Utilizing SSR Markers to Examine the Population Structure and Molecular Genetic Diversity of Walnut (*Juglans regia* L.) Genotypes in the Northwestern Himalayan Region of Jammu and Kashmir

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By using 16 simple sequence repeat (SSR) markers, the genetic relatedness of 21 exceptional walnut genotypes was assessed. A significant degree of genetic diversity was observed within a given population, as indicated by the number of alleles per locus ranging from 2 to 4. WGA-1, WGA-4, and WGA-79 contained the greatest number of alleles (4), followed by WGA-118, WGA-202, and WGA-42. Conversely, WGA-27, WGA-69, and WGA-32 contained the fewest alleles. The range of the PIC value was 0.11 to 0.38. Using model-based cluster analysis, all genotypes were categorized into two primary clusters according to the UPGMA dendrogram, with varying degrees of sub-clustering. All the genotypes were categorized into six genetically distant subpopulations. The genotypes were genetically distinct but had variable degrees of admixture. The anticipated heterozygosity at a specific locus ranged from 0.563 to 0.741. Additionally, population differentiation (Fst) ranged between 0.176 and 0.261. These findings highlight the importance of considering germplasm diversity in walnut breeding programs and conservation efforts aimed at enhancing walnut cultivation in the region. Overall, this study contributes to our understanding of walnut genetic diversity in the Northwestern Himalayan region of Jammu and Kashmir and informs future breeding and conservation strategies.

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INTRODUCTION

Walnut (*Juglans regia* L.), with 2n = 2x = 32 sporophytic chromosomes, is classified as a member of the Juglandaceae family. It is referred to as "Jupiter's royal acorn" and in Kashmiri as "Doan." *Juglans* comprise an estimated 20 species, including heartnut (*J. ailantifolia* var. *cordiformis*), eastern black walnut (*J. cinerea*), and butternut (*J. cinerea*). The reproductive characteristics of walnuts have contributed to their substantial genetic variability over an extended period in a complex environment (Wu *et al.* 2000; Yang and Xue 2005).

The extent to which diverse and distinct walnut germplasm is available globally remains unknown. Utilizing state-of-the-art technology is imperative to ascertain evolutionary connections and genetic diversity among identified species, thereby fully harnessing the immense potential of walnut diversity. Furthermore, genetic diversity within or between species could be ascertained by employing molecular and biochemical markers to genetically characterize walnut varieties, in addition to visual identification (Cervera *et al.* 2000).

Molecular markers, such as RAPDs (Randomly Amplified Polymorphic DNAs), RFLPs (Restriction Fragment Length Polymorphisms), ISSRs (Inter Simple Sequence Repeats) (Potter *et al*. 2002), and SSRs (Simple Sequence Repeats) (Victory *et al*. 2006), have emerged as indispensable tools in the characterization of walnuts. These markers offer a comprehensive approach to understanding the genetic diversity and population dynamics of this valuable tree species. In contrast to alternative marker types, SSR markers possess the ability to discern species genetics unaffected by environmental factors. In this regard, they have been utilized to examine genetic relationships among walnut germplasm and have been validated as the most effective (Dangl *et al*. 2005; Di *et al*. 2006; Bernard *et al*. 2018; Torokeldiev *et al.* 2018; Yuan *et al.* 2018; Balapanov *et al.* 2019; Lorenzo and Michelangelo 2020). Microsatellites have exhibited considerable ubiquity and interspecies transferability in the genomes of *Juglans* (Ebrahimi *et al.* 2016). Ahmed *et al.* (2012) observed that walnut populations in India exhibited considerable genetic heterogeneity, as determined by morphological, RAPD, and SSR markers.

SSR markers are increasingly recognized and utilized in the molecular characterization of various plant species (Litt and Luty 1989; Gupta and Varshney 2000). Cervera *et al.* (2000) define SSRs as a compilation of short motifs varying in length from 1 to 6 base pairs. Multiallelism, codominance, repeatability, and adequate genome coverage have emerged as potent instruments for integrating sequence-based physical mapping with physiological and genetic mapping in plants (Jacobs *et al.* 2006). The utilization of SSR markers in combination with RAPD markers has been demonstrated to be an effective method for evaluating molecular diversity and genetic relationships between various crop species. Prominent inheritance, ease of PCR detection, high polymorphism, and repeatability are all attributes that contribute to the high resolution that these markers offer for genetic investigations (Gupta and Varshney 2000). These markers have evolved into highly versatile tools that are commonly employed as genetic markers across various plant species. Markers with these characteristics—codominant, hypervariable, and multiallelic—are favored for use in conservation genetics, plant breeding, fingerprinting, and phylogenetic analyses. SSRs are tandemly repeated DNA sequences ranging from two to six nucleotides. Karimi *et al.* (2010) found its widespread application in breeding programs, and evolutionary and conservation biology. Multiple studies have identified SSRs in plants and other eukaryotic taxa, providing evidence of their utility and application (Mahmoodi *et al.* 2013).

With an emphasis on precision and reliability, microsatellite markers have become instrumental in elucidating genetic diversity. Notably, these markers have unveiled a remarkable aspect of walnuts, showcasing the highest level of polymorphism at an impressive rate of 89.6% (Hamza *et al.* 2004; Karimi *et al.* 2010; Ahmed *et al.* 2012; Salieh *et al.* 2013). Ahmed *et al.* (2012) identified, on average, two polymorphic SSR loci per SSR primer using 13 SSR primers. The walnut vegetation in the Kashmir region consists primarily of fortuitous seedlings, and to date, no characterization has been conducted on walnut plants originating from the study areas. Utilizing the information and efficacy

generated by microsatellite-based markers, the objective of this study was to assess the genetic population, genetic connections, and genotype identity of 21 walnut cultivars sourced from the north-west Himalayas in the Jammu and Kashmir region. Specifically focusing on *Juglans regia* L., commonly known as Persian walnut or English walnut, the aim was to elucidate the population structure and molecular genetic diversity of these walnut genotypes, providing insights into their genetic relationships and diversity within the region.

EXPERIMENTAL

Sample Collections of Leaves

Twenty-one walnut genotypes, aged between 50 and 100 years, were selected from the Ganderbal and Budgam valleys located in the Kashmiri district of Ganderbal for inclusion in this study. Leaf samples were collected from each genotype at a consistent height of 1.5 meters above ground level to ensure uniformity in sampling across all genotypes. These samples were subsequently analyzed at the Virus Indexing and Molecular Laboratory, CITH, Srinagar, and the Division of Biotechnology, SKUAST-Kashmir. Care was taken to avoid sampling from branches exhibiting visible signs of disease or damage, as these may introduce confounding factors into the genetic analysis. Samples were collected from multiple trees per genotype to account for within-genotype variability. All sampling procedures were conducted in accordance with established guidelines for genetic sampling to ensure the reliability and accuracy of the results.

DNA Extraction

Through employing the methods described by Doyle and Doyle (1990), genomic DNA with minor modifications was isolated. The leaf tissues (young leaf samples from 21 genotypes) were reduced to fine powder in a sterile mortar and pestle containing liquid nitrogen. Pre-warmed 2 X CTAB extraction buffer (65 °C) was mixed with 2.5 g of powdered leaf tissue powder in a sterile 50-mL polypropylene DNA extraction vial. The materials were thoroughly combined with the extraction buffer through multiple inversions of the tubes. Subsequently, the tubes were incubated at 65 °C in a water bath for a duration of 60 min. In three-quarters of the DNA extraction tubes, water was immersed. The contents of the containers were gently inverted every 5 to 10 min to facilitate mixing. Following incubation, the specimens were allowed to settle to room temperature for five min before their addition to a 5 mL solution of chloroform: isoamyl (24:1). Through repeated gentle rotations of the containers, the samples were thoroughly combined. Ten min of centrifugation was conducted at 15000 rpm. Following this, the uppermost aqueous phase was transferred into a pre-sterilized centrifuge tube. The DNA was spooled from ice-cold isopropanol precipitate in an equivalent volume, using sterile glass hooks, and subsequently immersed in 1 mL of 70% ethanol for a duration of 10 min. Following that, the tubes were centrifuged at 10000 rpm for 5 min; the supernatant was discarded and the DNA pellet settled to the lower end of the tube. The pellet was then desiccated at room temperature until any remaining ethanol was removed. Following the drying process, the particle was dissolved in a sufficient volume of TE buffer.

SSR Assay

Primers used for DNA amplification

A collection of 16 primers, chosen at random, was utilized to amplify genomic DNA. Following the findings of prior research, SSRs were chosen, as detailed in Table S1.

Amplification of DNA

A 25 µL PCR (polymerase chain reaction) reaction volume was utilized, which included the following:

1. HCl Tris 10 mM,
2. 0.1 mg collagen per mL
3. 50 mL M KCl,
4. 40 ng DNA genomics,
5. 200 M Mg Cl_2 in addition to
6. 0.5 units of Taq polymerase for DNA.

For DNA amplification, a thermal circler (Bio-Rad) was utilized in the PCR reaction. The agarose utilized for the analysis of the ethidium bromide-containing products was 2.0%. The resulting bands were subsequently evaluated for polymorphism using a scoring system based on their presence (1) or absence (0).

Data Analysis

Each character was assessed independently, with bands that were uniform, distinct, and reproducible being assigned a value of either 1 or 0. Based on character state data, NTSYS-PC software version 2.0e (Rohlf 1988), Arlequin 3.00, and Genalex were utilized to conduct cluster analysis, population structure, analysis of molecular variance (AMOVA), and Principal Coordinate Analysis (PCA) (Peakall and Smouse 2006). Furthermore, a comparison was made between visual interpretations of dendrograms generated by clustering methods that utilized similarity matrices and genetic connections. Dendrograms were generated by joining neighbours utilizing the UPGMA (unweighted pair group method with arithmetic average) technique (Rohlf 1988). The molecular data were collected and analyzed in the required 'allelic format' using the DARwin 5 computer software application to assess genetic diversity between genotypes. The approximate values of the number of private alleles (Np), expected heterozygosity (He), information index (I), and observed heterozygosity (Ho), effective alleles (Ne), and effective alleles (Ne) per locus were calculated using Genalex (Nei 1973; Peakall and Smouse 2006). The genetic organization and gene pool were deduced utilizing STRUCTURE v.2.3.4 (Pritchard *et al.* 2000). An additional ten fictitious populations (K) were utilized in the experiment, which was conducted in duplicate. The values for iterations and burn-in were 2,000,000, and 1,000,000, respectively. An admixture-free model with correlated allele frequencies was employed for each trial. By estimating the most probable number of groups with the 1K value, the optimal range of K was determined (Evanno *et al.* 2005). Genotypes that possess an affiliation probability (*i.e.*, inferred ancestry) exceeding 80% were categorized as "admixtures" within a given group. This denotes that these genotypes seem to be a synthesis of parental heritage originating from different gene pools or regions. The heterozygosity (gene diversity) and population differentiation (Fst) between individuals in a subpopulation were predicted utilizing the STRUCTURE software.

RESULTS

SSR Analysis

In the current investigation, 21 genotypes were examined; their identifiers are provided in Table S2. The SSR primers were utilized to assess the genetic diversity of these genotypes at the molecular level NWS-005, BWS-024, BWS-027, AWS-037, PWS-016, NWS-012, SWS-005, TWS-039, MWS-025, CWS-023, RWS-018, SWS-033, MWS-035, KWS-025, KWS-003, RWS-004, AWS-011, SWS-007, KWS-002, BWS-025, and GWS-030 were the genotypes utilized in this investigation. A total of sixteen SSR primers were designed to identify polymorphic SSR loci (Woeste *et al.* 2002; Dangl *et al.* 2005). The quantity of amplified bands generated by these primer pairs varied, with values spanning from 100 to 1100 base pairs. In all primers, two bands were generated per genotype, thereby validating the diploidy degree of this species. The PCR-amplified gel patterns of different walnut genotypes utilized in our investigation are given in Figs. S1 to S3. The genetic diversity analysis of walnut genotypes incorporated the following factors: gene flow (Nm), population differentiation or genetic variation (Fst), and per locus NA, Ho, He, PIC, and NPA. The NA observed at each locus ranged from 2 to 4 alleles, with an average of 2.94 alleles per locus, as shown in Table S2. The values of Ne and I in the populations of the Budgam and Ganderbal ranged between 1.00 and 1.80 and 1.24 and 1.68, respectively, with means of 1.37 and 1.45. WGA-4 exhibited the highest quantity of expected heterozygosity (0.89), followed by WGA-27 (0.83); WGA-27 displayed the lowest expected heterozygosity (0.52). The mean heterozygosity was 0.15, with observed values ranging from 0.03 to 0.23. Following the principal Ho in WGA-292 and WGA-332 were WGA-1, WGA-69, and WGA-76. In WGA-27, a minimum value was observed. An average PIC of 0.22 was determined. The range of the PIC value was 0.11 to 0.38. WGA-69 yielded the highest PIC value, which was subsequently surpassed by WGA-1, WGA-118, and WGA-276. The lowest PIC value was documented in WGA-202. The average value of private alleles was 1.17, with a range of 0.41 to 3.11. The WGA-376 variant exhibited the highest count of private alleles at 3.11, whereas the WGA-76 variant documented the lowest count at 0.41. The mean population differentiation (Fst) was 0.219, with values ranging from 0.156 to 0.343. The highest Fst value was detected in WGA-76, with WGA-118 and WGA-32 following suit. In WGA-4, the minimum value of heterozygosity (Fst) was observed.

Notably, the examination of germplasm effects on walnut trees provided valuable findings. Germplasm variation was observed to have a discernible impact on various phenotypic and genotypic traits, including growth characteristics, disease resistance, and nut quality attributes. Specifically, certain walnut genotypes exhibited distinct genetic profiles and phenotypic expressions, suggesting the influence of specific germplasm on tree morphology and performance. These observations underscore the importance of considering germplasm diversity in walnut breeding programs and conservation efforts aimed at enhancing walnut cultivation in the region. Further investigations into the specific mechanisms underlying germplasm effects on walnut trees are warranted to fully elucidate their implications for tree productivity and resilience in the Northwestern Himalayan Region of Jammu and Kashmir.

Assessing the genetic population, genetic relationships, and genotype identity of walnut trees has significant implications for their lifespan and conservation. Understanding the genetic diversity and population structure allows for the identification of unique genotypes with desirable traits, such as disease resistance, high yield, and superior nut

quality. By pinpointing these valuable genetic resources, breeders can develop improved cultivars that are better adapted to local environmental conditions and more resilient to biotic and abiotic stresses. Furthermore, assessing genetic relationships helps elucidate the evolutionary history of walnut populations, informing conservation strategies to preserve genetic diversity and prevent genetic erosion. Ultimately, by leveraging genetic information, stakeholders can implement targeted conservation efforts and sustainable management practices to ensure the long-term survival and productivity of walnut trees in the Northwestern Himalayan Region of Jammu and Kashmir.

Population Variation

The mean population variation (Fst) for each locus under investigation was 0.219, with values ranging from 0.156 to 0.343 (Tables S3 and S4).

Table 1. The Nucleotide Sequences of the SSR Primers Used to Screen Walnut Selections/Genotypes

S. No.	Primer	PIC*	MI*	RP*
1	WGA-69	0.38	0.38	0.98
2	WGA-118	0.29	0.28	1.89
3	WGA- 32	0.27	0.27	1.72
4	WGA-202	0.11	0.10	1.32
5	WGA-1	0.33	0.33	0.85
6	WGA-4	0.12	0.12	0.56
7	WGA-27	0.19	0.18	0.71
8	WGA-76	0.17	0.16	1.85
9	WGA-42	0.22	0.22	1.95
10	WGA-79	0.21	0.21	0.78
11	WG-72	0.28	0.28	0.44
12	WGA-71	0.13	0.13	0.73
13	WGA-276	0.29	0.29	1.62
14	WGA-225	0.14	0.14	1.04
15	WGA-376	0.23	0.22	1.83
16	WGA-332	0.16	0.16	2.12
Average		0.22	0.199	1.27

*PIC = Polymorphic Information Content
*Rp = Resolving power
*MI = Marker Index

The population exhibited a range of inbreeding levels (Fis) between 0.013 and 0.691, with an average value of 0.188. Similarly, the fit coefficient, which represents the overall inbreeding, fluctuated between a minimum of 0.167 in WGA-76 and a maximum of 0.756 in WGA-4. The gene flow (Nm) values are displayed in Table S3. The results indicate that WGA-4 had the highest gene flow (1.352 Nm), followed by WGA-225, with WGA-76 having the lowest (0.479 Nm). The maximum recorded value of the marker index was 0.38, while the minimum value was 0.10, achieved using the primers WGA69 and WGA202, which had a mean value of 0.199, respectively. The range of resolving power

(Rp) was between 0.44 and 2.12, with an average value of 1.27. WGA-332 contained the highest concentration of Rp, which was followed by WGA-42 and WGA-118. At the lowest level (Rp = 0.44), WGA-72 exhibited the results (Table 1).

Analysis of Clusters and Population Structure

Using the UPGMA and arithmetic average, a dendrogram was constructed to illustrate the genetic relationships among 21 walnut genotypes. The dendrogram provided evidence that there is substantial genetic diversity among all walnut accessions. The genetic connection among the genotypes was illustrated by the dendrogram derived from the DNA profile. Using the empirical data, a dendrogram was constructed.

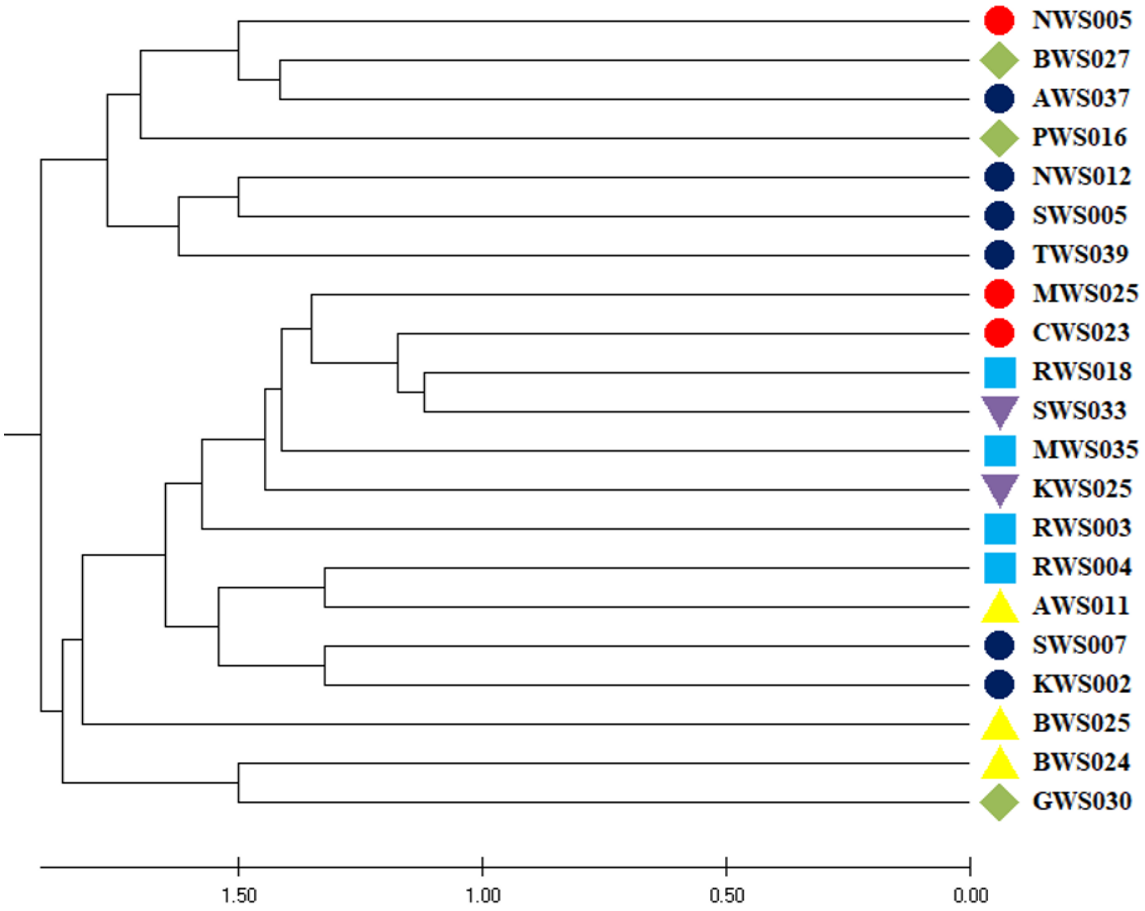

Fig. 1. Dendrogram depicting the genetic relationships among 21 *Juglans regia* L. genotypes based on microsatellite data analysis. Branch lengths reflect the degree of genetic similarity between genotypes, with shorter branches indicating closer genetic relationships. Genotypes sharing the same color and symbol are considered similar in terms of their genetic makeup.

The genotypes being studied exhibited a broad dispersion into two significant clusters, which, as indicated by the dendrogram, demonstrates the considerable genetic diversity and heterogeneity of the open-populated walnut genotypes in the districts of Budgam and Ganderbal in the Kashmir Valley. The highest bootstrap value was found in the node that partitioned clusters I and II, suggesting that the genotypes in clusters I and II exhibited considerable diversity. Cluster II comprised 14 of the genotypes, the plurality. The second cluster was divided into three subgroups, with each subgroup consisting of 3

genotypes and 12 in number. As illustrated in Fig. 1, the initial cluster was composed of two subgroups of seven genotypes. With respective contributions of 16.30%, 22.43%, and 9.8%, the first three axes accounted for 48.58% of the genetic similarity variance (Table 2; Fig. 2).

Table 2. Principal Coordinate Analysis of Different Walnut Genotypes from Districts Budgam and Ganderbal

	1	2	3
Axis %	9.85	16.30	22.43
Cum %	9.85	26.15	48.58

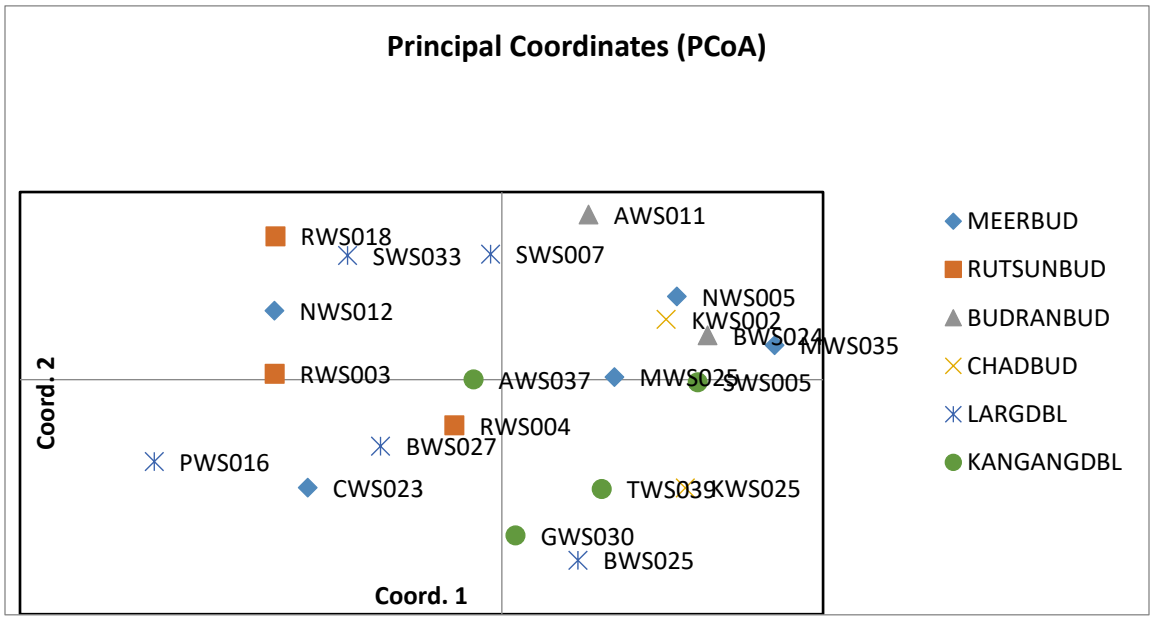

Fig. 2. Principal Coordinate Analysis (PCoA) plot illustrating the genetic relationships among 21 Juglans regia L. genotypes sourced from Budgam and Ganderbal Districts. The plot is based on a matrix of chord distances, with each point representing a genotype. The proximity of points on the plot reflects the degree of genetic similarity between genotypes, with closer points indicating greater genetic resemblance.

Population Structure

Using SSR markers, the population structure of 21 walnut genotypes was estimated (Fig. 3); the assignment procedure in STRUCTURE determined that the genotypes were categorized into six groups (Table 3). As demonstrated in Fig. 4, the clustering method indicated that the Ln probability of the data provided the most accurate prediction of delta K at K = 5. This substantiates the classification of 21 walnut genotypes into six distinct populations through substantial mixing, thereby illustrating the exceptionally diverse character of walnuts as a species. The expected heterozygosity, which quantifies the likelihood that two arbitrarily chosen individuals will be heterozygous at a specific locus, ranged between 0.563 and 0.741 in the third subpopulation, with an average of 0.672. The Fst exhibited variability, ranging from 0.176 in the fourth subpopulation to 0.176 in the third subpopulation.

Table 3. Heterozygosity and Fst Value Calculated for Nine Subpopulations of Walnut

S.NO	Sub-population (K)	Expected Heterozygosity	Fst Value
01	1	0.617	0.261
02	2	0.563	0.221
03	3	0.741	0.176
04	4	0.709	0.242
05	5	0.731	0.178
06	6	0.621	0.234
Average		**0.672**	**0.215**

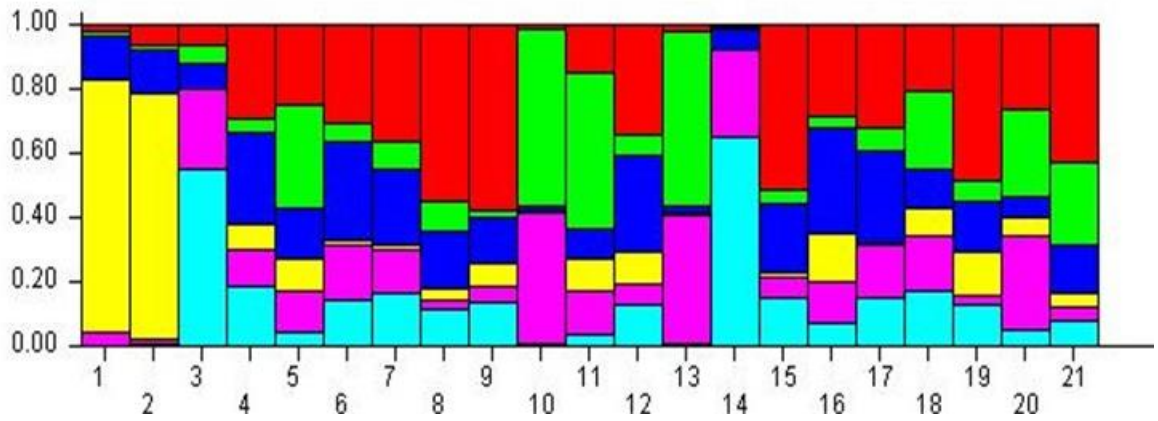

Fig. 3. This graphical representation visually depicts the population structure in walnut genotypes. Each vertical line represents an individual genotype, and the colors within each line indicate the estimated membership percentage of that genotype in different clusters (K clusters). Essentially, it illustrates the degree of admixture or genetic contribution from different clusters within each genotype. By examining the distribution of colors across the lines, one can gain insights into the genetic composition and admixture patterns present within the walnut population under study.

Analysis of Molecular Variance (AMOVA)

The amount of percent variation that exists between distinct populations of walnut genotypes was determined using an AMOVA. The research outcomes revealed a higher degree of variability (91%) among populations within Kashmir than between populations categorized by location, cultivar, and plant. This suggests that walnut genotypes in the region are exceptionally diverse. The genetic differentiation values (Fst) within and between populations were determined to be 0.04, suggesting a high degree of homogeneity. This conclusion is supported by the low Fst value, which signifies minimal genetic differentiation. Anomalous variations exist both among and within groups, as stated by AMOVA. In contrast to the 2% variation observed in the population, individual variation was 73% greater at 25% (Table 4).

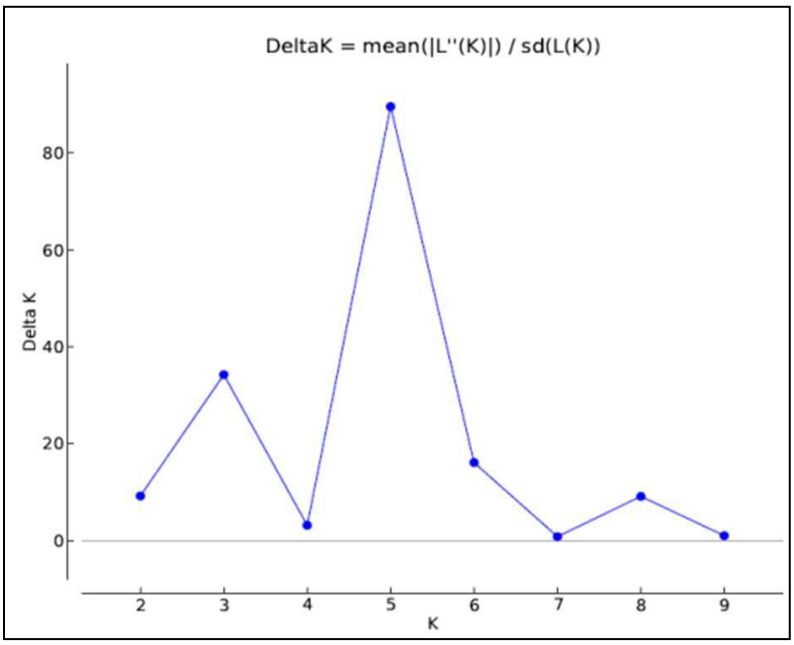

Fig. 4. Second order of change of the log-likelihood of the data (Delta K) as a function of K, calculated over two replications

Table 4. Statistical AMOVA

Source	Df	SS	MS	Est. Var.	%
Among Pops	5	10.920	2.184	0.160	9%
Within Pops	15	24.598	1.640	1.640	91%
Total	20	35.517		1.799	100%
Stat	**Value**	**P < 0.05**			
ϕst	0.089	0.001			

DISCUSSION

A substantial degree of genetic diversity is present in walnut trees, and significant divergence is observed in their germplasm (Shahi *et al.* 2023). The observed genetic heterogeneity and heterozygosity among walnut genotypes are primarily attributable to plantations cultivated from seeds (Buyuksolak *et al.* 2020). Forthcoming endeavors in crop development must evaluate the germplasm presently available (Pop *et al.* 2013; Akca *et al.* 2020). Critical information for selecting desirable genetic resources can be obtained by analyzing these resources for a variety of traits (Ebrahimi *et al.* 2015; Hassani *et al.* 2020). Molecular markers contain information regarding genetic structure and evolution (Cervera *et al.* 2000). In the domains of population genetics, linkage mapping, genotyping, determining parentage, and mapping qualitative and quantitative features, they are indispensable (Crespel *et al.* 2002).

Microsatellite markers have proven to be efficacious in genetic research, particularly in the examination of walnut genetic diversity (Hamza *et al.* 2004; Karimi *et al.* 2010; Ahmed *et al.* 2012), because of the area's exceptional conservation. The utilization of microsatellite markers in the current research identified a significant degree

of polymorphism (89.6%), which is consistent with prior discoveries in walnuts (Ahmed *et al.* 2012; Salieh *et al.* 2013). Other research has revealed that, on average, walnuts contain two polymorphic SSR loci per SSR primer. Ahmed *et al.* (2012) identified an average of two polymorphic SSR loci per primer when thirteen primers were utilized. The results obtained in the present investigation regarding various primer parameters align with those reported in prior research (Wani *et al.* 2017). It is imperative to comprehend the magnitude of genetic variation and the genetic interrelationships among genotypes within a population to optimize the utilization of germplasm resources. Additionally, this facilitates the formulation of suitable approaches to plant breeding, such as the establishment of the genetic map and the assessment of quantitative trait loci (Yilmaz *et al.* 2012).

The genetic diversity, population structure, and relationships of twenty-one walnut genotypes selected from Budgam and Ganderbal, two districts in the valley of Kashmir, were analyzed using SSR markers. The ongoing inquiry has uncovered valuable genetic data within the genotypes of walnuts that were gathered. An average of 2.94 alleles per locus was observed, with values ranging from 2 to 4. In 29 apricot genotypes, Messina *et al.* (2004) reported a range of allele numbers 2 to 9 using 20 SSR producers, whereas Khadivi-Khub *et al.* (2015) reported 3 to 7 for walnut genotypes. Walnuts (*Juglans regia* L.) exhibited allelic numbers ranging from two to four, according to Ebrahimi *et al.* (2016). The marker index is a characteristic that provides insight into the discriminatory capability of a marker. As such, it was computed for each of them, with the primers WGA69 and WGA202 having the lowest value of 0.10 and the highest value of 0.38 (with a mean of 0.199). The studies conducted by Shah *et al.* (2018) identified the highest (0.89) and lowest (0.00) values of MI for WGA32, WGA331, and WGA89, with a mean of 0.244. Lamia *et al.* (2010) examined 81 apricot accessions and determined that the average marker index per 24 SSR markers was 4.27. Moreover, the most crucial characteristic of a primer is its PIC, which signifies its capacity to differentiate among various individuals. The investigation yielded an average PIC value of 0.22. The range of the PIC value was 0.11 to 0.38. The WGAs with the highest PIC values were WGA-69, WGA-1, WGA-118, and WGA-276. At 0.22 per locus, WGA-202 exhibited the lowest PIC value, falling short of the 0.690 value reported by Bakir *et al.* (2019) for *Prunus armeniaca* (wild form) when 16 microsatellite primers were utilized and the 0.546 value reported by Bourguiba *et al.* (2012) when 24 SSR microsatellite primers were applied to *Prunus armeniaca*. The mean PIC value, as reported by Shah *et al.* (2018), was 0.168. Persian walnut populations in Iran were investigated and PIC values identified that varied between 0.56 and 0.82. (2015). This is consistent with the findings of Orhan *et al.* (2020), who obtained an average PIC value of 0.68 across 32 walnut genotypes using 21 SSR markers. Potential causes for the inconsistencies in these results include the use of a distinct set of SSR markers and the examination of separate genotypes. Informational markers include loci with a PIC value greater than 0.5, whereas loci with a PIC value greater than 0.7 are deemed optimal for mapping. Primer WGA-332 exhibited the maximum Rp (resolving power) in this investigation at 2.12, while primer WGA-72 displayed the lowest Rp (resolving power) at 0.44. The method of Rp is a valuable approach to assessing the discriminatory power of a primer against various genotypes (Shah *et al.* 2018). Furthermore, the current report provides comprehensive documentation of genetic diversity metrics, including the effective number of alleles, observed heterozygosity, anticipated heterozygosity, and unbiased heterozygosity, as well as gene diversity. The calculated values for the observed NA and Ne were 2.94 and 1.37, respectively. Li *et al.* (2014) identified 4 to 2 allelic

numbers and Ne in apricots and walnuts, respectively (4.5 and 3.7). The average Shannon's information index value for the microsatellite primers utilized in the current study was 1.45, which is comparable to the values reported by Ebrahimi *et al.* (2016) for walnut populations in southeast Iran (I = 1.49) and walnut accessions from Europe, Africa, and Asia (I = 1.22). Furthermore, the values are nearly identical (I = 1.05) as reported by Bourguiba *et al.* (2010). The mean Ho value of 0.15 indicates that the population under observation has the greatest degree of genetic diversity and may contain a greater number of genetic variants of crucial adaptive characteristics, as it ranged from 0.03 to 0.44. As mentioned earlier, the mean Fst estimate for each locus examined was 0.219, with a range of 0.156 to 0.343. The population exhibited a range of inbreeding levels (Fis) between 0.013 and 0.691, with an average value of 0.188. The minimum value of fit (total inbreeding coefficient) was 0.167 in WGA-76 and the maximum value was 0.756 in WGA-4. The largest gene flow (1.352) was identified in WGA-4, followed by WGA-225 (Table S3), whereas the smallest gene flow (Nm = 0.479) was identified in WGA-76. The lowest degree of heterozygosity (Fst) was observed in WGA-4. Fst values that are positive are commonly interpreted as indicators of genetic divergence or structure among subpopulations. This divergence can occur due to factors, such as drift, assortative mating, or natural selection, all of which are facilitated by the restriction of gene flow between populations.

Almost every tree species that undergo wind pollination has been investigated. The heterozygote deficit was significant at each of the polymorphism loci, as indicated by the inbreeding coefficient (FIS = 0.691, Table S3). This phenomenon was ascribed to high levels of inbreeding at every locus that was examined. Moreover, the inbreeding coefficient across populations (0.691) was lower than the overall inbreeding coefficient (FIT = 0.756). The Wahlund effect led to an underestimation of the mean genetic variability in this study. The Wahlund effect is a consequence of restricted gene flow leading to population fragmentation (Hartel and Clark 2007). In anemophilic species, Fis and Fst estimates continue to be extremely prominent, indicating substantial inbreeding. The results of the current investigation indicate that walnut genotypes (seed propagated) exhibit a significant level of heterozygosity. The heterozygosity values we observed were in agreement with those documented by Vahdati *et al.* (2015) and Khokhlov *et al.* (2019) for walnut genotypes. Those researchers documented heterozygosity values ranging from 0.00 to 0.85 and 0.50 to 0.88, with mean values of 0.23 and 0.73, respectively. The current findings are consistent with the maximal observed heterozygosity of 0.62 reported by Mahmood *et al.* (2013). Comparable to the current findings, Balapanov *et al.* (2019) examined 62 walnut genotypes containing 11 microsatellite loci and discovered high levels of polymorphism and heterozygosity, with values ranging from 0.44 to 0.86 and a mean of 0.67. Additionally, in the present study, the calculated mean value of predicted heterozygosity was 0.68, ranging from 0.52 to 0.89. The mean and variability of anticipated heterozygosity values identified in this research align with those reported in prior investigations of walnut genotypes (Wang *et al*. 2008; Ebrahimi *et al.* 2011; 0.86 to 0.87). The performance of the markers employed in this study in differentiating 21 walnut genotypes was supported by the number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and information index (I). These results indicate that the walnut germplasm of Kashmir, specifically in the study area of Budgam and Ganderbal, possesses a considerable degree of genetic diversity, which is crucial for the development, maintenance, and analysis of parent mixtures for plan implementation. Moreover, information regarding the genetic relationship among the collected germplasm could

potentially be utilized to identify parental and superior genotypes to augment genetic material and improve walnut germplasm. The authors analyzed the population structure, connectivity, and genetic diversity of twenty-one walnut genotypes in the current report by employing three clustering techniques: the PCoA plot, the neighbor-joining (NJ) dendrogram, and population structure analysis. The dendrogram derived from the DNA profile unequivocally illustrated the genetic connection that existed among the genotypes. Following the disclosure of the investigative information, the dendrogram was constructed.

The genotypes being studied exhibited a broad dispersion into two significant clusters, which, as indicated by the dendrogram, demonstrates the considerable genetic diversity and heterogeneity of the open-populated walnut genotypes in the districts of Budgam and Ganderbal in the Kashmir Valley. The highest bootstrap value was found in the node that separated clusters I and II, indicating that the genotypes in clusters I and II were extremely diverse. Cluster II comprised 14 of the genotypes, the plurality. A total of 14 genotypes were identified in three subgroups comprising the second cluster. Seven genotypes comprised the initial cluster, which was further subdivided into two groups. Therefore, genotypes collected from diverse geographic locations tended to cluster into similar clusters. This suggests that the clustering process was independent of geographic sites, suggesting that the majority of genotypes share a common gene pool and that morphological similarities may exist between genotypes in the same cluster. Aside from genotypes collected from the same location and the characteristics of walnuts, there was no correlation between geographical proximity and germplasm genetic proximity.

The distinct clustering of genotypes collected from various districts in the Kashmir Valley may be accounted for by outcrossing. The aforementioned findings are in immediate correlation with each other (Simsek *et al.* 2010; Ahmad *et al.* 2012; Orhan *et al.* 2020). The first three axes accounted for 9.8%, 16.3%, and 22.4%, respectively, of the genetic similarity variance, or 48.5%, as shown in Table 5. The outcomes generated by PCoA closely resemble those obtained from the UPGMA dendrogram. The geographical differentiation went unnoticed as the PCoA assemblage's location prevented it from displaying a vibrant clustering outline. Nevertheless, the PCoA structure analysis undergoes some minor modifications as a result of the implementation of distinct algorithms. This finding is consistent with the research conducted by Ahmad *et al.* (2012), who observed similarities in walnut cultivars originating from Turkey by employing SSR markers. Consistent findings were reported by Shah *et al.* (2018) for walnut genotypes exposed to SSR markers, Dar *et al.* (2017), for common bean genotypes exposed to SSR markers, and Ebrahimi *et al.* (2016) for walnut genotypes exposed to SSR markers. Comparable results were obtained from the population STRUCTURE analysis, dendrogram, and PCoA. The STRUCTURE study did not differentiate between populations based on their residential location. Based on the maximal natural log probability and the utmost average possibility of likelihood value L (K) = 0.672 derived from STRUCTURE over individual association coefficient at K = 5, five (K = 5) subpopulations were confirmed in twenty-one walnut genotypes. The findings of the STRUCTURE study, which identified five distinct populations from twenty-one walnut accessions with high levels of admixtures, indicate that walnuts are inherently diverse by random mating (cross-pollination) and the absence of a zygotic barrier. The range of population divergence (Fst) values observed in the first subpopulation is 0.261 in the 4^{th} subpopulation and 0.176 in the 3^{rd} subpopulation. The genetic differentiation between the groups from the Caserta and Sorrento peninsulas in Sorrento walnut was detected using microsatellite primers. This resulted in the formation of two distinct clusters, which were

closely but not precisely associated with the geographic origin of each sample (Foroni *et al.* 2007).

The present study employed structure analysis to illustrate genetic divergence or differentiation among walnut genotypes originating from various topographical regions. This regional disparity might have been the consequence of selection pressure exerted by the reproduction and selection process. In the future, the insights obtained from population structure analysis will prove to be advantageous when conducting association mapping in walnuts across various attributes. Conversely, prior investigations have delineated apricot populations into two to four subpopulations (Khadivi-Khub *et al.* 2015; Li *et al.* 2020). The blending of walnut genotypes may take into account the cross-cultural and geographical barriers that hinder commercial objectives. Moreover, the findings indicate that the sixteen SSRs employed in this study have the potential to facilitate a rational evaluation of genetic diversity, which would be beneficial for marker-assisted reproduction and parental crossing-line identification. The AMOVA demonstrated that substantial variations exist both among and within groups. The proportion of variation present within a given population was significantly greater (91%), in contrast to the mere 9% variation observed between populations. Similar conclusions were reached by Hu *et al.* (2018) and Bourguiba *et al.* (2012), who discovered that molecular variation was less within populations than between populations. Furthermore, Aradhya *et al.* (2010) discovered that walnut genotypes exhibit a greater degree of population variance (86%) compared to alternative genotypes. Similar results were reported by Wang *et al.* (2008) regarding the diversity of Chinese walnut populations, 89% and 81%, respectively, within populations. Further research objectives and management operations about walnuts will be significantly enhanced by the results obtained from these inquiries.

In addition to its implications for walnut genetic diversity, this research may also have implications for the practical properties of walnut wood. While the primary focus of this study was on the population structure and molecular genetic diversity of walnut genotypes, the genetic variability observed among the studied genotypes could potentially influence various wood traits, such as density, strength, and color. Understanding the genetic basis of these traits could inform breeding programs aimed at developing walnut varieties with desired wood characteristics for applications in furniture making, construction, and woodworking industries. Further research exploring the relationship between genetic variation and wood properties in walnut trees could provide valuable insights into optimizing walnut cultivation for both timber and nut production, thereby enhancing the economic viability and sustainability of walnut farming in the region.

CONCLUSIONS

In conclusion, this study utilized 16 simple sequence repeat (SSR) markers to evaluate the genetic relatedness of 21 exceptional Persian walnut (*Juglans regia*) genotypes. The analysis revealed a significant degree of genetic diversity within the population, with alleles per locus ranging from 2 to 4. Notably, WGA-1, WGA-4, and WGA-79 exhibited the greatest number of alleles (4), while WGA-27, WGA-69, and WGA-32 contained the fewest. The range of the polymorphic information content (PIC) value was 0.11 to 0.38. Model-based cluster analysis and STRUCTURE HARVESTER categorized all genotypes into two primary clusters and six genetically distant subpopulations, respectively, reflecting their distinct genetic makeup and variable degrees

of admixture. Anticipated heterozygosity ranged from 0.563 to 0.741, while population differentiation (Fst) ranged between 0.176 and 0.261. These results are anticipated to aid in the development of association mapping studies for walnut characteristics. Moreover, our findings underscore the importance of considering germplasm diversity in Persian walnut breeding programs and conservation efforts to enhance walnut cultivation in the region. Overall, this study significantly contributes to our understanding of Persian walnut genetic diversity in the Northwestern Himalayan Region of Jammu and Kashmir, providing valuable insights for future breeding and conservation strategies.

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APPENDIX

Supplementary Tables

Table S1. Thermal Profiles Used for DNA Amplification

Step	Temperature (°C)	Time	No. of Cycles
Initial denaturation	95	5 min	1
Denaturation	94	1min	35
Annealing	53 to 60	1 min 40 s	
Elongation	72	40 s	
Final Extension	72	5 min	1

Table S2. SSR Primers Used for DNA Amplification

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temperature (°C)	Expected Amplicon Size (bp)
WGA- 42	F: GTGGGTTCGACCGTGAAC	R: AACTTTGCACCACATCCACA	55	100 to 380
WGA- 1	F :ATTGGAAGGGAAGGGAAATG	R : CGCGCACATACGTAAATCAC	56	180 to 310
WGA-376	F:GCCCTCAAAGTGATGAACGT	R:TCATCCATATTTACCCCTTTCG	56	850
WGA-71	F: ACCCGAGAGATTTCTGGGAT	R:GGACCCAGCTCCTCTTCTCT	57	310 to 600
WGA-276	F: CTCACTTTCTCGGCTCTTCC	R: GGTCTTATGTGGGCAGTCGT	60	230 to 420
WGA-72	F : AAACCACCTAAAACCCTGCA	R : ACCCATCCATGATCTTCCAA	55	300 to 480
WGA-79	F : CACTGTGGCACTGCTCATCT	R : TTCGAGCTCTGGACCACC	55	420 to 580
WGA-76	F : AGGGCACTCCCTTATGAGGT	R : CAGTCTCATTCCCTTTTTCC	58	100 to 180
WGA-202	F: CCCATCTACCGTTGCACTTT	R: GCTGGTGGTTCTATCATGGG	62	150 to 380
WGA-4	F: TGTTGCATTGACCCACTTGT	R: TAAGCCAACATGGTATGCCA	62	180 to 200
WGA-69	F: TTAGATTGCAAACCCACCCG	R: AGATGCACAGACCAACCCTC	55	180 to 300
WGA-32	F:CAGTTTGTCCCACACCTCCT	R: AACCCATGGTGAGAGAGTGAGC	62	100 to 1100
WGA-118	F:TGTGCTCTGATCTGCCTCCC	R: GGGTGGGTGAAAAGTAGCA	60	175 to 490
WGA-332	F:ACGTCGTTCTGCACTCCTCT	R:GCCACAGGAACGAGTGCT	57	320 to 580
WGA- 27	F: AACCTCACGCCTTGATG	R: TGC TCA GGC TCC ACT TCC	57	580 to 600
WGA- 225	F:AATCCCTCTCCTGGGCAG	R:TGTTCCACTGACCACTTCCA	58	800

where N_A – number of alleles, N_E - No. Effective Alleles, I-

Table S3. Detail of Genetic Diversity After Subjecting Walnut Genotypes to SSR Marker Analysis

Locus	N_A	N_E	H_O	H_E	I	PIC	N_{PA}	Fst
WGA-69	2	1.31	0.21	0.71	1.36	0.38	1.5	0.168
WGA-118	3	1.43	0.14	0.68	1.27	0.29	1.3	0.300
WGA- 32	2	1.80	0.08	0.58	1.30	0.27	1.07	0.331
WGA-202	3	1.38	0.23	0.75	1.55	0.11	0.47	0.176
WGA-1	4	1.51	0.22	0.72	1.56	0.33	1.3	0.178
WGA-4	4	1.48	0.44	0.89	1.68	0.12	1.12	0.156
WGA-27	2	1.26	0.03	0.83	1.63	0.19	0.46	0.178
WGA-76	2	1.32	0.16	0.68	1.27	0.17	0.41	0.343
WGA-42	3	1.42	0.12	0.60	1.57	0.22	1.21	0.215
WGA-79	4	1.52	0.11	0.61	1.54	0.21	0.54	0.208
WG-72	2	1.13	0.07	0.52	1.30	0.28	1.31	0.266
WGA-71	3	1.43	0.12	0.64	1.24	0.13	0.48	0.199
WGA-276	2	1.00	0.09	0.62	1.53	0.29	1.12	0.242
WGA-225	3	1.39	0.08	0.59	1.34	0.14	2.12	0.158
WGA-376	2	1.34	0.14	0.65	1.53	0.23	3.11	0.179
WGA-332	3	1.18	0.23	0.72	1.57	0.16	1.31	0.198
Mean±SD	**2.94 ± 1.52**	**1.37 ± 0.21**	**0.63 ± 0.08**	**0.68 ± 0.08**	**1.45 ± 0.25**	**0.22 ± 0.04**	**1.17 ± 1.72**	**0.219 ± 0.07**
SE	**0.18**	**0.13**	**0.03**	**0.01**	**0.12**	**0.01**	**0.81**	**0.015**

Information Index, H_O- Observed Heterozygosity, H_E- Expected Heterozygosity, PIC- Polymorphic information content, N_{PA}- number of private alleles, F-statics per Locus (Fst), SE-Standard error.

Table S4. Statistics of Genetic Structure and Gene Flow for the 16 Polymorphic Loci in Walnut Populations

All Pops.	Locus	Fis[A]	Fit[B]	Fst[C]	Nm[D]
	WGA-69	0.165	0.453	0.168	1.240
	WGA-118	0.193	0.435	0.300	0.582
	WGA-32	0.182	0.305	0.331	0.504
	WGA-202	0.045	0.213	0.176	1.171
	WGA-1	0.103	0.263	0.178	1.155
	WGA-4	0.691	0.756	0.156	1.352
	WGA-27	0.116	0.274	0.178	1.155
	WGA-76	0.013	0.167	0.343	0.479
	WGA-42	0.051	0.201	0.215	0.913
	WGA-79	0.124	0.424	0.208	0.949
	WG-72	0.102	0.341	0.266	0.688
	WGA-71	0.289	0.430	0.199	1.005
	WGA-276	0.151	0.356	0.242	0.783
	WGA-225	0.431	0.553	0.158	1.332
	WGA-376	0.238	0.375	0.179	1.144
	WGA-332	0.113	0.288	0.198	1.012
	Average	0.188	0.365	0.219	0.966
	SE ±	0.042	0.037	0.015	0.071

[A] Level of inbreeding due to non-random mating within the population
[B] overall inbreeding coefficient; [C] Population sub-division; [D] Gene flow

Supplementary Figures

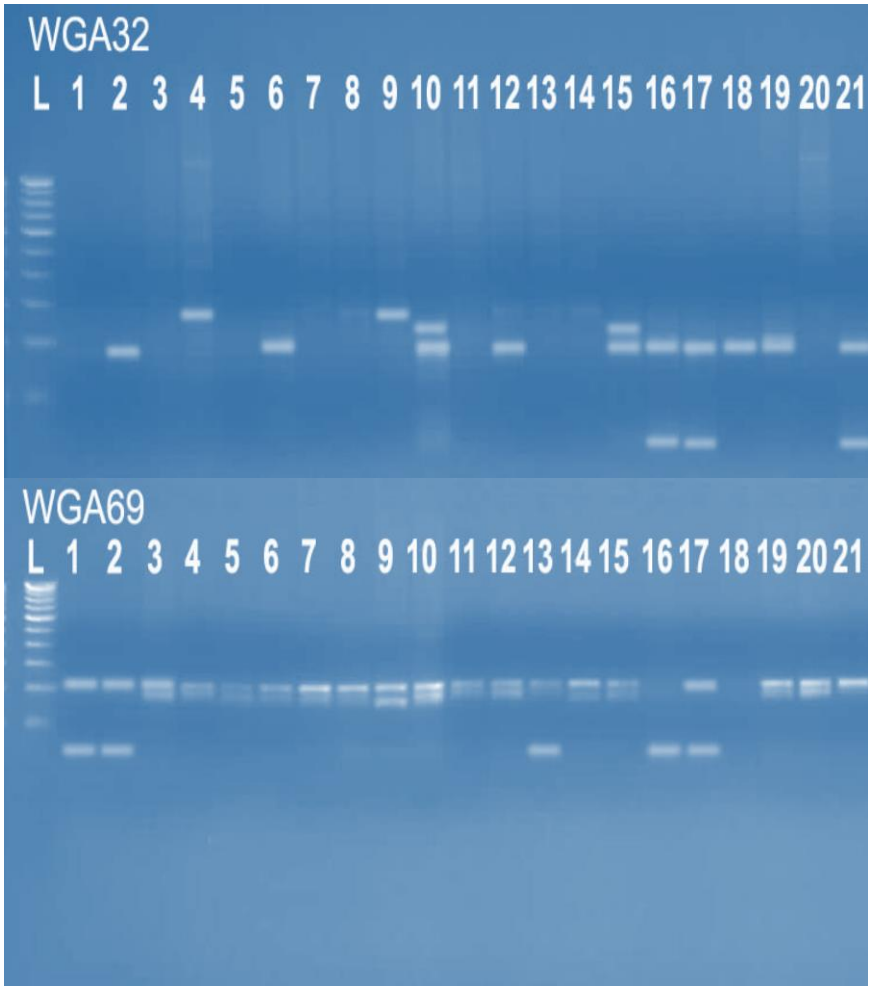
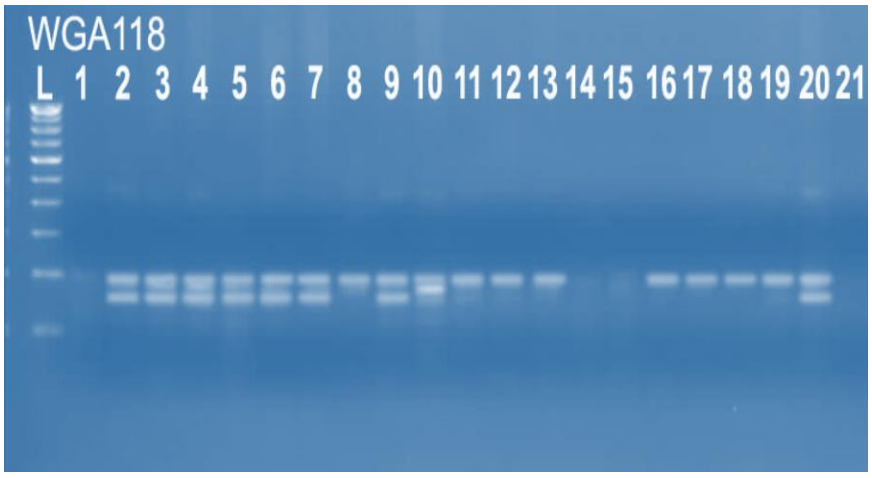


Fig. S1. PCR amplified pattern of walnut using primer WGA 32 and WGA 69. L:100 bp ladder. Lanes 1 to 21 represent genotypes



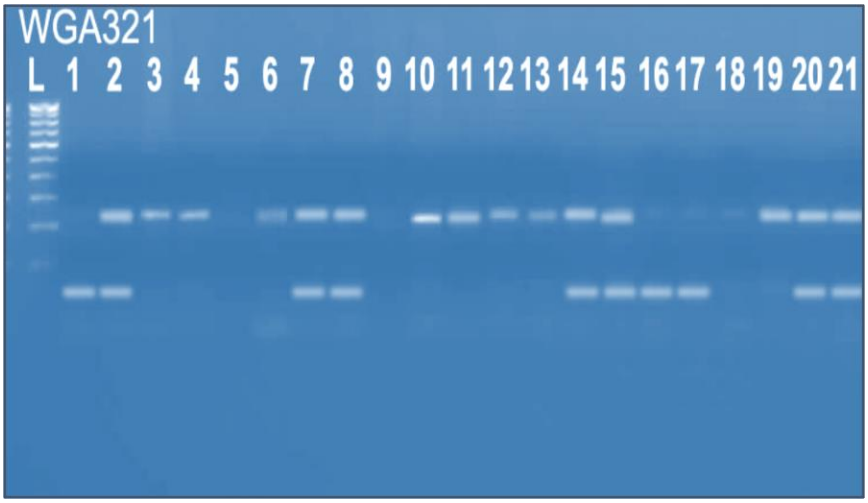


Fig. S2. PCR amplified pattern of walnut using primer WGA 118 and WGA 321. L:100 bp ladder. Lanes 1 to 21 represent genotypes

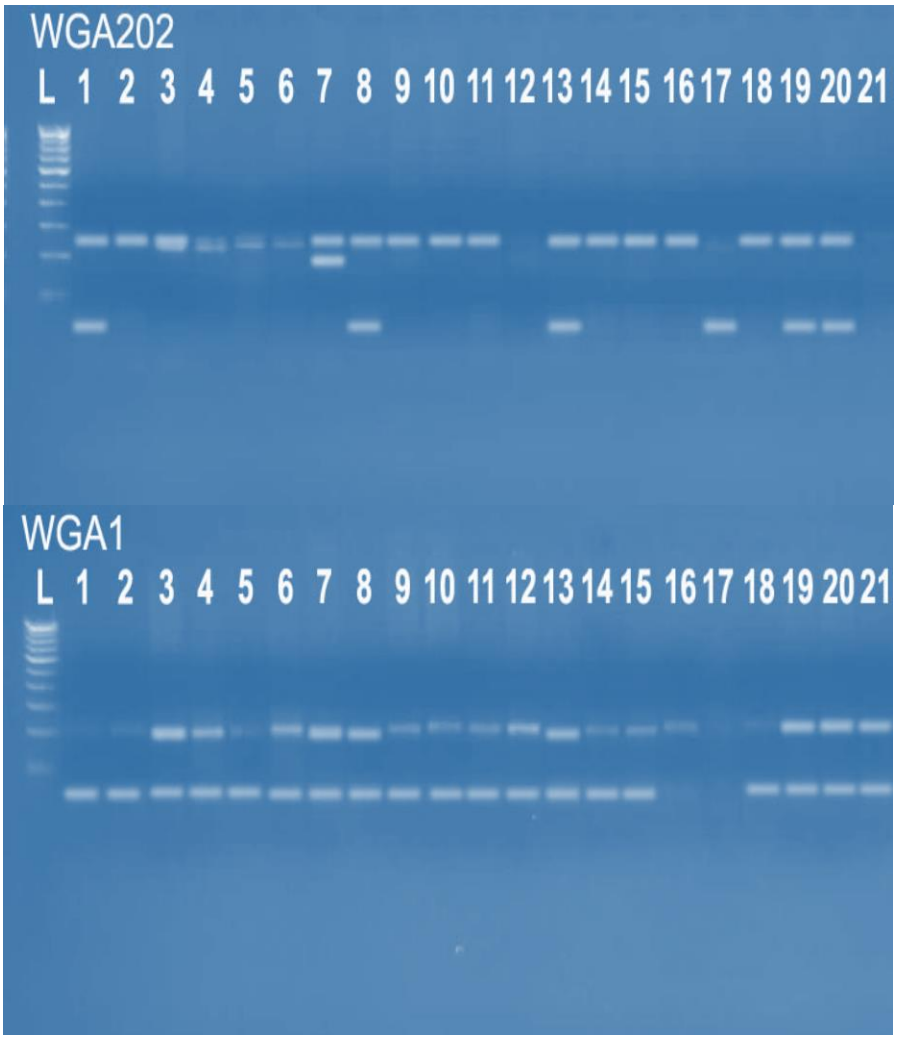


Fig. S3. PCR amplified pattern of walnut using primer WGA202 and WGA1. L:100 bp ladder. Lanes 1 to 21 represent genotypes