers targeting different microsatellite sequences to increase the coverage of the genome and

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polymorphisms. A more comprehensive description of the methodology, which starts with the isolation of genomic DNA with the help of CTAB, PCR amplification with the help of the specific annealing temperatures of each primer, separation of products by electrophoresis on 2% agarose gel, visualization of the products by means of UV light, and the binary scoring of the bands, would enhance a better understanding of the technical processes used.

Carry out the reaction following the procedure below: start with 1 cycle of 30 seconds at 94 °C, then 45 cycles of amplification, each consisting of 30 seconds at 94 °C, 45 seconds at 52 °C, and 90 seconds at 72 °C. Finish with another 5-minute cycle at 72 °C (Figure 1).

To amplify the resulting products, they were separated on a 2% agarose gel (containing RedSafe dye) for 90 minutes at 5 V/cm, and then visualized under ultraviolet light. A Gel Documentation System was used to capture images of the gel and analyze the bands formed, noting their molecular sizes for each primer. Each 20 µL polymerase chain reaction (PCR) mixture comprised 1× buffer, 2.0 mM magnesium chloride ($MgCl_2$), 200 µM of each deoxynucleotide triphosphate (dNTP), 0.3 µM of the inter-simple sequence repeat (ISSR) primer, 1 unit of Taq DNA polymerase, and approximately 20 ng of genomic DNA. Cycling conditions were 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds, a primer-specific annealing temperature (Ta) ranging from 48 °C to 55 °C (optimized for each primer) for 45 seconds, and 72 °C for 90 seconds, concluding with a final extension at 72 °C for 7 minutes. For each primer tested, biological duplicate amplifications were performed along with a no-template control. Only bands that were consistently present in both replicates were recorded (Table 1).

2.4 Statistical analysis

Binary matrices were constructed through conservative band matching with a size tolerance of 1.0%–1.5%. A band was accepted only if it was identified in both technical replicates. The locus-specific error rate was determined as the ratio of inconsistent replicate scores and was ≤2% across primers; loci with error rates>5% were omitted. The molecular fingerprinting data were converted into a binary matrix, with the presence of a band scored as 1 and its absence as 0. This binary data was analyzed using the NTSYS-pc software [17]. Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to generate a dendrogram.

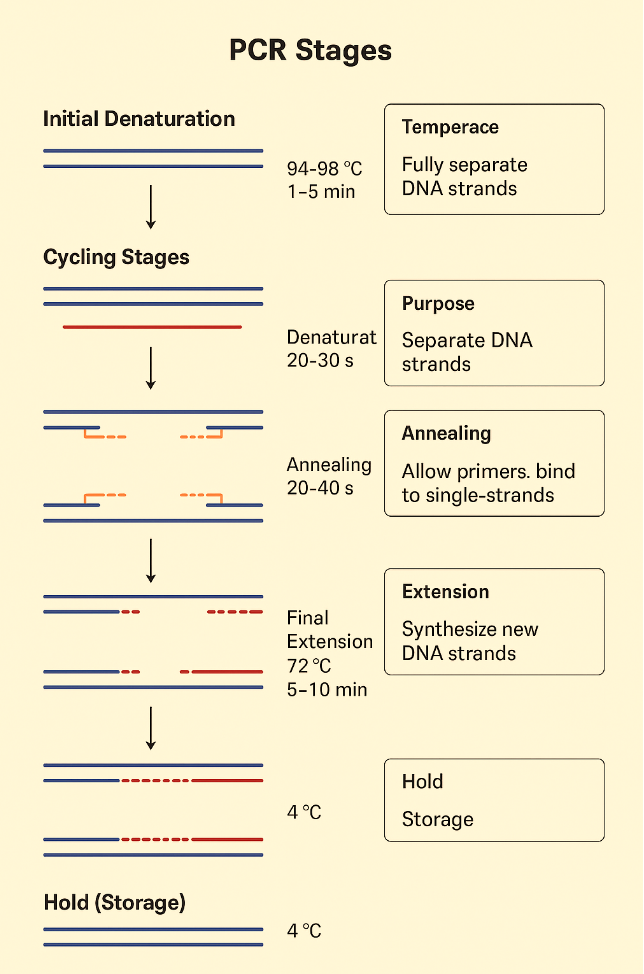

Fig. 1 PCR Stages [18, 19]

Table 1 ISSR Fingerprinting with Ten Primers Used for Ten Olive Cultivars

Sr. No.	Primer	Sequence (5,-3,)	Annealing Temperature (°C)
1	UBC 817	(CA)7.CAA	45
2	UBC 826	$(AC)_7$ AAC	45
3	17899B	(CA)6GG	35
4	SLISSR 14	$(GA)_5$ GG	35
5	ISSR 8932805	(CA)6GC	44
6	ISSR 8932806	(CA)6A	44
7	ISSR 8932807	(CA)6A	42
8	ISSR 8932809	$(GT)_6$TG	42
9	ISSR 8932811	(GT) 6 CT	44
10	ISSR 8932812	$(GT)_6$AT	39

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3 RESULTS AND DISCUSSION

Figure 2 represents the PCR products displayed by electrophoresis on a 2% agarose gel for the ten cultivars of Olea europaea using ISSR primers. Lane M: 100 bp DNA ladder (ranging from 1500 to 100 bp), with major band sizes noted on the right side. Lanes 1-10 represent the cultivars Ashrasi, Bashiqa, Manzanilla, Dakel, Al-Khudairi, Darmalali, Sorani, Qaisi, Nabali, and Ascolano.

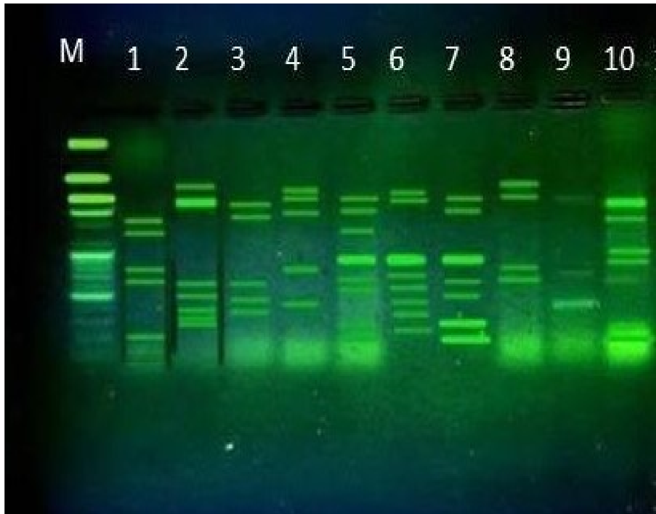
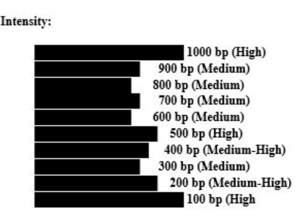


Fig. 2 PCR electrophoresis products on an agarose gel

Because the primers used produced bands primarily within the 100-1200 bp range (consistent with typical ISSR amplicons), a 100 bp ladder is suitable and encompasses all detected fragments. The ladder image used includes the following band sizes: 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp (Figure 3). These sizes are clearly indicated on the right side of the lane, facilitating visual comparison with the gel bands.



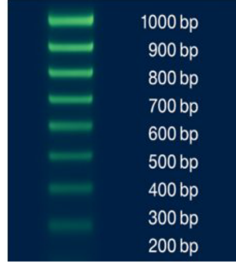


Fig. 3 standard molecular ladder

3.1 ISSR Primer Efficiency in Revealing Genetic Variation

Table 2 presents the effectiveness of ten different primers used in ISSR (Inter-Simple Sequence Repeat) analysis based on three main parameters: total number of bands, number of polymorphic bands, and percentage of polymorphism. The objective of this analysis was to identify the individual capacity of each primer in displaying genetic variation among the samples used in the study.

Total bands: The total number of bands obtained by all primers was 98, with an average of 9.8 bands per primer. This indicates broad amplification of DNA fragments across different genomic regions, suggesting high primer efficiency in targeting multiple genetic loci.

Polymorphic bands: The number of polymorphic bands ranged from 4 to 8 per primer, yielding a total of 64 polymorphic bands. This value indicates substantial genetic variation among the tested samples and supports the reliability of ISSR markers for detecting diversity.

Polymorphism percentage: Polymorphism is an important indicator of primer efficiency because it reflects the ability of primers to distinguish genetic differences and, therefore, provides a clear picture of the degree of genetic diversity among samples. The percentages ranged from 57% (as observed with primers UBC 817 and SLISSR 14) to 88% (achieved with ISSR 8932807). The overall average polymorphism was 64.7%, indicating a generally acceptable level of primer efficiency.

This value exceeds the previously reported 53.3% by [20], who investigated the genetic diversity of 22 native and Egyptian olive cultivars. In comparison, [21] observed a higher polymorphism rate of 94.9% among 40 Turkish genotypes (eight cultivars, each represented by five clones).

Table 2 Number of bands and polymorphism of the used primers

Primers	Bands number	Polymorphism	Polymorphism%
UBC 817	7	4	%57
UBC 826	10	6	%60
17899B	9	6	%66
SLISSR 14	7	4	%57
ISSR 8932805	9	6	%66
ISSR 8932806	10	6	%60
ISSR 8932807	9	8	%88
ISSR 8932809	12	8	%66
ISSR 8932811	13	8	%61
ISSR 8932812	12	8	%66
Total	98	64	
Average	9.8	6.4	%64.7

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The numerical data presented in Table 2 and Figure 4 provide a graphical overview of ISSR primer performance. The bar chart shows the total number of bands and the number of polymorphic bands, whereas the line graph shows the percentage of polymorphism for all primers. The evident differences among primers indicate that they differ in their ability to detect genetic polymorphisms. For example, ISSR 8932807 and ISSR 8932811 exhibited higher counts of polymorphic bands and higher polymorphism percentages, supporting their usefulness for differentiating among genotypes.

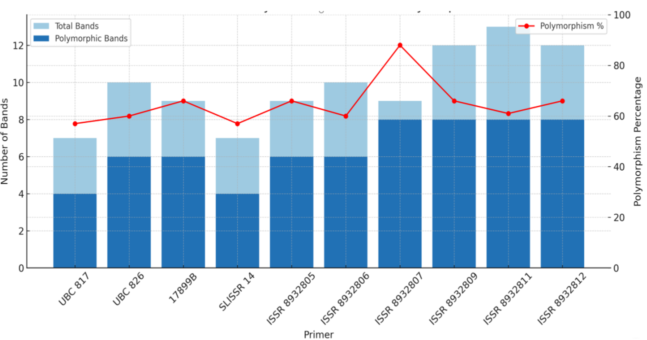

Fig. 4 Analyze the number and proportion of polymorphic bands generated by each ISSR primer, to determine how well each primer detects genetic variations

This work found that the primers employed were highly effective at tracing genetic diversity in the examined samples and that the ISSR marker method is reliable for evaluating genetic diversity. Differences in polymorphism rates among primers suggest that primers should be carefully selected to suit the genetic properties of the target species or strain. The mean polymorphism rate was 64.7%, which is satisfactory in real-world applications, especially for research aimed at genetic characterization and biodiversity conservation programs [22].

These findings support earlier evidence affirming the usefulness of the ISSR technique in molecular research, as it is easy to perform, inexpensive, and capable of generating a large number of reproducible markers [23]. Nonetheless, variation in primer efficiency underscores the need for preliminary studies to select the most suitable primers for a given species, thereby increasing the validity and reliability of the findings [24].

3.2 Genetic similarity analysis

Similarity between pairs based on genetic similarity was assessed using Jaccard (or Dice/Nei–Li), which is applicable to dominant data. A UPGMA dendrogram was constructed using 1,000 bootstrap pseudoreplicates. Cophenetic correlation was used to evaluate tree accuracy using the Mantel test. We also performed PCoA of the Jaccard distances to depict the multivariate structure.

We determined the Polymorphism Information Content (PIC) of dominant markers for each primer using the formula PIC = 2f (1 - f), as well as the Effective Multiplicity Ratio (EMR) = count of polymorphic bands × fraction of polymorphic bands, Marker Index (MI), and Resolving Power (Rp), to evaluate the informativeness of each primer.

The genetic distance between the ten olive cultivars was calculated using the formula given by [25, 26]. The genetic similarity coefficients presented in Table 3 ranged from 0.11 between Nabali and Dakel to 0.83 between Nabali and Al-Khudairi, suggesting a wide range of genetic association among the studied germplasm.

High similarity coefficients were also observed in certain cultivar pairs, such as Nabali and Al-Khudairi (0.83) and Darmalali and Al-Khudairi (0.80). This high degree of similarity may indicate shared ancestry, similar selection pressures, or exposure to similar environmental conditions. In contrast, the similarity index between Nabali and Dakel (0.11) and between Ashrasi and Baziq (0.14) was much lower, suggesting substantial divergence that may be useful in breeding programs to enhance genetic diversity.

It is also notable that Ashrasi consistently exhibited low similarity values relative to most other cultivars, indicating its status as one of the most genetically distinct genotypes. This uniqueness underscores its potential value in breeding initiatives to improve the genetic diversity of Iraqi olive varieties. Despite greater similarity with a few cultivars, such as Darmalali and Al-Khudairi (0.80), Ascolano still showed distinct separation in the dendrogram, likely reflecting its distinct origins.

These results indicate that the genetic diversity of these cultivars should be preserved to retain valuable genetic resources and to support future breeding efforts. A reservoir of genetic diversity can contribute to improved agronomic outcomes (productivity, tolerance to stress extremes, adaptation, and ecological niche compatibility). These results parallel those of [19, 27–29].

3.3 Cluster analysis

Phylogenetic insights and genetic diversity of ten native Iraqi olive cultivars (Olea europaea L.) were assessed using Inter-Simple Sequence Repeat (ISSR) markers. The derived dendrogram showed that the studied

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Table 3 Genetic similarity values between 10 olive cultivars

Ashrasi	Ashrasi	Bashiqa	Manzanilla	Dakel	Al-Khudairi	Darmalali	Sorani	Qaisi	Nabali	Ascolano
Bashiqa	1									
Manzanilla	0.14	1								
Dakel	0.12	0.50	1							
Al-Khudairi	0.66	0.42	0.22	1						
Darmalali	0.50	0.12	0.42	0.37	1					
Sorani	0.80	0.12	0.25	0.57	0.66	1				
Qaisi	0.28	0.80	0.42	0.57	0.25	0.25	1			
Nabali	0.80	0.28	0.11	0.83	0.42	0.66	0.42	1		
Ascolano	0.33	0.33	0.50	0.42	0.28	0.28	0.28	0.28	1	
Ashrasi	0.60	0.14	0.28	0.42	0.80	0.50	0.28	0.50	0.33	1

cultivars split into two major clusters, Group A and Group B, exhibiting different degrees of genetic relationships, similarities, and differences.

Four cultivars, Nabali, Manzanilla, Bashiqa, and Sorani, formed Group A. Notably, Nabali and Manzanilla had highly cosegregating genotypes (similarity coefficient > 0.85), forming a distinct subcluster (A1). This close association may reflect common ancestry or a long-shared cultivation history under similar conditions. Further supporting this pattern, Bashiqa and Sorani formed sub-cluster A2. Overall, the genetic relationships indicated limited differentiation among some members of Group A, implying a distinctive population structure that may reflect adaptation and stable cultivation over time in Iraqi olive-growing regions.

In contrast, six cultivars, Dekel, Qaisi, Darmali, Ashrasi, Al-Khudairi, and Ascolano, comprised Group B, which showed higher levels of heterogeneity, with Qaisi as the sole exception. Meanwhile, Darmali, Ashrasi, and Al-Khudairi displayed moderate internal variation, although they clustered within the same group. The cultivar Ascolano appeared as an outlier (B2), occupying a more distant branch from the others, which aligns with its known foreign origin and distinct genetic background.

To summarize these relationships, Table 4 presents the genetic grouping of the studied cultivars along with their approximate similarity values (Figure 5). These findings highlight a meaningful level of genetic diversity among the analyzed olive cultivars, which is valuable for breeding programs and the conservation of genetic resources. The results also underscore the need to verify local cultivar names, especially among those with high similarity, to avoid redundancy in propagation and nursery practices. The results of the study agreed with [30–35].

Table 4 Genetic grouping of Iraqi olive cultivars based on ISSR analysis

Group	Sub-cluster	Cultivars	Approx. Genetic Similarity	Remarks
A	A1	Nabali, Manzanilla	0.85-0.90	Very high similarity, likely a shared origin or environment
	A2	Bashiqa, Sorani	0.80-0.85	Local genetic background or environmental adaptation
	-	Group A (overall)	0.55-0.60	Generally high similarity, limited genetic differentiation
B	B1	Dakel, Qaisi	0.80	Strong genetic affinity
	-	Darmali, Ashrasi, AlKhudairi	0.65-0.75	Moderate variation within the cluster
	B2	Ascolano	0.50-0.60	Genetically distinct, likely of foreign origin
	-	Group B (overall)	0.50	Greater variability, possible introduced or hybrid cultivars

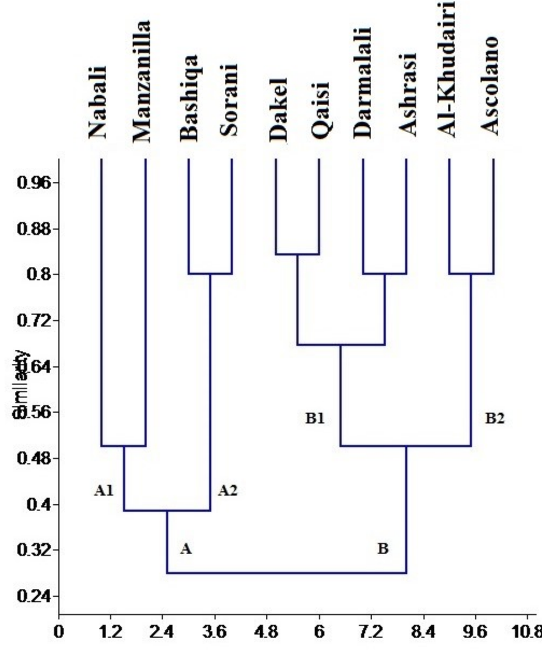

Fig. 5 Dendrogram of Olive cultivar genotypes

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4 CONCLUSION

This study confirms that ISSR markers are effective in assessing the genetic diversity of olive cultivars. The observed levels of polymorphism indicate significant genetic variation within the group. Remarkably, the Ashrasi cultivar was the most genetically differentiated and may therefore serve as an important resource for breeding programs. Phylogenetic analysis divided the cultivars into two major clusters, indicating different levels of genetic similarity. Overall, the Ashrasi and Ascolano cultivars may have potential to contribute to genetic improvement in Iraqi olive breeding.

Acknowledgement

N/A

Author contributions

All authors mentioned above have made a significant contribution to the work. A.A. and I.A.designed the study, A.A. and I.A., B.A. , A.K. ,M.M. conducted experiments, I.A. analyzed the data, A.A. and I.A. wrote the manuscript.

Funding source

No funds received.

Data availability

N/A

DECLARATIONS

Conflict of interest

The authors declare no competing interests.

Consent to publish

N/A

Ethical approval

N/A

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How to cite this article

Alalwani AK, AL-Jaf IHM, Ahmed MM, kamis A, Abed BM. Genetic diversity and molecular characterization of olive cultivars (Olea europaea L.) in Iraq using ISSR markers . Journal of University of Anbar for Pure Science. 2026; 20(1):110-118. doi:10.37652/juaps.2025.162802.1527

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