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Molecular detection of the mutations in Khoderi Olive cultivar (*Olea europaea L.*)

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Abstract

The characterization of olive cultivars using molecular markers is necessary for genetic diversity conservation, cultivar verifiction, and the identification of locally adapted genotypes. This study searches for genetic variation in the Khoderi olive cultivar from Karbala, Iraq, utilization two genetic molecular regions: the nuclear ribosomal ITS1 spacer and the plastid matK gene. Through DNA was extracted using a modified CTAB protocol, amplified by PCR, and get sequenced utilization Sanger sequencing. Alignment and comparative analysis with reference sequences deposited in Gen-Bank the results revealed 92.3% similarity of the ITS1 sequence with the Khoderi-Bakraju cultivar with serial number (OQ134700.1), indicating genetic affinity but insufficient similarity for confirmed cultivar identity. The matK region showed 96% similarity with an Olea europaea subsp. europaea plastid genome with serial number (MG372119.1), we notice reflecting high conservation of the chloroplast coding sequences. Multiple SNPs and small indels were detected in both regions, representing intraspecific polymorphisms. The combined data suggest that the studied sample belongs to the broader O. europaea gene pool but may represent a distinct local genotype. Further analyses using high-resolution molecular markers (SSR, SNP-based GBS) are recommended to refine cultivar differentiation.

Keywords: SNP detection, *Olea europaea L.* , Khoderi olive, matk gene, its1 region

Introduction

Olive (Olea europaea L.) represents a fundamental agricultural species within the Mediterranean basin and its neighboring regions in the Middle East to the Oleaceae family (Besnard, 2016) (Julca et al., 2020) where cultivated varieties (cultivars) constitute the foundation for both local agricultural systems and regional agrobiodiversity. The precise genetic characterization of these cultivars is paramount for the purposes of germplasm conservation, cultivar identification, breeding initiatives, and the authentication of product origins. In the past decade, molecular marker systems have transitioned from low-throughput markers (RAPD, ISSR, SSR) to high-throughput, genome-wide single nucleotide polymorphism (SNP) identification facilitated by genotyping-by-sequencing (GBS) and analogous reduced-representation sequencing methodologies (Kaya et al., 2019; Marchese et al, 2023). These genomic strategies facilitate the identification of both population-level diversity and intricate point mutations that traditional markers frequently overlook. (Jahja, 2023; D'Agostino et al., 2018). The Khoderi olive a cultivar of significant local importance in various, particularly in Iraq and all Middle Eastern contexts. However, it is still underrepresented in genome scale mutation investigations studies(Al-Kilani et al., 2024; Casilla García et al., 2025)

This procedure ought to be obligatory prior to the dissemination of any plant materials from germplasm repositories, thereby leads to mitigating confusion regarding varietal identity and promoting the dissemination of true-to-type cultivars constitutes an essential initial step in the preservation of genetic diversity (Sion et al., 2021). Another issue relates to the vagueness in varietal nomenclature arising from adaptation of cultivars to the novel climatic conditions and the using of local designations for newly introduced plant materials.

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The ambiguity in olive nomenclature has underscored the imperative to validate cultivars by reliable methodologies, including morphological and molecular markers (Corrado et al., 2009).

The integration of all markers constitutes a beneficial approach for the genotyping of regional varieties and the identification (Ibtissem et al., 2017). The investigation of genetic diversity is critically significant and is associated with many various factors, such as the selection of desirable characteristics within breeding programs and the advancement of disease resistance to diverse stressors in crops. Furthermore, under conditions of climatic extremes, it is vital to conserve plant genetic resources as a precious reservoir of gene pools for future use. (Sion et al., 2021). From fragment markers to genome-wide SNPs — a methodological transition and underlying rationale.

Initial molecular investigations concerning olive employed markers such as RAPD, ISSR, SSR, and AFLP to evaluate cultivar interrelationships, diversity, and clonality; these markers continue to be advantageous for low-cost, rapid assessments and for comparisons against legacy data sets. Nevertheless, as many agriculturally significant mutations occur as single-nucleotide alterations or minor indels, SNP-based methodologies have now emerged as the preferred approach for mutation discovery and high-resolution population genomics in olive. Genotyping-by-sequencing (GBS) and related reduced-representation sequencing protocols yield thousands to tens of thousands of SNPs across the genome, thereby enabling both population structure analyses and the identification of rare or de-novo variants that remain undetectable by multi-locus fragment markers. (Islam et al., 2021; Slobodova et al., 2023).

Multiple investigations have substantiated the efficacy of Genotyping-by-Sequencing (GBS) in Olea europaea: genotype-by-sequencing methodologies have yielded extensive Single Nucleotide Polymorphism (SNP) catalogs that elucidate the relationships among cultivars as well as the population structure within Italian and broader Mediterranean cohorts, thereby facilitating linkage mapping and Genome-Wide Association Studies (GWAS) for agronomic characteristics. These scholarly contributions illustrate that GBS can consistently discern thousands of high-quality SNPs appropriate for subsequent analyses, including but not limited to population genetics, marker development, phylogenetic studies, and association analyses.

The GBS offers the requisite sensitivity to identify variant alleles across a many of individuals while also prioritizing candidate loci for subsequent validation during targeted sequencing (Agostino et al., 2018; Casilla García et al., 2025). Khoderi cultivar, agronomic investigations and local documentation allude to its inclusion in pollination and germplasm inventories; however, genome-scale mutation scans focusing on Khoderi are notably limited—thereby presenting an evident opportunity for concentrated GBS/SNP or the amplicon sequencing endeavors targeting this cultivar (Al-Kilani et al., 2024). The study of Applied mutation suggests that the merger of marker systems expedites the discovery process: fragment markers facilitate the delineation of candidate mutant lineages, where as GBS/SNPor amplicon analyses precisely identify specific variant loci within genomic regions or candidate genes. In instances where mutagenesis is employed experimentally, targeted resequencing of candidate pathways in conjunction with comprehensive population SNP scans has demonstrated its ability in associating genotype alterations with phenotypic manifestations. These precedents equip a practical framework for mutation detection initiatives fixated on the Khoderi cultivar (Tekin et al., 2022). Single Nucleotide Polymorphism (SNP) constitutes a variation characterized by a singular nucleotide substitution within DNA sequences. These genetic markers exhibit codominance, good reproducibility, and a widespread distribution throughout the genome. Recent advancements in Next Generation Sequencing (NGS) of the O. europaea genome have facilitated the prompt and extensive utilization of SNPs in phylogenetic analyses (Rao et al., 2021), and the genetic mapping, and varietal identification demonstrating the effectiveness of SNP primers in the caliber assessment and authenticity verification of olive identification. (Ayed et al., 2019).

In their study, Mariotti et al. (2020) identified 124 candidate genes that influence the processes of flower development, metabolic pathways, and responses to environmental stressors through analysis of Expressed Sequence the Tag-Single Nucleotide Polymorphisms (EST-SNP), which has helped to an enhanced understanding of the genomic diversity of olives, as well as the reveal of polymorphic genes, thereby underscoring the significance of germplasm conservation. SNPs are recognized for their very high reliability, attributable to their prevalence and diallelic nature.

Advancement in sequencing technologies have rendered SNPs a preferred marker in the genetic analysis of olives, easing the construction of genetic maps and the exploration of geographical relationships, with

anticipated benefits for future research endeavors (Sion et al., 2021).

Materials and Methods

Plant Material Sampling

On the 7rd of January 2025, leaf samples from the Khoderi - olive cultivar were collected from situated in the olive orchard at the Al-Fadk farm in Karbala, Iraq. Three expanded young leaves were harvested from the mid-canopy of the tree, immediately placed into labeled sterile plastic bags,and transported on ice to the laboratory, and stored at a temperature of -20 °C.

DNA Extraction and Quantification

The Genomic DNA was isolated from 100 mg of foliar tissue employing a modified cetyl-trimethyl-ammonium-bromide (CTAB) methodology. Through, the leaf samples were meticulously pulverized in a liquid nitrogen environment, subsequently suspended in 1 mL of CTAB extraction buffer comprising 2% CTAB (w/v), 100 mM TrisHCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, and 0.2% β-mercaptaethanol, followed by incubation at a temperature of 65 °C for 30 minutes. Subsequent to the extraction with a chloroform-isoamyl alcohol mixture (24:1), the DNA underwent precipitation with the isopropanol, was rinsed in 70% ethanol, air-dried, and subsequently re-suspended in TE buffer. (Sahu et al., 2012).

The purity and concentration of the extracted DNA were quantified use a UV spectrophotometer whereby the ratios of A_{620}/A_{280} and A_{260}/A_{230} were evaluated; acceptable thresholds were established at approximately 1.8 and greater than 1.5, respectively. The isolated DNA was preserved at -20 °C to until the initiation of Polymerase Chain Reaction (PCR).

PCR Amplification of Nuclear Regions

In order to ascertain sequence variability within genomic and nuclear genomic regions. Use the specific primer pairs were to target the cpDNA regions matK F: ACCCAGTCCATCTGGAAATCTTGGTTC, matK R: CGTACAGTACTTTTGTGTTTACGAG) and nuclear DNA ITS1 regions Forward: 5'-AAAAGGTAGACCCAGAACTCG-3'- Reverse: 5'-TCGCATTTTGCTGCGTTCTTC-3' (Khamy and Yousi, 2024). Total volume reaction. 25 µL PCR reaction encompassed: 50 ng of template DNA, 2.5 µL of 10× PCR buffer), 0.4 µM of each forward and reverse primer ,1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTP), and 1.25 U of high-fidelity Taq DNA polymerase. The thermocycling protocol included: initial denaturation at 94 °C for 3 minutes; 35 cycles of 94 °C for 30 seconds, annealing at 55-60 °C for 30 seconds, and elongation at 72 °C for 45 seconds; concluding with a final extension at 72 °C for 5 minutes. PCR products were visualized on the 1.5% agarose gel (Gupta, 2019).

Sequencing and Bioinformatics Analysis

PCR-purified amplicons were sent to a sequencing laboratory in South Korea for Sanger sequencing conducted in a bidirectional manner. The sequences were compared through the Basic Local Alignment Search Tool (BLAST) against the NCBI nucleotide database to identify the closest matches and to annotate the cultivar sequences. Following this the sequences derived from the Khoderi samples were systematically aligned with reference sequences of documented olive cultivars (sourced from public databases) employing the ClustalW algorithm and subsequently examined for the presence of single-nucleotide variants (SNVs) or minor insertions/ deletions.

Results and Discussion

The CTAB-based protocols have been shown to yield genomic DNA of superior quality from plant species (Sahu et al., 2012).The result showed of the nrDNA sequence alignment utilizing the ITS1 region of the specimen 92.3% genetic similarity between the examined specimen and the Khoderi-Bakraju-Sulaymaniya olive cultivar in Sulaymaniya-Iraq, registered in the GenBank accession number OQ134700.1. This degree of genetic resemblance suggests an underlying genetic association between the two specimens; nevertheless, it does not fulfill the criteria requisite for classification as the same cultivar. This finding implies that the studied specimen may signify a locally differentiated isolate or a distinct genetic variant within the extensive genetic diversity of olive trees in Iraq. The noted 7.7% sequence divergence in the ITS1 region could be ascribed to point mutations (SNPs) or insertion/ deletion events (indels) mutation (Abdallah et al., 2023; Casilla et al., 2025). Which have the potential to genomic architecture and influence gene expression although the specimen is not categorized as Khoderi-Bakraju-Sulaymaniya, it appears to be evolutionarily proximate and may epitomize a locally adapted isolate. sourced from Gen Bank unveiled a distinctive pattern of genetic variability within the ITS1 region. Molecular alignment of sample under study against Seven point mutations were discerned at positions 118 (G→A), 124 (C→G), 130 (G→T), 139 (A→G), 161 (A→G), 207 (G→A), and 205 (T→A), alongside three sequence gaps at positions 123, 129, and 217 that mutations in these regions are indicative of evolutionary divergence among cultivars. (Zhang et al. (2025). Which is recognized for its accelerated

evolutionary rate compared to chloroplast regions (Casilla et al., 2025).

Recent study proved have ITS sequence variations are prevalent among indigenous groups olive cultivars and serve as indicators of environmental adaptation and local selective pressures (Firouzi et al., 2025).

This result very importing and findings highlight the significance of meticulous molecular analysis for cultivar differentiation, as the documented mutations furnish a distinctive genetic fingerprint that can be dependably use for the identification of local olive cultivars and molecular classification (**Figure 1** and **2**).

Molecular Mutation Analysis of the ITS1 Region in Olea europaea

The mutations identified encompass single-nucleotide substitutions and short indels, which are characteristic of non-coding ribosomal spacers that tend to accumulate neutral mutations devoid of selective pressure (Said & Hassan, 2023). These polymorphisms function as neutral molecular markers rather than inducing functional alterations (Niu et al., 2020). Effect on Protein Function the ITS1 region is part of the nuclear ribosomal DNA cluster, positioned between the 18S and 5.8S rRNA genes, and is transcribed without being translated into

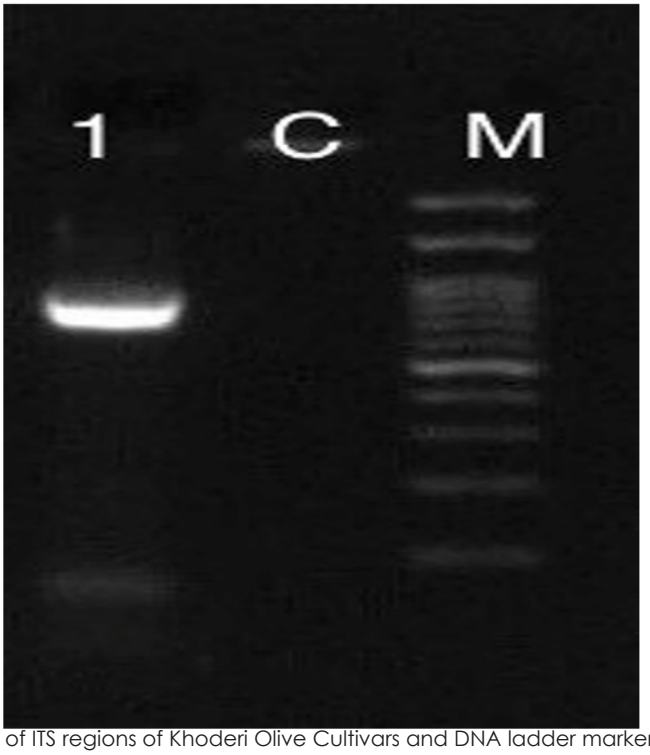

Figure 1. explains the Amplification of ITS regions of Khoderi Olive Cultivars and DNA ladder marker (M 100-3000bp) by migration on agaroes gel after PCR reaction

Olea europaea subsp. europaea cultivar Khoderi-Bakraju-Sulaymaniya internal transcribed spacer 1, partial sequence

Sequence ID: OQ134700.1 Length: 132 Number of Matches: 1

Range 1: 1 to 131 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
183 bits(99)	6e-45	121/131(92%)	3/131(2%)	Plus/Minus

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Query  107  TGTTCCTTGACACTTT-GACGTC-TGGTTTGTTGGGCCGAGCGAGTCTCCGACTCAGGAT  164
            ||||||||||| ||||  |||||  |||||||| ||||||||||||||||||||| |||
Sbjct  131  TGTTCCTTGACGCTTTTCACGTCAGGGTTTGTTAGGCCGAGCGAGTCTCCGACTCAAGAT  72

Query  165  GATCGTTCAGTTACACCCGTGGCATCCTCCCGTACGTGTCAAGAATCGAGTTC-TGGGTC  223
            ||||||||||||||||||||||||||||||||||||||||| | ||||||||| ||||||
Sbjct  71   GATCGTTCAGTTACACCCGTGGCATCCTCCCGTACGTGTCATGGATCGAGTTCGTGGGTC  12

Query  224  TACCTTTTAGA  234
            ||||||||||
Sbjct  11   TACCTTTTAGA  1
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Figure 2. alignment between the sample under study and the reference sample Khoderi-Bakraju-Sulaymaniya, registered in the GenBank accession number OQ134700.1. with the identification of mutations.

protein (Niu et al., 2020). As such, point mutations and indels within the ITS1 region do not modify amino acid sequences or enzymatic functionality. Minor nucleotide variations may have a slight impact on rRNA secondary structure or processing efficacy; however, such influences are infrequently substantial. Therefore, mutations in ITS1 are regarded as evolutionarily neutral (Edger et al., 2014; Nafisi et al., 2023). The Genetic variation and Phylogenetic Relationship Sequences of ITS are widely employed for clarifying phylogenetic relationships among closely related cultivars of Olea europaea due to their inherent variability (Niu et al., 2020; Said & Hassan, 2023).

Mutation Analysis in the matK Region of Olea europaea

The result showed of cpDNA sequence alignment, specifically the matK region of the sample under study , indicated a 96% similarity with the Olea europaea subsp. isolate Oeiras 1 mitochondrial genome, as found in GenBank under accession number Sequence ID: MG372119.1. and showed This degree of similarity signifies a genetic between the two samples. Such a relationship implies that the sample may embody a locally adapted isolate or a genetic variant within the extensive genetic diversity observed among olive cultivar in Iraq. The detected 4% sequence difference in the matK region could be returns to insertion/deletion events (indels), or single nucleotide polymorphisms (SNPs) which may have change_for gene expression, Eighteen point mutations were identified and three gaps (**Figure 3** and **4**). Recent investigations suggest that mutations within these regions are indicative of evolutionary divergence among cultivars **Table 1**, (Zhang et al. (2025) non-coding regions within plant genomes serve of genetic variation, which can be used to evolutionary relationships among species.

The few variations are in alignment with research regarding olive plastid genes: the plastid genomes exhibit a high degree of conservation, characterized is low mutation rates and a limited degree of polymorphism (Mariotti et al., 2010). The matK gene, which encodes the plastid maturase K enzyme.

Effect on the Protein the absence of substantial frameshifts or not wide insertions and deletions, it is plausible that the majority of nucleotide variations are indicative of neutral mutations that have no influence on the functionality of the enzyme.

Mutation rate Analysis indicates a ~4% divergence within a ~509 bp alignment The nucleotide composition and variability were evaluated using a chi-square test ($\chi^2 \approx 6.51$, df = 3, $p > 0.05$), which compared observed base frequencies against equal expected frequencies. Given the non-significant result of the test, the deviation in base composition does not reach a statistically extreme level, thereby reinforcing the concept of normal plastid variability as opposed to the existence of mutational hotspots. the substitution rates and the absence of significant variation are containing with studies indicating exceedingly low cpDNA substitution rates in olive less than 0.07% divergence between plastomes and a slow pace of molecular evolution (Kaya et al., 2018). Genetic Origin and Phylogenetic Implications - The elevated sequence similarity endorses the classification of the Iraqi olive specimen within the gene pool of O. europaea subsp.

Table 1. Types and location of the Mutation determined through cpDNA sequence alignment useing the matK region of the sample under study and reference sample, accession number MG372119.1 in the GenBank

n	Approximate Query Position	Mutation Type	Observed Change (approximate)
1	659	Single nucleotide substitution	A →G
2	796	Single nucleotide substitution	G →T
3	834	Single nucleotide substitution	A →G
4	839	Single nucleotide substitution	A →C
5	868	Single nucleotide substitution	A → G
6	894	Single nucleotide substitution	A →G
7	895	Single nucleotide substitution	C →A
8	915	Single nucleotide substitution	C →T
9	917	Single nucleotide substitution	C →T
10	937	Single nucleotide substitution	C →G
11	944	Single nucleotide substitution	C →A
12	946	Single nucleotide substitution	G →A
13	988	Single nucleotide substitution	C → A
14	990	Single nucleotide substitution	C →G
15	993	Indel (Gap observed Deletion / insertion detected Gap 1	--- →A
16	1002	Single nucleotide substitution	C →T
17	1003	Single nucleotide substitution	C →G
18	1004	Indel (Gap observed Deletion/ insertion detected Gap 2	--- →T
19	1013	Indel (Gap observed Deletion/ insertion detected Gap 3	A →----
20	1032	Single nucleotide substitution	C →G

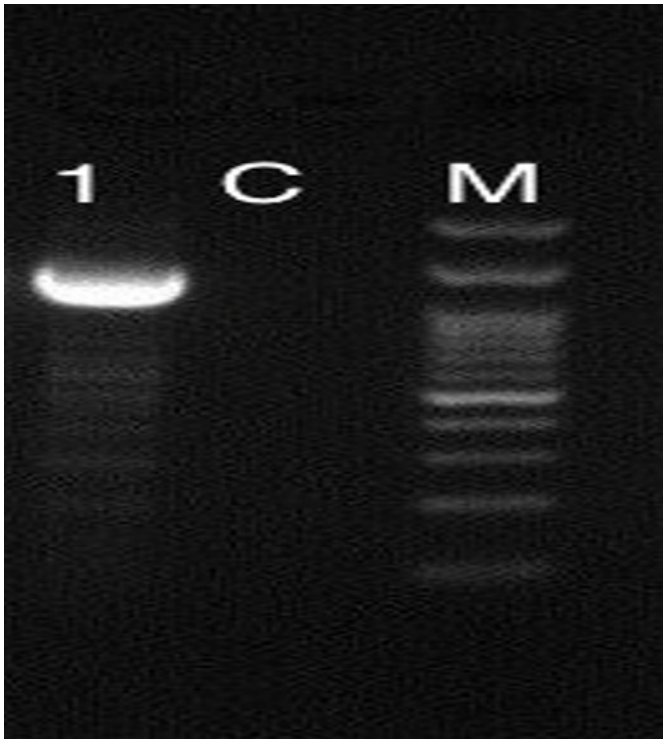

Figure 3. Explains the Amplification of matk regions of Khoderi Olive Cultivars and DNA ladder marker (M 100-3000bp) by migration on agaroes gel after PCR reaction

Olea europaea subsp. europaea isolate Oeiras 1 mitochondrion, complete genome
Sequence ID: MG372119.1 Length: 769995 Number of Matches: 1

Range 1: 326353 to 326859 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
826 bits(447)	0.0	489/509(96%)	3/509(0%)	Plus/Plus

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Query  545     GGGGTGATCTCGTAGTTCTTGGTCTGTGAAGATGCGTTGTTAGGTGCTCCATTTTCTTTT  604
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  326353  GGGGTGATCTCGTAGTTCTTGGTCTGTGAAGATGCGTTGTTAGGTGCTCCATTTTCTTTT  326412

Query  605     CCCATTGAGGCCGAACCTAAACCTGTGCTCGAGAGATAGCTGTCCATACACTGATGAGGG  664
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  326413  CCCATTGAGGCCGAACCTAAACCTGTGCTCGAGAGATAGCTGTCCATACACTGATAAGGG  326472

Query  665     ATGTATGGATTCTCGAGAAGAGAGGAGCCGTGGTGGTccccccGGACCGCCCGGATCCC  724
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  326473  ATGTATGGATTCTCGAGAAGAGAGGAGCCGTGGTGGTCCCCCCCCGGACCGCCCGGATCCC  326532

Query  725     ACGAGTGAATCGAAAGTTGGATCTACATTGGATCTCACCCGAATCGCCCCATCTATCCTC  784
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  326533  ACGAGTGAATCGAAAGTTGGATCTACATTGGATCTCACCCGAATCGCCCCATCTATCCTC  326592

Query  785     CTGAGGAGAGTTTTGGTTTCAAACCCCGGTTCGAACAGGAGGAGTACGCCCTGCTCATGT  844
               ||||||||| ||||||||||||||||||||||||||||||||||||||||| |||| ||||
Sbjct  326593  CTGAGGAGAGGTTTGGTTTCAAACCCCGGTTCGAACAGGAGGAGTACGCCATGCTAATGT  326652

Query  845     GCCTTGGATGATCCACATCTCAAGGTCAGGCGCCGATGAGCACATTGAGATATCCATGTG  904
               |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  326653  GCCTTGGATGATCCACATCTCAGGGTCAGGCGCCGATGAGCACATTGAACTATCCATGTG  326712

Query  905     GCTGAGAGCTCTTACAGCCCAGGCACAACGACGGAATTATAAAGGGCGCGCTCTACCACT  964
               |||||||| || |||||||||||||||||| |||||| | ||||||||||||||||||||
Sbjct  326713  GCTGAGAGCCCTCACAGCCCAGGCACAACGACGCAATTATCAGGGGCGCGCTCTACCACT  326772

Query  965     GAGCTAATAGCCCGTCGTGCGAGCATGCCAACTGGGGGCTGTGCTATGCC-AAAGCGAGA  1023
               |||||||||||||||||||||||||| ||| |||||||| |||||||| |||||||||||
Sbjct  326773  GAGCTAATAGCCCGTCGTGCGAGCCTCCCA-CTGGGGGCCC-GCTATGCCAAAAGCGAGA  326830

Query  1024    GAAACCCGATCCCTCTCTTTCCTTTTTTC  1052
               ||||||| ||||||||||||||||||||||
Sbjct  326831  GAAACCCCATCCCTCTCTTTCCTTTTTTC  326859
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Figure 4. Alignment between the sample under study and the reference sample Khoderi-Bakraju-Sulaymaniya, registered in the GenBank accession number OQ134700.1. with the identification of mutations.

europaea and implies only negligible divergence from the European reference Oeiras 1. may signify intraspecific variation (Slobodova et al., 2023).

Conclusions and Recommendations

The comparative molecular analysis of both matK and regions in the studied Olea europaea L. biological sample disclosed complementary patterns of genetic variation. The ITS1 region showed and exhibited moderate polymorphism (92 % similarity) with the Khoderi-Bakraju-Sulaymaniya cultivar, characterized by neutral point mutations and small. In contrast, the matK gene showed high conservation (~96 % similarity) with the reference genome in bank gene, with no frameshift or deleterious mutations, indicating that the maturate K enzyme remains functionally intact. Together, these results confirm that ITS1 variability reflects nuclear-level driven by genetic drift, while the matK regions maintains plastid stability under purifying selection. But use of both markers very enhances the resolution of intraspecific genetic relationships in olives.

Ethical statement and data availability

The olive leaves samples collected from the Al-Fadk farm in Karbala, Iraq,mafter take the approval from the administration of the farm's management

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