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DNA Barcoding of Pomegranate (*Punica granatum* L.) Cultivars in Duhok Province- Kurdistan Region/ Iraq Using 18S–28S rRNA and ITS Region

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RESEARCH ARTICLE

Abstract

Pomegranate is a tree species with a significant plant diversity, therefore molecular methods are necessary to define and verify approaches to recognize rapidly and correctly. This research looks at a genetic strategy for identifying pomegranate varieties in Duhok KRG. The approach is based on application of ITS region, PCR-RFLP, sequencing and SNP identification. For this study, 14 pomegranate accessions were taken from various regions, namely the Center of Duhok, Amedi, Akre, Zaxo, the South of Duhok, and Sulav. The PCR product of the 18S–28S rDNA intergenic spacer was 854bp, and the sequence analysis revealed a 99.94 percent similarity with other accession numbers in NCBI, demonstrating the use of the 18S–28S rDNA intergenic spacer for identifying and barcoding pomegranate cultivars. The PCR product of the ITS region was 700bp. This result was then employed for PCR-RFLP using two restriction enzymes namely RsaI GT/AC and HaeIII GG/CC which helped grouping as well as genetic similarities. This study Further involved sequencing examined genes were compared using the NCBI BLASTn tool and clustalo (Version 1.2.4) to determine gene location and SNP. According to this study, the result of PCR-RFLP revealed poor association between pomegranate physical morphology and genetic features, while SNP identification was identified between studied cultivars. Moreover, this result showed possible DNA barcoding of pomegranate cultivars under the study.

Keywords: Pomegranate; DNA barcoding; 18S–28S rDNA; ITS region; PCR- RFLP; Sequence and SNP

INTRODUCTION

Pomegranate (*Punica granatum* L.) is an economically important fruit crop grown globally for its delectable fruits and health advantages.Yet, due to their significant physical and genetic variety, pomegranate identification is challenging (Holland et al., 2019). Molecular markers have been utilized in pomegranate and many other plants to study genetic diversity and cultivar identification. These markers enable study on the genetic makeup of individual plants and compare them to plants in the same population or variety (Hussein & Jubrael 2021). One of the most commonly used types of molecular markers for pomegranate research is simple sequence repeats (SSRs), which are short, repetitive DNA sequences found throughout the genome (Sinjare and Jubrael 2022; Hasnaoui et al., 2011). Other types of markers, such as single nucleotide polymorphisms (SNPs) and amplified fragment length polymorphisms (AFLPs), have also been used in pomegranate research (Sinjare and Jubrael 2020). In addition to cultivar identification, molecular markers have been used to study genetic diversity within pomegranate populations. This information can be used to develop conservation strategies and to identify plants with unique genetic traits that may be useful for breeding programs. DNA

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barcoding, a molecular identification tool based on short DNA sequences, has emerged as an effective method to distinguish different plant species and cultivars (Dong et al., 2015; Wang et al.,2015). The internal transcribed spacer (ITS) region and 18S-28S rRNA gene (Figure 1) are two commonly used DNA barcodes for plants. The ITS region is a non-coding DNA sequence located between the 18S and 5.8S rRNA genes and is highly variable among species and cultivars, making it an ideal marker for inter and intra species identification and genetic diversity analysis (Chen et al., 2010; Tripathi et al., 2013). The 18S-28S rRNA gene, on the other hand, is a highly conserved coding sequence that has been used for phylogenetic analysis and taxonomic classification (Álvarez and Wendel, 2003).

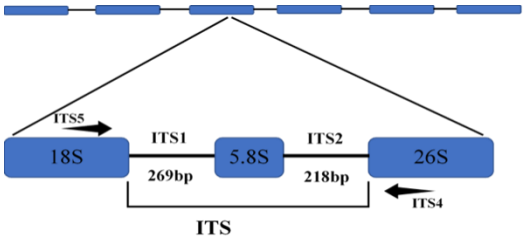

Figure 1. The Internal transcribed spacer (ITS) sequences diagram (Zhang et al., 2015)

PCR-RFLP is a PCR-based technique that combines PCR amplification of target DNA regions and restriction enzyme digestion of PCR products to produce unique DNA fragment patterns. PCR-RFLP also has been widely used for cultivar identification and genetic diversity analysis in pomegranate (Gharbi et al., 2017). Using specific restriction enzymes, PCR-RFLP can identify single nucleotide polymorphisms (SNPs) within the amplified DNA fragments and provide valuable genetic information for cultivar identification and breeding programs. For the genetic characterization of pomegranate cultivars, PCR-RFLP analysis along with DNA barcoding utilizing the ITS region and 18s-28s rRNA is a powerful method. This technique may be applied to the investigation of genetic diversity and the identification of SNPs within cultivars, as well as the identification and categorization of cultivars (Riaz et al., 2019).

The primary goals of this work were to analyze DNA barcoding utilizing the 18S–28S rDNA intergenic spacer and ITS region, and apply PCR-RFLP to pomegranate in Duhok province- Kurdistan region/ Iraq.

MATERIALS AND METHODS

Collecting Plant Samples

Leaf samples collected from 14 *Punica granatum* (pomegranate) cultivars used in this study were collected from different region of Duhok (Table 1; Figure 2) and brought to the laboratory and prepared for genomic DNA isolation. The reasons for selecting those cultivars from the mentioned areas were due to their specificity, local preferences and their large cultivation. In addition, to the fact that genetic diversity was sought in this work, the ecological patterns in this region are mountainous, in most of Kurdistan, the climate is semi-arid continental: very hot and dry in summer, and cold and wet in winter with an average tempreture of 2- 45 degrees Celsius. The main difference between these areas is the high altitude.

Table 1. Cultivars used in this study

no.	Cultivar name	Area of collection
1	Soormiz	Amedi
2	Rash dindik heer	Amedi
3	Radisho	Duhok
4	zeena var. nana	Duhok
5	Rawa	Duhok
6	Sin aljamel	South of Duhok
7	Slami	South of Duhok
8	Kapepan	Akre
9	Arbeli 1	Sulav- Bebade
10	Babe haji	Sulav- Bebade
11	Mrad	Duhok - Bu2
12	Armesht	Zaxo - Bu5
13	var. nana 1	Duhok - Am(U)
14	var. nana 2	Duhok - Mo (V)

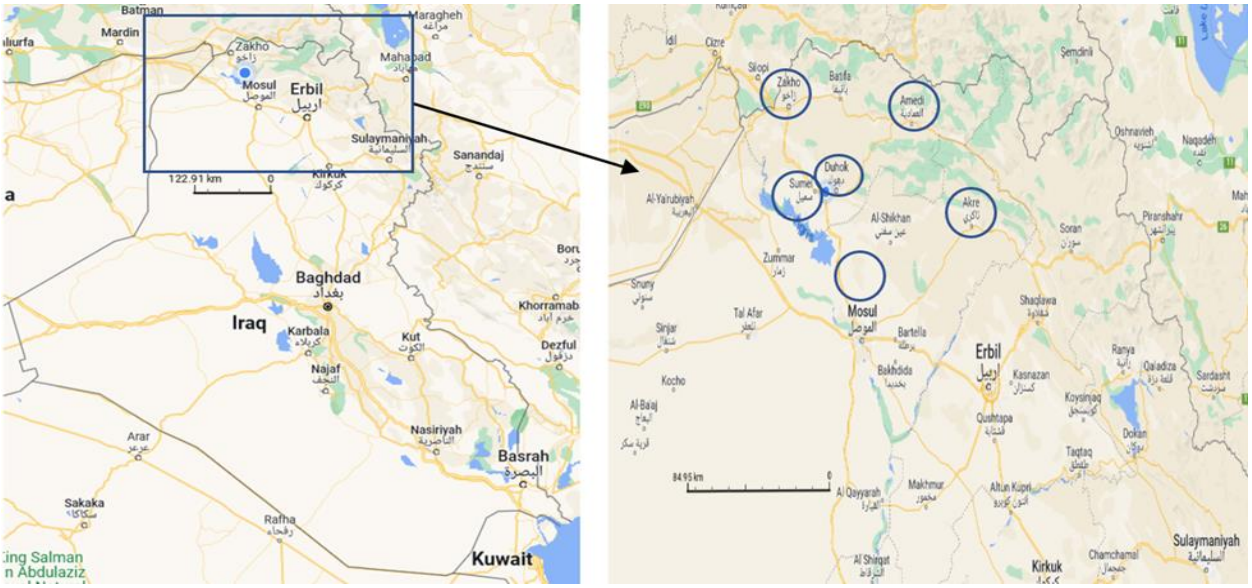

Figure 2. Geographic locations of the different pomegranate populations in the Duhok province

Genomic DNA isolation and PCR amplification:

The genomic DNA was extracted using the CTAB methods described by Weigand et al., (1993), with minor changes by Jubrael et al. (2005) and, Hussein & Jubrael (2021).

For amplification of both region using two set of primers (Table 2), A 20µl PCR reaction mixture was prepared containing 2 µl of DNA (25-50 ng), 2 µl (10 pmol) of each forward and reverse primers and 10 µl of (Go Taq G2 green) (Promega, USA) along with 4 µl of double deionized sterile water. The following settings were used to conduct the PCR amplification in an ABI Applied Biosystems PCR System 2720 thermal cycler. For ITS region the optimized thermocycler conditions for the reaction were 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. While 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 primers used in this study

primer name	sequence of Forward primer	sequence of Reverse primer	amplicon size bp.
18S–28S rDNA intergenic spacer	5'TTGTACACACCGCCCGTCGC3'	5'AGTTTCTTTTCCTCCGCTTA3'	854
ITS region	5'TCCTCCGCTTATTGATATGC3'	5'TCCGTAGGTGAACCTGCGG3'	700

PCR-Restriction Fragment Length Polymorphism (PCR- RFLP)

All PCR product of ITS region (gene) were further used for PCR- RFLP analyses. Enzymes that generated smaller and lower-molecular-weight fragments were chosen. Using these criteria, the enzymes RsaI GT/AC and HaeIII GG/CC (Jena bioscience, Germany) were selected to digest the amplified 700bp amplicon. Individual digestion conditions were performed in a final volume of 50 µl, as described by the manufacturer, 5µl of 10x universal buffer, 1µg of PCR product, 10 units of enzyme then the reaction was made up to 50µl with PCR grade water, the mixture then incubate at 37 ^{0}C for 2 to 4 hours. Then the product of digested results was analyzed using 3% agarose gel electrophoresis.

Capillary Standard Sequencing

The PCR products were sent to Macrogen Inc (Seoul, South Korea http:// dna.macrogen.com) for bi-directional Sanger sequencing using the same primers used for PCR. The sequencing was conducted for both PCR results of ITS gene and 18s – 28s nuclear DNA, Following the manufacturer's instructions, the illustra GFX PCR DNA and Gel Band Purification Kit from (GE healthcare) was used to purify the PCR amplification product. The purified product was

submitted to Macrogen, a public biotechnology company in South Korea, for sanger sequencing per the business's instructions. Using Big Dye chemistry in conjunction with capillary electrophoresis on an ABI 3730xl.

Data analysis

Based on the electrophoresis results, DNA fragments were scored as present (1) or absent (0) in allele size. Genetic diversity was analyzed based on these scores. To verify the potential of the selected markers and discriminate them among the cultivars analyzed, as well as evaluate genetic diversity, various parameters were measured.

POPGENE 4.2 software (Rousset, 2008) and CERVUS v.3.0.3 software 2006 (Marshal et al., 1998) were used to calculate the number of observed alleles per locus (Na), major allele frequency, observed heterozygosity (Ho), and expected heterozygosity (He). To evaluate the informativeness of the polymorphism information content (PIC) was calculated based on the allele frequencies of all the varieties analyzed (Weir, 1990). The NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) software version 2.1. (Rohlf, 1998) was used for clustering and construction of dendrogram. The data were analyzed using the Similarity for Qualitative Data routine to generate genetic similarity index (Nei and Li, 1979) with the unweighted pair-group method with arithmetic mean (UPGMA).

Sequencing data analysis (Phylogenetic analysis)

The assembly and analysis of sequencing data was conducted using BioEdit version 7.0.5.3 (Hall, T.A. 1999. Nucl. Acids. Symp. 41:95-98.). BioEdit: a user-friendly biological sequence alignment editor and analysis application for Windows 95/98/NT. 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) was used to align the sequenced segments of the gene with comparable sequences and additional sequences of other species within the same genus. The Neighbor-Joining (NJ) statistical approach was used to undertake phylogenetic tree analysis.

RESULTS AND DISCUSSIONS

In this study, 14 pomegranate cultivars were identified and categorized using the internal transcribed spacer (ITS) region and 18s-28s rRNA markers. The 18s-28s rRNA showed less variability than the ITS region, which was effectively amplified and sequenced for the studied cultivars. DNA barcoding has been used widely in plant biology to resolve phylogenies of related taxa. DNA barcoding has been suggested as the method of choice for species differentiation purposes of pomegranate.

The 18S–28S rDNA intergenic spacer showing (854 bp) Figure 3, while for ITS region was invariant in length for pomegranate genotypes (700 bp) Figure 4.

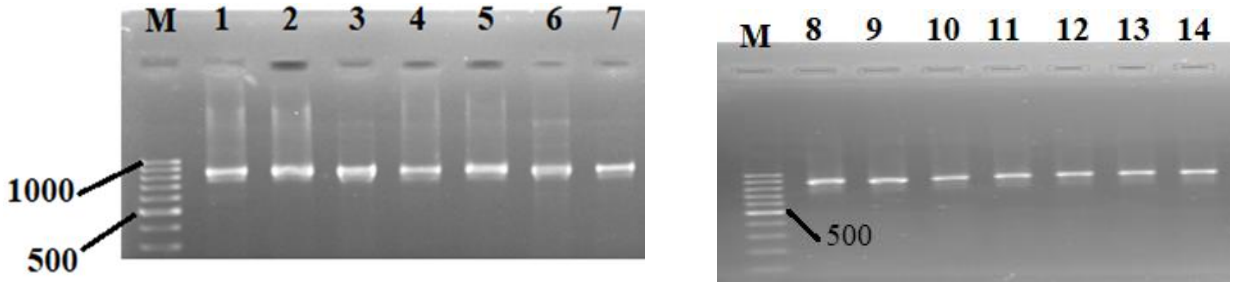

Figure 3. The18S–28S rDNA intergenic spacer for 14 samples of pomegranate

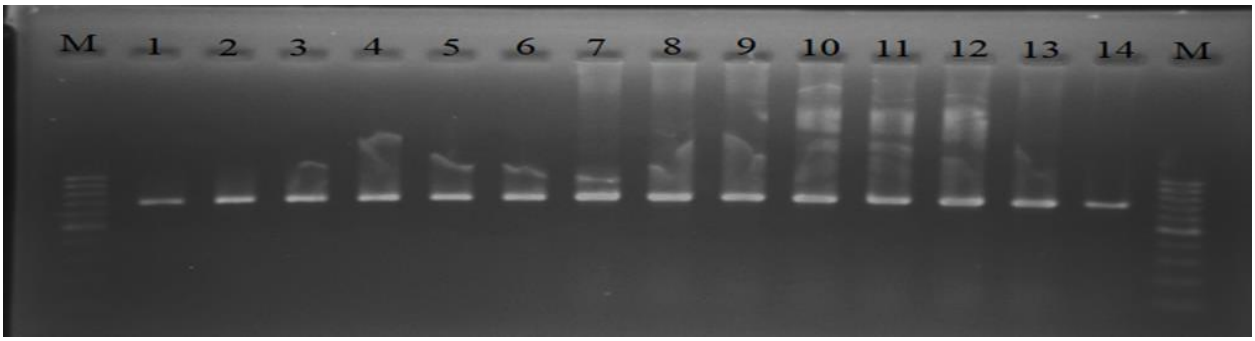

Figure 4. ITS region of 14 samples of pomegranate

The results of restriction enzymes digestion using Rsa I and Hae III to digest 700bp fragment of ITS region obtained across all cultivars of pomegranate are shown in Figure 5 and 6, and Table 3.
The PCR-RFLP profiles revealed different banding patterns, all samples produced relatively the same banding pattern except cultivar no. 9, 10, and 12. This result could therefore identify polymorphism between studied samples. Meanwhile the most informative results were obtained using Rsa I enzyme which revealed clear and reproducible DNA bands ranging between 440bp to 135bp across tested cultivars.

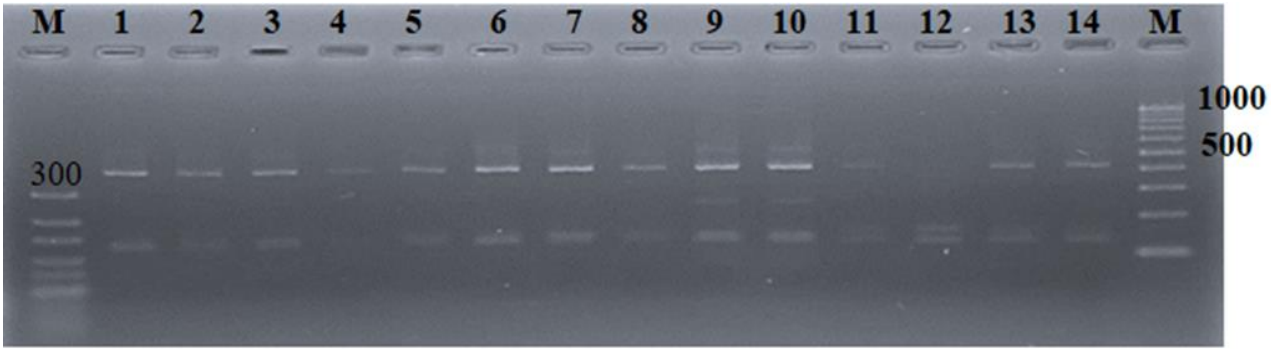

Figure 5. Rsal digestion for 14 pomegranate RFLP result with ITS region pcr product

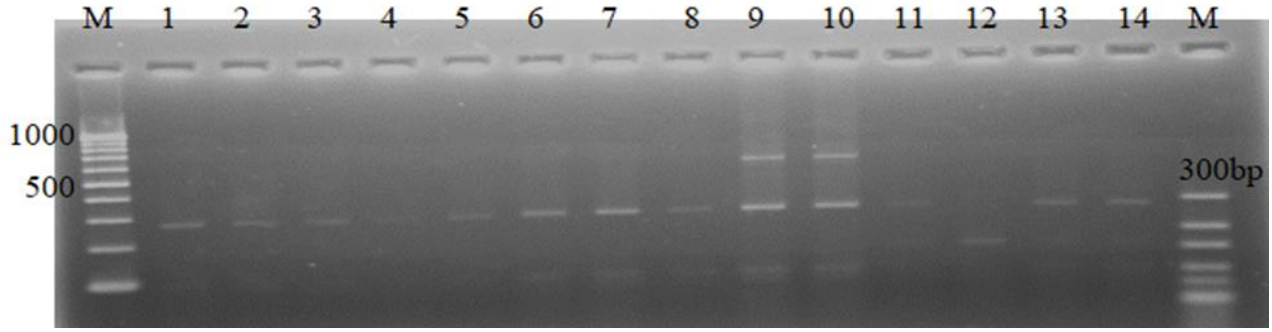

Figure 6. HaeIII digestion for 14 pomegranate RFLP result with ITS region pcr product

Table 3. fragment result from both restriction enzyme RsaI and HaeIII

sample no.	RsaI	HaeIII
1	440, 135	280
2	440, 135	280
3	440, 135	280
4	440	280
5	440, 135	280
6	440, 135	280
7	440, 135	280
8	440, 135	280
9	440, 300, 135	600, 280
10	440, 300, 135	600, 280
11	440, 190, 135	280, 170
12	190, 135	170
13	440, 135	280
14	440, 135	280

The PCR-RFLP results obtained in this study were used to construct a dendrogram, which demonstrated the distinction between most of pomegranate samples. The genetic similarity coefficient ranged from 0.45 to 1.00 similarity, the dendrogram revealed (4) clusters according to the statistical analysis performed using version 2.02

of NTSYS software. The most distinct cluster was cluster 1 which involved most of the samples as 1,2,3,5,13 and 14 showing 100% similarity. This cluster also contained sample no.4 but with similarity of 89%. While cluster 2 involve cultivar 6, 7, 8 and 11 showing similarity of 100% and 77% respectively. Meanwhile, clusters 3 and 4 involved cultivar 9, 10 and 12 showing low similarity with other clusters reveal 55% and 44% respectively as shown in (Figure 7 and Table 4).

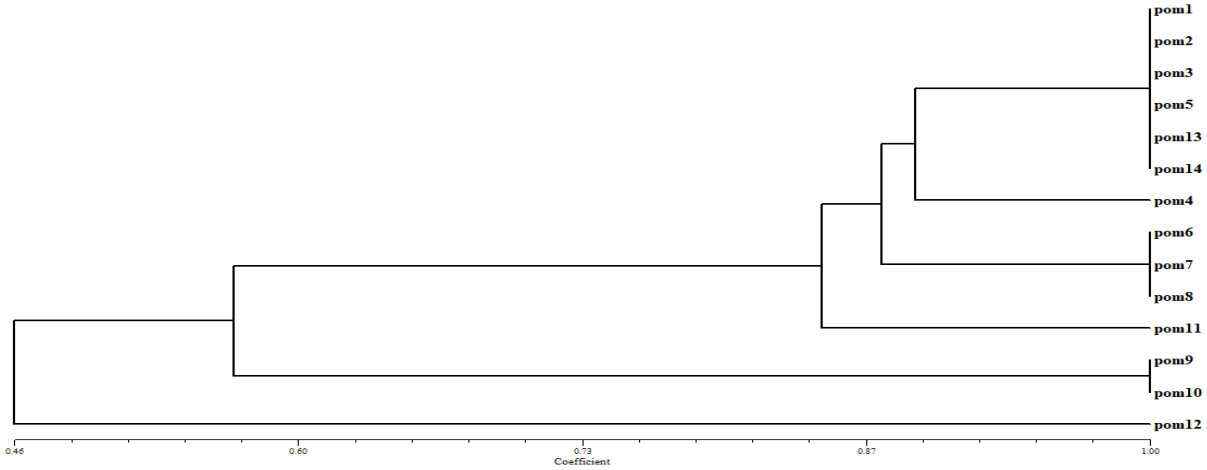

Figure 7. Clustering data of pomegranate cultivars

Table 4. Genetic similarity among pomegranate cultivars

1.0000000												
1.0000000	1.0000000											
1.0000000	1.0000000	1.0000000										
0.8888889	0.8888889	0.8888889	1.0000000									
1.0000000	1.0000000	1.0000000	0.8888889	1.0000000								
0.8888889	0.8888889	0.8888889	0.7777778	0.8888889	1.0000000							
0.8888889	0.8888889	0.8888889	0.7777778	0.8888889	1.0000000	1.0000000						
0.8888889	0.8888889	0.8888889	0.7777778	0.8888889	1.0000000	1.0000000	1.0000000					
0.5555556	0.5555556	0.5555556	0.4444444	0.5555556	0.6666667	0.6666667	0.6666667	1.0000000				
0.5555556	0.5555556	0.5555556	0.4444444	0.5555556	0.6666667	0.6666667	0.6666667	1.0000000	1.0000000			
0.8888889	0.8888889	0.8888889	0.7777778	0.8888889	0.7777778	0.7777778	0.7777778	0.4444444	0.4444444	1.0000000		
0.5555556	0.5555556	0.5555556	0.4444444	0.5555556	0.4444444	0.4444444	0.4444444	0.1111111	0.1111111	0.6666667	1.0000000	
1.0000000	1.0000000	1.0000000	0.8888889	1.0000000	0.8888889	0.8888889	0.8888889	0.5555556	0.5555556	0.8888889	0.5555556	1.0000000
1.0000000	1.0000000	1.0000000	0.8888889	1.0000000	0.8888889	0.8888889	0.8888889	0.5555556	0.5555556	0.8888889	0.5555556	1.0000000	1.0000000

The mean observed (Ho) and expected (He) heterozygosity frequencies was 0.607 and 0.68, respectively. Given that (He) at a locus increases with increasing (Na) and equalizing allele frequencies, the criteria for low (He) and low (Na) were met for most loci (Alamuti et al., 2012). The PIC value ranged from 0.286 to 0.510 with an average of 0.398 as shown in Table 5.

Table 5. Data analysis of genetic diversity using popgen and CERVUS program

Loci (Restriction enzyme)	Polymorphism information content (PIC)	Allele no. (Na)	Observed heterozygosity (Ho)	P- value	Expected heterozygosity (He)
HaeIII	0.286	3	0.214	0.041	0.320
RsaI	0.510	4	1.000	0.461	0.616
Mean	0.398	3.5	0.60	0.251	0.468

To assess the heterogeneity in length and/or in nucleotide sequence within the ITS regions in pomegranate, the ITS regions and 18S-28S rRNA were successfully amplified and aligned with reference sequences MT007597.1 of complete pomegranate ITS regions in NCBI data base. The result showed 100% query cover and 99.95% identity of homogenous in length and structure. The full length of ITS1-5.8S-ITS2 region reveal a number of SNP with reference data in the NCBI data base apparently showing base substitution upon direct sequencing using ITS-1 and ITS-4 universal primers in three cultivars.

Upon multiple sequence alignment of submitted ITS regions of pomegranate sequences with accession number

(OQ704339.1, OQ704338.1, OQ704337.1), it could detect SNPs at number of sites within cultivar Zina however other cultivar showed high similarity with reference used for alignment (Figure 8 and 9).

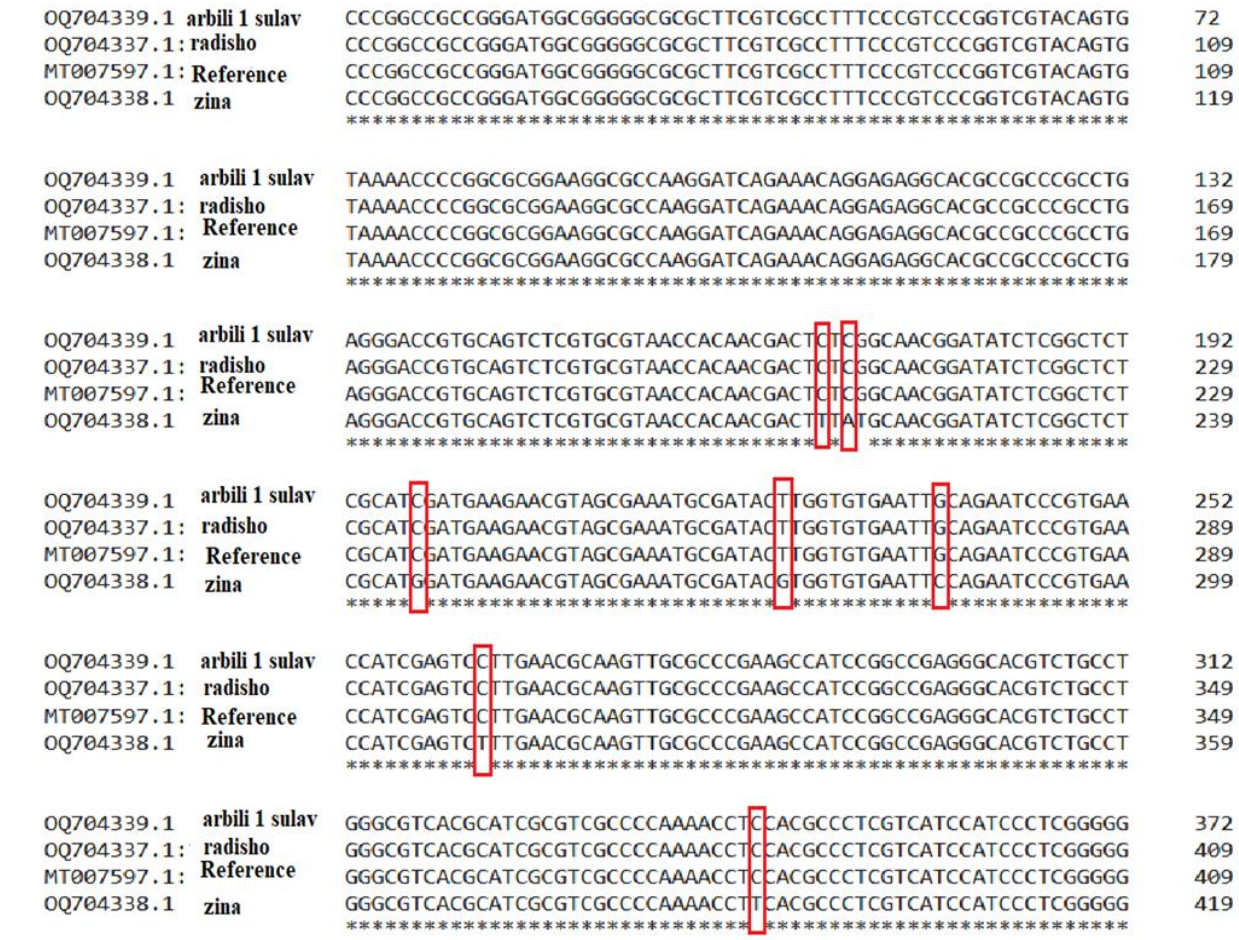

Figure 8. showing SNPs within used pomegranate cultivars and reference using ITS region.

Figure 9. SNPs clustering and similarity analysis of ITS region

Further the 18S-28S rRNA PCR product were aligned with reference sequences JX911348.1 showing 99% query cover and 99.58 identity, these sequences were deposited and published in the GenBank database with the accession numbers (OQ720931.1, OQ720932.1). The SNP also reveals with this region which exhibited INDELS at two places and SNPs at two places in term of replacement a single nucleotide as shown in (Figure 10 and 11).

The ITS region was shown to be significantly varied among the cultivars. In addition to the successful amplification and sequencing of the ITS region, the study also used the 700 bp PCR product of both universal primer ITS1/ ITS4 of the ITS region for PCR-RFLP analysis, using HaeIII and RsAI restriction enzymes. As a result of the research, each cultivar was shown to have a unique pattern of restriction fragments that allowed for reliable identification and differentiation.

This pattern related with sequence alignment and indicated the existence of a number of SNPs at the restriction sites and either within studied cultivars. This result is reliable with previous studies that used PCR-RFLP to identify pomegranate cultivars (Gharbi et al., 2017; Hajimehdipoor et al., 2015).

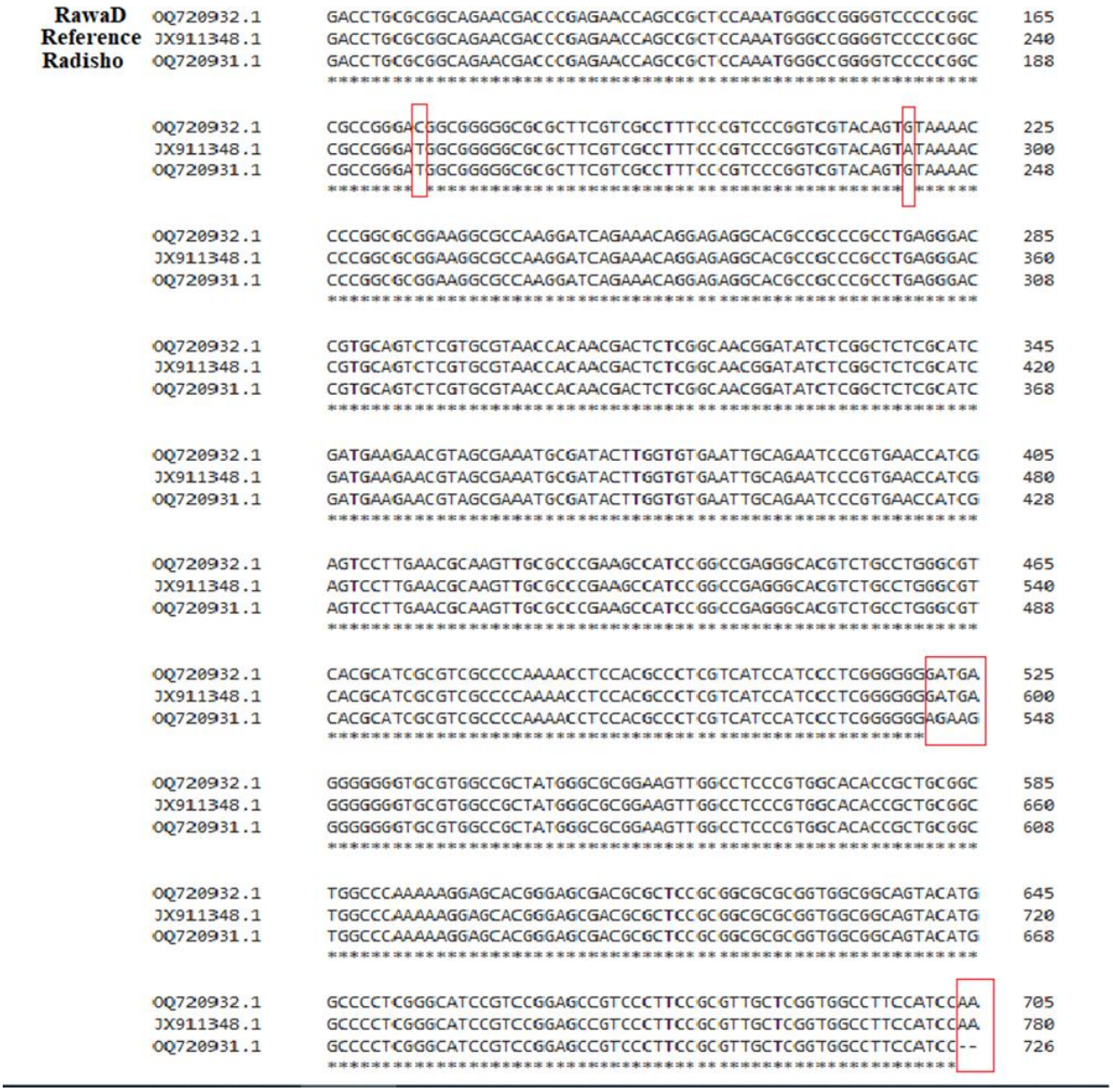

Figure 10. showing SNPs within used pomegranate cultivars and reference using 18S-28S rRNA.

Figure 11. SNPs clustering and similarity analysis of 18S-28S rRNA

For plant breeding, conservation, and production, cultivar identification and categorization are crucial. One of the most used nuclear markers for phylogenetic reconstructions at the species level or lower is the nuclear ribosomal internal transcribed spacer region or a portion of it. Recently, the ITS region has been promoted as a possible barcode for animals, protists, and potentially even plants (Moniz & Kaczmarska 2010; Yao et al., 2010; Li

et al., 2011; Schoch et al., 2012). The broad availability of reference sequence libraries is essential for the successful application of DNA barcoding in species identification.

The use of DNA barcoding and PCR-RFLP in pomegranate cultivar identification and genetic diversity analysis has important implications for breeding programs and quality control in the pomegranate industry. The ability to accurately identify and distinguish cultivars can facilitate the development of improved varieties and ensure the authenticity and quality of pomegranate products in the market (Martinez et al., 2006). The ITS length variants and polymorphism have been reported in several plant species (Mir et al., 2010; Raturi et al., 2012). Saini et al., 2008 reported heterogeneity in nuclear rDNA ITS region in Vigna radiata which did not cause any phylogenetic errors at species level. Based on both markers, phylogenetic analysis revealed a clear division of the cultivars into four primary clusters, with certain sub clusters suggesting genetic relatedness between particular cultivars. The results also revealed a significant level of genetic variety within the cultivars, 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 pomegranate cultivars in this study highlights the usefulness of these markers in genetic analysis of plants. The high variability of the ITS region makes it a valuable tool for differentiating closely related cultivars, while the less variable 18s-28s rRNA can be used for phylogenetic analysis and identifying the evolutionary relationships between cultivars.

CONCLUSION

In conclusion, the use of ITS region and 18S-28S rRNA gene as DNA barcodes and PCR-RFLP as a genetic diversity analysis tool have shown great potential for identifying and characterizing pomegranate cultivars. These techniques could facilitate the development of improved pomegranate varieties and ensure the authenticity and quality of pomegranate products in the market.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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