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BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS OF POMEGRANATE (*Punica granatum* L.) USING HYPERVARIABLE AND CHLOROPLAST SSR MARKERS

*Hedi Wshyar Ibrahim[1] , Emad Omer Hama-Ali[2]
[1]Dept. of Biotech. Crop Sci. – Coll. Agric. Engin. Sciences – Univ. Sulaimani
[2]Dept. of Envi. Sci. – Coll. Envi. Sciences – Univ. Sulaiman

ABSTRACT

Pomegranate is a self-pollinated species crop, and its improvement programmes depend on molecular genetics and phenotypic variation. However, inadequate information on genetic diversity and population structure among pomegranate genotypes from this region and the variations in phytochemicals hinder its improvement. In this study, 40 pomegranate samples were chosen from 11 locations to represent the genetic spectrum from the Halabja Province. The pomegranate samples were tested using 23 Hypervariable SSRs (HvSSRT) and 19 newly developed Chloroplast SSRs (cpSSR) markers. The highest values of pH (4.40), TSS (16.774 °Brix), density (1066.5 kg/m^3), glucose (72.408 mg/ml), fructose (60.071 mg/ml), cyanidin-3-glucoside (71.984 mg/L), and cyanidin-3, 5-diglucoside (155.701 mg/L) were recorded among the pomegranate samples. SSR markers genotyping of pomegranate samples generated 114 alleles, and the MAF, Shannon's information index (I), and Nei's gene diversity for HVSSRTs were 0.570, 0.915, and 0.537, and for cpSSRs 0.618, 0.668, and 0.460. The polymorphic information content ranged from 0.129 to 0.723, with an average of 0.467 for HVSSRTs and 0.360 for cpSSRs. Finally, high levels of genetic diversity (66%) were recorded among individuals within populations, suggesting that the Halabja pomegranates are diverse.

Keywords: Anthocyanin, Chloroplast, Genetic diversity, High-Performance Liquid Chromatography, Microsatellite, Pomegranate.

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INTRODUCTION

Pomegranate (*Punica granatum L.)* is an ancient fruit native to Iran that then spread to the surrounding areas in Central Asia and the Mediterranean (Fawole et al., 2026; Liu et al., 2025). It has been cultivated for thousands of years, but the exact timeline and the processes of pomegranate domestication are not well-documented (Ward, 2003). According to available records, the pomegranate fruit has been mentioned in ancient documents, such as the Bible and Koran (Włodarczyk & Kapczyńska, 2025). Because of its importance, it is cultivated in many parts of the world today (Polat et al., 2024). In Iraq, fertile lands and favourable environmental conditions provide suitable conditions for pomegranate cultivation and pomegranates are considered a vital fruit crop (Abdulrahman et al., 2025). Pomegranates are cultivated in various regions of Iraq, in the central, southern and northern parts of Kurdistan, where the climate ranges from warm to semi-arid, and in the mountain regions. In the Kurdistan region, and especially in Halabja Province, pomegranate cultivation is becoming increasingly popular among farmers, serving as a source of income, pomegranate cultivation in Halabja served domestic consumption in the past, while in the last five years, it began serving export markets (Toptancî, 2024). In addition, the pomegranate of Halabja Province has more consumer preferences, market demands and superior aspects than other regions of Iraq (Al-Jabbari et al., 2019). The pomegranate's genome size ranges between approximately 320.31 and 361.76 Mb (Usha et al., 2022; Zhang et al., 2025). Its genome is similar in size to the genomes of other Rosaceae species, such as apples and peaches. Furthermore, pomegranate genome assembly or hybrid sequencing technology can resolve complex simple sequence repeats (SSRs) and provide essential genomic data for breeding new varieties (Usha et al., 2022). SSRs, also known as microsatellites, are valuable markers among molecular genetic markers for studying pomegranate diversity (Patil, Singh, et al., 2020). Numerous studies reported that SSR was used to investigate pomegranate diversity in the countries they are most cultivated in: Iran (Polat et al., 2025; Sadeghi Seresht et al., 2024), India (Parashuram et al., 2022; Patil, Singh, et al., 2020; Patil et al., 2021) and China (Wang et al., 2023). Moreover, several studies examined pomegranate genetic resources from different countries (Aziz et al., 2020; Gunnaiah et al., 2021; Patil, Jamma, et al., 2020; Yuan et al., 2024).A study of (Patil et al., 2021) identified and characterised highly variable SSRs in pomegranates. Hypervariable SSRs (HvSSRT) are chromosome-specific SSR regions found in the whole genome of the pomegranate cultivar 'Tunisia'. Moreover, to get comprehensive information on pomegranate genetics, it is essential to use other molecular markers, such as chloroplast SSRs (cpSSR), to provide additional information on the overall genetic makeup of pomegranate plants (Chen et al., 2022; Singh et al., 2021).Chloroplast genome sequencing is a valuable area of study that led to the discovery and development of cpSSRs, which provides a highly informative resource on the maternal lineage and diversity analysis within populations (Singh et al., 2021). There is an important need for information about the comprehensive genetics of cultivated pomegranates in Kurdistan. Their farmers only have traditional information about pomegranates, such as morphological and flavour profiles, which they get by consulting local agricultural sources and agricultural extension services, or by connecting with other farmers and agricultural organisations. Therefore, analyses of pomegranates at the biochemical and molecular levels would be beneficial and have more influence on investigating the diversity and genetic makeup of the fruit in this region. This study will serve as a benchmark for future work on pomegranate breeding and creating new varieties with desirable traits.

MATERIALS AND METHODS

Experimental site and plant material

Fresh pomegranate leaves and fruit samples were collected in October 2021 from Halabja in Kurdistan; 40 pomegranate samples were collected from 11 locations (Khurmal, Ahmad Awa, Zalm, Byara, Tawella, Siwan, Eneb, Sazan, Bawa Kochak, Twawshk and Ababayle) as shown in **(Figure 1)**. The samples were classified based on the morphometric parameters of fruits, seeds and leaves, the pomegranate's local name, and the geographic area. Fresh leaf samples were ground into liquid nitrogen directly and kept at −80°C until further molecular analysis.

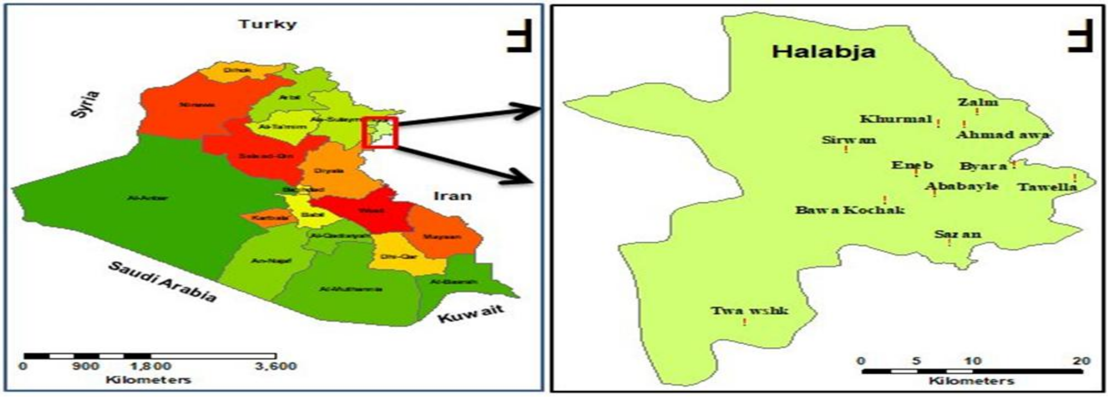

Figure 1. Map of Iraq and the Halabja region showing locations where pomegranate samples were collected

Juice extraction: Two kg of fruits from each sample were harvested at the ripeness stage and then transported to the laboratory. Fruits were sanitised with chlorinated tap water; pomegranate fruits were hand-peeled to obtain the juice from the arils. The juice was extracted from all samples using an extractor machine.

pH, TSS, and density analysis

pH measurements were performed using Mettler Toledo's SevenCompact Duo pH/Cond S213 (Belgium) digital pH meter. The total soluble solids (TSS; °Brix) were measured with a digital refractometer RX-5000i (ATAGO Co., Ltd., Tokyo, Japan) calibrated using distilled water. Results were reported as °Brix at 21°C. The water displacement approach determined density, and the weight density of the fruit was obtained by the ratio of weight to volume by weighting 10 ml of a pomegranate sample in a cylinder on a precision digital balance (Sartorius Lab Instruments GmbH & Co. KG, Gottingham, Germany) and then converted to average true density.

HPLC analysis of sugars

The following steps were conducted based on (Jalaludin & Kim, 2021) with modifications to determine the content of sugars (glucose and fructose) in pomegranate juice. The composition of sugars was identified with an HPLC on a Waters e2695 separation module equipped with a Waters 2414 refractive index detector and a Waters 2998 photodiode array detector (Waters, Milford, MA, USA). With Hypersil GOLD™ Amino HPLC columns, cat no. 25703-154630 (Thermo Scientific). For the mobile phase, 0.557 g/L of monobasic sodium phosphate (sodium dihydrogen phosphate anhydrous CAS 7558-80-7) from Sigma-Aldrich was added to 500 ml of HPLC grade water and filtered through a magnetic stirrer. 400 ml of this solution was mixed with 1,600 ml of acetonitrile (Sigma-Aldrich). 5 ml of each pomegranate juice sample was diluted with 50 ml of water (5:50) in a 50 mL volumetric flask filtered at the end through a 0.45 µm filter (Millipore) into a vial for injection in HPLC mode: LC use of Detector: Refractive index Column: 4.6-mm × 15-cm; 3-µm packing L8. In the temperatures of Column: 40 ± 1°C, Detector: 40 ± 1°C, in the flow rate: 1.3 ml/min, with the injection volume of 20 µL in vials. The different sugars in the samples were measured and identified by comparison of their retention times and peak areas with those of authentic standards obtained from Sigma Aldrich (St. Louis, Missouri, USA) under the same conditions.

U-HPLC analysis of anthocyanins

15 ml of each pomegranate juice sample was centrifuged at 5000 rpm for five minutes and filtered through a 0.45 µm filter (Millipore). The amount of 1.008 (Cy3,5) and 1.077 (Cy3) mg of two major anthocyanins with a potency of 98% were weighted and dissolved in 1 ml of acidified methanol (CH_3OH/HCl) (98/2), then diluted with 5 ml of Aqua H_3PO_4(177ml H_2O and 13.45 ml H_3PO_4) in an amber volumetric flask. Next, to prepare mixed anthocyanin standards, 1 ml from each standard stock solution (Cy3 and Cy3, 5) was diluted in 5 ml of Aqua H_3PO_4 in an amber volumetric flask and filtered through a 0.45 µm filter, then placed into a vial for injection in HPLC; we made five readings for each standard for more accuracy. We obtained the average of the standard peaks. The HPLC mode was according to the methodology of chromatographic analysis described by Thermo Scientific use Dionex, UltiMate 3000 (U-HPLC), RS Variable Wavelength Detector (VWD) for a Thermo Scientific AccucoreTM C18, 2.6 µ Particle Sz, Dim (mm) Analytical 150 × 2.1 (P/N-17126-152130, Lithuania) column as in (Neamtu et al., 2020), with modification in sample preparation in this study.

Genomic DNA extraction and PCR amplification: The DNeasy Plant Mini Kit (Qiagen) was utilised to extract the samples' genomic DNA. The quantity and quality of DNA were determined by a NanoDrop spectrophotometer and represented by 1% agarose gel in 1X TBE buffer. The gel was viewed using the gel documentation system – MiniDoc Plus (Herolab GmbH Laborgeräte, Germany). In total, 42 SSR primers were used in this study. A set of 23 chromosome-specific hypervariable SSR (HVSSRT) primers selected from (Patil et al., 2022) were used in conjunction with 19 newly-developed chloroplast SSRs (cpSSR) in the genotyping of 40 pomegranate samples (Appendix 1). The complete chloroplast genomes of pomegranate cultivar Nana (MK603513), Tunisia (MK603512, MN833212), Daihong1 (MN630639), Sanbaitian (MN630637), and Bhagwa (MK635347.1, KY635883.1) were downloaded from NCBI GenBank and aligned. Then, SSRIT (Temnykh et al., 2001) was used to find repeats (motifs) from the chloroplast sequences. CpSSR primer pairs were designed using Primer3 (Untergasser et al., 2012) from the sequences flanking the SSR loci. The total PCR reaction was optimised to be 20 µl; it included 2 µl of about 15 ng DNA template, 10 µl PCR master mix of 2X Add Taq Master (Addbio, Korea), 1.5 µl of each primer (forward and reverse), and 5.0 µl of nuclease-free water. The PCR amplification was carried out with Applied Biosystems Veriti™ 96 Well Fast Thermal Cycler (Model 9902), and the PCR conditions were five minutes at 95°C, 35 cycles of 30 second at 95°C, 30 second at annealing temperature (Appendix 1), 30 second at 72°C, plus another 10 minutes at 72°C, followed by infinity holding at 4°C. The amplified products were visualised using 5 µl ethidium bromide-stained concentration of 10 mg/mL in H_2O on 2.0% agarose gels in 1% TBE. The gel was examined using the gel documentation system. The gel picture was interpreted using the sample band with a 100 bp plus (Addbio, Korea) DNA ladder for the band size of each SSR primer. POPGENE v1.32 software assessed the SSR data allele frequency (Yeh, 1999).

Structure and genetic diversity analysis

STRUCTURE 2.3.4 software (Pritchard et al., 2000) detected the genotypes' subpopulations. The parameters were 100,000 for the burn-in period and 100,000 as the number of Markov chain Monte Carlo replications after burn-in. Cluster values are set as 1 to 10, with ten replicates (Paul et al., 2022). Structure Harvester was used to determine the value of K (subpopulation) (Earl & VonHoldt, 2012). A dendrogram of genotypes was generated using the unweighted pair group method with arithmetic mean (UPGMA) through PowerMarker v3.25 (Cheng et al., 2024), and then visualised using MEGA X (Kumar et al., 2018). Moreover, principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA) were implemented through GenAlEx V6.5 (Peakall & Smouse, 2012; Rai et al., 2025).

RESULTS AND DISCUSSION

Quality and biochemical analysis

The results of the pH, Brix, and density analysis of 40 pomegranate juice samples are shown in **(Figures 2a, b, c and Appendix 2)**. The mean pH value was 3.57, S11 (in Zalm) had the lowest pH at 2.97, and S5 (in Ahmad Awa) had the highest pH at 4.40. The TSS mean value was 14.682 °Brix, ranging from 12.158 to 16.774 °Brix for the samples S4 (in Ahmad Awa) and S31 (in Bawa Kochak), respectively. In comparison, the mean density was 1044.818 kg/m^3, with S17 (in Byara) recording the lowest value at 1030 kg/m^3 and S39 (in Ababayle) recording the highest value at 1066.5 kg/m^3. HPLC was used to estimate this study's two primary sugar contents (glucose and fructose). The results indicated that glucose concentrations were higher than fructose in all samples **(Figures 3a, c, and Appendix 2)**.

Samples S22 (from Sirwan) and S37 (from Eneb) recorded the highest values of glucose and fructose, with 72.408 and 60.071 mg/ml, respectively. Meanwhile, S29 (from Ababayle) recorded the lowest values of glucose and fructose, with 30.119 and 25.597 mg/ml, respectively. In addition, two individual anthocyanins (cyanidin-3-glucoside and cyanidin-3,5-diglucoside) were selected to be identified in pomegranate juice samples using U-HPLC **(Figures 3b, d, and Appendix 2)**. The average cyanidin-3-glucoside in all samples was 10.741 mg/L, ranging from 0.123 mg/L (S6 and S32) to 71.984 mg/L (S35). The average cyanidin-3,5-diglucoside was 35.508 mg/L, ranging from 0.331 mg/L (S6) to

155.701 mg/L (S23). The concentration of cyanidin-3,5-diglucoside was higher than that of cyanidin-3-glucoside in all samples.

HVSSRT and cpSSRs diversity

All 42 SSR primers (23 HVSSRT and 19 newly developed cpSSRs) were polymorphic for genotyping 40 pomegranates from the Halabja region. A total of 114 bands were generated for all HVSSRT and cpSSR markers, and the number of observed alleles (Na) ranged from two to six alleles, with an average of 3.217 for HVSSRTs and 2.105 for cpSSRs **(Table 1)**. Most cpSSR markers produced two alleles per locus, except cpSSR5 and cpSSR14, which had three alleles per locus. The effective number of alleles (Ne) ranged from 1.280 (cpSSR9) to 4.222 (HvSSRT_100), with an average of 2.402 for HVSSRTs and 1.887 for cpSSRs across all markers. The average values of major allele frequency (MAF), Shannon's information index (I), and Nei's gene diversity for HVSSRTs were 0.570, 0.915, and 0.537, and for cpSSRs were 0.618, 0.668, and 0.460. The polymorphic information content (PIC) ranged from 0.129 to 0.723, averaging 0.467 for HVSSRTs and 0.360 for cpSSRs. Finally, the results showed that HvSSRT_100 and HvSSRT_490 were the most informative loci because they had the highest PIC values, with values of 0.723 and 0.706.

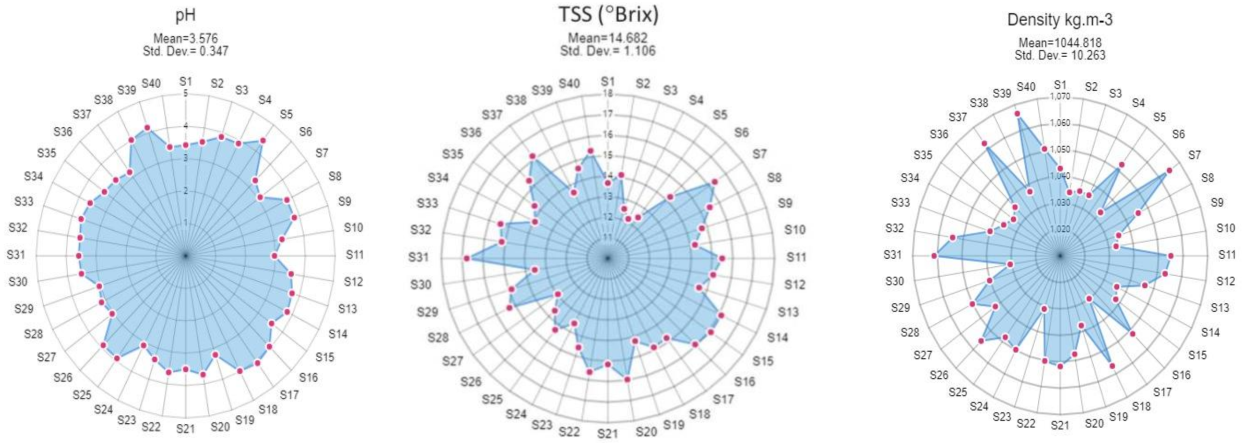

Figure 2. Quality parameters identified in 40 pomegranates from the Halabja region: (a) pH, (b) TSS; (c) Density

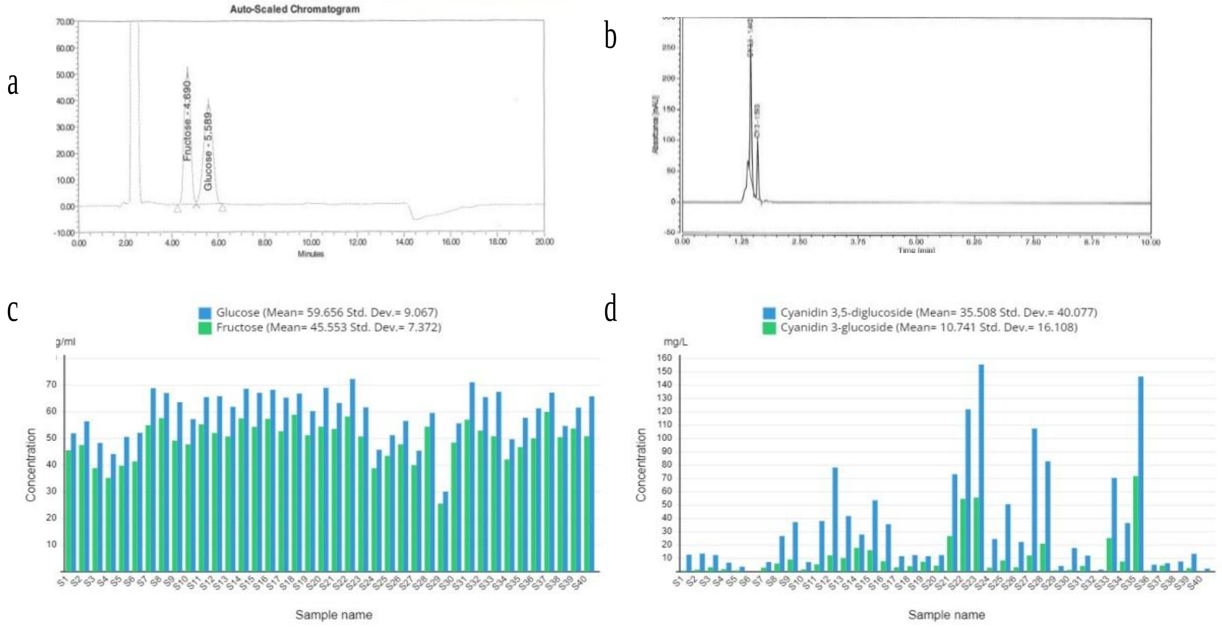

Figure 3. Chromatograms of: (a) glucose and fructose; (b) chromatograms of cyanidin-3-glucoside and cyanidin-3,5-diglucoside; (c) content of glucose and fructose; (d) content of cyanidin-3-glucoside and cyanidin-3,5-diglucoside in 40 pomegranates from Halabja

Table 1. Genetic diversity of pomegranate samples based on 23 HVSSRT and 19 cpSSR markers

Marker	Na	Ne	MAF	I	Gene Diversity	PIC
HvSSRT_92	3	2.608	0.475	1.017	0.617	0.538
HvSSRT_100	6	4.222	0.288	1.529	0.763	0.723
HvSSRT_110	3	2.062	0.650	0.887	0.515	0.460
HvSSRT_200	2	1.956	0.575	0.682	0.489	0.369
HvSSRT_217	3	1.427	0.825	0.555	0.299	0.271
HvSSRT_269	3	1.421	0.825	0.536	0.296	0.265
HvSSRT_317	2	1.663	0.725	0.588	0.399	0.319
HvSSRT_324	3	1.865	0.700	0.815	0.464	0.417
HvSSRT_348	2	1.724	0.700	0.611	0.420	0.332
HvSSRT_460	4	3.828	0.325	1.363	0.739	0.690
HvSSRT_483	3	1.802	0.700	0.746	0.445	0.381
HvSSRT_490	6	3.922	0.388	1.506	0.745	0.706
HvSSRT_571	4	2.759	0.475	1.116	0.638	0.569
HvSSRT_592	4	1.995	0.650	0.877	0.499	0.432
HvSSRT_648	2	1.980	0.550	0.688	0.495	0.372
HvSSRT_697	2	1.923	0.600	0.673	0.480	0.365
HvSSRT_700	3	2.111	0.575	0.836	0.526	0.431
HvSSRT_714	4	2.640	0.550	1.155	0.621	0.572
HvSSRT_750	3	2.020	0.650	0.857	0.505	0.442
HvSSRT_820	4	3.765	0.313	1.355	0.734	0.685
HvSSRT_840	2	1.835	0.650	0.647	0.455	0.351
HvSSRT_879	2	1.835	0.650	0.647	0.455	0.351
HvSSRT_893	4	3.884	0.275	1.37	0.743	0.694
Mean (HvSSRT)	3.217	2.402	0.570	0.915	0.537	0.467
cpSSR1	2	1.956	0.575	0.682	0.489	0.369
cpSSR2	2	1.995	0.525	0.692	0.499	0.374
cpSSR3	2	1.835	0.65	0.647	0.455	0.351
cpSSR4	2	1.882	0.625	0.662	0.469	0.359
cpSSR5	3	2.180	0.575	0.88	0.541	0.455
cpSSR6	2	1.663	0.725	0.588	0.399	0.319
cpSSR7	2	1.882	0.625	0.662	0.469	0.359
cpSSR8	2	1.995	0.525	0.692	0.499	0.374
cpSSR9	2	1.280	0.875	0.377	0.219	0.195
cpSSR10	2	1.995	0.525	0.692	0.499	0.374
cpSSR11	2	1.835	0.650	0.647	0.455	0.351
cpSSR12	2	1.536	0.775	0.533	0.349	0.288
cpSSR13	2	1.835	0.650	0.647	0.455	0.351
cpSSR14	3	2.558	0.438	0.998	0.609	0.526
cpSSR15	2	1.782	0.675	0.631	0.439	0.342
cpSSR16	2	2.000	0.500	0.693	0.500	0.375
cpSSR17	2	1.995	0.525	0.692	0.499	0.374
cpSSR18	2	1.761	0.684	0.624	0.432	0.339
cpSSR19	2	1.882	0.625	0.662	0.469	0.359
Mean (cpSSR)	2.105	1.887	0.618	0.668	0.46	0.360

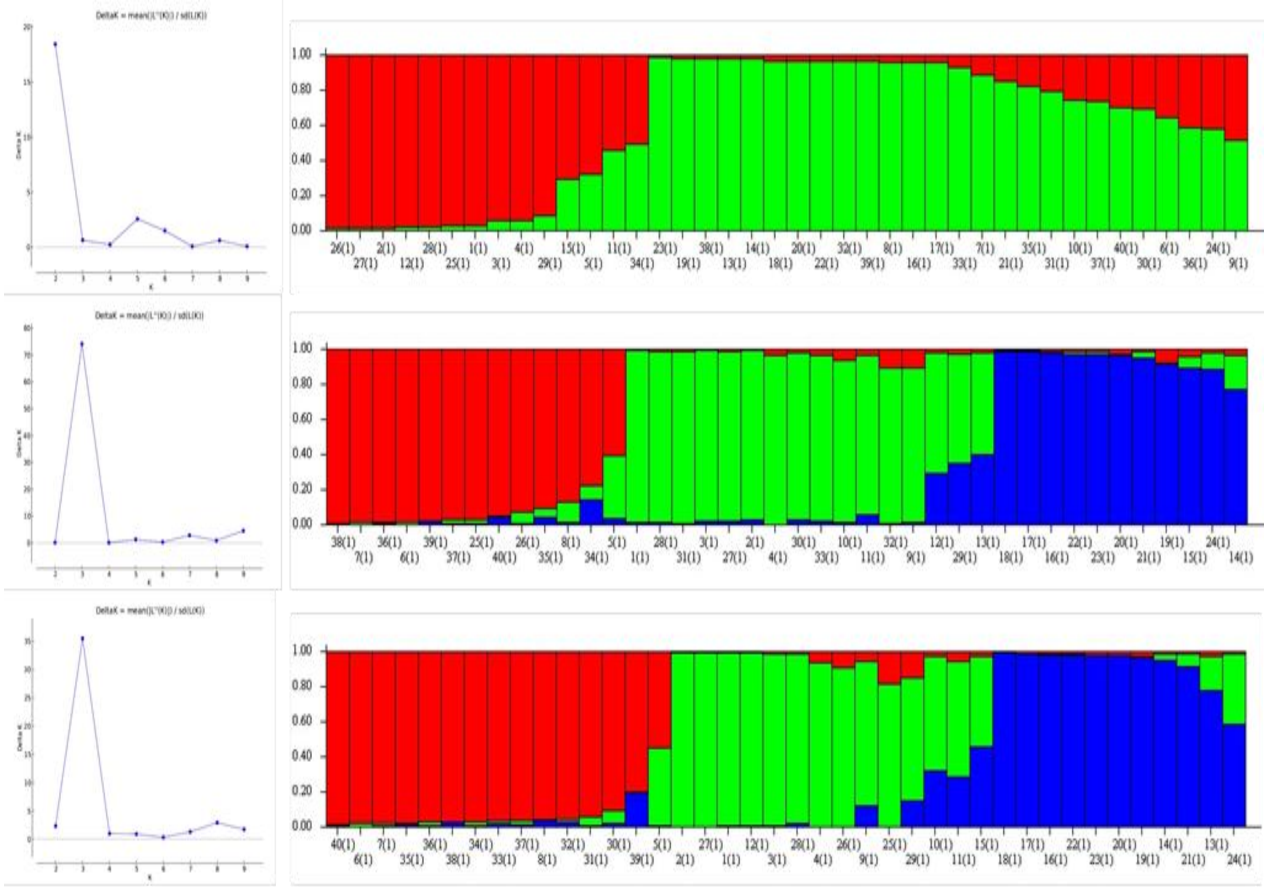

Figure 4. (a) Number of assumed Delta K = 3 and population structure of 40 pomegranate samples using HVSSRT markers; (b) Number of assumed Delta K = 3 and population structure of 40 pomegranate samples using cpSSR markers; (c) Number of assumed Delta K = 3 and population structure of 40 pomegranate samples using HVSSRT and cpSSR markers

Phylogenetic analysis

Phylogenetic analysis was performed to investigate genetic diversity further and was based on the dissimilarity matrix using PowerMarker Field (Shahsavari et al., 2022). The resulting tree (UPGMA method) was visualised using MEGA X (Kumar et al., 2018; Polat et al., 2025). The pomegranates were clustered into two major groups **(Figure 5a)**. The first significant group included 15 pomegranates, the same as G1 in the structure result, and the second major group included 35 pomegranates, divided into two subgroups identical to G2 and G3 of the structure result. However, unlike the structure analysis, the admixture pomegranate sample (15) is grouped under G3. Moreover, Nei's genetic distance **(Figure 5a and Table 2)** shows that G1 is a separate Group, while G2 and G3 are under one major group. G2 and G1 had the lowest level of genetic identity (0.768), while G2 and G3 displayed the highest levels of genetic identity (0.804). Furthermore, the data from previous analyses of this study were gathered to conduct a principal coordinate analysis (PCoA). The PCoA (1 vs. 2) interpreted 11.53% and 10.45% of the variance in the genotype data, respectively **(Figure 5b)**. From the PCoA below, we can see those three categories (red, green, and blue), and the admixture pomegranate sample (15) is grouped under G2, but it is located in quadrant plot two and closer to G1. The results of the PCoA of pomegranate samples were confirmed by the assigned cluster of structure and phylogeny tree analysis.

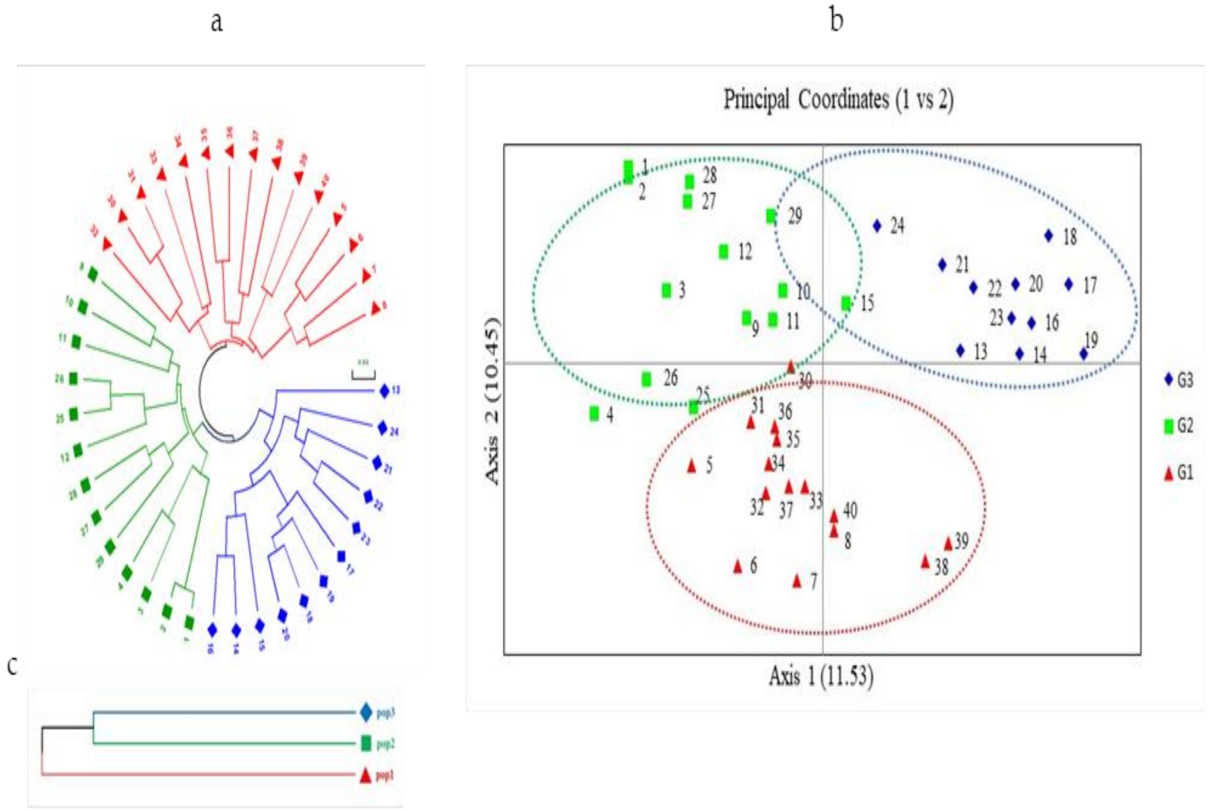

Figure 5. (a) Phylogenetic tree of 40 pomegranate samples using the UPGMA method; (b) PCoA of individual pomegranate samples using HVSSRT and cpSSR data; (c) Nei's genetic distance for the populations

Table 2. Nei's original measures of genetic identity (above diagonal) and genetic distance (below diagonal) between different populations (G1, G2, and G3).

Population	G1	G2	G3
G1	1.00	0.771	0.768
G2	0.260	1.00	0.804
G3	0.264	0.219	1.000

From the dissimilarity matrix output data, a heatmap and hierarchical clustering were generated With Morpheus software-(https://software.broadinstitute.org/morpheus). According to the cluster heatmap **(Figure 6)**, we can infer a precise similarity level among pomegranate samples based on the colour level. The most similar samples were S1 and S2 (from Khurmal), while the highest dissimilarity was between sample S13 (from Byara) and S36 (from Eneb). On the other hand, the tree generated with the heatmap shows that pomegranate samples are divided into two major clusters. The first cluster consisted of G1 (red), and the second cluster consisted of G2 (green) and G3 (blue). These results provide informative value about genetic similarity among the studied samples and support structure analysis and phylogeny tree results. Finally, the AMOVA for SSR markers **(Table 3)** revealed 18% of molecular variance (among populations), 66% (among individuals within populations), and 16% (within individuals). A high genetic differentiation F_{ST} value of 0.178 ($p \leq 0.001$) and gene flow (Nm) 1.151 was recorded between populations.

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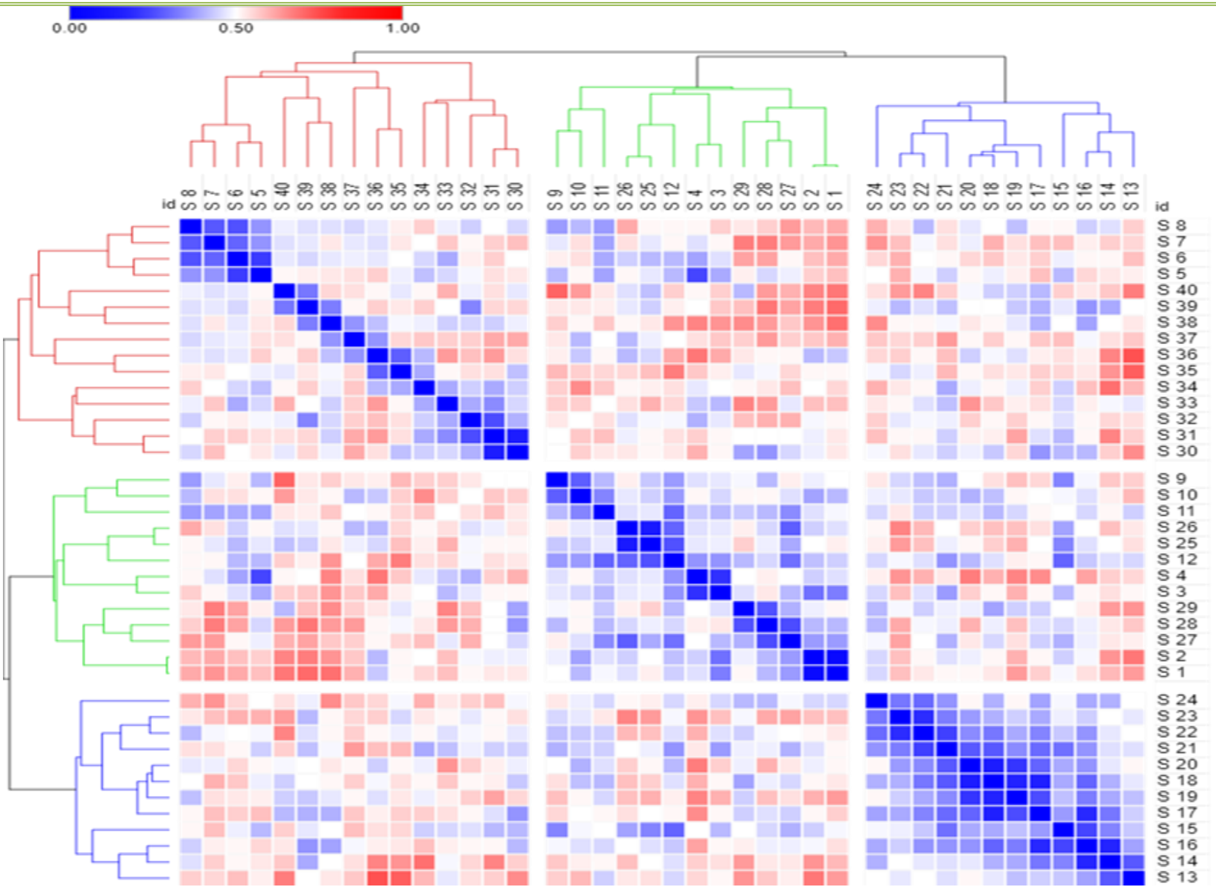

Figure 6. Heatmap showing the genetic distances among the studied pomegranate. The highest dissimilarity (red) and the lowest genetic distance (blue).

Table 3. Summary analysis of molecular variance based on HVSSRT and cpSSR data

Source of Variation	df	SS	MS	Est. Var.	%
Among Populations	**2**	**142.257**	**71.128**	**2.047**	**18%**
Among Individuals within Populations	**37**	**628.681**	**16.991**	**7.564**	**66%**
Within Individuals	**40**	**74.500**	**1.863**	**1.863**	**16%**
Total	**79**	**845.438**		**11.474**	**100%**
FST (fixation index)	**0.178 (p ≤ 0.001)**				
Nm	**1.151**				

The results of this study show a wide range of pomegranates' quality index (pH, TSS, and density) in the Halabja region. According to these data and the farmers' observations, we can deduce that the sour pomegranates have a pH range of (2.97 to 3.4), sour-sweet ones (3.42 to 3.91), and sweet ones (3.96 to 4.40) in this study. This finding is consistent with (Calín-Sánchez et al., 2011), who categorise some Spanish pomegranate cultivars based on pH mean into sour, sour-sweet, and sweet. The density of pomegranate juice mean value for our samples was 1044.818 kg.m^{-3}. Regarding the TSS, our samples ranged from 12.158 to 16.774 °Brix. These results are similar to those TSS reported in (Cirillo et al., 2022). Abdel-Sattar et al. (2023) argued that a TSS of 12 °Brix was the minimum acceptable value for commercial use of pomegranates. Here, we can see why pomegranates in the Halabja Province have more consumer preferences. The present study has demonstrated that glucose concentrations are higher than fructose in all 40 pomegranate samples. Previously published studies on the higher amount of primary sugar content between glucose and fructose are varied. Some studies have shown that fructose is higher than glucose (Montefusco et al., 2021; Nafees et al., 2020), but others have shown that glucose is higher (Arlotta et al., 2022; Jihad Al-Aslan et al., 2023). Regarding the anthocyanin in this study, the content of cyanidin-3-glucoside in all samples ranged from 0.123–71.984 mg/L.

and cyanidin-3,5-diglucoside (0.331 to 155.701 mg/L). Overall, the quantities of cyanidin-3,5-diglucoside were higher than cyanidin-3-glucoside in all samples. The present study's findings agree with (Bernini et al., 2024; Budiene et al., 2021; Hasnaoui et al., 2011). We selected 23 chromosome-specific highly variable SSR markers (HvSSRT) identified and developed by (Patil et al., 2021) from a survey of the 'Tunisia' genome. Moreover, 19 cpSSR markers were newly designed from the online chloroplast genome in this study. Allele frequency data generated from HvSSRTs shows higher values of Na (3.217), Ne (2.402), I (0.915), gene diversity (0.537) and PIC (0.467), which were higher compared to those of cpSSR. In contrast, the MAF mean (0.570) was higher in cpSSRs. The most likely cause of this difference is the nature of SSRs because genomic SSRs generate more alleles than chloroplast SSRs. This also aligns with our earlier observations, which showed that most cpSSR has two alleles. In the same vein, (Norouzi et al., 2012), in their diversity investigation of Iranian pomegranates using chloroplast microsatellites, found that the number of alleles ranged from two to three, with an average of 2.44. Previous studies have also reported similar Na, Ne, and PIC values in pomegranates using SSR markers (Patil, Singh, et al., 2020; Patil et al., 2021; Zeinalabedini et al., 2012). However, our observations were higher than those obtained by (Zarei & Sahraroo, 2018).

In comparison, other studies reported higher SSR allele frequencies (Aziz et al., 2020; Perveen et al., 2023). The STRUCTURE programme was implemented to detect the clusters of studied pomegranates using 23 HVSSRT, 19 cpSSR, and combined all 42 SSR (HVSSRT, cpSSR) allele data. The programme generated a population structure with two groups using HVSSRT, three groups using cpSSR, and three groups using all markers together (HVSSRT, cpSSR). But the members of three groups based on cpSSR were different form the three groups based on all markers (HVSSRT, cpSSR). Because all markers combined show more partition in clusters than HVSSRT and different than cpSSR. As a result, all markers (HVSSRT, cpSSR) analysis-based population structure of pomegranates was chosen for other analyses in this study. The Bayesian model-based clustering separates the pomegranates into three colour groups (G1, G2, and G3). Furthermore, the percentages of admixture samples in each group were 6% (one sample) in G1, 21% (three samples) in G2, and 18% (two samples) in G3. A similar population structure of three clusters was observed in a previous research (Luo et al., 2018) in a collection of 136 pomegranate cultivars in China. Population structure analysis in different pomegranate diversity panels has indicated the existence of two to six clusters in pomegranates (Gunnaiah et al., 2021; Hasnaoui et al., 2011; Patil, Jamma, et al., 2020; Patil, Singh, et al., 2020; Singh et al., 2015; Zarei & Sahraroo, 2018). The UPGMA phylogenetic tree was valuable in that it indicated new results rather than the structure analysis by gathering G2 and G3 in one major group, separating them from G1. The tree results are also supported by Nei's genetic distance **(Table 2),** which indicates the highest level of gene identity between G2 and G3. Regarding PCoA, axis 1 and 2 results accounted for 21.98% of the total variability of the 40 pomegranates. Axis 1 contains individuals from G2 and G3, while Axis 2 comprises individuals from G1. Our results are comparable with the two-dimensional PCoA of (Zeinalabedini et al., 2012), where they obtained 30.43% for the first two axes of plotting 25 pomegranate genotypes based on 23 SSRs. The heatmap **(Figure 6)** shows apparent similarities and dissimilarities between the studied pomegranate samples based on colour. The dendrogram generated in the heatmap follows the UPGMA tree's clustering method **(Figure 5a).** AMOVA showed a high percentage of 66% variance among individuals within populations. Meanwhile, (Zarei & Sahraroo, 2018), an assessment of the genetic diversity among 50 pomegranate accessions from five different regions of Iran using 16 SSR markers revealed low variation among individuals within

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populations (16%). Also, a low value among individuals within populations (26%) was observed by testing 60 SSR markers on 96 pomegranates from different countries and the Himalayan wild pomegranate (Gunnaiah et al., 2021). In addition, high genetic differentiation (F_{ST} = 0.178) reveals a high degree of genetic differentiation among the currently studied pomegranates. A similar pattern regarding population differentiation index (F_{ST} = 0.18) was observed in the study of (Singh et al., 2015) for 88 wild-type and cultivated pomegranate accessions using 44 microsatellite primers from India. However, (Luo et al., 2018) identified a lower value of F_{ST} = 0.11 for 136 pomegranate varieties, most of which were from China. With these molecular analysis methods, software, and SSR data suggest that Halabja pomegranates are diverse. The findings indicate this region is among the oldest places where pomegranates have been cultivated. In addition, this informative genetic background is essential for future pomegranate breeding programmes and germplasm preservation.

Appendix 1. Sequence details of 42 SSR markers: 23 chromosome-specific Hypervariable SSR (HVSSR) primers from (Patil et al. 2022) and 19 newly developed chloroplast SSRs (cpSSR) in this study.

Primers ID	Size range bp	Forward primer	Reverse primer	Annealing temp °C	References
HvSSRT_92	145-173	GTTTTCGGTCAAAAGCTCC	AAGAAAACCCAAAACAGGC	54	(Patil et al., 2022)
HvSSRT_100	117-140	GAGAGAGTCATCTGTTCCG	AGAGAGAAGAGAGAGAGGG	57	
HvSSRT_110	286-300	GGAGGAAAAGCTGTACACG	GATCTATCGGTCGGTTTCC	57	
HvSSRT_200	147-181	ATGTTGCATGTTGTTGTCC	TCTTCCTTCTTCCTCACCC	55	
HvSSRT_217	117-139	AAACATGTGGCATTCTCCC	CAAATATGGAAAACGGGCC	55	
HvSSRT_269	160-177	AGGGTAAAACTCAACCACC	GGAGAGATCGAACTTTCCC	56	
HvSSRT_317	181-200	AAATATCTGAAGTTCGGCG	ACATTGTAGGGAATCGTGG	54	
HvSSRT_324	165-196	AGGTTTCATCTGGTTTCGG	CTGTCCTATGGTAGAGGGG	57	
HvSSRT_348	198-290	ATGCCAAAAATTAGCGAGC	CTTATAAGTGAGCTCCCCC	55	
HvSSRT_460	126-162	ATTCGTTTAGTGTACAAGGC	CAATGAGGATCGGACTTCC	56	
HvSSRT_483	100-115	TGGATGGTTTTAACGACCG	GGATCTACCGAGGAAGAGG	57	
HvSSRT_490	100-128	TTTTCCCCAATATGCTTGC	AACAAAGGAGATATCCGGC	54	
HvSSRT_571	170-190	ATCGAAAAGAGCTCATCCC	GCGAATGAACAGCTAAGG	54	
HvSSRT_592	164-210	TGCTCATATACATGCCAGC	ATTACACATGAAAGTGCGG	54	
HvSSRT_648	267-297	TCCGCACTAATGTCATTCC	AGAAAATTTGACACTTCCCC	55	
HvSSRT_697	108-120	TGGGAATCATACTGTTGGC	TCCAAACCAATACCACACC	55	
HvSSRT_700	180-199	GTAAGACTTAAGGCGGAGG	AAAAGGAAAACTGCCGAGG	56	
HvSSRT_714	105-144	GAACAATTCTTTGCCTCGG	TGTCTAATGAGCTGGAACC	55	
HvSSRT_750	128-176	TCTATATCATGCAACGCCC	TTGAATTATTGAAGCGGCC	54	
HvSSRT_820	152-178	CTTTCCCCAGTACACAACC	CAGATCCCATGTTCATCCG	57	
HvSSRT_840	170-213	CGGGTTCTATGCTTCTCG	CGACAAAAATACACGTTTCC	55	
HvSSRT_879	174-275	CTTTCTCCCTCCCTTTTCC	TGATCACCTCACTGAAACC	56	
HvSSRT_893	168-198	TCCACTATGAGTTGCTTGG	CATTTTGGACAAAGACTTGG	55	
cpSSR1	321	AGCAAAGATTAGGGACGAA	AGAGAGAGAAGAGGGTGGG	56	newly developed chloroplast SSRs (cpSSR) in this study
cpSSR2	286	ACAACAATCAGAAGACGGAC	GGAATAGGAATCAAACCGA	55	
cpSSR3	136	CGCTCAATTACTTCAACCA	TTCTGGTCTTGGATTAGGG	54	
cpSSR4	239	ATGAACTCCTGCTTCCATC	TGGGTCATTTGATGTTGTC	54	
cpSSR5	282	TTTCGGGTTGTGAGACATA	TACTTTCTTCAGGGCGATT	53	
cpSSR6	333	ACCCACAAAATACCAGAGG	GAAGGAATTGACCCACCTA	55	
cpSSR7	330	ATTTTCACCTCATACGGCT	CTAAGAAGAATGGTTGGGC	54	
cpSSR8	239	CACTAGGCTCGAAGGAAAG	GTTAAAGGTGCGGAAAGAG	56	
cpSSR9	339	TAGTAATTTTGATCCCGGC	AGGTTAGGACATGAAACCG	54	
cpSSR10	246	ACTAAAAGGATTGAGCCGA	AAGAAGTTGGGATACGTGC	54	
cpSSR11	349	CAAGATGAAAGAAATCCCC	CAACATCGAATTGAACCAC	53	
cpSSR12	290	AAAGAACTCGCAGGTTGAT	AGGAAGGATGGTAATTGGA	53	
cpSSR13	398	GCTGGTATTGACCCAGATT	AGATCCATTCGAGGAACAC	55	
cpSSR14	400	TGTCCCTACCCATGATAAAC	AAACGTGACAACGGTATGA	55	
cpSSR15	354	TCGATGAAAGGGAAGAGAT	ATTACAAATGGGTTCGGTC	53	
cpSSR16	100	TTATGTGTACGCGCTCTTT	TCAAAACCCAATCCTTACC	53	
cpSSR17	370	GGTAAGGATTGGGTTTTGA	AATTCCCGGTTGAAAGAG	52	
cpSSR18	364	TATTCCCGACTCCTTTGA	GGATCATAGTGCATAGTGTGA	54	
cpSSR19	392	TACTTGGACCCTATTTGCC	GCCTTTGTGAGAACCATTT	54	

Appendix 2. Identification and quantification of of some quality and biochemical traits of pomegranates in Halabja Region

Id	PH	TSS (°Brix)	Density kg.m^3	Glucose mg/ml	Fructose mg/ml	cyanidin 3,5-diglucoside mg/L	Cyanidin 3-glucoside mg/L
S1	3.420	13.669	1043.0	51.989	45.603	12.886	0.933
S2	3.560	14.114	1034.3	56.547	47.615	13.723	1.726
S3	3.860	12.538	1035.7	48.390	38.969	12.613	3.540
S4	3.890	12.158	1035.7	44.213	35.265	7.007	2.149
S5	4.400	12.467	1052.6	50.663	39.888	3.874	1.074
S6	3.290	14.231	1033.2	52.189	41.472	0.331	0.123
S7	3.080	16.339	1064.9	68.972	55.085	7.358	3.135
S8	3.800	15.486	1045.6	67.135	57.732	26.881	6.217
S9	3.820	14.744	1035.0	63.714	49.278	37.314	9.264
S10	3.260	14.231	1033.0	57.340	47.910	7.338	1.832
S11	2.970	15.486	1055.0	65.648	55.435	38.132	5.671
S12	3.570	15.116	1053.2	66.003	52.181	78.308	12.452
S13	3.740	14.604	1046.2	61.915	50.862	42.006	10.427
S14	3.810	16.109	1035.8	68.724	57.633	28.010	18.053
S15	3.550	16.109	1037.9	67.243	54.457	53.685	16.309
S16	3.960	15.925	1051.7	68.348	57.404	35.874	8.084
S17	4.090	14.791	1030.0	65.398	52.800	11.757	3.435
S18	3.990	14.860	1056.7	66.924	59.062	12.691	4.280
S19	3.210	14.231	1037.7	60.333	51.309	11.737	7.679
S20	3.710	15.971	1047.6	69.084	54.526	12.613	4.791
S21	3.500	15.162	1051.8	63.417	53.695	73.344	26.965
S22	3.640	15.602	1050.2	72.408	58.365	122.124	54.899
S23	3.360	14.558	1031.1	61.818	50.835	155.701	55.886
S24	3.100	13.552	1049.8	45.801	38.880	24.721	3.206
S25	3.910	14.301	1048.0	51.290	43.572	50.882	8.630
S26	3.900	13.599	1055.5	56.668	47.947	22.482	3.487
S27	3.040	12.963	1042.6	45.502	40.071	107.603	12.294
S28	3.160	15.301	1050.1	59.675	54.505	83.038	21.241
S29	3.040	14.860	1040.2	30.119	25.597	4.496	1.268
S30	3.530	13.552	1030.6	55.782	48.571	17.888	1.515
S31	3.580	16.774	1061.3	71.147	57.119	12.282	4.474
S32	3.580	15.162	1054.3	65.652	53.056	1.849	0.123
S33	3.680	15.417	1040.0	67.549	50.883	70.561	25.433
S34	3.590	13.927	1035.8	49.798	42.214	36.614	7.802
S35	3.360	14.348	1033.5	57.849	46.865	146.688	71.984
S36	3.310	15.347	1036.0	61.379	50.137	5.450	1.039
S37	3.190	16.155	1062.5	67.267	60.071	6.482	4.861
S38	4.010	13.599	1037.2	54.758	50.577	7.805	0.247
S39	4.160	14.604	1066.5	61.671	53.793	13.606	2.730
S40	3.400	15.301	1050.9	65.924	50.877	2.550	0.370
Mean	3.567	14.682	1044.818	59.656	49.553	35.508	10.741
Std. Dev.	0.347	1.106	10.263	9.067	7.372	40.077	16.108

CONCLUSIONS

This study aimed to better understand the structure and diversity of pomegranates in the Halabja region. Our multi-character approaches of genetic software analysis using phytochemical and molecular data highlight the similarities and dissimilarities between the studied pomegranate samples. We demonstrated the existence of high genetic diversity in the central region (Halabja) of pomegranate production. This result will help identify the pomegranates' ancestry in the Halabja region hereafter. Our results have not reached a specific conclusion, but various hypotheses can be put forward. One hypothesis would be that the Halabja region is one of the origins of the pomegranate. Another hypothesis would be that the Halabja region has a close border to Iran, and that pomegranates were introduced there long ago.

Advancing molecular studies in the future would require a complete genome sequence of pomegranates in the Halabja region. Alternatively, a genetic diversity study of some of our studied samples with other international cultivars, such as Tunisia, Dabenzi and Bhagwa, could assist in providing a more comprehensive understanding of the origins of pomegranates in our region.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received funding.

AUTHOR/S DECLARATION

We confirm that all Figures and Tables in the manuscript are original to us. Additionally, any Figures and images that do not belong to us have been incorporated with the required permissions for re-publication, which are included with the manuscript.

AUTHOR’S CONTRIBUTION STATEMENT

All authors contributed equally to all aspects of the study and manuscript preparation.

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التحليل الكيميائي و الوراثي الجزيئي للرمان (*Punica granatum* L) باستعمال واسمات SSR ذا تباينا كبيرا والكلوروبلاست

هيدي وشيار إبراهيم [1] ، عماد عمر حمه علي[2]

[1] قسم التقنية الحيوية و علوم المحاصيل الحقلية – كلية علوم الهندسة الزراعية – جامعة السليمانية

[2] قسم علم البيئة – كلية العلوم البيئية – جامعة السليمانية

المستخلص

الرمان من المحاصيل ذاتية التلقيح، وتعتمد برامج تحسينه على الوراثة الجزيئية والتنوع المظهري. ومع ذلك، فإن المعلومات غير الكافية حول التنوع الوراثي و البنية العشيرة بين الأنماط الجينية للرمان والاختلافات في المواد الكيميائية النباتية في هذه المنطقة تعيق تحسينها. في هذه الدراسة ، تم اختيار 40 عينة من الرمان من 11 موقعًا لتمثيل الانواع الجيني الموجودة في محافظة حلبجة. تم اختبار عينات الرمان باستخدام 23 البادئة SSR ذا تباينا كبيرا (HvSSRT) و19 البادئة الكلوروبلاست Chloroplast SSR (cpSSR) التي تم تطويرها حديثًا. أعلى قيم الأس الهيدروجيني كانت (4.40)، TSS(16.774 Brix°)، الكثافة (1066.5 كجم/م3)، الجلوكوز (72.408 مجم/مل)، الفركتوز (60.071 مجم/مل)، السيانيدين-3-جلوكوزيد (71.984 مجم/لتر) وتم تسجيل السيانيدين-3،5-ديجلوكوزيد (155.701 ملغم/لتر) بين عينات الرمان. أنتج التنميط الجيني لعينات الرمان 114 أليلًا البادئة SSR، وكان متوسط قيم MAF و دليل معلومات شانون (I) و Neiالتنوع الجيني ل HVSSRTs كانت 0.570 و0.915 و0.537 وبالنسبةل cpSSRs كانت 0.618 و0.668 و0.460. تراوح محتوى PIC من 0.129 إلى 0.723، و بمتوسط 0.467 لـ HVSSRTs و0.360 لـ cpSSRs. وأخيراً، تم تسجيل مستويات عالية من التنوع الوراثي (66%) بين الأفراد ضمن العشائر، مما يشير إلى أن رمان حلبجة متنوع.

الكلمات المفتاحية: انثوسيانين، كلوروبلاست، التنوع الجينى،الكروماتوغرافيا السائلة عالية الأداء، الرمان، الميكروساتلايت.