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GENETIC DIVERSITY AND PHYLOGENETIC ANALYSIS OF REGIONAL POMEGRANATE (*PUNICA GRANATUM* L.) GENOTYPES FROM SOUTHEASTERN TÜRKIYE USING ISSR MARKERS AND *TRNL–F* CHLOROPLAST DNA SEQUENCES

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Abstract

Pomegranate (*Punica granatum* L.) is a significant fruit crop that has high nutritional, economic and genetic values. The genetic relationships among 24 pomegranate accessions (18 local genotypes from Mardin, 5 registered cultivars and 1 Siirt Seedless genotype) were analyzed using chloroplast trnL-F sequence and ISSR markers. The fragments of the trnL-F region were amplified and the fragments were around 450 bp. All accessions were clustered together in the Neighbor-Joining analysis, except genotype 15 (accession 47 N 15), which was on a separate branch. Maximum likelihood analysis gave higher resolution, and yielded three large clusters with genotype 15 appearing separately. Initially, a total of 23 ISSR primers were screened, and six of them were selected because they produced clear, reproducible, and polymorphic band patterns. A total of 30 amplified bands were generated by these primers, with an overall polymorphism level of 60%, which included 18 polymorphic bands. Polymorphism rates ranged from 33% to 83%, and PIC values varied between 0.13 and 0.37, with the highest values obtained from primer UBC845. UPGMA clustering and PCoA revealed genetic differentiation among the ac-cessions, with genotype 1 (accession 47 N 01) showing the most distinct position in the ISSR dendrogram. Overall, ISSR markers provided higher discriminatory power than the chloroplast trnL-F region. The results indicate that southeastern Turkish pomegranate germplasm contains detectable genetic diversity and includes divergent accessions that may be useful for future conservation and breeding studies.

Key words: Germplasm characterization; Molecular markers; Genetic resources; UPGMA; Principal coordinate analysis Cultivar differentiation

Introduction

Pomegranate (*Punica granatum* L.) belongs to the family Lythraceae and the order Myrtales. The genus Punica includes two species, *P. protopunica* and *P. granatum* (Mars & Marrakchi, 1999), of which the latter is the principal cultivated species (Onur, 1988). Pomegranate is a deciduous or semi-deciduous shrub or small tree whose fruits contain numerous fleshy arils. The fruit is consumed fresh or processed into products such as juice, jam, and pomegranate molasses. It is a valuable source of vitamins, minerals, polyphenols, and other bioactive compounds (Gil *et al*., 2000; Ebcioğlu, 2003; Şimşek & İkinci, 2017). These compounds contribute to its nutritional and commercial importance and have attracted interest because of their reported antioxidant and other health-related properties (Singh *et al*., 2023; Valero-Mendoza *et al*., 2023).According to popular belief, the pomegranate, i.e., a species that can grow both in tropical and subtropical climatic zones, originated from Central Asia, particularly the Transcaucasian-Caspian region of Iran, and spread from there to the rest of the world (Levin, 1994; Verma *et al*., 2010). While it is evergreen in tropical climates, it tends to shed its leaves partially or completely in winter in subtropical climates. Its high adaptability to adverse conditions and especially its drought resistance make it an excellent tree for cultivation in arid regions, as well.

Pomegranate is widely cultivated in Mediterranean and Asian countries under warm, dry climatic conditions and across diverse soil types (Ismail *et al*., 2014; Şimşek, 2017). Türkiye is among the major pomegranate-producing countries, with cultivation concentrated mainly in the Mediterranean, Aegean, and Southeastern Anatolia regions (Dursun *et al*., 2019). Breeding perennial fruit species is a lengthy process because of their long generation times. Molecular markers can be used to help characterize genetic variation and to select breeding material, which is genetically different (Yıldırım & Kandemir, 2001). Random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs), amplified fragment length polymorphisms (AFLPs), and sequence-related amplified polymorphisms (SRAPs) are some of the different marker systems used to estimate genetic diversity and relationships among and within plant populations (Reddy *et al*., 2002; Andersen & Lübberstedt, 2003; Kaçar, 2004; Vardar-Kanlıtepe *et al*., 2010). Sequences of chloroplast DNA (cpDNA) are also common in plant systematics (Hao *et al*., 2010). Regions of the chloroplast genome, including rbcL, trnL and trnL-F, can be useful for studies of the phylogenetic relationships, and geographic origins, of germplasm of plants (Shaw *et al*., 2007). The chloroplast trnL–trnF region includes the trnL intron and the intergenic spacer located between the 3′ exon

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of trnL and the trnF gene. The universal e and f primers specifically amplify the trnL-trnF intergenic spacer and have facilitated the application of this region in plant systematics and germplasm characterization (Taberlet *et al*., 1991). Because non-coding chloroplast regions generally accumulate more sequence variation than coding plastid genes, trnL-trnF can provide useful information about phylogenetic relationships. Nevertheless, its relatively conserved nature may provide limited discrimination among closely related genotypes or cultivars (Shaw *et al*., 2007).

Inter-simple sequence repeat (ISSR) markers amplify anonymous nuclear DNA regions located between adjacent microsatellite repeats using repeat-anchored primers; therefore, prior genomic sequence information is not required (Zietkiewicz *et al*., 1994; Reddy *et al*., 2002). ISSR analysis produces multilocus fingerprints and is relatively simple, cost-effective, and capable of detecting polymorphism, making it useful for germplasm characterization and genetic-diversity studies. However, ISSRs are dominant markers, meaning that the presence of a band cannot distinguish a heterozygous genotype from a dominant homozygous genotype. Their reproducibility and interpretation also depend on consistent PCR conditions and careful band scoring (Reddy *et al*., 2002).

The genetic diversity described by chloroplast trnL–trnF sequences and ISSR markers are complementary. The primers used in this region are universal and can be used to amplify DNA from a variety of plant taxa; the region is relatively conserved and could be used to reliably distinguish closely related genotypes, but was selected because it has been broadly employed in plant-systematics studies and because it can be compared directly with previous investigations of the molecular diversity of the Turkish germplasm of pomegranate. Furthermore, trnL–trnF is a generally uniparentally inherited plastid region that offers information on maternal-lineage relationships which cannot be derived from multilocus nuclear ISSR profiles. The ISSRs, on the other hand, are able to sample several areas of the recombining nuclear genome and can detect more intraspecific variation. Also, Sevindik & Efe, (2021) observed that higher polymorphisms were detected using ISSR markers when compared to trnL–trnF sequences in Turkish populations of pomegranate. Thus, both the marker systems were integrated to give a wider preliminary evaluation of the regional germplasm of pomegranate than an individual one could do.

Although several studies have investigated the genetic diversity of Turkish pomegranate germplasm, regional accessions from Mardin and neighboring areas of southeastern Türkiye remain insufficiently characterized at the molecular level. Moreover, relatively few studies have jointly employed chloroplast sequence data and multilocus nuclear markers to compare maternal-lineage variation with broader nuclear genetic diversity in this regional germplasm. Therefore, the present study combined chloroplast trnL–trnF sequence analysis with ISSR markers to characterize genetic diversity and relationships among 24 pomegranate accessions.

Although several studies on diversity have been conducted in Turkey on pomegranate using ISSR, SSR, ITS or chloroplast markers, germplasm from the city of Mardin is not adequately represented in national diversity studies based on molecular markers. In the present study, the use of nuclear ISSR variation was combined with chloroplast trnL–F sequence differentiation in the same regional collection. The aim of the study was to characterize the genetic diversity and relationships among the 18 local Mardin genotypes, Siirt Seedless genotype and five registered cultivars, and to identify some of the potentially divergent accessions for further evaluation for conservation and breeding purposes.

Materials and Methods

Plant material: Eighteen different genotypes, naturally growing in the region and collected from Artuklu (Kabala, Yardere neighborhoods and Ahmetli village) and Kızıltepe (Ayaz, Uluköy and Erdem villages) districts of Mardin province, along with five registered varieties named Zivzik, Esin, Yılmaz, Hicaz and Onur, and the genotype 'Siirt Seedless' from the Siirt region, were used as plant sources in this study (Fig. 1, Table 1). Leaf samples obtained from these were immersed in liquid nitrogen at -196°C, wrapped in aluminum foil, and stored at -80°C until used in the studies.

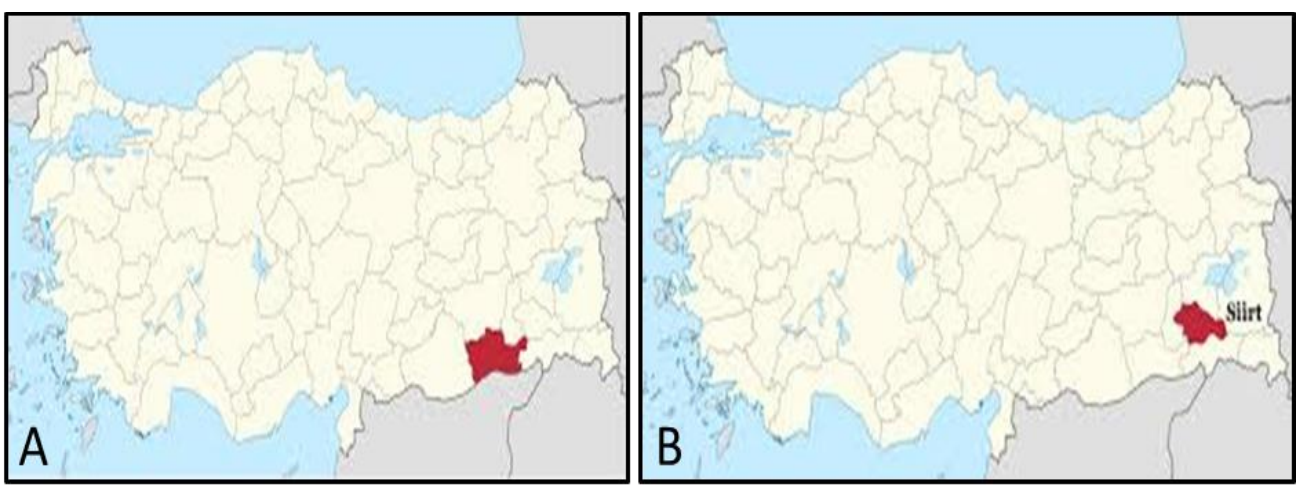

Fig. 1. Geographical locations of the pomegranate sampling areas in southeastern Türkiye. (A) Mardin Province, where 18 local genotypes were collected from; (B) Siirt Province, the source of the Siirt Seedless genotype. The highlighted areas indicate the locations of Mardin and Siirt provinces within Türkiye.

Table 1. Local pomegranate genotypes and registered cultivars used in the study, including their collection districts and sampling locations.

No.	Name of the sample	District	Neighborhood / Village
1.	47 N 01	Kızıltepe	Erdem
2.	47 N 02	Kızıltepe	Erdem
3.	47 N 03	Kızıltepe	Erdem
4.	47 N 04	Kızıltepe	Erdem
5.	47 N 05	Kızıltepe	Erdem
6.	47 N 06	Kızıltepe	Uluköy
7.	47 N 07	Kızıltepe	Ayaz
8.	47 N 08	Artuklu	Kabala
9.	47 N 09	Artuklu	Kabala
10.	47 N 10	Artuklu	Kabala
11.	47 N 11	Artuklu	Kabala
12.	47 N 12	Artuklu	Kabala
13.	47 N 13	Artuklu	Yardere
14.	47 N 14	Artuklu	Ahmetli
15.	47 N 15	Artuklu	Ahmetli
16.	47 N 16	Artuklu	Yardere
17.	47 N 17	Artuklu	Yardere
18.	47 N 18	Artuklu	Yardere
19.	Siirt Seedless	Siirt	Center
20.	cv. Zivzik	Siirt	Zivzik
21.	cv. Esin	Antalya	Center
22.	cv. Yılmaz	Antalya	Center
23.	cv. Hicaz	Antalya	Center
24.	cv. Onur	Antalya	Center

Genomic DNA Isolation: DNA isolation was performed using the CTAB (cetyltrimethylammonium bromide) method, adapted from Doyle and Doyle, (1987) and modified by Karaca *et al*., (2005). The resulting pellet was dissolved in 100 µl of TE buffer (pH: 8.0) and stored at +4 or -20ºC for later use.

PCR for chloroplast-specific markers: The chloroplast trnL–trnF intergenic spacer was amplified using the universal primers e and f developed by Taberlet *et al*., (1991). The PCR cycling protocol followed Altıntaş *et al*., (2019), with modifications to the reaction mixture. Amplification was performed in a total volume of 50 µL containing genomic DNA, Taq reaction buffer, $MgCl_2$, dNTPs, forward and reverse primers, bovine serum albumin, Taq DNA polymerase, and nuclease-free water. A no-template negative control, in which genomic DNA was replaced with nuclease-free water, was included in each PCR run to detect possible contamination and verify the reliability of the amplification results. The primer sequences were as follows:

Forward primer (trnL-e) 5′-GGTTCAAGTCCCTCTATCCC-3′;
Reverse primer (trnF-f): 5′-ATTTGAACTGGTGACACGAG-3′.

PCR was performed by denaturating the samples at 95°C for 7 min, followed by 35 cycles of three steps: (i) 1 min of denaturing at 94°C, (ii) 1 min of annealing at 52°C, and (iii) 2 min of elongation at 72°C. A final extension was performed at 72°C for 10 min, after which the reactions were held at 10°C until retrieval from the thermocycler.

Amplification products were separated on 1.5% agarose gels in 1× TAE buffer and visualized using ethidium bromide staining and a UV gel documentation system. Because ethidium bromide is a hazardous mutagen, all contaminated materials were handled and disposed of according to institutional laboratory-safety procedures.

The purified PCR products were sent to Hibrigen for Sanger sequencing in both the forward and reverse directions using the trnL-e and trnF-f primers, respectively. The forward and reverse chromatograms were analyzed and edited using BioEdit; subsequently, the complementary read data were merged to generate a consensus sequence for each accession number. The resulting consensus sequences were aligned using ClustalW prior to phylogenetic analysis to determine the kinship relationships of species whose sequences were aligned, a phylogenetic tree was constructed using the MEGA6 computer program. For this purpose, Neighbour-Joining (NJ) and Maximum-Likelihood distance-based methods were used. The trnL-F sequences obtained in this study have not been deposited in GenBank.

PCR for ISSR markers: A total of 23 ISSR primers were initially screened for amplification quality and polymorphism. Six primers-UBC807, UBC810, UBC820, UBC845, UBC847, and UBC850-were selected for the final analysis because they produced clear, reproducible, and polymorphic banding profiles across the examined pomegranate accessions. ISSR amplification reactions were performed in a total volume of 25 µL. PCR was performed as follows: (i) denaturation at 95°C for 3 min, (ii) 35 cycles of; (ii-a) denaturation at 95°C for 45 sec, (ii-b) annealing at 55°C for 1 min, (ii-c) elongation at 72°C for 45 sec, (iii) final extension at 72°C for 7 min, and (iv) being held at 4°C until used. Amplification products were resolved by electrophoresis on 2% agarose gels in 0.5% 1X TAE buffer and stained with 0.1% ethidium bromide at 90 V for 3 hours and visualized on an UV transilluminator.

ISSR analysis was carried out as follows: each band was scored as present (1) or absent (0) and the obtained data were analyzed using the PAST version 4.09 (Paleontological statistics software package for education and data analysis) computer package program developed by Hammer *et al*., (2001). Ambiguous bands and reactions showing unsuccessful amplification were recorded as missing data rather than as band absence and were excluded from the corresponding pairwise comparisons during the similarity analysis. Cluster analyses were performed using the UPGMA (Unweighted Pair Group Method Using Arithmetic Average) method with the similarity index, and dendrograms were obtained. The similarity matrix was calculated with the Mantel Matrix Correspondence Test (Mantel, 1967) and the cophenetic correlation coefficient (r) value was obtained. In this study, *Rotala indica* was used as the outgroup in all dendrograms.

AMOVA analysis: The molecular variance analysis (AMOVA) was conducted using the binary matrix of ISSR to analyze the genetic variations among and within the Kızıltepe and Artuklu sampling districts. The 18 local genotypes, seven genotypes from Kızıltepe and 11 from Artuklu were included in the analysis. The five cultivars that were registered and Siirt Seedless were not included because they were not comparable district-level population samples. If ISSR scores were missing, they were filled in with the mean score of the appropriate locus. 9,999 permutations were used to evaluate the significance of ΦPT in R software.

Results

Phylogenetic analysis based on the chloroplast trnL-F region: Amplification of the chloroplast trnL-F region produced PCR fragments of approximately 450 bp for all analyzed pomegranate genotypes. Both Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods were used to reconstruct the phylogenetic relationships among 24 pomegranate accessions, comprising 18 local genotypes from Mardin, the Siirt Seedless genotype, and five registered cultivars.All the analysed genotypes were clustered in a single major cluster except Genotype 15 (accession 47 N 15), which was separated from the other accessions (Fig. 2).

Accession genotype 15 (47 N 15) was placed in a single cluster, not among the other accessions, in the NJ and ML trees. The other accessions revealed different clustering patterns in the two analyses, with the NJ tree clustering them into one large group, and the ML tree forming several subsets. The difference in the topologies might be due to the limited sequence variation in the relatively conserved chloroplast trnL–trnF region, as well as to the different assumptions used in NJ and ML methods. Unfortunately, no bootstrap resampling was done, so the statistical support for internal branches and the separate placement of accession 47 N 15 could not be assessed. Thus, the pattern observed should be considered preliminary sequence affinities and should be confirmed with the help of bootstrap-supported analyses and other more informative chloroplast or nuclear markers (Fig. 3).

Genetic diversity analysis using ISSR markers: Genetic diversity among the analysed pomegranate germplasm was initially screened with 23 ISSR primers. These were chosen to give clear, reproducible, polymorphic amplification profiles: UBC807, UBC810, UBC820, UBC845, UBC847 and UBC850. The descriptive parameters of the informative primers such as total number of amplified bands, number of polymorphic bands, band size range, polymorphism percentage and polymorphism information content (PIC) are summarized in Table 2.

All six of the informative ISSR primers gave rise to 30 amplified bands, with the 18 polymorphic bands giving rise to a range of polymorphisms of from 33% to 83%. Amplified bands ranged in size from 300 to 1500 bp and the number of amplified bands per primer ranged between 3 and 7. The polymorphism rate for Primer UBC845 was 83% with the highest PIC value (0.37), while for UBC847 the polymorphism rate was 33%, and the lowest PIC value (0.13). PIC values were relatively low to moderate, which meant that markers were relatively informative. The result should be viewed with the understanding that ISSR markers are dominant so that the presence or absence of a band will score a locus as either the presence of the dominant band or the absence of the recessive band, resulting in lower resolution of genetic-diversity estimates.

Genetic relationships based on ISSR markers: UPGMA analysis of ISSR data showed genetic subclustering of 24 pomegranate accessions into distinct genetic groups (Fig. 4). The local genotypes and registered cultivars were not grouped in two distinct clusters, indicating a shared origin or historical exchange of propagation material, or genetic similarity between cultivated and locally-maintained germplasm in the same major clusters. Meanwhile, several local genes were present in relatively distinct positions, suggesting that the region germplasm could show genetic variation that is not adequately sampled by registered cultivars. Such divergency accessions can be utilized to expand the genetic base of the future germplasm of pomegranate breeding but their breeding value needs to be confirmed with complementary morphological, biochemical and agronomic evaluations.

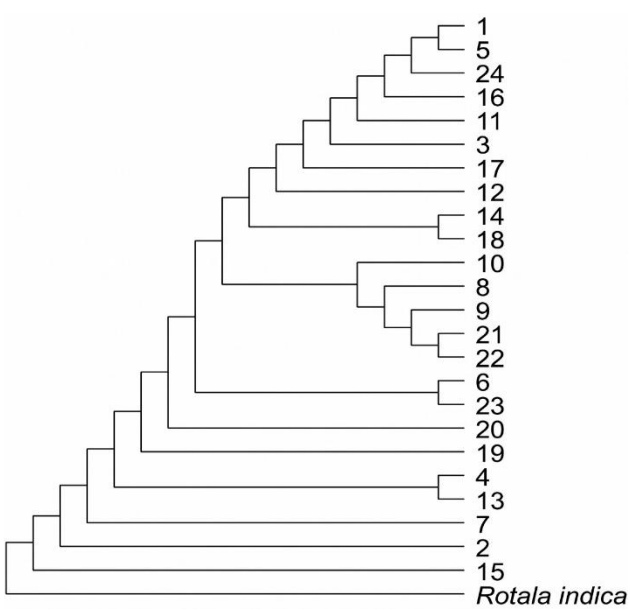

Fig. 2. Relatedness of 24 pomegranate accessions according to aligned chloroplast trnL-F sequences using neighbor-joining (NJ) phylogenetic tree. As an outgroup for rooting the tree, *Rotala indica* was added. The accession numbers are assigned to the pomegranate genotypes and registered cultivars in the Table 1.

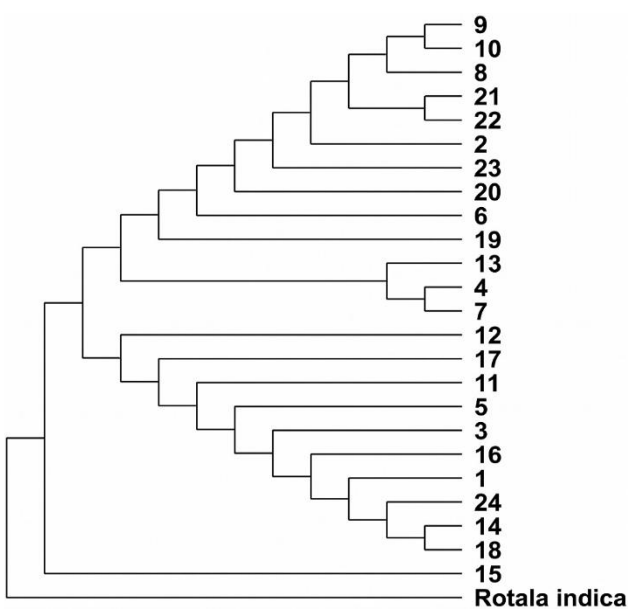

Fig. 3. Maximum Likelihood (ML) phylogenetic tree showing the relationships among 24 pomegranate accessions based on aligned chloroplast trnL-F sequences. *Rotala indica* was used as an outgroup to root the tree. The accession numbers are the same as the pomes listed in Table 1, with the exception of registered cultivars which are numbered as in the accession list.

Table 2. The total and polymorphic band numbers, amplified fragment-size ranges, polymorphism rates and polymorphism information content (PIC) values of the six informative ISSR primers used to evaluate the genetic diversity among the pomegranate accessions were presented.

Primer	Total no. of bands	No. of polymorphic bands	Length of band (bp)	Polymorphism rate (%)	PIC
UBC807	7	5	300-1050	71	0.30
UBC810	6	2	350-1100	33	0.17
UBC820	4	2	350-1100	50	0.19
UBC845	6	5	300-1250	83	0.37
UBC847	3	1	750-1000	33	0.13
UBC850	4	3	500-1500	75	0.20

Table 3. Analysis of molecular variance (AMOVA) of ISSR-based genetic variation among and within the Artuklu and Kızıltepe sampling districts.

Source of variation	df	Sum of squares	Mean square	Variance component	Variation (%)
Among districts	1	3.902	3.902	0.038	1.05
Within districts	16	57.253	3.578	3.578	98.95
Total	17	61.155	3.597	3.616	100.00

ΦPT = 0.010; p = 0.358, based on 9,999 permutations. df, degrees of freedom

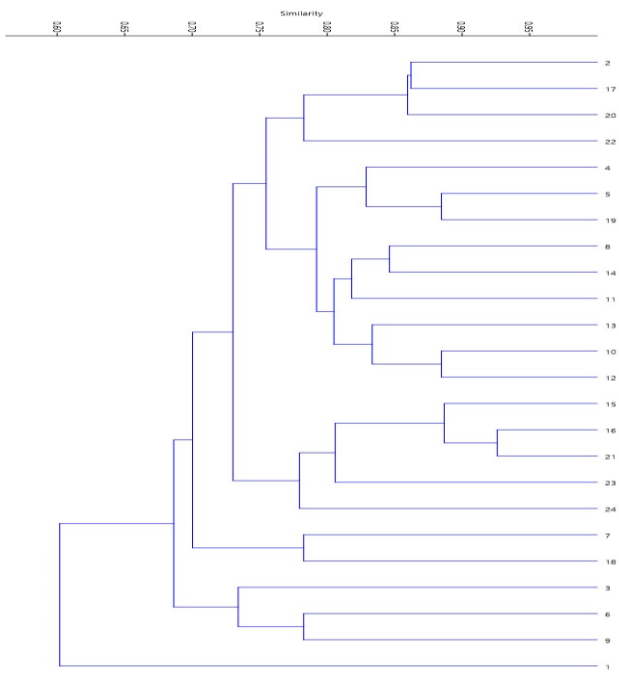

Fig. 4. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram showing the genetic relationships among 24 pomegranate accessions-18 local genotypes from Mardin Province, the Siirt Seedless genotype, and five registered cultivars-based on ISSR marker data.

In the dendrogram, several pairs or small group of genotypes had relatively high genetic similarity, such as genotype 5 and 19, 10 and 12, 16 and 21 and 7 and 18. Genotypes 1, 3, 6, and 9, on the other hand, were located on branches that were genetically more distant from the other genotypes. Typical of accessions, genotype 1 (accession 47 N 01) was the most distinct in the dendrogram and thus one of the most divergent accessions among the germplasm analyzed. Local genotypes and registered cultivars are intermingled within the same clusters, which is indicative of shared genetic background or propagation material exchange in the past. However, the somewhat distinct positions of some of the local genotypes suggest they may offer further genetic variation for expanding parental diversity in future breeding. The six accessions selected using the ISSR primers resulted in enough variation for initial discrimination among the accessions. However, ISSRs are dominant markers and cannot differentiate between heterozygous and dominant homozygotes and thus do not offer much information about allelic diversity. Therefore, the relationships and breeding implications identified should be confirmed with the help of codominant or genome-wide markers (SSRs or SNPs) in combination with phenotyping.

A two dimensional coordinate analysis was also carried out to support the hierarchical cluster analysis carried out with the ISSR data (Fig. 5). The results of the coordinate plot generally corroborated the relationships seen in the UPGMA dendrogram, with accessions genetically different from the remainder of the plot being separated from the group and genetically similar accessions being grouped together. A few genotypes, such as 1, 18, 23, 24 and 9, were away from the central area of the plot, indicating a higher level of genetic differentiation compared to the rest of the plot. Another point of comparison between the dendrogram and coordinate analysis confirms the reliability of using the ISSR marker system to assess genetic diversity among the genotypes analysed. The first two axes of PCoA accounted for 38.00% of the total variation, with 20.01% and 17.99% explained by Coordinate 1 and Coordinate 2, respectively.

It was found that Artuklu-Kızıltepe difference accounted for 1.05% of the total ISSR variation, while 98.95% of the total ISSR variation was within the sampling districts. There was little genetic difference between the two districts, and not significant (ΦPT = 0.010, p = 0.358; Table 3). The results showed that the two districts did not have a strong difference in the genotypes obtained according to the investigated ISSR markers.

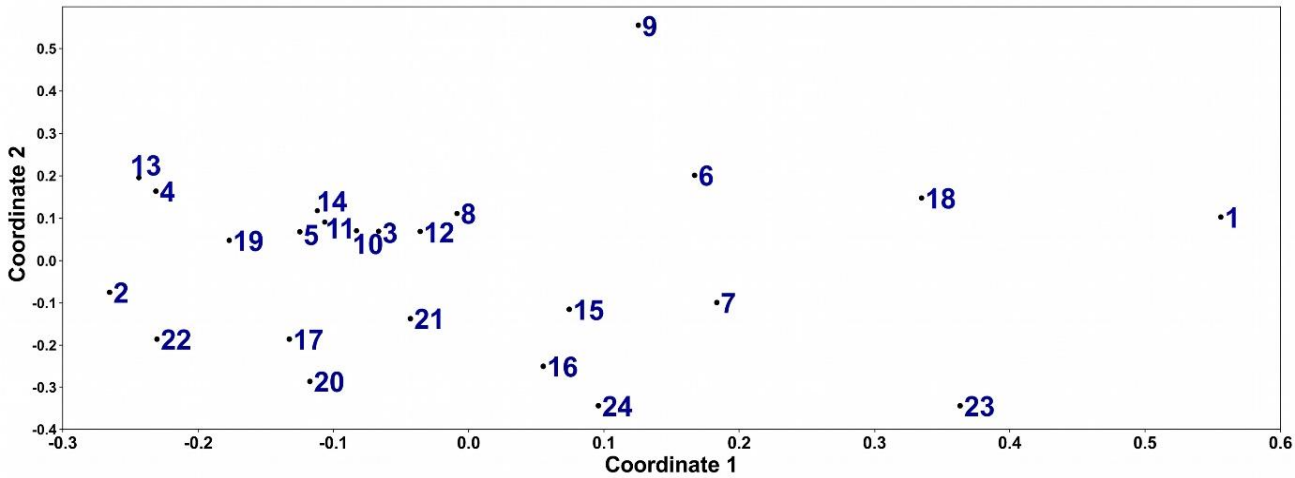

Fig. 5. Principal Coordinate Analysis (PCoA) of the distribution of 24 pomegranate accessions based on the ISSR marker data. The first coordinate, Coordinate 1, accounted for 20.01% of the variation and the second coordinate, Coordinate 2, accounted for 17.99% of the variation. Accession numbers are from the list of genotypes and registered cultivars in Table 1.

Discussion

In the present study, chloroplast trnL-F sequence analysis and ISSR markers were used to assess the genetic relationships among 24 accessions of pomegranate. The two marker systems provided complementary but different levels of resolution. In angiosperms, chloroplast DNA is uniparentally (and mostly maternally) inherited, with low levels of recombination, and is monophyletic. Thus, the trnL-F region is relatively conserved and mostly reflects differences in maternal ancestry and is of limited capacity to discriminate between closely related accessions. However, in contrast, ISSR markers target several polymorphic regions located all over the nuclear genome. These ISSRs can identify a wider range of genetic variability due to the nuclear genome receiving genetic material from both parents and being subject to genetic recombination. Similar marker resolution differences were found in Asparagus, where nuclear ITS sequences were more informative for the phylogenetic analysis than the chloroplast trnL-F region (Altıntaş *et al*., 2019). On the other hand, İnal *et al*., (2017) found that the trnL-F region discriminated between the various Turkish apple genotypes from the local region and relationships among them were inferred based on 1000 bootstrap replicates. The results of the two studies combined show that the discriminatory power of trnL-F is taxon-specific and depends on the degree of the sequence variation of the germplasm under study. This seems to account for the higher number of discriminations that could be obtained from the ISSR markers in the present pomegranate collection when compared with the chloroplast trnL-F region.

The results of previous molecular study of the pomegranate germplasm from the southeastern region of Türkiye also illustrate the difficulties that can be faced in distinguishing pomegranate accessions that are very close together from a single sequence region. A Neighbor-Joining analysis based on nuclear ITS sequence was used to assess 30 Pomegranate (*Punica granatum* L.) genotypes collected from Siirt Province, Turkey with the bootstrap method performed by Pakyürek (2019). The study showed that most accessions were very similar genetically, and that there was little variability in ITS, even though there were differences in their pomological characteristics. In this context, the author suggested the use of nuclear and chloroplast markers along with morphological, phenological and pomological assessments to better define the germplasm of pomegranate of a particular region. These results are consistent with the present findings, and support the conclusion that the resolution is restricted at the intraspecific level.

Paleotypographic differentiation was generally poor in the chloroplast trnL-F region but genotype 15 was always clearly differentiated from the others. The pattern indicated that there may be a different chloroplast haplotype for genotype 15, which may indicate that the maternal lineage is different or that there is a different source from a seed origin. It is an accession which may offer valuable germplasm as well as further characterization due to its unique location. Due to the lack of bootstrap support in the single conserved chloroplast region, the genetic distinctness of genotype 15 should be treated with caution and needs to be further verified with other chloroplast regions and more discriminatory nuclear markers. A similar pattern of limited chloroplast resolution was reported by Sevindik and Efe, (2021), who found that ISSR markers detected higher polymorphism than trnL-F sequences in Turkish pomegranate populations. Their finding that the use of trnL-F alone did not prove to be very effective to distinguish closely related pomegranate populations is similar to the results of the present study.

ISSR markers provided greater discriminatory power than the chloroplast sequence analysis. The six informative primers generated 30 bands, of which 18 were polymorphic, corresponding to an overall polymorphism rate of 60%. This value was lower than the approximately 78% ISSR polymorphism reported for pomegranate populations from the Aegean region of Türkiye by Sevindik and Efe (2021). This difference may reflect variation in primer selection, sample size, geographic coverage, and germplasm composition between the studies. PIC values in the present study ranged from 0.13 to 0.37, with an average of 0.23, indicating moderate marker informativeness. UBC845 exhibited the highest polymorphism rate and PIC value, whereas UBC847 had the lowest values.

The polymorphism detected in the present study was moderate compared to other studies on pomegranate. In Morocco, 27 cultivars were tested using 8 ISSR primers, and 87.14% polymorphism was detected by Ajal *et al*., (2014) while Madadi *et al*., (2017) found 83.23% polymorphism amongst the 18 Iranian commercial cultivars. Recently, Hajiyeva *et al*., (2018) found an average of 78% ISSR polymorphism in 90 wild pomegranate genotypes of Azerbaijan. Similarly, substantially high ISSR diversity was observed within and among the wild populations of PUN in Pir Panjal Himalaya by Dar *et al*., (2022) with 58% variation between the populations and 42% within populations. On the other hand, Ahmed, (2018) and Ismail *et al*., (2014) reported 67.12% and 53% of polymorphism in Egyptian material, respectively. The differences may be due to differences in geographical coverage, germplasm type, sample size, number of primers used, and the genetic diversity of the collectable samples that were used for evaluation.

The moderate PIC results for the present study must also be interpreted with care. The figures are not as high as some SSR based studies but are within reasonable limits for dominant ISSR markers and for a geographically restricted germplasm set. In a study of 60 pomegranate genotypes, Pirseyedi *et al*., (2010) calculated the PIC values ranging from 0.26 to 0.61 which was an average of 0.43. In 127 Turkish pomegranate genotypes, Polat *et al*., (2025) observed an average PIC of 0.48 while Patil *et al*., (2020) observed an average PIC of 0.44 in this study using hyper variable SSRs. The higher values are predicted due to the multi-allelic and codominant nature of SSRs as compared to the absence/presence data of ISSRs. However, Kadam *et al*., (2023) reported values of SSR between 0.13 and 0.37 with an average of 0.25, almost similar to the present ISSR data which showed marker informativeness between 0.15 and 0.36 with an average of 0.21. Hence, moderate PIC values found in the present study should not be considered as a limitation of the study but as the representative of the marker type and germplasm composition.

Based on the dendrogram generated by the ISSR-based UPGMA algorithm, there were several pairs of closely related genotypes such as genotypes 5 and 19, 10 and 12, 16 and 21, and 7 and 18. Meanwhile, genotypes 1, 3, 6 and 9 were placed on more isolated branches, with genotype 1 being the most distant from most of the accessions. This trend was further evident in the PCoA, in which several accessions (1, 18, 23, 24, 9) were separated from the central cluster. This UPGMA-PCoA agreement enhances the reliability of the ISSR data and indicates that the markers selected were biologically meaningful and showed variation among the accessions analyzed.

The clustering pattern was not entirely discriminating between local genotypes and registered cultivars. Pomegranate biology and cultivation history suggest that the flow is likely to continue in this direction, as it has been for centuries and is currently ongoing. The same results were obtained by Ajal *et al*., (2014) when they sampled pomegranate cultivars from different areas in Morocco, which did not group in a strict geographical order. Additionally, Madadi *et al*., (2017) demonstrated that commercial Iranian cultivars might have different clones that points to the cultivar names do not always represent genetically uniform material. However, the population structure based on geographical origin was well established in a larger population of indigenous, exotic, and wild accessions by Patil *et al*., (2020). These distinctions imply the geographic structure is more prominent when wider ecological and domestication and wild-cultivated contrasts are included.

It should therefore be interpreted with caution that there is no strict geographical separation in the present study. Çetinkaya *et al*., (2019) also observed clustering of Turkish pomegranate cultivars into two groups using SSR markers and some cultivars being closely genetically similar. More recently, Polat *et al*., (2025) analysed 127 Turkish pomegranate genotypes and identified two principal genetic clusters using both UPGMA and STRUCTURE analyses. The present analysis was based on a smaller number of accessions (24) from a fairly small geographic area as compared to these general studies carried out in Turkey. The size and geographical restriction of the sample restricts the potential to identify rare alleles, to draw generalizations about the larger population structure and to draw generalizations about the relationships observed among Turkish pomegranate germplasm. Nevertheless, the study provides preliminary molecular information on Mardin and neighboring germplasm, which remains underrepresented in national pomegranate diversity studies.

Recent studies demonstrate that marker choice strongly influences the resolution of pomegranate diversity analyses. SSR-based investigations of large germplasm collections have revealed population structure and supported the development of representative core collections (Wang *et al*., 2023), while studies integrating molecular markers with morphological and biochemical traits have identified both genetically divergent and agronomically valuable accessions (Parashuram *et al*., 2022; Lozano-Soria *et al*., 2025). Compared with dominant ISSRs, codominant SSRs and genome-wide SNPs provide more detailed information on allelic diversity and population structure. However, molecular divergence alone does not establish breeding value; molecular data must be combined with pomological, biochemical, and agronomic evaluations to identify useful parents and support effective germplasm management.

The molecular findings can also be considered alongside the previously published pomological characterization of the same regional germplasm. Öztürk *et al*., (2019) identified six Mardin genotypes-47 N 02, 47 N 05, 47 N 07, 47 N 10, 47 N 14, and 47 N 17-as promising material for fresh consumption based on their pomological characteristics. These phenotypically selected genotypes did not completely correspond to the most divergent accessions identified in the present molecular analysis. This difference demonstrates that molecular distinctness and agronomic performance represent complementary dimensions of germplasm value and should be evaluated together when determining breeding and conservation priorities. The genetically divergent accessions identified in this study may have particular value for pomegranate germplasm conservation. In particular, genotype 1 was well separated in the ISSR analysis, and genotype 15 had a separate position in the chloroplast analysis.

From the regional conservation standpoint, accession genotype 1 (47 N 01) that was relatively different in ISSR analysis and accession genotype 15 (47 N 15) that was isolated in the chloroplast analysis should be given special attention for conservation in the local field collections and germplasm repositories. The retention of these accessions together with representatives of the major genetic groups will contribute to the conservation of nuclear diversity as well as perhaps of distinct chloroplast variation within the regional gene pool. The high similarity at the molecular level found in some accessions may also aid in the development of a representative regional core collection without the need for undue duplication. Genetically divergent accessions might be assessed as candidate parents for commercial cultivar development to expand the genetic base of the breeding population, while accessions with molecular distinctiveness and desirable fruit quality, yield, and adaptation traits might be advanced for selection to serve as elite parents for commercial cultivar development. However, the commercial value of these candidates cannot be determined solely based on molecular divergence and they should be subjected to standardized pomological, biochemical, agronomic and multi-environmental assessments prior to their use in cultivar development.

The present ISSR analysis was used to characterize the germplasm of the region for pomegranate using a relatively simple and cost effective marker system. ISSRs are dominant markers, however, and cannot differentiate between heterozygous and dominant homozygotes, so they are not very informative regarding allelic diversity and population structure. An increased degree of accuracy in the estimation of allelic diversity, population structure, heterozygosity, and genetic differentiation would be possible with the use of codominant genome-derived SSR markers or genome-wide SNPs. The relationships found here should thus be confirmed in the future through the use of these higher resolution markers and combined with pomological, biochemical and agronomic traits to incorporate the molecular data. In addition, the increasing genomic resources of pomegranate, such as the reference genome and polymorphic SSR markers specific to each chromosome (Luo *et al*., 2020; Patil *et al*., 2021), lay a solid foundation for these analyses. The present study extends the study on the pomegranate diversities in Turkey by supplementing the existing information in the study with complementary nuclear and chloroplast molecular data for germplasm from Mardin region which is poorly represented in the previous Turkish germplasm study. The results from the combined analyses demonstrated a partial genetic overlap in the local genotypes and the registered cultivars as well as the presence of potentially divergent local accessions. Accession 47 N 15 (genotype 15) consistently had its unique position in both chloroplast-based phylogenetic analyses, and accession 47 N 01 (genotype 1) was among the most distinct accessions in the ISSR analysis. These accessions could be useful for germplasm conservation and evaluation that is undertaken for breeding purposes. Their genetic distinctiveness and breeding value must, however, be verified by bootstrap-supported phylogenetic analyses, by higher resolution nuclear and/or genome-wide markers and by standardized pomological, biochemical and agronomic evaluations.

The high level of variation within districts (98.95%) and the non-significance of ΦPT value indicate that there is significant genetic similarity or gene flow between the Artuklu and Kızıltepe pomegranate populations. This low level geographical structuring may also be due to exchange of planting material between adjacent cultivation areas. However caution should be used in interpreting this because of the relatively small and unequal sample sizes of the districts, and limited number of ISSR markers.

The most significant limitation of the chloroplast analysis was that bootstrap resampling was not performed for the NJ and ML trees. Consequently, the robustness of the internal branches and the distinct positioning of the 47 N 15 samples could not be statistically evaluated. Therefore, these findings should be considered preliminary until they are replicated using bootstrap-supported analyses based on additional chloroplast regions and higher-resolution nuclear markers.

The distinctiveness of accession 47 N 15 (genotype 15) may be determined with a much greater resolution using whole genome sequencing or complete plastome sequencing. A completed plastome would be used to decide if it is one of several distinct chloroplast haplotypes or a distinct maternal lineage, while whole genome sequencing would be used to determine if the apparent difference is also in the nuclear genome.

Conclusions

In this study, the genetic relationships among 24 pomegranate accessions were evaluated using chloroplast trnL-F sequence analysis and ISSR markers. The trnL-F region showed limited sequence diversity among the accessions; however, genotype 15 was consistently distinguished from the other genotypes in both the Neighbor-Joining and Maximum Likelihood analyses, potentially indicating a distinct maternal lineage. In contrast, the ISSR markers revealed a higher level of polymorphism and were more effective in distinguishing closely related accessions from one another. Six informative ISSR primers generated 30 bands; 18 of these were polymorphic, with an average polymorphism rate of 60% and PIC values ranging from 0.13 to 0.37. UPGMA and PCoA analyses further confirmed the presence of genetic diversity within the examined germplasm; genotype 1 emerged as one of the most distinct accessions. Generally, the combined use of cpDNA and ISSR markers provided complementary information and revealed the presence of detectable genetic diversity among the pomegranate genetic resources studied from southeastern Turkey. One limitation of the present study was that only 24 accessions were tested, a relatively small number of interests and restricted geographically, and there was only one conserved chloroplast region used, along with six dominant ISSR primers. Furthermore, the NJ and ML trees were not supported by a bootstrap, so no statistical analyses of branch robustness could be performed. These constraints limited the ability for allelic and phylogenetic resolution and generalizability of the results to the rest of the Turkish and Mediterranean pomegranate germplasm. There is a need for future research that can validate the observed relationships, by using larger and geographically more representative collections from other parts of Türkiye and neighbouring countries, bootstrap-supported phylogenetic analysis, codominant SSR or genome-wide SNP markers, complete plastome or whole genome sequencing and complementary pomological, biochemical and agronomic assessments.

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