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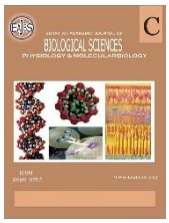

DNA Barcoding of Almond (*Prunus dulcis*) in Duhok Province-Kurdistan Region / Iraq Using 18S- 28S rRNA and ITS Region

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ABSTRACT

Almond is an economically important plant with a high genetic diversity, therefore molecular methods are reliable approaches to define and verify rapidly and correctly such diversity. This research sought the use of molecular markers for identifying and barcoding Almond genotypes in Duhok Province KRG- Iraq. It involved the application of Internal Transcribed Spacer region,18S-28S intergenic spacers, sequencing and Single Nucleotide Polymorphism identification. For this study, 8 genotypes were collected from various regions, namely the Barwari, Zakho, Bajlor, Kora, Kemeka, Pirumara, Qirqrava and Deragzhnic. The PCR amplification products of the ITS region and 18S–28S rDNA intergenic spacer were 700 bp. and 850 bp. respectively. To determine the types and the number of SNPs in the different genotypes, the sequencing data obtained in this study were compared to those in the NCBI. The SNPs results in the ITS region revealed 6 transversions within the Bajlor genotype (sequence three) and the clustering analysis showed that the eight almond genotypes were grouped into three main groups. Whereas SNPs results in the 18S–28S rDNA intergenic spacer detected a total number of 38 positions. 16 transition and 22 transversion sites within the Bajlor genotype (sequence three) while clustering analysis showed that the eight almond genotypes were grouped into two main groups. This study showed the feasibility of using those markers for possible DNA barcoding and identification of Almond genotypes in this region.

INTRODUCTION

Almond *Prunus dulcis* is a small deciduous tree belonging to the *Prunoideae* subfamily of the Rosaceae family with a chromosome number 2*n* =16, and a genome size of 246 Mb (Dirlewanger *et al*., 2004). Almond is an agronomically important crop as it is a pleasant nut worldwide and is cultivated mostly for its kernel and oil with many applications in the processed food, pharmaceutical and cosmetic industries (Colic *et al*., 2012; Zeinalabedini *et al*., 2008).

For genetic variability analyses, pedigree determinations, or cultivar identification in almonds and many other plant species different molecular markers have been used (Sánchez-Pérez *et al*., 2004). Out of many different types of common PCR-based DNA markers that have been used in almond research are the RAPD (random amplified polymorphic DNA), the SSR (Simple Sequence Repeat), Amplified Fragment Length Polymorphisms (AFLPs) (Sorkheh *et al*., 2009).

Each of these techniques may have its own benefits according to the target application because of their many advantages such as low cost, simplicity, rapidity, and the requirement for a minimal amount of DNA (Farinha *et al*., 2000; Khadivi-Khub, 2014).

The initial crucial step in preserving plant genetic resources involves precisely recognizing the specific species being targeted. One of the potential methods for such purposes issue is DNA barcoding (Wang *et al*., 2015). This technique involves the identification of species based on a short universal DNA sequence that demonstrates a significant degree of variation, enabling differentiation between species (Sayed *et al*., 2023) The internal transcribed spacer (ITS) region and 18S-28S rRNA gene are two commonly used DNA barcodes for plants (Sinjare *et al*., 2023). The ITS region is a non-coding DNA sequence situated between the 18S and 5.8S rRNA genes. It exhibits significant variability across species and cultivars, giving it a suitable indicator for identifying and analysing genetic diversity within and between species(Tripathi *et al*., 2013). The 18S-28S rRNA gene, however, is a highly conserved coding sequence that has been used for phylogenetic analysis and taxonomic classification(Álvarez & Wendel, 2003). This method can be used to study genetic variation and detect Single Nucleotide Polymorphisms (SNPs) in cultivars. It is also useful for identifying and classifying different cultivars (Goonetilleke *et al*., 2018). DNA barcoding has been found to be a powerful strategy for genetically characterizing almond cultivars through the use of PCR analysis combined with specifically targeting the ITS region and 18s-28s rRNA (Ding *et al*., 2020). The data obtained from such analysis may be used in developing conservation strategies and identifying plants with distinctive genetic characteristics that could prove valuable for breeding programs (Khadivi *et al*., 2023).

Almond is cultivated and/ or grown in natural populations in the different mountainous regions of Kurdistan- Iraq. This region is ecologically characterised by a semi-arid continental climate, which is hot and dry in summer and cold and wet in winter with an average temperature of 2–45 degrees Celsius, the primary distinction between these locations is high altitude. To the best of our knowledge, no genetic diversity analysis based on scientific data has been reported in almond populations. Therefore, the main objective of this study was to examine the use of DNA barcoding with the 18S–28S rDNA intergenic spacer and ITS region to almond in Duhok province- Kurdistan region/ Iraq.

MATERIALS AND METHODS

Samples Collection:

Leaf samples obtained from 8 *Prunus dulcis* (Almond) plants were gathered from various regions of Duhok (Table 1; Fig. 1). These samples were then transported to the laboratory and prepared to isolate genomic DNA. The cultivars were chosen based on their distinctiveness, local preferences, and extensive cultivation.

Table 1: Represent the areas of plant sample collection used in this study.

No.	Area of sample collection
1	Barwari
2	Zakho
3	Bajlor
4	Kora
5	Kemeka
6	Pirumara
7	Qirqrava
8	Deragzhnic

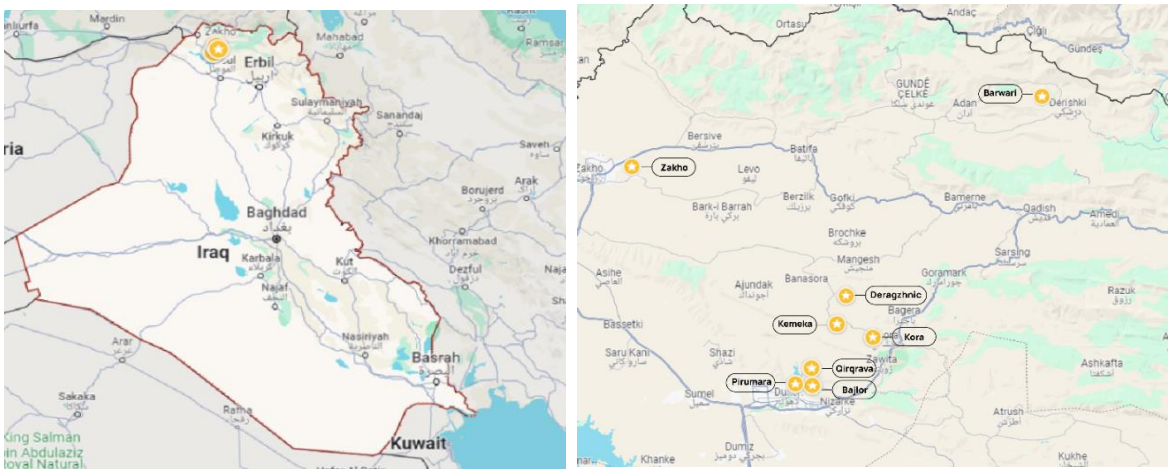

Fig. 1. The geographic locations of the various almond populations in Duhok province.

Genomic DNA Isolation and PCR Amplification:

The genomic DNA was extracted from 3g of fresh almond leaves using the CTAB methods described originally by (Weigand *et al*., 1993), with some modifications by (Jubrael *et al*., 2005) and, Hussein & Jubrael, (2021). For PCR amplification of both regions, two sets of primers were used (Table 2), For this a 20µl PCR reaction mixture was prepared containing 10 µl of Addbio/ Korea master mix, 2 µl of DNA (50-100 ng), 2 µl (10 pmol) of forward and reverse primers and 6 µl of double deionized sterile water. The PCR amplification was performed in an ABI Applied Biosystems PCR System 2720 thermal cycler using the following settings. For the ITS region the optimized thermocycler conditions were an initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 10 min. For 18S–28S rDNA intergenic spacer an Initial denaturation step at 94ºC for 3 min, 40 cycles consisting of 94°C for 20 sec (denaturation), 50ºC for 30 sec(annealing), 72 °C for 1 min (primer extension) and final extension of 72°C for 5 min.

Table 2: The ITS region and 18S-28S rDNA intergenic spacer primer sequences used in this study.

Primer name	The sequence of forward primer	The sequence of the reverse primer	Amplicon size bp.
ITS region	5’TCCTCCGCTTATTGATATGC3’	5’TCCGTAGGTGAACCTGCGG3’	700
18S-28S rDNA intergenic spacer	5’TTGTACACACCGCCCGTCGC3’	5’AGTTTCTTTTCCTCCGCTTA3’	850

Capillary Standard Sequencing:

The amplified PCR products of both ITS region and 18S-28S rDNA intergenic spacer were sent to Macrogen Inc., located in Seoul, South Korea (http://dna.macrogen.com), for bi-directional Sanger sequencing using the original amplification primer as the sequencing primer.

Sequencing Data Analysis:

The assembly and analysis of sequencing data were conducted using BioEdit version 7.0.5.3 (Hall, T.A. 1999. Nucl. Acids. Symp. 41:95-98.). The sequencing findings were examined online using the BLAST tool on the NCBI nucleic acid database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple NCBI alignment and clustalo (Version 1.2.4) were used to align the

sequenced segments of the gene with comparable sequences. The sequences were submitted to Gene Bank (NCBI) using the BankIt portal (https://www.ncbi.nlm.nih.gov/WebSub/).

RESULTS AND DISCUSSION

In this investigation, attempts were carried out to barcode and classify a total of 8 almond genotype varieties. To achieve this, they were subjected to several experiments using the internal transcribed spacer (ITS) region and 18s-28s rRNA markers. The results of these experiments are described individually. The amplification results of ITS region in these experiments were found to be of the same length in all tested almond genotypes, measuring 700 bp as shown in (Fig. 2),

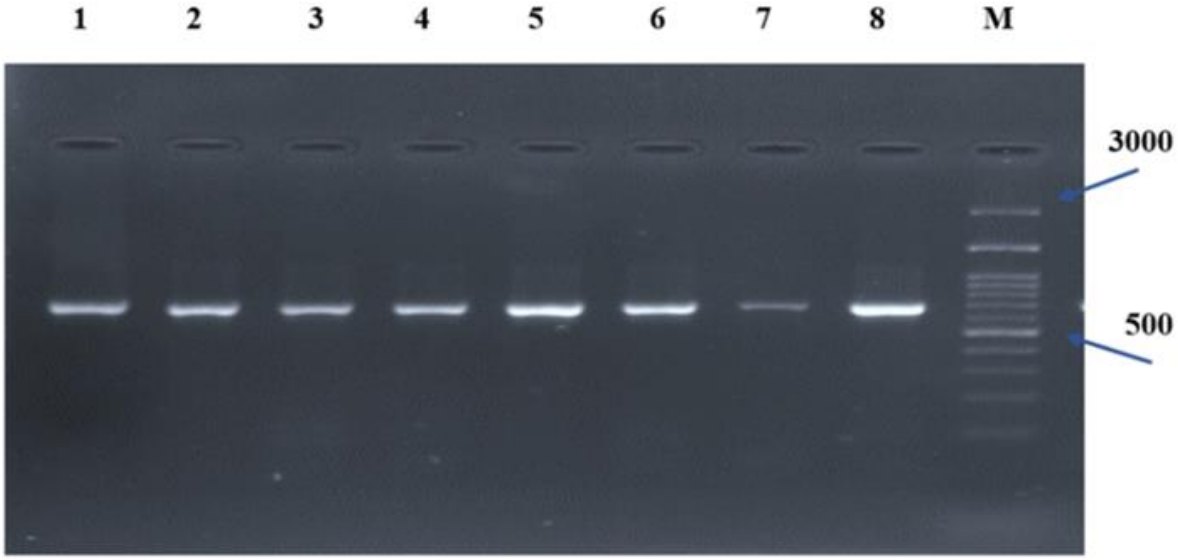

Fig. 2. Represents 2% agarose gel electrophoresis of ITS region for 8 samples of almond genotypes (1-8). Lane **M** Represent 100 bp DNA molecular weight marker.

To assess the heterogeneity in length or nucleotide sequences, the obtained ITS region sequences of the eight almond genotypes were (PP515900, PP515901, PP515902, PP515903, PP515904, PP515905, PP515906, PP515907) aligned with reference sequences AP021837.1 of complete almond ITS regions in NCBI database. The results (Fig. 3) showed 94% query cover and 96.77% identity of homogenous in length and structure for sequence (3) three representing Bajlor, genotype on the other hand, all other sequences showed 99.52% identity of homogenous in length and structure using ITS-1 and ITS-4 universal primers. The results of SNPs detection revealed 6 transversions within sequence three (Bajlor genotype), as follows; in location 185 C changed to G, location 189 G to C, location 191 C changed to A, location 195 T changed to G, location 210 C changed to G and location 212 T changed to G). However, all other genotypes showed high similarity with each other used for alignment (Fig. 3). The results of clustering analysis (Fig. 4) showed that the eight almond genotypes grouped into three main groups, The first one included sequence seven (Qirqrava), the second included sequence four (Kora) and group three included sequence three Bajlor, sequence six Pirumara, sequence eight Deragzhnic, sequence five Kemeka, sequence one Barwari and sequence two Zakho.

Fig. 3. Showing SNPs within used almond cultivars using ITS region.

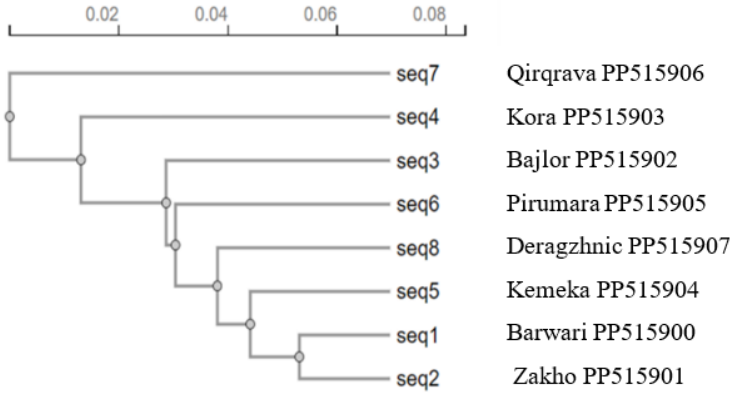

Fig. 4. SNPs clustering and similarity analysis of ITS region.

The revealed SNPs within the studied almond genotypes were found to be somewhat similar to those reported by (Wang *et al*., 2015) who studied the internal transcribed spacer (ITS/ITS2) region in Ascomycetes and Monocotyledons and proposed the standard DNA barcode for fungi and seed plants and has been widely used in DNA barcoding analyses for other biological groups. DNA barcoding has been proposed as the preferred method using other markers for distinguishing almond species underscoring the importance of evaluating *trnH-psbA* loci sensitivity for barcoding, shedding new light on Prunus species phylogeny notably, the *trnH-psbA* locus exhibited variable length, with phylogenetic analysis revealing sweet almond's association with *Prunus dulcis* and bitter almond forming a separate group (Hassan, 2023).

Utilizing DNA barcoding for identifying almond genotypes and analysing genetic diversity has significant implications for almond industry breeding programs and quality control. Precisely identifying and differentiating cultivars might aid in the advancement of enhanced variations and guarantee the authentic quality of almond products in the market (Rizwan *et al*., 2024). Length variations and polymorphism of the ITS have been documented in several plant species (Katzir *et al*., 1996; Vijayan & Tsou, 2010, Karacaoğlu, 2021).

The PCR amplification results of the 18S–28S rDNA intergenic spacer in the tested almond genotypes, produced a band of 850 bp in length as shown in (Fig. 5).

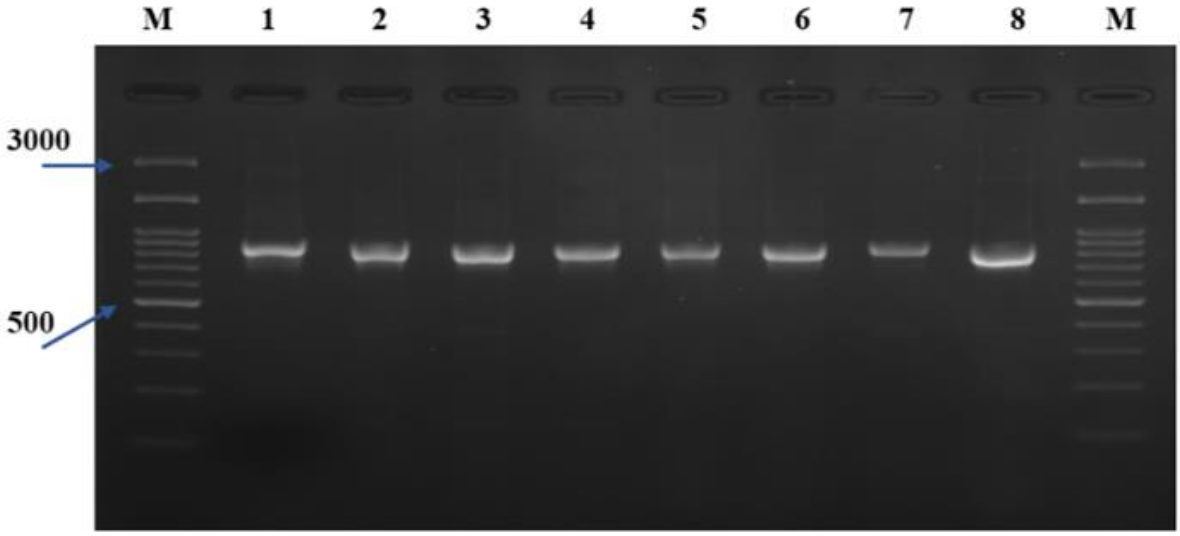

Fig. 5. Represents 2% agarose gel electrophoresis of the 18s-28s r DNA intergenic spacer for 8 samples of almond genotypes (1-8). Lane **M** Represent 100 bp DNA molecular weight marker.

The generated sequences of 18S-28S rRNA (PP690968, PP690969, PP690970, PP690971, PP690972, PP690973, PP690974, PP690975) in these experiments were aligned with the reference sequence AP020792.1 of complete almond 18S rRNA in NCBI database. The results (Fig. 6) revealed that Barwari (sequence one) genotype showed 100% query cover and 97.08% identity of homogenous in length and structure and sequence two (Zakho) show 97.96 similarity and 100% query cover compared to the reference sequence AP020792.1, also other sequences, sequence four (Kora), sequence five (Kemeka), sequence six (Pirumara), sequence seven (Qirqrava), sequence eight (Deragzhnic) all of them showed high identity of homogenous to the reference sequence AP020792.1. However, the Bajlor genotype (sequence three) showed 81.25% similarity and 95% query cover to the standard reference AP020792.1, and only in this genotype SNPs were detected with a total number of 38 positions. 16 transition and 22 transversion sites as shown in Table 3.

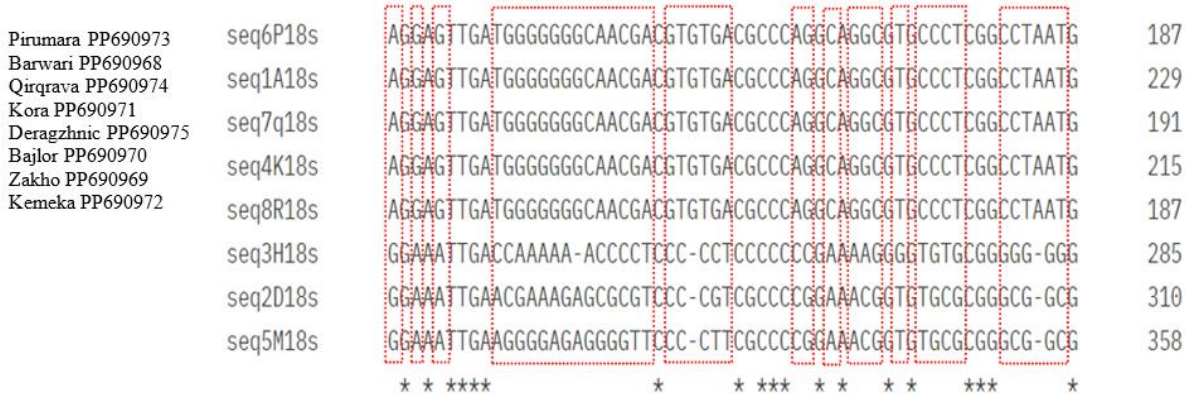

Fig. 6. Showing SNPs within used almond cultivars using 18s-28S rRNA.

Table (3): Represent point mutation of noncoding 18S-28S rRNA.

Location	Transition in sequence seq3H18S of almond in Bajlor	Transversion in sequence seq3H18S of almond in Bajlor
226	(A to G)	
228	(G to A)	
230	(G to A)	
235	(T to C)	
236		(G to C)
237		(G to C)
238	(G to A)	
239	(G to A)	
240	(G to A)	
241	(G to A)	
243		(C to A)
244		(A to C)
245		(A to C)
247		(G to C)
248		(A to T)
250		(G to C)
251	(T to C)	
253	(T to C)	
254		(G to C)
255		(A to T)
257		(G to C)
261		(A to C)
262		(G to C)
264		(C to A)
266	(G to A)	
267	(G to A)	
268		(C to G)
270		(T to G)
272	(C to T)	
273		(C to G)
274	(C to T)	
275		(T to G)
279		(C to G)
280		(C to G)
281		(T to A)
283	(A to G)	
284	(A to G)	
285		(T to G)

The results of cluster analysis of the eight almond genotypes according to NCBI sequence alignment showed that they fold into two main groups. Group one included sequence six (Pirumara), sequence one (Barwari), sequence seven (Qirqrava), sequence four (Kora), and sequence eight (Deragzhnic). While the second group included sequence three (Bajlor), sequence two (Zakho,) and sequence five (Kemeka) as shown in (Fig. 7).

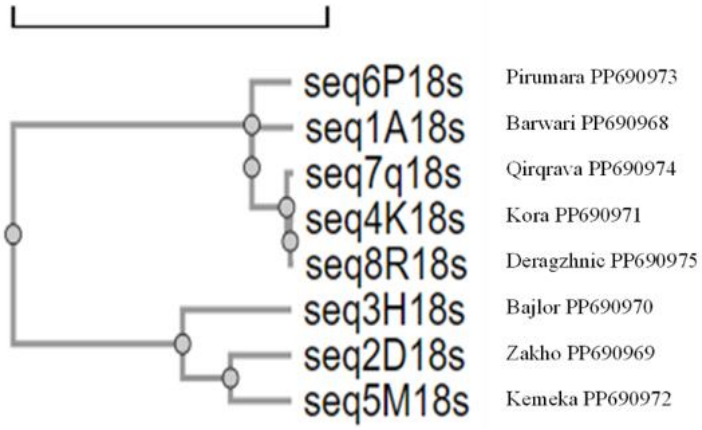

Fig. 7. SNPs clustering and similarity analysis of 18S-28S rRNA.

The 18S-28S rRNA has been proposed barcode and performed well for NGS metabarcoding of diatoms produce standard sequences using accurately recognized specimens, ideally type specimens, as a significant percentage of sequences deposited in GenBank were mislabelled (Liu *et al*., 2020).

The overall results revealed a significant level of genetic variety within the eight genotypes, indicating their future breeding and enhancement potential, the successful use of the ITS region and 18s-28s rRNA markers for the identification and classification of almond genotypes in this study highlights the usefulness of these markers in genetic analysis of plants. The high variability of the ITS region and 18s-28s rRNA makes it a valuable tool for differentiating closely related genotypes, phylogenetic analysis and identifying the evolutionary relationships between genotypes (Sinjare *et al*., 2023).

Conclusion

In this study, the ITS region and the 18S-28S rRNA marker have demonstrated their potential application as DNA barcode tools for genotype identification and characterization in the almond population in this region. These methods could make it easier to create enhanced types of almonds and guarantee the authenticity and quality of almond products available for the market.

Declarations:

Ethical Approval: Not applicable

Conflict of interests: The authors declare no conflict of interest.

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Availability of Data and Materials: The data underpinning the findings of this study are accessible upon request from the corresponding author.

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