



Genetic Diversity and Population Structure Analysis of Drought Tolerance in Walnut (*Juglans regia* L.) Using SSR Markers

Ayda Taheri¹ · Nasrin Seyedi² · Babak Abdollahi Mandoulakani³ · Ghader Mirzaghaderi⁴ · Solmaz Najafi⁵ · Kourosh Vahdati⁶ · Müge Şahin⁷ · Harlene Hatterman-Valenti⁸ · Ozkan Kaya^{8,9}

Received: 13 November 2024 / Accepted: 22 October 2025 / Published online: 5 November 2025
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Abstract

Walnut biodiversity represents a critical genetic resource for developing climate-resilient cultivars capable of withstanding increasing drought stress under changing environmental conditions. Understanding the genetic basis of drought tolerance mechanisms is essential for sustainable nut production and conservation of valuable genetic resources. This study investigated the genetic and physiological basis of drought tolerance in walnut (*Juglans regia* L.) saplings, providing crucial insights for breeding programs aimed at developing climate-resilient cultivars. A comprehensive analysis was conducted using 65 walnut samples collected from diverse geographic locations, employing SSR (simple sequence repeat) markers for genetic characterization combined with detailed morphological and physiological assessments under controlled drought stress conditions. Our findings revealed significant variability in drought stress responses among walnut saplings. Morphological and physiological traits exhibited substantial variation under drought stress, reflecting the genetic diversity present in natural populations. Population structure analysis performed using STRUCTURE software identified three distinct genetic clusters within the studied population, with varying degrees of admixture indicating complex evolutionary relationships and gene flow patterns across different populations. Some individuals showed strong affiliation to a single cluster, while others exhibited significant admixture patterns. Mixed Linear Model analysis identified significant marker-trait associations between SSR markers and drought tolerance traits. Multiple markers showed strong correlations with physiological parameters including chlorophyll content, leaf characteristics, and stress response indicators. Hierarchical clustering and phylogenetic analyses confirmed the genetic relationships among populations and revealed patterns consistent with geographic distribution and environmental adaptation. This research elucidates the genetic architecture underlying drought tolerance in walnuts and demonstrates the effectiveness of SSR markers for identifying drought-responsive genes. The identified marker-trait associations provide valuable tools for marker-assisted selection in breeding programs. Furthermore, the observed genetic diversity and its correlation with drought-related traits offer a foundation for developing more resilient walnut cultivars. These findings contribute to the conservation and utilization of walnut genetic resources while supporting the development of climate-adaptive breeding strategies for sustainable nut production under water-limited conditions.

Keywords Association mapping · Cluster analysis · Mixed Linear Model · Zagros forests

✉ Nasrin Seyedi
n.seyedi@urmia.ac.ir

✉ Ozkan Kaya
ozkan.kaya@ndsu.edu; kayaozkan25@hotmail.com

¹ Faculty of Natural Resources, Urmia University, Urmia, Iran

² Department of Forestry, Faculty of Natural Resources, Urmia University, Urmia, Iran

³ Department of Plant Production and Genetics, Faculty of Agriculture, Urmia University, Urmia, Iran

⁴ Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

⁵ Department of Field Crops, Faculty of Agriculture, Van Yuzuncu Yil University, Van, Turkey

⁶ Department of Horticulture, College of Aburayhan, Tehran University, Tehran, Iran

⁷ Department of Horticulture, Faculty of Agriculture and Natural Sciences, Bilecik Şeyh Edebali University, Bilecik 11230, Turkey

⁸ Department of Plant Sciences, North Dakota State University, Fargo, ND 58102, USA

⁹ Republic of Turkey Ministry of Agriculture and Forestry, Erzincan Horticultural Research Institute, 24060 Erzincan, Turkey

Introduction

Walnut (*Juglans regia* L.), a member of the Juglandaceae family, is indigenous to the Zagros forests of Iran. This wind-pollinated, metamorphic species serves as a valuable rootstock (Shamlo et al. 2016). Within the Juglandaceae family, which comprises 60 species, the genus *Juglans* accounts for 20 species, with *Juglans regia* emerging as the most prominent (Ducci et al. 1997; McGranahan et al. 1998). The Persian walnut, a diploid species with 32 chromosomes, represents the most economically significant member of the genus *Juglans* (McGranahan et al. 1998). Global walnut production has shown significant growth in recent years, with China maintaining its dominant position as the world's largest producer. The increasing economic importance of walnut cultivation worldwide has intensified research efforts to understand genetic diversity patterns and stress tolerance mechanisms (Bernard et al. 2020). Climate variability and water stress have become major limiting factors for walnut production, particularly in semi-arid regions, necessitating the development of drought-tolerant cultivars (Arab et al. 2022). The cultivation of walnuts in arid and semi-arid regions necessitates a comprehensive understanding of drought tolerance mechanisms and the identification of resistant genotypes. Recent molecular studies have revealed that drought tolerance in walnuts involves complex regulatory networks, including transcription factors such as JrWRKY7 and JrERF2-2, which interact to regulate glutathione S-transferases (GSTs) in drought stress responses (Yang et al. 2021). Additionally, the JrMYB44 transcription factor has been identified as a key regulator for polyphenol accumulation and drought tolerance in *Juglans regia* (Gao et al. 2025). Genome-wide association studies have provided insights into the genetic basis of photosynthetic responses to drought stress, revealing candidate genes and pathways involved in walnut adaptation to water limitation (Arab et al. 2022). Research efforts have intensified to elucidate these mechanisms through molecular approaches (Du et al. 2011), with the goal of genetic engineering applications (Abebe et al. 2003). The development of high-quality draft genome sequences has facilitated the identification of genes involved in polyphenol biosynthesis and stress responses, providing valuable resources for molecular breeding programs (Martinez-Garcia et al. 2016).

The development of effective methods for identifying and selecting superior stress-resistant genotypes has become increasingly crucial. Association mapping, or association analysis, has emerged as a promising methodology that enables precise and reliable identification of genes and loci controlling quantitative traits. Recent genome-wide association studies (GWAS) in walnut have

successfully identified candidate genes involved in fruit quality traits and phenological characteristics using multi-locus models (Bernard et al. 2021; Marrano et al. 2020). These studies have demonstrated the effectiveness of both linkage mapping and association mapping approaches in unraveling the genetic architecture of complex traits in Persian walnut (Marrano et al. 2020). This approach circumvents the limitations of traditional QTL mapping by investigating genotype-phenotype correlations in unrelated individuals across large population sources, eliminating the need for dispersing population preparation (Ersoz and Bukler, 2007; Myles et al. 2009). Population structure analysis and consideration of kinship relationships are essential prerequisites for minimizing false associations in mapping studies (Yu and Buckler 2006; Yu et al. 2006). Two principal analytical methods have been developed: the General Linear Model (GLM), which incorporates population structure (Q matrix), and the Mixed Linear Model (MLM), which considers both population structure and kinship relationships (K matrix). The MLM approach has gained widespread adoption in plant research due to its superior ability to minimize false marker-trait associations (Ghavami et al. 2011). Multi-locus GWAS methods have shown improved performance in detecting small-effect loci and reducing false positive rates in walnut genetic studies (Bernard et al. 2021). These advanced statistical approaches have been successfully applied to identify QTLs for shell suture strength and other important traits in walnut breeding programs (Pham et al. 2020). This methodology has been successfully implemented across various plant species, including both agricultural crops and trees such as barley (Kraakman et al. 2006), corn (Andersen et al. 2007), wheat (Ghavami et al. 2011; Zhang et al. 2012), sunflower (Paknia et al. 2018; 2020), and various tree species including *Pinus taeda* (Chang et al. 1996), *Malus domestica* Borkh. (Cevik et al. 2009), and Eucalyptus species (Thumma et al. 2005). Recent applications in walnut have utilized the Axiom™ *J. regia* 700 K SNP array for high-density genotyping, enabling precise mapping of phenological traits and lateral bearing characteristics (Marrano et al. 2020). Simple Sequence Repeat (SSR) markers remain highly valuable tools for genetic diversity assessment in walnut populations despite the advent of SNP-based technologies. Recent studies have demonstrated the effectiveness of SSR markers in revealing genetic relationships and population structure in worldwide walnut germplasm collections (Bernard et al. 2020; Dang et al. 2025). Large-scale SSR-based studies have successfully characterized genetic diversity patterns across different geographic regions, with studies analyzing up to 253 worldwide accessions using 13 SSR markers revealing significant genetic differentiation among populations (Bernard et al. 2020). Additionally, genome-wide

SSR marker development has been facilitated by whole-genome sequencing, with recent studies identifying over 198,924 SSR loci and developing 110 polymorphic markers for genetic diversity analysis (Itoo et al. 2023). The combination of morphophysiological trait evaluation with SSR marker analysis has proven particularly effective for comprehensive genetic characterization of walnut germplasm (Dang et al. 2025).

The present investigation aims to advance our understanding of *Juglans regia* in the context of drought tolerance and genetic diversity, particularly given its significance in arid and semi-arid regions. Considering the species' economic importance as a major agricultural crop and its natural presence in the Zagros forests of Iran, this study pursues the following objectives: I) assess the genetic diversity of 65 *Juglans regia* seedlings using 20 SSR markers to identify valuable genotypes for breeding and conservation. II) characterize morphophysiological traits linked to drought tolerance, enhancing understanding of adaptive mechanisms under water stress. III) identify marker-trait associations for drought tolerance using association mapping, supporting marker-assisted selection. IV) apply findings to conservation and breeding strategies, emphasizing drought-resilient genotype identification and efficient genetic resource utilization.

Materials and Methods

Cultivation and Drought Stress

Sixty-five one-year-old *Juglans regia* L. seedlings were collected from natural populations in the Zagros Forest region (West Azerbaijan Province, Iran) and established in standardized growing conditions with three replications per treatment group. The drought stress treatment was implemented using tensiometric measurements to precisely monitor soil moisture conditions. All experiments were conducted in a controlled greenhouse environment maintained at 25 ± 2 °C with a 16:8 h light to dark photoperiod and 60–70% relative humidity. Soil water potential was quantified at various moisture contents, and a soil moisture characteristic curve was generated to determine the requisite water volumes for both field capacity and 50% moisture drainage conditions. The experimental specimens were maintained at 50% moisture content for a 30-day period under controlled conditions. Comprehensive physiological and morphological analyses were conducted on the experimental specimens. The physiological parameters evaluated included membrane stability through electrolyte leakage measurements and detailed photosynthetic pigment quantification, specifically analyzing Chlorophyll a, Chlorophyll b, total chlorophyll content, and carotenoid

levels. Additionally, proline content was determined as an indicator of stress response. Morphological characteristics were extensively assessed through multiple parameters: seedling vertical growth and collar diameter measurements were recorded to evaluate overall plant development; foliar measurements encompassed green leaf enumeration, leaf biomass (both fresh and dry weight determinations), leaf surface area, and leaf mass per unit area calculations. Furthermore, stomatal density assessment was performed to evaluate the adaptation of leaf morphological characteristics under drought conditions. Genomic DNA was isolated from all 65 saplings utilizing the CTAB extraction protocol (Doyle and Doyle 1990), followed by SSR marker analysis. Mixed Linear Model (MLM) analysis was employed to identify associations between molecular markers and the assessed morphophysiological traits. All molecular analyses were conducted at the Genomics Laboratory of the Biotechnology Research Institute, Urmia University.

Evaluation of Morphology and Physiology

Following one month of drought stress treatment, morphological and physiological parameters were quantified in all 65 experimental saplings. Each measurement was replicated three times per individual, and mean values were used for subsequent analyses. Morphological measurements were conducted using standardized protocols: seedling height was determined from soil surface to terminal bud utilizing a metric ruler (measured in cm), while seedling collar diameter was assessed using a Vernier caliper (measured in cm). Foliar measurements included fresh and dry weight determinations using a high-precision electronic balance (accuracy: 0.001 g). Leaf area was quantified through digital image analysis, whereby individual leaves were scanned and processed using ImageJ software (expressed in cm²). Leaf mass per area was calculated as the ratio of leaf dry weight to leaf area (expressed in g·m⁻²). Stomatal density was determined using light microscopy at 400x magnification (40x objective lens, 10x ocular lens), with field calibration performed using a graduated slide. Physiological parameters were assessed using established methodologies. Membrane integrity was evaluated through electrolyte leakage measurements using a Consort c533 model EC meter, following the protocol described by Dogan et al. (2022). Photosynthetic pigments, including chlorophyll and carotenoid content, were quantified according to the Lichtenthaler and Wellburn (1985) method. Free proline content in leaf tissue was determined following the methodology established by Bates et al. (1973).

Table 1 Indices related to genetic diversity for SSR primers in the studied walnut population

Primer sequence	Primer	Primer sequence	Primer
ACGACAACACTATCTTTGCTTCG GTTTGTAAAGACAGATGGGACAAG	JRHR217037	ATTGGAAGGGAAGGAAATG CGCGACATACGTAAATCAC	WGA1
ATCTCGTACAGTCTTGCTCTTGC TGGTGCTGTAGCCATCTATTTATC	JRHR207652	TGTTGCATTGACCCACTTGT TAAGCCAACATGGTATGCCA	WGA4
GGGGAGGCATCATCTTAGC GGGGAAAGAACTTAACCGTACAC	JRHR211298	CATCAAAGCAAGCAATGGG CCATTGCTCTGTGATTGGG	WGA9
ATTTCCAACCAGATGCCTAGC TCATGTGAGGTCGTTATTCCA	JRHR225189	TTAGTTAGCAAACCCACCCG AGATGCACAGACCAACCCTC	WGA69
ACAAGCACGGAAGGAATTTT AGCAAGACGGGAATAAATGGA	JRHR225564	ACCCGAGAGATTTCTGGGGAT GGACCCAGCTCCTCTTCTCT	WGA71
TGGTCATGGAATATTGAACC GAA TAGCGAGCAAGAGATATGGA	JRHR214591	ACCCATCTTTCACGTGTG TGCCTAATTAGCAATTTC	WGA89
AGGGACTACCTCTTGAAAACCTGG CTCAGTGCTAAATCAATCTGTCTC	JRHR212067	TGTGCTCTGATCTGCCTCCC GGGTGGGTGAAAAGTAGCA	WGA118
ATGGTCTCGCGCGTTTATATT ACAAGGACACGACCAATTATCAC	JRHR209244	CCATCTACCGTTGCACTTTT GCTGGTGGTTCTATCATGG	WGA202
CTTTTGGTGGGCTTTGTAAACT CTTTATATCGATCTCGGGCTTC	JRHR211717	CTCACTTCTCGGCTCTTCC GGTCTTATGTGGGCACTCGT	WGA276
AAAGAAACATGGAGCTAGGGTCT CTACCTCTACGGCTCTGGTGAAT	JRHR217272	TCCAATCGAAACTCCAAAGG TGTCCAAAGACGATGATGGA	WGA321

Microsatellite Markers

In this study, a set of 20 Simple Sequence Repeat (SSR) primer pairs, previously described by Woeste et al. (2002), Dang et al. (2015), and Eser et al. (2019), was utilized (Table 1). The selection of these markers was based on their proven polymorphism in walnut species, high reproducibility, and strategic distribution across different linkage groups to ensure comprehensive genome coverage for association mapping. The selection of markers was based on their distribution across different linkage groups in the walnut genetic map, as reported by Kefayati et al. (2019), to ensure broad genomic coverage. Polymerase chain reactions were performed following the protocol outlined by Dang et al. (2015) in a total volume of 20 μ L. Each reaction mixture contained 20 ng of genomic DNA, 1X PCR buffer, 25 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of Taq DNA polymerase, and 1 μ M of each forward and reverse primer. The thermal cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at the primer-specific temperature for 40 s, and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 7 min. The amplified PCR products were resolved by electrophoresis on 3% agarose gels at 80 V for 2–3 h. The gels were subsequently stained with ethidium bromide, and the DNA fragments were visualized and documented using a Gel Logic 212 Pro imaging system (Carestream, Bio-Rad, USA). This methodology allowed for the efficient amplification and detection of SSR markers, facilitating the genetic analysis of the walnut samples under investigation.

Statistical Analysis

The statistical analysis and genetic structure investigation of the study were conducted using a combination of software tools and methodologies. Descriptive statistics and correlation analyses among traits under drought stress conditions were performed using SPSS version 26 software, providing a comprehensive overview of the phenotypic data. Statistical significance was determined at $p < 0.05$ for all analyses. Population structure analysis was carried out using STRUCTURE software version 2.3.1 (Pritchard et al. 2000). The analysis employed 50,000 Markov Chain Monte Carlo (MCMC) repetitions and a burn-in period of 50,000 iterations in Admixture mode. The number of potential subpopulations (K) was evaluated over a range of 1 to 10, with 10 independent runs for each K value. This software was utilized to estimate the membership coefficient matrix (Q), which quantifies the proportion of each individual's genome originating from each inferred subpopulation. Additionally, the average fixation index (F_{ST}) was calculated for potential subgroups using the same software. The optimal number of subpopulations (K) was determined using the delta K method (Evanno et al. 2005), which evaluates the rate of change in the log probability of data between successive K values. For the optimal K value, the mean fixation index (F_{ST}) and the population structure coefficient matrix (Q) were computed. To identify markers associated with the studied traits under stress conditions, a Mixed Linear Model (MLM) approach was employed using TASSEL 3.0 software (Bradbury et al. 2007).

Table 2 Descriptive statistics for the evaluated traits under drought stress condition

Traits	Minimum	Maximum	Mean	Standard error	Standard deviation	Coefficient of variation (%)
Number of green leaves (<i>n</i>)	0	145	46.09	3.26	26.31	57.08
Height (cm)	23	101	62.26	1.94	15.66	25.15
Diameter of breast height (cm)	0.71	2.75	1.59	0.06	0.46	28.92
Leaf fresh weight (g)	0.07	0.60	0.33	0.02	0.14	43.87
Leaf dry weight (g)	0.03	0.29	0.13	0.01	0.07	50.46
Leaf area (cm ²)	5.72	62.90	29.05	1.79	14.42	49.63
Leaf mass area (g/m ²)	21.97	73.02	46.60	1.26	10.17	21.82
Stomatal density (n/mm ²)	144.49	535.80	287.68	11.50	92.69	32.22
Electrolyte leakage (%)	24.78	84.41	56.68	2.11	16.99	29.98
Proline (mg/gDW)	0.41	0.81	0.51	0.01	0.08	15.54
Chlorophyll a (mg/gFW)	2.91	23.42	12.07	0.60	4.85	40.23
Chlorophyll b (mg/gFW)	1.87	10.46	5.68	0.23	1.88	33.08
Carotenoids (mg/gFW)	2.17	8.77	4.96	0.18	1.47	29.70
Total chlorophyll (mg/gFW)	4.78	33.89	17.75	0.81	6.55	36.92

Results

Morphological and Physiological Trait Variation

The morphological and physiological traits measured in walnut trees under drought stress conditions showed substantial variation (Table 2). The number of green leaves ranged from 0 to 145, with a mean of 46.09 ± 3.26 and the highest coefficient of variation (CV) at 57.08%. Plant height varied from 23 to 101 cm, averaging 62.26 ± 1.94 cm with a CV of 25.15%. Diameter at breast height ranged from 0.71 to 2.75 cm, averaging 1.59 ± 0.06 cm with a CV of 28.92%. Leaf fresh weight averaged 0.33 ± 0.02 g and dry weight averaged 0.13 ± 0.01 g, with CVs of 43.87% and 50.46%, respectively. Leaf area ranged from 5.72 to 62.90 cm² with a mean of 29.05 ± 1.79 cm² and CV of 49.63%. Leaf mass area averaged 46.60 ± 1.26 g/m² with a CV of 21.82%. Stomatal density ranged from 144.49 to 535.80 stomata/mm² with a mean of 287.68 ± 11.50 stomata/mm² and CV of 32.22%. Electrolyte leakage averaged $56.68 \pm 2.11\%$, ranging from 24.78 to 84.41%. Proline content showed a mean of 0.51 ± 0.01 mg/g DW with a CV of 15.54%. Chlorophyll a ranged from 2.91 to 23.42 mg/g FW (mean 12.07 ± 0.60 mg/g FW), chlorophyll b from 1.87 to 10.46 mg/g FW (mean 5.68 ± 0.23 mg/g FW), and carotenoids from 2.17 to 8.77 mg/g FW (mean 4.96 ± 0.18 mg/g FW). Total chlorophyll content averaged 17.75 ± 0.81 mg/g FW.

Correlation Analysis of Drought Stress Traits

Correlation analysis revealed significant relationships between traits under drought stress conditions (Fig. 1).

Number of green leaves correlated positively with diameter at breast height ($r = 0.425$, $p < 0.01$) and negatively with leaf area ($r = -0.401$, $p < 0.01$). Height correlated positively with leaf fresh weight ($r = 0.329$, $p < 0.01$), leaf dry weight ($r = 0.330$, $p < 0.01$), and leaf mass area ($r = 0.319$, $p < 0.01$). Leaf fresh weight and leaf dry weight showed a strong positive correlation ($r = 0.938$, $p < 0.01$). Both correlated positively with leaf area ($r = 0.874$ and $r = 0.902$ respectively, $p < 0.01$) and negatively with electrolyte leakage ($r = -0.608$ and $r = -0.611$ respectively, $p < 0.01$). Leaf area correlated negatively with carotenoids ($r = -0.332$, $p < 0.01$) and positively with stomatal density ($r = 0.411$, $p < 0.01$). Leaf mass area correlated positively with stomatal density ($r = 0.479$, $p < 0.01$) and carotenoids ($r = 0.301$, $p < 0.05$). Stomatal density correlated positively with electrolyte leakage ($r = 0.389$, $p < 0.01$), chlorophyll a ($r = 0.365$, $p < 0.01$), chlorophyll b ($r = 0.470$, $p < 0.01$), carotenoids ($r = 0.455$, $p < 0.01$), and total chlorophyll ($r = 0.405$, $p < 0.01$). Electrolyte leakage correlated positively with chlorophyll b ($r = 0.269$, $p < 0.01$) and carotenoids ($r = 0.382$, $p < 0.01$). Strong positive correlations existed among photosynthetic pigments: chlorophyll a with chlorophyll b ($r = 0.867$, $p < 0.01$) and carotenoids ($r = 0.729$, $p < 0.01$); chlorophyll b with carotenoids ($r = 0.846$, $p < 0.01$); and total chlorophyll with chlorophyll a ($r = 0.990$, $p < 0.01$), chlorophyll b ($r = 0.929$, $p < 0.01$), and carotenoids ($r = 0.783$, $p < 0.01$). Proline showed no significant correlations with measured traits.

Population Genetic Structure and Geographic Distribution

Structure analysis of 65 walnut samples from different geographic locations (Table 3) identified three distinct genetic

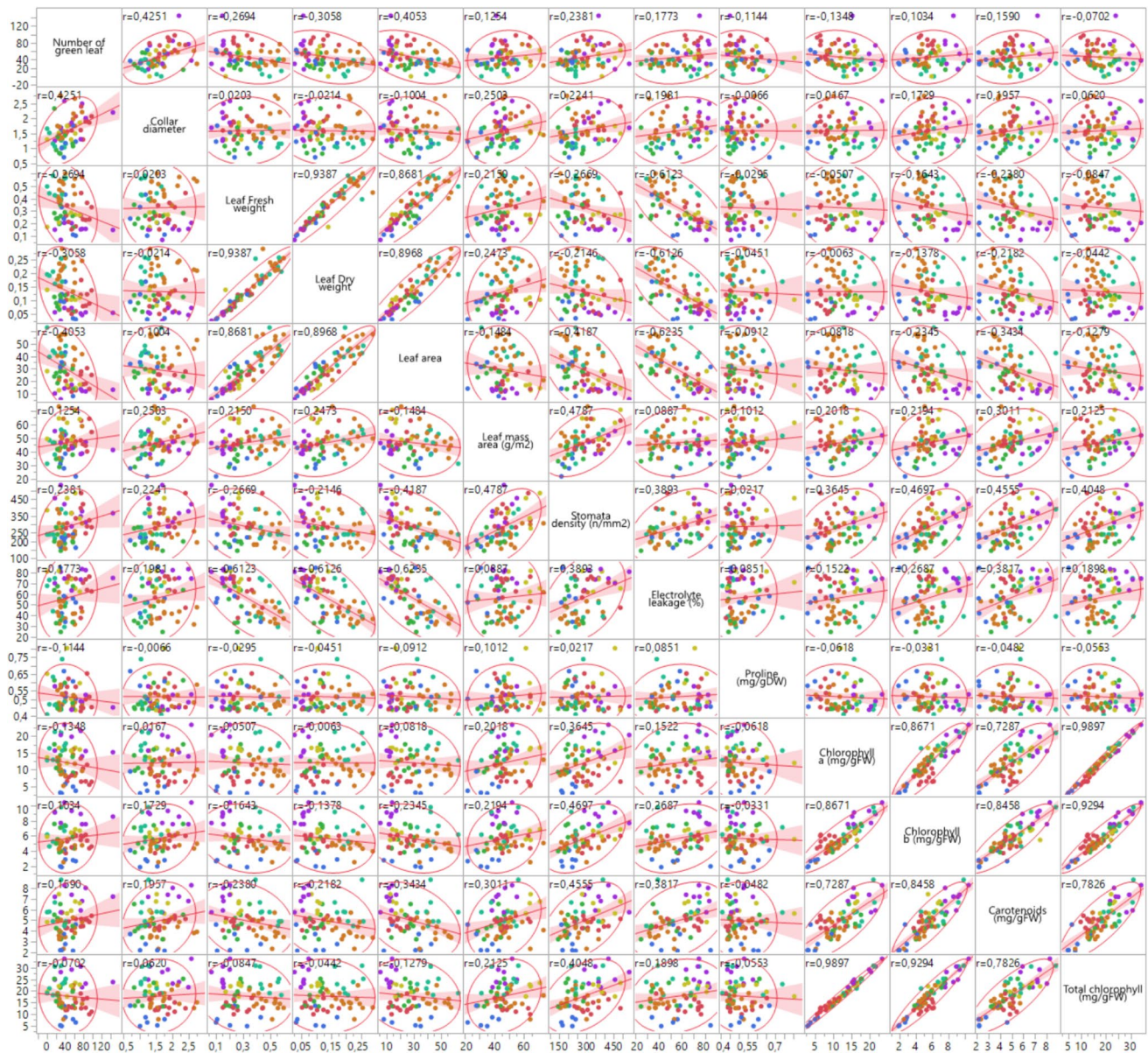


Fig. 1 Correlation matrix showing relationships between various physiological and morphological traits in walnut (*Juglans regia* L.) saplings under drought stress conditions. The scatter plots display pairwise correlations between measurements including number of green leaves, collar diameter, leaf fresh weight, leaf dry weight, leaf

area, leaf mass area, stomata density, electrolyte leakage, proline content, and chlorophyll parameters. Each plot includes correlation coefficients (r values) and trend lines highlighting the strength and direction of relationships between traits

clusters ($K=3$) with varying degrees of admixture. The Q matrix revealed samples W1, W7, and W16 with strong affiliation to cluster $Q1$ (>0.95), W40 and W41 to cluster $Q2$ (>0.90), and W55, W57, and W60 to cluster $Q3$ (>0.95). Sample W23 showed equal proportions from all clusters ($Q1=0.30$, $Q2=0.40$, $Q3=0.30$). Geographic analysis revealed cluster $Q1$ dominated samples W1–W18 from northern provinces (elevation >1200 m), cluster $Q2$ was prominent in samples W31–W53 from central regions (600–1200 m elevation), and cluster $Q3$ prevailed in samples W54–W65 from

southern locations (<600 m elevation). Admixed individuals W5 ($Q1=0.57$, $Q3=0.41$) and W21 ($Q1=0.42$, $Q3=0.37$) originated from transitional elevation zones between major geographic regions (Table 3).

Population Structure Visualization and Geographic Correlation

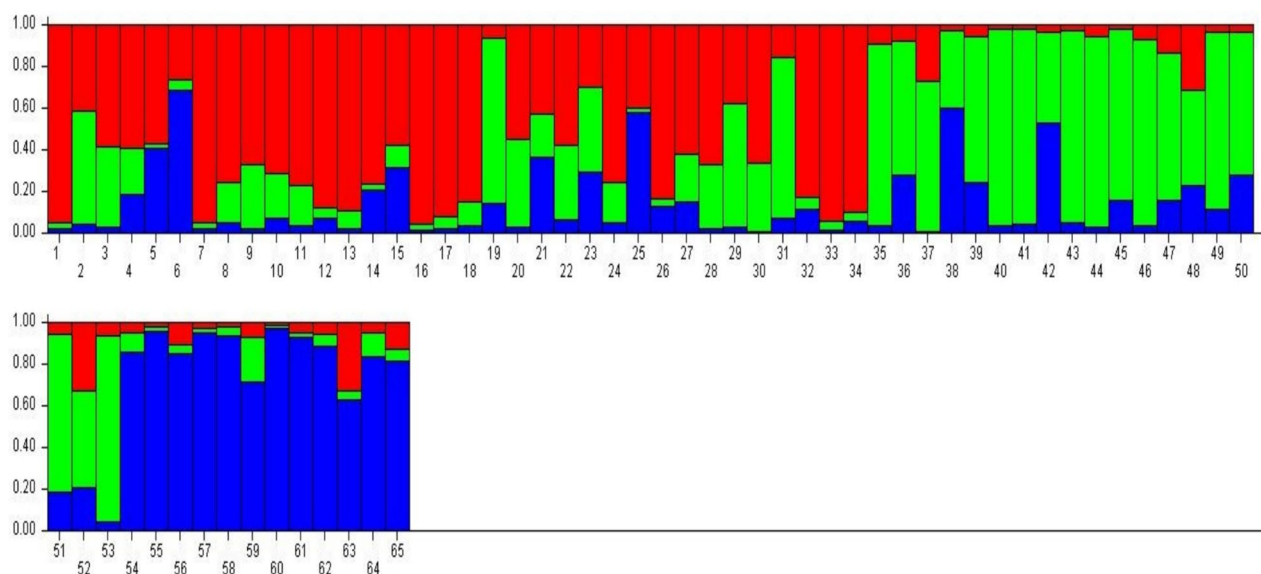
Population structure visualization revealed complex genetic composition across 65 samples with three distinct clusters

Table 3 Membership share matrix (Q matrix) calculated by Structure 2.3.1 software based on $K=3$

Sample	Q_1	Q_2	Q_3
W1	0.95	0.03	0.02
W2	0.41	0.55	0.05
W3	0.59	0.38	0.03
W4	0.59	0.22	0.19
W5	0.57	0.02	0.41
W6	0.26	0.05	0.69
W7	0.95	0.03	0.02
W8	0.76	0.19	0.05
W9	0.67	0.31	0.02
W10	0.71	0.22	0.07
W11	0.77	0.20	0.04
W12	0.88	0.05	0.08
W13	0.89	0.08	0.03
W14	0.76	0.03	0.21
W15	0.58	0.11	0.32
W16	0.95	0.03	0.02
W17	0.92	0.06	0.03
W18	0.85	0.11	0.04
W19	0.06	0.80	0.15
W20	0.54	0.43	0.03
W21	0.42	0.21	0.37
W22	0.57	0.36	0.07
W23	0.30	0.40	0.30
W24	0.75	0.20	0.05
W25	0.39	0.02	0.59
W26	0.84	0.04	0.13
W27	0.62	0.23	0.15
W28	0.67	0.31	0.03
W29	0.37	0.60	0.03
W30	0.66	0.32	0.01
W31	0.15	0.77	0.08
W32	0.83	0.05	0.12
W33	0.94	0.04	0.02
W34	0.90	0.04	0.06
W35	0.09	0.87	0.04
W36	0.08	0.64	0.28
W37	0.27	0.72	0.01
W38	0.02	0.38	0.60
W39	0.06	0.70	0.25
W40	0.02	0.94	0.04
W41	0.02	0.93	0.05
W42	0.03	0.44	0.53
W43	0.02	0.92	0.05
W44	0.06	0.91	0.03
W45	0.02	0.83	0.16
W46	0.07	0.90	0.04
W47	0.13	0.71	0.16
W48	0.31	0.46	0.24
W49	0.03	0.85	0.12
W50	0.03	0.69	0.28
W51	0.05	0.76	0.19

Table 3 (continued)

Sample	Q_1	Q_2	Q_3
W52	0.33	0.47	0.21
W53	0.06	0.89	0.05
W54	0.05	0.10	0.86
W55	0.02	0.02	0.96
W56	0.11	0.04	0.86
W57	0.02	0.02	0.96
W58	0.02	0.04	0.94
W59	0.07	0.22	0.72
W60	0.01	0.01	0.98
W61	0.05	0.02	0.93
W62	0.05	0.06	0.89
W63	0.33	0.04	0.63
W64	0.05	0.11	0.84
W65	0.13	0.05	0.82

**Fig. 2** The bar plot was drawn by Structure 2.3.1 software, based on data from 20 SSR locations, showing the horizontal axis of the saplings and the vertical axis of each seedling's share of each group

represented by red, green, and blue colors (Fig. 2). Samples 1–33 from high elevation regions (> 1200 m) showed red cluster predominance. Samples 1, 7, and 16 displayed near-complete membership to this cluster, while samples 6, 15, and 21 exhibited admixtures with other clusters. Samples 34–50 from mid-elevation regions (600–1200 m) demonstrated green cluster dominance. Samples 40, 41, and 43 showed strong affiliation with this cluster, while samples 36, 38, and 42 presented notable admixture. Samples 51–65 from lower elevation areas (< 600 m) marked blue cluster dominance. Samples 55, 57, 60, and 61 displayed high membership coefficients for this cluster. Highly admixed individuals (samples 23, 48, 52) originated from contact zones

between different populations. Mantel test confirmed isolation by distance patterns ($r=0.67$, $p<0.001$).

SSR Marker-Trait Associations

Mixed Linear Model analysis identified significant associations between SSR markers and drought stress traits (Table 4). The number of green leaves associated with JRHR211717 on linkage group 12 ($R^2=0.39$, $P=0.01$) and JRHR217272 on linkage group 14 ($R^2=0.41$, $P=0.02$). Beneficial alleles for leaf retention showed higher frequency in populations from water-stressed environments. Chlorophyll a content associated with JRHR217037 ($R^2=0.52$, $P=0.00$)

Table 4 SSR markers related to studied traits under drought stress conditions in walnut saplings in MLM mode

Trait	Marker	Linkage group	R^2	P -value
Number of green leaves	JRHR211717	LG12	0.39	0.01*
	JRHR217272	LG14	0.41	0.02*
Chlorophyll a	JRHR217037	LG1	0.52	0.00*
	JRHR209244		0.32	0.03*
Chlorophyll b	JRHR217037	LG1	0.61	0.00*
Total chlorophyll	JRHR217037	LG1	0.56	0.00*
	JRHR209244	LG10	0.31	0.04*
Height	JRHR209244	LG10	0.31	0.03*
Diameter of breast height	WGA202		0.37	0.04*
Leaf fresh weight	WGA4	LG14	0.20	0.03*
	WGA118		0.36	0.05*
	JRHR217272		0.38	0.04*
Leaf fresh weight	WGA4		0.26	0.01*
	WGA118		0.42	0.01*
Leaf area	WGA4		0.19	0.03*
	WGA118		0.36	0.03*
Leaf mass area	JRHR214591	LG6	0.34	0.02*
	JRHR217272	LG14	0.37	0.03*
%Electrolyte leakage	WGA4		0.18	0.03*
	WGA118		0.41	0.01*

and JRHR209244 ($R^2=0.32$, $P=0.03$) on linkage group 1. Chlorophyll b content associated with JRHR217037 on linkage group 1 ($R^2=0.61$, $P=0.00$). Total chlorophyll content linked to JRHR217037 on linkage group 1 ($R^2=0.56$, $P=0.00$) and JRHR209244 on linkage group 10 ($R^2=0.31$, $P=0.04$). Favorable chlorophyll alleles were more prevalent in populations from regions with intense solar radiation. Plant height associated with JRHR209244 on linkage group 10 ($R^2=0.31$, $P=0.03$). Diameter at breast height associated with WGA202 ($R^2=0.37$, $P=0.04$). Growth-related markers showed differential allele frequencies across geographic gradients. Leaf fresh weight associated with WGA4 ($R^2=0.20$, $P=0.03$), WGA118 ($R^2=0.36$, $P=0.05$), and JRHR217272 on linkage group 14 ($R^2=0.38$, $P=0.04$). Leaf dry weight associated with WGA4 ($R^2=0.26$, $P=0.01$) and WGA118 ($R^2=0.42$, $P=0.01$). Leaf area linked to WGA4 ($R^2=0.19$, $P=0.03$) and WGA118 ($R^2=0.36$, $P=0.03$). Leaf mass area associated with JRHR214591 on linkage group 6 ($R^2=0.34$, $P=0.02$) and JRHR217272 on linkage group 14 ($R^2=0.37$, $P=0.03$). Electrolyte leakage associated with WGA4 ($R^2=0.18$, $P=0.03$) and WGA118 ($R^2=0.41$, $P=0.01$). Populations from arid regions showed higher frequencies of stress-tolerance alleles.

Hierarchical Clustering and Phylogenetic Analysis

Hierarchical clustering analysis revealed seven major clusters differentiated by phenotypic characteristics and

physiological responses (Fig. 3). Clustering patterns reflected both genetic relationships and geographic origins. The first cluster (genotypes 1, 27, 58, and 72) exhibited strong positive correlations with leaf mass and chlorophyll content parameters. These genotypes originated from high-altitude regions with intense solar radiation. The second cluster (genotypes 123, 122, and 126) displayed moderate to high correlations with physiological traits. This cluster contained genotypes from intermediate elevation zones. The third cluster (genotypes 108, 105, and 111) showed unique patterns in leaf characteristics and electrolyte leakage parameters. These genotypes were collected from areas with variable water availability. The orange cluster (genotypes 136, 131, and 107) from semi-arid regions showed specialized water-use efficiency adaptations. The purple cluster (genotypes 28, 89, 90, and 70) exhibited variation in stress tolerance indicators. This diversity reflected heterogeneous environmental conditions in their collection regions. The yellow cluster (genotypes 25, 43, 14, and 109) formed a separate clade with unique trait combinations. Geographic isolation of these populations led to unique adaptive strategies.

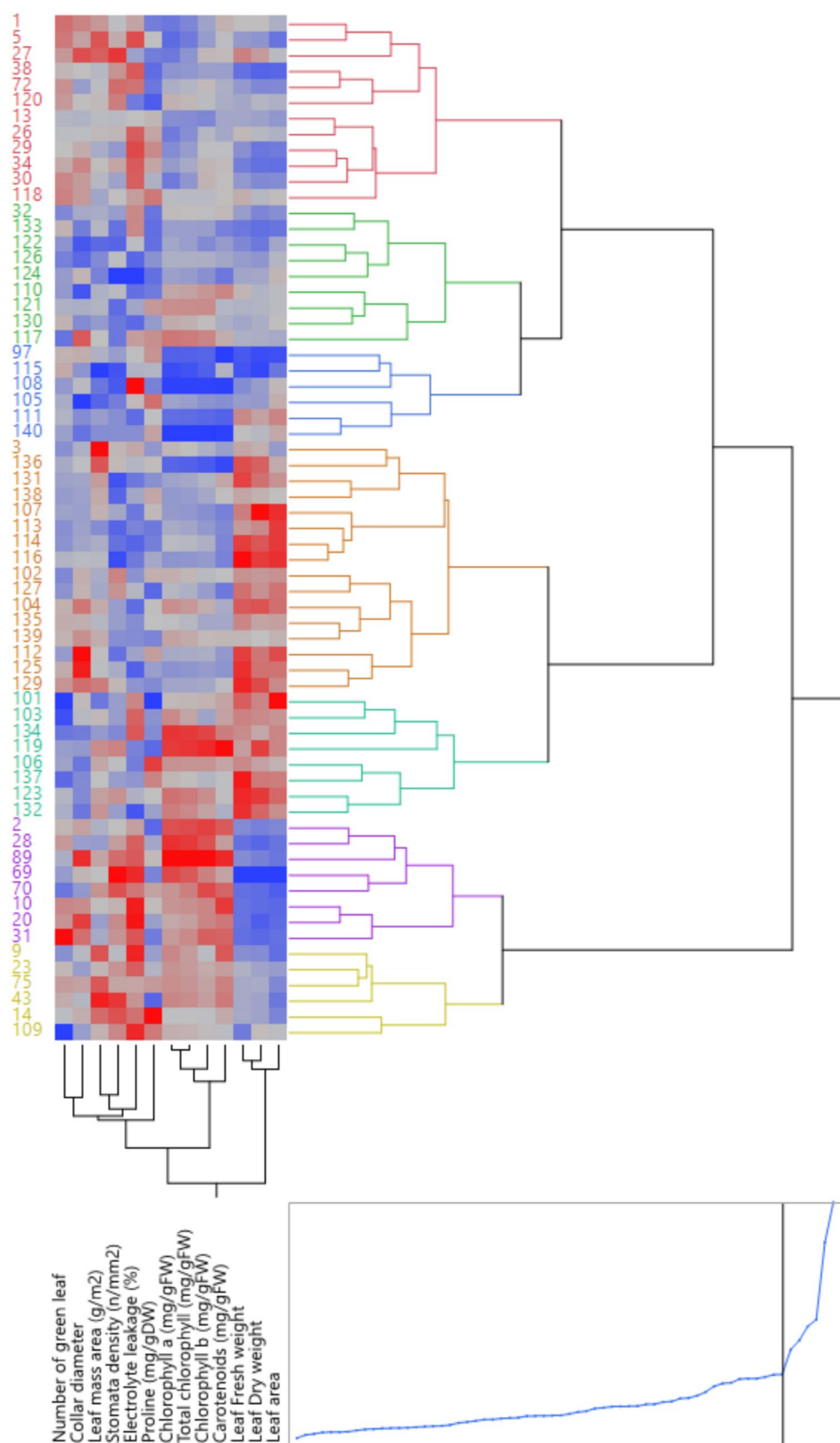
Phylogenetic Relationships and Geographic Patterns

Radial phylogenetic tree analysis revealed genetic relationships and evolutionary patterns among genotypes (Fig. 4). Phylogenetic structure reflected geographic distribution patterns and isolation by distance. The analyzed accessions formed distinct monophyletic clades in a circular arrangement. The purple clade (genotypes 28, 40, and 69) originated from northwestern mountain ranges. The red cluster (genotypes 1, 5, 58, and 72) represented populations from the central plateau region. The green cluster (genotypes 122, 124, 1 and 30) corresponded to coastal region populations, while the blue group (genotypes 97, 105, and 111) represented inland valley genotypes. The orange/brown cluster (genotypes 107, 114, and 136) and yellow cluster (genotypes 14, 75, and 109) represented populations from ecologically unique areas with distinct genetic heritage.

Discussion

Our findings revealed substantial phenotypic variability within the walnut population under drought stress conditions, particularly in leaf characteristics and physiological responses (Table 2). These results demonstrate the potential for genetic improvement through selective breeding programs targeting enhanced drought tolerance in walnut cultivation. The high variability observed in the number of green leaves ($CV=57.08\%$) suggests differential leaf senescence

Fig. 3 Hierarchical clustering analysis of physiological and morphological traits in walnut (*Juglans regia* L.) saplings under drought stress



Electrolyte leakage, an indicator of membrane integrity and cellular damage under stress, showed considerable variation among individuals. This aligns with the findings of Lotfi et al. (2010), who reported genotype-dependent differences in membrane stability under drought stress in walnut. The wide range of electrolyte leakage values (24.78 to 84.41%) clearly demonstrates varying degrees of cellular protection mechanisms, with some genotypes possessing inherently superior membrane stabilization systems. Interestingly, proline content showed the lowest variability (CV = 15.54%) among the measured traits. Proline accumulation is a well-documented response to drought stress, serving as an osmolyte and protective compound (Szabados and Savouré, 2010). The relatively uniform proline response across the population suggests a conserved biochemical adaptation mechanism that has been evolutionarily maintained in walnut species. However, it is important to note

that the absolute levels of proline accumulation, rather than the variability, may be more indicative of stress tolerance. The variability patterns observed in photosynthetic pigments (chlorophyll a, b, and carotenoids) indicate genotype-specific responses of the photosynthetic apparatus to drought stress conditions. This is consistent with the findings of Chakhchar et al. (2015), who reported genotype-specific alterations in photosynthetic pigments under water stress in another tree species. The maintenance of elevated chlorophyll content under drought conditions appears to be a key indicator of stress tolerance and sustained photosynthetic function. The correlation analysis revealed intricate relationships among morphological and physiological parameters, providing mechanistic insights into drought response strategies (Table 3). The moderate positive correlation between the number of green leaves and diameter at breast height ($r=0.425$, $p<0.01$) suggested that larger trees tend to retain more leaves under drought stress. This relationship indicates that tree size may serve as a predictor of drought resilience, potentially reflecting enhanced resource allocation capacity in more established individuals, aligning with findings by Ebrahimi et al. (2018) in Persian walnut. However, the negative correlation between the number of green leaves and leaf area ($r=-0.401$, $p<0.01$) implies a potential trade-off between leaf number and size, possibly as a drought avoidance strategy to reduce overall transpirational surface area.

The positive correlations between tree height and leaf parameters suggest that vertical growth confers advantages in maintaining leaf function under water stress conditions. This could indicate better access to water resources or more efficient water use in taller individuals, as observed by Vahdati et al. (2009) in walnut saplings. The strong positive correlations among leaf fresh weight, dry weight, and area, coupled with their negative correlations with electrolyte leakage, demonstrate that genotypes maintaining larger, more hydrated leaves also exhibit enhanced cellular protection mechanisms. This aligns with the findings of Lotfi et al. (2010), who reported that drought-tolerant walnut genotypes maintained better leaf water status and membrane integrity under water deficit conditions. The negative correlation between leaf area and carotenoids ($r=-0.332$, $p<0.01$) suggests a compensatory mechanism whereby genotypes with reduced leaf area invest more heavily in photoprotective compounds. This adaptive response has been observed in other woody species under drought stress (Chakhchar et al. 2015). The complex relationships between stomatal density and physiological parameters reveal trade-offs between gas exchange capacity and water conservation strategies. While higher stomatal density might allow for better gas exchange and potentially higher photosynthetic rates, it also correlates with increased electrolyte leakage, indicating potential costs associated with maintaining high stomatal densities under drought conditions. Interestingly, proline showed no

significant correlations with other measured traits, suggesting that osmoregulation mechanisms operate independently of morphological adaptations in this walnut population. This lack of correlation does not necessarily negate proline's role in drought tolerance but might indicate that its accumulation is independent of other measured parameters. Szabados and Savouré (2010) have noted that while proline accumulation is a common drought response, its relationship with stress tolerance can be complex and species-specific.

The genetic structure analysis revealed three distinct clusters ($Q1$, $Q2$, and $Q3$), indicating substantial genetic diversity and historical demographic complexity within the walnut population. The identification of individuals with strong affiliations to single clusters suggested the presence of relatively pure genetic lineages, aligning with the work of Pollegioni et al. (2017), who observed similar patterns of genetic differentiation in walnut populations across Eurasia. The notable admixture observed in certain individuals provides evidence of ongoing gene flow between genetically distinct groups, contributing to the population's adaptive potential. This admixture pattern was reminiscent of findings reported by Aradhya et al. (2017), who documented extensive hybridization in walnut populations. The Mixed Linear Model analysis identified multiple SSR markers significantly associated with drought stress-related traits, providing valuable targets for marker-assisted selection programs. The identification of markers associated with chlorophyll content (JRHR217037 and JRHR209244) is particularly noteworthy, as these loci may play crucial roles in maintaining photosynthetic capacity under drought stress. The marker JRHR217272 showed pleiotropic effects on multiple traits, suggesting that this genomic region harbors genes involved in coordinating overall plant responses to water limitation. This finding aligns with the work of Bartholomé et al. (2013), who observed similar pleiotropic effects in maritime pine. The hierarchical clustering analysis revealed seven distinct clusters with unique physiological profiles, demonstrating the complex genetic architecture underlying drought tolerance in walnut. The clear separation of monophyletic clades in the phylogenetic analysis suggests historical isolation events and adaptation to specific environmental conditions, supporting observations by Aradhya et al. (2017) regarding genetic diversity patterns in walnut populations. These findings collectively indicate that the studied walnut population harbors significant genetic resources that could be strategically utilized in breeding programs aimed at developing drought-tolerant cultivars for sustainable walnut production under changing climatic conditions.

Conclusion

This investigation successfully elucidated the genetic and physiological mechanisms underlying drought tolerance in walnut (*Juglans regia* L.), providing a foundation for

climate-adaptive breeding strategies. Our study demonstrated significant phenotypic variability under drought stress, with high coefficients of variation for leaf characteristics and biomass parameters. These findings establish clear targets for breeding programs aimed at improving drought resilience. The correlation analysis revealed coordinated trait networks defining drought response mechanisms, while population structure analysis identified three distinct genetic clusters with varying admixture patterns. This genetic architecture represents valuable resources for crop improvement initiatives and explains the observed phenotypic diversity. The Mixed Linear Model analysis identified multiple SSR markers significantly associated with drought-related traits, establishing the first comprehensive marker-trait association map for walnut drought tolerance. The identified markers, particularly those linked to chlorophyll content and leaf morphology, offer immediate applications in marker-assisted selection programs. Future research should focus on validating these associations across diverse environments and genetic backgrounds. Functional studies of identified genomic regions and advanced approaches including genome-wide association studies would enhance understanding of drought adaptation mechanisms. This research provides a comprehensive framework for developing climate-resilient walnut cultivars under increasingly challenging environmental conditions. The integration of genetic diversity assessment, physiological evaluation, and molecular marker development offers practical solutions for maintaining walnut productivity. These findings have immediate applications for breeding programs worldwide, contributing to sustainable nut production strategies in the face of climate change and water scarcity challenges.

Acknowledgements The authors hereby consider it necessary to express their sincere gratitude to the Biotechnology Research Institute of Urmia University, which provided the necessary facilities for this research.

Author Contributions Author Contributions: A.T., N.S., B.A.M., G.M., S.N., K.V., M.S., H.H.V and O.K conceived the study and designed the experiments. B.A.M., G.M., S.N., K.V., M.S and O.K performed data collection and analysis. N.S and O.K contributed to the manuscript writing and revisions. All authors have read and approved the final manuscript.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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