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Genetic Diversity of Olive Varieties in Northern Iraq Using Microsatellite Markers

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THE OLIVE (*Olea europaea* L.) is a member of the Oleaceae family, which includes approximately 30 genera and 600 species. In this study collected 88 leaf samples from 17 olive varieties from five geographical regions in Iraq. The Deoxyribonucleic acid (DNA) was extracted from 17 varieties. The DNA samples were analyzed by polymerase chain reaction (PCR) using 9 microsatellite (SSR) primers (DCA7, DCA9, DCA11, DCA16, DCA18, EMO90, GAPU71A, GAPU71B, and GAPU103). For statistical analysis, Power Marker v.3.25 was used to assess different molecular genetics parameters. Mega software was used to generate a phylogenetic tree.

The size of the alleles ranged from 91 bp in DAC11 to 245/270 bp in DAC9. The computed mean number of allele was 44.33, and the allele frequency was 0.0892. The observed heterozygosity was 0.2577, with heterozygosity 0.258. Cluster analysis of the total 9 microsatellite markers was used to study the genetic relationships among the 88 different olive genotypes, and they were divided into two main clusters and five sub-clusters. The local cultivars and the foreign cultivar shared the same SSR alleles and they were genetically similar. There were small molecular variations (1%) among the studied geographical regions. The study aimed to investigate the genetic connections among these cultivars and offer valuable insights that can guide forthcoming efforts in cultivation expansion and breeding initiatives.

Keywords: Genetic diversity, *Olea europaea,* Phylogenetic analysis, Population structure, SSR.

Introduction

The Olive (*Olea europaea* L.) is a member of the Oleaceae family, which includes approximately 30 genera and 600 species (Hashmi et al., 2015). The Olea europaea, contains approximately 40 specific and sub-specific taxa distributed across four continents (Africa, Asia, Europe, and Oceania) (Calabrese et al., 2012). Olive is an important tree economically within the Mediterranean basin (Langgut et al., 2019). It has a high degree of genetic diversity because of its ancient domestication, spread, long lifespan, and self-incompatibility (Sánchez-Pérez et al., 2002). The genetic diversity within a population may probably be attributed to the gene flow among different olive cultivars. The gene flow can be caused by domestication, introduction, hybridization, and other related breeding manipulations between olive cultivars (Breton et al., 2006). Different techniques have been used to evaluate olive diversity, (Ouazzani et al., 1993) found a high level of isozyme polymorphism for cultivar identification. Genomic DNA-based markers have enormously empowered the ability to characterize genetic variation in crop plants. Several molecular markers (Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Single Nucleotide Polymorphsim (SNP), Diversity Arrays Technology (DArT), Microsatellite (SSR) etc.) have been employed for the genetic characterization of olive cultivars. Microsatellite

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markers are the most recognized to be successful in studying olive diversity (Sefc et al., 2000; (Bracci et al., 2009) SSRs are hypervariable short (1–6 bp) repeat motifs that show a high level of length polymorphism due to insertion or deletion mutations of one or more repeat motifs, which are highly distributed throughout the genome (Powell et al., 1996). SSRs, have proven to be effective tools for identifying and characterizing of olive cultivars. Their high level of polymorphism and transferability make them suitable for various applications, such as constructing genetic maps, conducting linkage analysis, facilitating marker-assisted selection, and investigating genetic diversity, phylogeny, population structure, and phylogeography of olive cultivars (Bracci et al., 2011; Hmmam et al., 2018; Muzzalupo et al., 2009). The objective of this research was to analyze the genetic variation of some olive cultivars of Iraq. Additionally, the study aimed to investigate the genetic connections among these cultivars and offer valuable insights that can guide forthcoming efforts in cultivation expansion and breeding initiatives.

Materials and Methods

Plant material

Eighty-eight separated leaf samples were collected from 17 distinct cultivars across diverse geographical zones in the northern region of Iraq including Kurdistan Regional Governance also (Table1).

DNA extraction

DNA was obtained from young and healthy leaves by grinding with liquid nitrogen. Lyophilized powdered leaves were used for DNA extraction using a rapid plant genomic DNA extraction kit (N1191) from Dongsheng Biotech Company, China. The purity and concentration of the DNA were assessed using Thermo Nanodrop 2000. The extracted DNA was stored at -20°C for future applications. Genotyping involved the utilization of nine microsatellite markers (Table 2). The selection of the most appropriate and consistent SSR markers for the study was based on their informativeness, as reported in previous studies (Sefc et al., 2000; Carriero et al., 2002; De la Rosa et al., 2002).

TABLE 1. Different Types of Olive Trees: Their Origin, Identification Numbers, Number of Replicas, and Locations for Collection. 1- Zakho in Duhok, 2- Bestane in Erbil, 3- Bakraju in Sulaymaniyah, 4- Hay-Almuhandsine in Ninawa, 5- Alqush in Ninawa

No.	Varieties	Origin	Collection code with several replicates	Places of collection
1	Dahkan	Iraq	Dh (3)	1
2	Hojiblanca	Spain	Hb (3)	1,2
3	Arbequina	Spain	Arb (3)	1,2,3,4
4	Dgl	Iraq	Dg (3)	1,4
5	Santa Catrina	Italy	Sk (3)	1,4,5
6	Picual	Spain	Pic (3)	1,2,3,4
7	Qaysi	Syria	Qs (3)	3,5
8	Khoderi	Iraq	Khd (3)	3
9	Baashiqi	Iraq	Bsh (3)	3,4,5
10	Labeeb	Iraq	Lab (1)	4
11	Suorie	Syria	Sur (3)	4,5
12	Sorani	Syria	Sor (3)	3
13	Nepali	Palestine	Nb (3)	1,4
14	Zurafa	Iraq	Zur (2)	4
15	Ashrasi	Iraq	Ash (3)	1,4
16	Khastawi	Iraq	Khs (3)	4,5
17	K18	Spain	K18 (3)	2,3

TABLE 2. Illustrate the Information regarding the nine utilized polymorphic SSR markers, including their respective origins

Marker	Type	Forward (5′-3′)	Reverse (5′- 3′)	Reference
DCA7	NuSSR	GGACATAAAACATAGAGTGCTGGGG	AGGGTAGTCCAACTGCTAATAGACG	(Sefc et al., 2000)
DCA9	NuSSR	AATCAAAGTCTTCCTTCTCATTTCG	GATCCTTCCAAAAGTATAACCTCTC	(Sefc et al., 2000)
DCA11	NuSSR	GATCAAACTACTGCACGAGAGAG	TTGTCTCAGTGAACCCTTAAACC	(Sefc et al., 2000)
DCA16	NuSSR	TTAGGTGGGATTCTGTAGATGGTGG	TTTTAGGTGAGTTCATAGAATTAGC	(Sefc et al., 2000)
DCA18	NuSSR	AAGAAAGAAAAAGGCAGAATTAAGC	GTTTTCGTCTCTCTACATAAGTGAC	(Sefc et al., 2000)
EMO90	NuSSR	CATCCGGATTTCTTGCTTTT	AGCGAATGTAGCTTTGCATGT	(De la Rosa et al., 2002)
GAPU71A	NuSSR	GATCATTTAAAATATTAGAGAGAGAGA	TCCATCCATGCTGAACTT	(Carriero et al., 2002)
GAPU71B	NuSSR	GATCATTTAAAATATTAGAGAGAGAGA	TCCATCCATGCTGAACTT	(Carriero et al., 2002)
GAPU103	NuSSR	TGAATTTAACTTTAAACCCACACA	GCATCGCTCGATTTTTATCC	(Carriero et al., 2002)

The polymerase chain reaction conditions

The PCR reaction mixture, with a total volume of 25µL, contained the following components: 3µL of DNA genome, 10µL of master mix, 10µL of deionized water, 1µL of forward primer, and 1µL of reverse primer. The PCR program followed the protocol outlined below: an initial denaturation step at 94°C for 5min, followed by 36 cycles of denaturation at 94°C for 45sec. To improve the PCR efficiency and eliminate any nonspecific products, the annealing temperature at 60°C for DAC9, GAPU103, EMO90, and GAPU71A, at 55°C for DAC7, DAC11, DAC16, and DAC18, and at 53°C for GAPU71B. The extension of the primers occurred at 72°C for 1min, followed by a final extension at 72°C for 10min.

The amplified DNA fragments were run on 1.5% agarose gel electrophoresis (1.5h/80 Voltages) to detect successful amplifications. After that, the PCR products were run on 6% denaturing polyacrylamide gels (PAGE). To detect the size of the DNA bands a 50bp DNA ladder was run with the PCR products. The DNA bands were visualized by silver staining (Bassam & Gresshoff, 2007).

Data analysis

To assess the genetic variability among the olive cultivars, the software Power Marker v 3.25 was employed (Liu & Muse, 2005). Allele information was recorded for each genotype across all repetitions. The genetic dissimilarity was examined following the methodology described by Nei (1987). Using the similarity matrix, the dendrogram was fashioned utilizing the unweighted pair group method arithmetic averages (UPGMA) technique (Sokal & Rohlf, 1962). For the construction of the phylogenetic tree, Mega software (Version 10.0.5) was utilized, employing the Neighbor-Joining approach as outlined by Kumar et al. (2018).

Results

Microsatellite polymorphism and cultivar identification

Nine microsatellite loci were used to analyze genetic relationships among 88 olive accessions in different regions of the northern part of Iraq. Figures 1 and 2 indicated that the GAPU71A, GAPU71B, GAPU103, EMO 90, DC7, DC9, DC11, DC16, DC16 and DC18, microsatellite marker banding patterns were at 17 olive accessions.

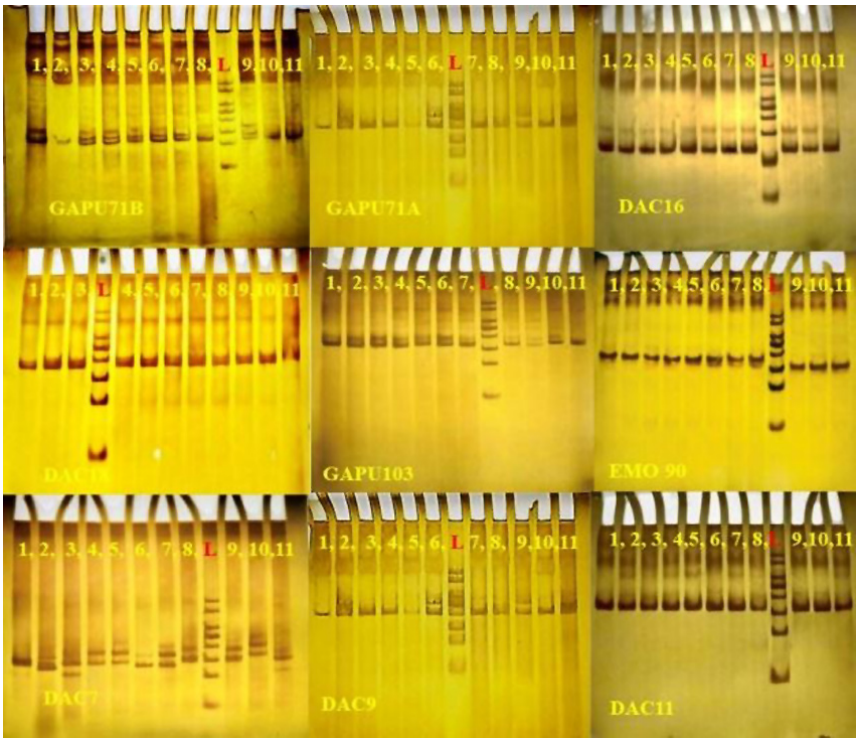

Fig. 1. Amplified PCR product bands of nine microsatellites [The bands were as follows: Lane 1 - Dahkan, Lane 2 – Santa Catrina, Lane 3 - Qaysi, Lane 4 - Sorani, Lane 5 - Nepali, Lane 6 - Picual, Lane 7 - Khastawi, Lane 8 - Labeeb, Lane 9 - Khoderi, Lane 10 - Arbequina, Lane 11 - Hojiblanca, all belonging to *Olea europaea* L. Lane L represents the 50 bp DNA ladder]

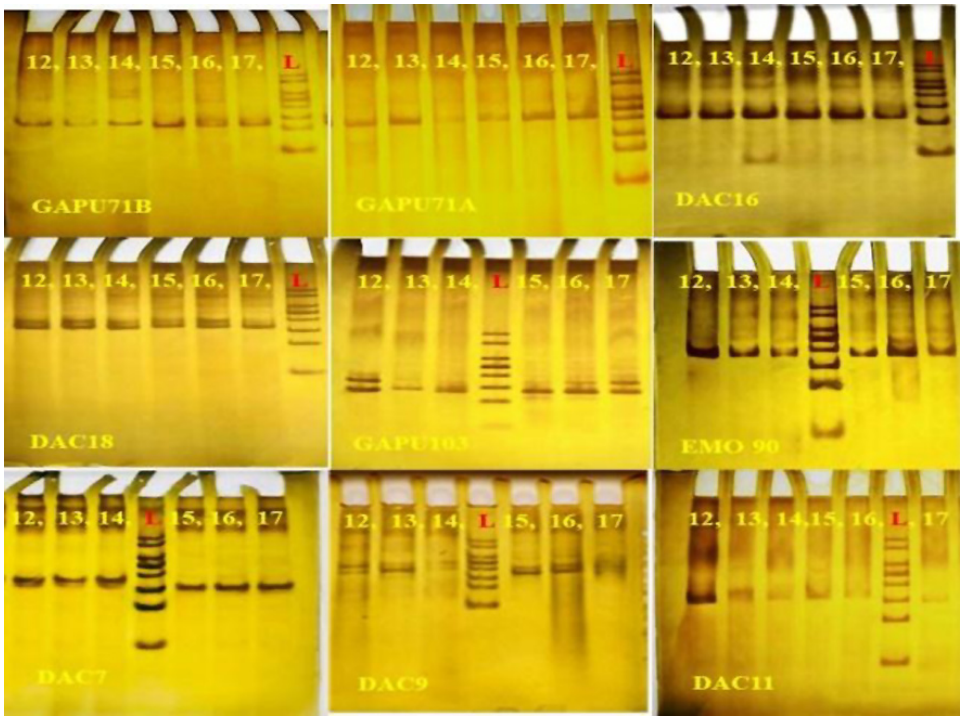

Fig. 2. Amplified PCR product bands of nine microsatellites [The bands were as follows: Lane 12 - Dgl, Lane 13 - Souri, Lane 14 - Zurafa, Lane 15 - K18, Lane 16- Ashrasi, Lane 17 - Baashiqi, all belonging to *Olea europaea* L. Additionally, Lane L represents the 50 bp DNA ladder]

The information presented in Table 3 demonstrated the span of allele sizes at each genetic locus. The greatest allele size was observed at the DAC9 locus in the Hay-Almuhandsine population, measuring 245/270bp. Conversely, the smallest allele size appeared at the DAC11 locus in the Bakraju population, ranging from 91 to 188bp. Allele frequencies signify the proportion of a specific allele within a population's genetic pool. These frequencies ranged from 0.058 at the GAPU71B locus to 0.121 at the GAPU71A locus, with an average frequency of 0.089.

The total number of identified alleles in all populations was reveled in (Table 4), it was 399 alleles. The result showed that the number of alleles per locus was highest at the GAPU103 locus (55) and lowest at the GAPU71B locus (32) with an average of 44.333 alleles per locus (Table 4). Gene diversity is often referred to as expected heterozygosity, as well as a measure of genetic variation present in a population. Its values in this study ranged from 0.953 at the DAC16 locus to 0.971 at the GAPU71B locus, with a mean of 0.963. The detected high level of polymorphism can be partially attributed to the out-crossing pollination mechanism in this species, which likely enhances the degree of polymorphism, this phenomenon is evidenced by the considerable molecular variation within cultivars (27%) depicted in Fig. 3. Heterozygosity values in the present study ranged from 0.00 to 0.727 with a mean value of 0.258 across all genotypes. In this study, PIC values ranged from 0.970 to 0.950 for the DAC16 and GAPU71B loci, respectively (Table 4).

The mean polymorphism information content (PIC) value was 0.961. In general, the analysis revealed significant molecular variation among cultivars (72%), followed by variation within cultivars (27%) and molecular variations between populations were very small (1%). This could be attributed to the shared origin of the studied cultivars in Fig. 3.

TABLE 3. Allele size variation across five populations: Zakho, Bestane, Bakraju, Alqush, and Hay- Almuhandsine

	Range of allele size, bp					
Locus	**Zakho**	**Bestane**	**Bakraju**	**Alqush**	**Hay-Almuhandsine**	**Allele size bp**
DCA7	124-174/186	113/150-156/173	120/140-165/175	137/154-190	125/143-172/188	120/140-172/188
DCA9	160-204/238	125-215	122-204	145/155-209	158/178-245/270	122-245/270
DCA11	94-165	94-167	91-188	92-193	142-165	91-193
DCA16	112-148	115-132/145	119-152	111-116/135	112/125-125/142	111-152
DCA18	158-188/192	150-210	160-192	160/185-200	158-166/199	150-210
EMO90	181-200	181-190	158-203	173-210	168-194	158-210
GAPU71A	148-212	179-225	156-214	150-200	130-240	130-240
GAPU71B	105-153	127-152	102-148	105-128	107-159	102-159
GAPU103	118/132-170/200	142-168/190	125/146-170/195	118/133-170/195	108/137-162/179	108/137-170/200
Range of all allele size bp	**94-204 /238**	**94-225**	**91-214**	**92-209**	**107-245/270**	**91-245/270**

TABLE 4. Allele frequency, genotype count, observed count, allele number, data availability, gene diversity, heterozygosity, and polymorphic information content (PIC) across the five populations

Marker	Allele frequency	Genotype No	No. of observed genotypes	Allele No	Avail ability	Gene diversity	Hetero zygosity	PIC
DAC7	0.108	70.000	88.000	50.000	1.000	0.965	0.636	0.964
DAC9	0.081	65.000	80.000	53.000	0.909	0.968	0.450	0.967
DAC11	0.084	42.000	83.000	42.000	0.943	0.963	0.000	0.962
DAC16	0.092	40.000	87.000	32.000	0.989	0.953	0.115	0.950
DAC18	0.098	65.000	87.000	44.000	0.989	0.961	0.391	0.960
EMO90	0.082	34.000	85.000	34.000	0.966	0.955	0.000	0.953
GAPU71A	0.121	45.000	83.000	45.000	0.943	0.962	0.000	0.960
GAPU71B	0.058	44.000	87.000	44.000	0.989	0.971	0.000	0.970
GAPU103	0.080	79.000	88.000	55.000	1.000	0.967	0.727	0.966
Mean	**0.089**	**53.778**	**85.333**	**44.333**	**0.970**	**0.963**	**0.258**	**0.961**

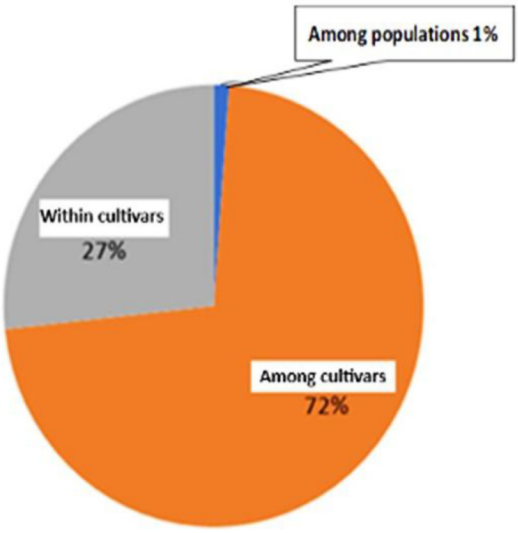

Fig. 3. The proportions of genetic variations within cultivars, among different cultivars, and across five geographical population regions

Cluster analysis

The phylogenetic tree presented in Fig. 4 illustrated the separation of the olive genotypes into two main clusters denoted as Cluster I and Cluster II. Cluster I can be further divided into two sub-clusters. The first sub-cluster encompasses two populations, Zakho (Dohuk) and Alqush (Ninawa) populations. Zakho population included 14 genotypes out of 24 genotypes, the remaining 10 genotypes distributed across other populations. Notably, the Zakho cultivars group appears to exhibit higher inference, indicative of plant material exchange with other regions of the country. The second sub-cluster contained all 12 genotypes from Bestane (Erbil) population. Cluster II was also divided into two sub-clusters, the first one comprises 14 genotypes originating from Bakraju, and the second sub-cluster contains 15 genotypes from Hay- Almuhandsine (Ninawa). Few olive genotypes from all populations mainly the recently imported cultivars (such as Hojiblanca, Arbequina, Santa Catrina, Nepali), were mixed with other sub-clusters. This can be attributed to their shared origin and that the same genotypes had been spread to different regions of the country. The dendrogram results and the estimated genetic diversity indicated that these cultivars are closely related to each other since they were in the same groups with different proportions. The results showed that local cultivars and imported ones shared the SSR alleles; this is another indicator of the genetic similarity.

Discussion

The use of molecular markers especially SSR has becoming the preferred choice in olive identification because of their high discriminatory power and usually straight forward interpretation. The research results reported here SSR markers can be successfully used in studying genetic diversity among the Iraqi olive cultivated varieties. These conclusions are similar to those of Abdelhamid

et al. (2017) and Abuzayed et al. (2018). The 9 SSR markers used in this study were shown to be highly polymorphic and repeatable as previously described by Sefc et al. (2000), Carriero et al. (2002) and De la Rosa et al. (2002). The highest allele size value was at the DAC9 locus and the lowest allele size value was found at the DAC11 locus. Similar alleles size has been reported by Poljuha et al. (2008) and Gomes et al. (2009).

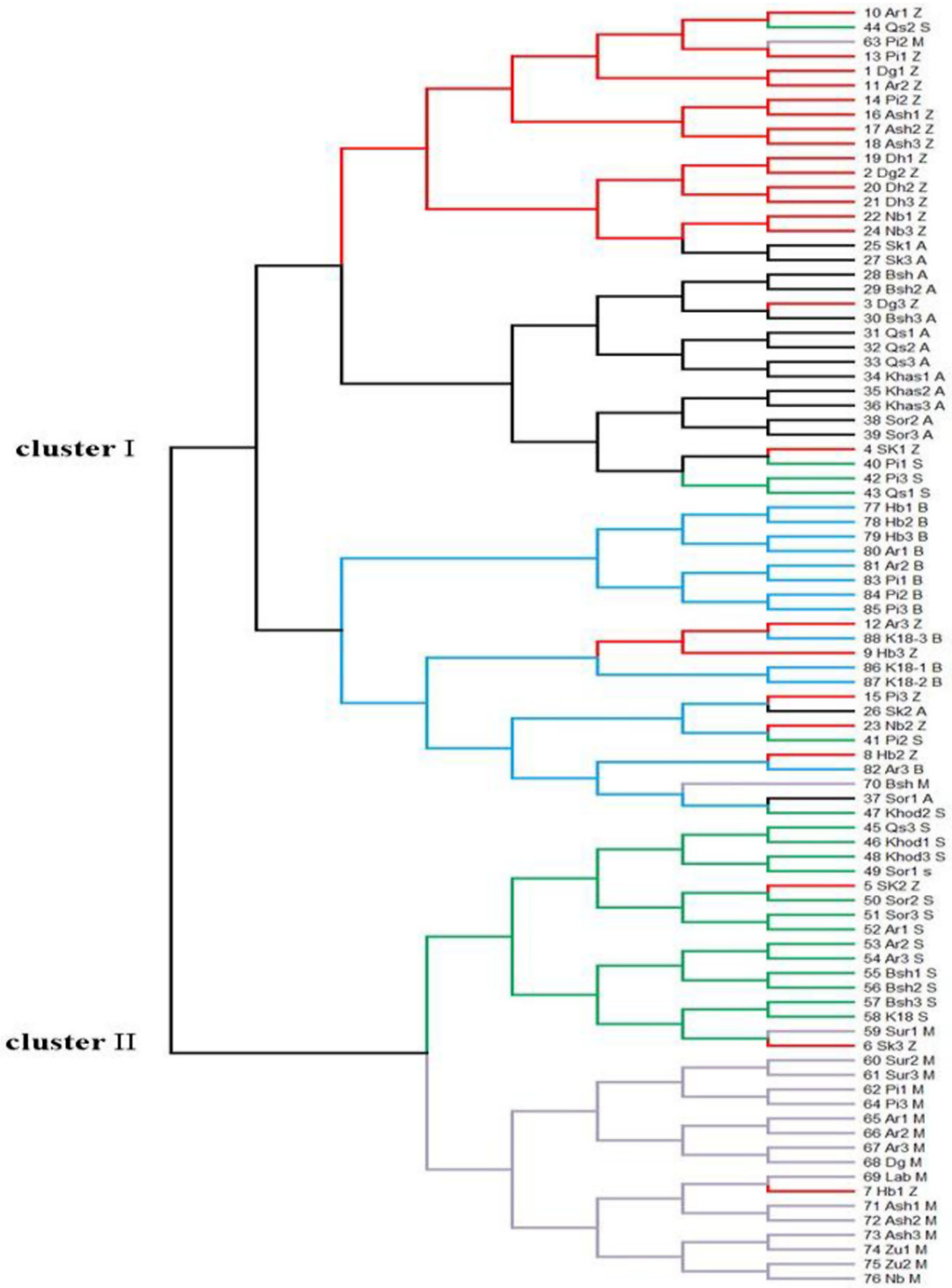

Fig. 4. Phylogenetic tree of 88 olive genotypes created using 9 SSR markers [The dendrogram encompassed genotypes from various populations, including 24 from Zakho (denoted as 'Z'), 15 from Alqush ('A'), 12 from Bestane ('B'), 19 from Bakraju ('S'), and 18 from Hay-Almuhandsine ('M')]

The allele frequencies refer to the proportion of a particular allele within a population's gene pool. The average allele frequency value was 0.089. Las Casas et al. (2014), Abdessemed et al. (2015) and Mohamed et al. (2017) reported higher values compared to the results of this experiment. The obtained value of mean alleles number per locus (44.333). These figures are significantly higher than those reported by Poljuha et al. (2008), Abdessemed et al. (2015), and Falek et al. (2021). The co-dominant nature of SSR markers enabled the detection of a large number of alleles per locus. This diversity is associated with the variation in the loci as well as several genotypes and their location (Las Casas et al., 2014). Indeed, these loci showed the average of expected heterozygotes 0.963. Values close to this mean were observed by many other researchers such as Alba et al. (2009) with a mean value of 0.710, Erre et al. (2009) with mean value of 0.748 and Mohamed et al. (2017) with a mean value of 0.775. The genetic diversity in olive cultivars presented in this study can be attributed to their diverse origin.

The heterozygosity values ranged from 0.00 to 0.727. Similar values have been reported by Yadav et al. (2021), but they were notably lower than those of Falek et al. (2021). The Polymorphism Information Content (PIC) is commonly used to assess the informativeness of a genetic marker. The mean PIC value in this study was 0.961, indicating that the 9 SSR primers used in this study were highly informative and suitable for identifying olive varieties and conducting more genetics studies. This data is consistent with the findings of Muzzalupo et al. (2018) and Aksehirli-Pakyurek et al. (2017), who reported mean PIC values of 0.736 and 0.728, respectively. The analysis uncovers substantial molecular variation among cultivars (72%), with subsequent variation within cultivars (27%). In contrast, molecular variations between populations were minimal (1%). This phenomenon could be attributed to the shared origin of the investigated cultivars. Similar results were also found in other studies which revealed that the olive genotypes from different countries closely clustered in a group and discovered no categorization of olive genotypes can be produced based on geographical origin (Besnard et al., 2001; Öz & Tangu, 2009; Abdessemed et al., 2015).

Cluster analysis, as shown in the dendrogram in Fig. 4 illustrated the separation of the olive genotypes into two main clusters denoted as Cluster I and Cluster II. A small number of olive genotypes across all populations, particularly the recently imported cultivars (such as Hojiblanca, Arbequina, Santa Catrina, Nepali), intermingle with other sub-clusters. This phenomenon is likely due to their common origin and the dispersion of identical genotypes to various regions of the country. The dendrogram results and the inferred genetic diversity reinforce the proximity of these cultivars, as they cluster together within the same groups, albeit with varying proportions. The results showed that local cultivars and imported ones shared the SSR alleles, this is another indicator of a genetic similarity. Similar outcomes are reported in other studies. Li et al. (2020) reported that the Koroneiki cultivar from Greece and the Arbequina cultivar from Spain clustered closely and displayed limited genetic distances. Similar outcomes are also reported in the results of Noormohammadi et al. (2014), Biton et al. (2015), Mousavi et al. (2017).

Conclusions

The use of molecular markers such as SSRs is imperative to build a database for cultivar analysis and appropriate management of olive cultivars collections. This work is the first study with such a large number of olive cultivars analyzed using the same set of SSR markers which allowed the characterization of the genetic structure and diversity. The results showed that the majority of molecular variations are among cultivars and there are little variations among regions as well substantial variations within the cultivars which were attributed to the outcrossing pollination nature of the olive trees. The local cultivars and the imported ones clustered in the same groups indicated the little effects of the geographical region on olive diversity.

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Authors' contributions: YMF was responsible for designing the experiments and analyzing the data using MEGA X software. KJA gathered samples, conducted the experiments, analyzed the data, and authored the manuscript.

Ethics approval: Not applicable.

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دراسة التباين الوراثي في اصناف الزيتون العراقيه المزروعة في شمال العراق باستخدام المعلمات الجزيئية

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الزيتون (*Olea europaea* L.) هو من عائلة Oleaceae ، التي تشتمل على حوالي 30 جنسًا و 600 نوعا. في هذه الدراسة، تم جمع 88 عينة من الأوراق من 17 صنافا من الزيتون مزروعه في خمس مناطق جغرافية في العراق. تم استخراج الـ DNA من هذه الاصناف. كذلك تم تحليل عينات الـ DNA باستخدام تفاعل البلمرة المتسلسل (PCR) باستخدام 9 بادئات SSR كالتالي :

(DCA7 ، DCA9، DCA11، DCA16، DCA18، EMO90، GAPU71A، GAPU71B ، GAPU103).

تم التحليل الإحصائي باستخدام برنامج Power Marker v.3.25 لتقييم مختلف المعلمات الوراثية الجزيئية ، كذلك استخدم برنامج Mega لإنشاء الشجرة التطورية.

اظهرت النتائج ان حجم الأليلات تراوحت من 91 قاعدة زوجية في DAC11 إلى 245/270 قاعدة زوجية في .DAC9 كان متوسط عدد الأليلات 344.33 ، وتردد الأليلات بلغ 0.0892. القيمة الهجينيه الملاحظه بلغت 0.2577. تم استخدام التحليل العنقودي لمجموع 9 معلمات لدراسة العلاقات الوراثية بين 88 نمطا وراثيا مختلفًا من أصناف الزيتون. تم تقسيمها إلى مجموعتين رئيسيتين وخمس مجموعات فرعية. شاركت الأصناف المحلية والأصناف الأجنبية نفس الأليلات لتكون متشابهة وراثياً. كانت هناك تغيرات جزيئية صغيرة (1%) بين المناطق الجغرافية المدروسة. الدراسة كانت تهدف إلى التحقيق في الروابط الوراثية بين هذه الأصناف وتقديم رؤى قيمة يمكن أن توجه الجهود القادمة في التوسع في زراعة الزيتون ومبادرات التربية.