Agric Res J **61** (1): 39-48, *February* 2024
DOI: 10.5958/2395-146X.2024.00006.7

ASSESSING THE CONGRUENCE OF MOLECULAR DIVERSITY USING SSR MOLECULAR MARKERS WITH KEY PHENOLOGICAL TRAITS AMONG PERSIAN WALNUT (*JUGLANS REGIA* L.) GENOTYPES

Shailja Sankhyan[1*], Rajinder Kaur[1] and Krishan Kumar[2]

[1]Department of Biotechnology, [2]Department of Fruit Science,
Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan-173 230, India

ABSTRACT

Walnut (*Juglans regia* L.) is an important food crop with significant economic value. However, there has been no systematic work on the genetic characterization of indigenous and exotic walnut germplasm in India. Therefore, the present study aimed to genetically characterize walnut germplasm using molecular markers (SSR). A total of 29 primers were tested on a group of 37 walnut genotypes (maintained in Ochghat fields of Solan, Himachal Pradesh), out of which 19 markers showed robust amplification and generated polymorphism. A high level of genetic diversity was detected by the SSR primer S2. UPGMA cluster analysis classified the germplasm into two groups. Comparison between morphological traits and molecular markers was conducted using the Mantel test, showing a positive correlation (r=0.85). Thus, the molecular markers and morpho-physical characters used in this study revealed a high level of polymorphism in the 37 *J. regia* genotypes demonstrating their efficiency for diversity analysis studies.

Keywords: Genetic diversity, Genotypes, Morphological characters, SSR, Walnut germplasm

Walnut (*Juglans regia* L.), is an important nut belonging to the family Juglandaceae, commonly known as *'Akhrot'* in India. It is also referred to as Persian walnut or English walnut. Indigenous to Eurasia, it is cultivated worldwide in temperate regions for its high-quality wood and edible nuts. This monoecious and heterodichogamous species possesses a 2n chromosome number = 32. Major producers include China, Iran, the USA, and Turkey (FAO, 2012), with limited cultivation in Indian hilly regions such as Arunachal Pradesh, Himachal Pradesh, Uttarakhand, and Jammu and Kashmir (Laddha *et al*., 2020) (Fig 1). Walnut stands are found in various regions including Kopet Dagh, the lowlands along the southern Caspian Sea, and sporadic locations throughout the western and southern ranges of Iran (McGranahnan and Leslie, 1991).

Persian walnuts, the most common type, exhibit a distinct nutrient profile compared to black walnuts. Walnut oil is predominantly composed of polyunsaturated fatty acids, notably alpha-linolenic acid and linoleic acid (Ros and Mataix, 2006), contributing to its effectiveness in reducing cardiovascular disease risk. Furthermore, walnuts contain a rich spectrum of antioxidants, surpassing almonds, peanuts, and hazelnuts, including those bound to fiber.

Various techniques, including morphological characteristics (Atefi, 1997) and molecular markers, have been developed to estimate walnut genetic diversity. SSRs (Simple Sequence Repeats), in particular, have gained prominence due to their simplicity, efficiency, and ability to detect polymorphisms across the genome. These markers, comprising repeating units of 2-6 base pairs, offer codominant inheritance, multi-allelic variation, and high reproducibility, making them powerful tools for genetic analysis (Maggie *et al.*, 2022; Itoo *et al.*, 2023). They are classified into Genomic SSRs and EST or Genic SSRs, both of which exhibit high levels of polymorphism. SSR markers have proven useful in examining genetic redundancies and propagation errors within collections (Mitchell *et al.*, 1997; Phippen *et al.*, 1997; Dangl *et al.*, 2001).

Despite the extensive variation in native walnut germplasm, there has been a lack of systematic genetic characterization in India. Therefore, this study aims to assess genetic diversity among walnut genotypes using molecular markers (SSRs) and morphological traits. The identified genetic stocks can be used in future breeding programs.

MATERIALS AND METHODS

Source and DNA Isolation

Different samples of walnut were collected from the field located at Ochghat, within the Fruit Science Department of Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan. Young and healthy leaves from thirty-seven walnut genotypes were excised from

*Corresponding author: shailjabiotech@gmail.com
Date of receipt: 10.02.2023, Date of acceptance: 13.02.2024

Fig. 1. Geographic location of walnut cultivation in India

the plants in the field and transported to the laboratory. The leaves were stored at -80ºC until further use. Genomic DNA from the collected leaves was isolated using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987), followed by further purification.

PCR amplifications and gel electrophoresis

The PCR protocol was standardized for amplification using ISSR primers. A 20 µL mixture for PCR-SSR analysis was prepared, comprising 10X PCR buffer, 2mM $MgCl_2$, 1mM dNTPs, 0.2µM of each primer, 3U Taq DNA Polymerase, and 50ng of template DNA. The amplification followed a thermal profile consisting of an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at a temperature specific to each primer's Tm for 1 minute, and extension at 72°C for 2 minutes. This was further followed by a final extension step at 72°C for 5 minutes. The amplified DNA was thoroughly mixed with 6X loading dye (0.25% Bromophenol blue, 40% Sucrose) and electrophoresed in a 2% agarose gel in 1X TAE buffer (40mM Tris-acetate, 1.0mM EDTA). The gel was run at a constant voltage, with a rate of 5V/cm, for approximately 3 hours. Ethidium Bromide at a concentration of 0.5µg/mL was incorporated into the gel.

Data Analysis

Primers demonstrating polymorphism among walnut genotypes were selected. Genetic diversity, quantified through Polymorphism Information Content (PIC) as defined by Anderson *et al.* (1993), served to assess allelic diversity at each Genomic SSR locus. PIC values were computed as follows:

$$PIC = 1 - \Sigma pi2$$

where pi was the frequency of the i^{th} allele in the set of genotypes analyzed. Then the percentage of polymorphism was calculated. A binary code, where 0 represented absence and 1 represented presence of bands, was employed to depict the molecular markers. The Jaccard's similarity coefficient matrix was generated using NTSYSpc Version 2.02h software. Dendrograms were constructed based on the results obtained, and the efficiency of polymorphism generation by Genomic SSR was compared.

Morphological Studies

The UPOV (2000) test guidelines were followed for the characterization of Persian walnut based on morpho-physical features. Observations were recorded for three plants in each case. The mean value for each parameter (trait) in each of the 37 genotypes was utilized for statistical analysis of morphological traits where applicable. The timing of leaf emergence was noted when vegetative buds fully opened with green tips visible. The duration of leaf emergence for each genotype was determined by the time taken from the first bud leafing to the last vegetative bud leafing.

The dates of first and last catkin bud bursts were recorded, and the time gap between them was designated as the duration of male flowering. Similarly, data on female flowering was collected at the opening of the first and last female flowers, and the interval between them represented the duration of female flowering. Observations were made to determine the nature of dichogamy, categorizing trees as: a) Protandrous, b) Protogynous, c) Homogamous.

The average weight of five randomly collected sun-dried nuts from each of the 37 genotypes was measured using an electrical balance and expressed in grams. Shell hardness was visually assessed and characterized as: Hard, Very Hard, Intermediate, or Soft. The weight of the kernel was determined from five sun-dried nuts, and the average was calculated and expressed in grams.

To record data on kernel percentage for each genotype, the weight of five nuts was measured, and then the weight of their kernels was recorded. Kernel percentage was calculated using the following formula

$$\text{Kernel percentage} = \frac{\text{Kernel Weight}}{\text{Nut Weight}} \times 100$$

Table 1. Genomic SSR primers used in the study on *Juglans regia* L.

S. No.	Primer Name	Primer Code	Sequence	Tm	GC	Length (bp)
1.	Primer 1	S1	F: GTGGGTTCGACCGTGAAC	63.2	50.00	20
			R: AACTTTGCACCACATCCACA	64.0	60.00	20
2.	Primer 2	S2	F: ACGTCGTTCTGCACTCCTCT	64.1	50.00	20
			R: GCCACAGGAACGAGTGCT	64.3	55.00	20
3.	Primer 3	S3	F:GTGGCGAAAGTTTATTTTTTGC	63.3	50.00	22
			R: ACAAATGCACAGCAGCAAAC	63.9	55.00	22
4.	Primer 4	S4	F: AAACCACCTAAAACCCTGCA	63.2	55.00	20
			R: ACCCATCCATGATCTTCCAA	64.1	55.00	20
5.	Primer 5	S5	F: CACTGTGGCACTGCTCATCT	64.3	50.00	20
			R: TTCGAGCTCTGGACCACC	64.3	40.90	18
6.	Primer 6	S6	F: AGGGCACTCCCTTATGAGGT	63.7	45.00	20
			R: CAGTCTCATTCCCTTTTTCC	60.1	50.00	20
7.	Primer 7	S7	F: ACCCGAGAGATTTCTGGGAT	63.7	55.00	20
			R: GGACCCAGCTCCTCTTCTCT	63.8	55.00	20
8.	Primer 8	S8	F: AACCCTACAACGCCTTGATG	63.8	50.00	20
			R: TGCTCAGGCTCCACTTCC	64.4	50.00	20
9.	Primer 9	S9	F: AATCCCTCTCCTGGGCAG	64.3	50.00	18
			R: TGTTCCACTGACCACTTCCA	64.4	55.00	20
10.	Primer 10	S10	F: TCCCCCTGAAATCTTCTCCT	63.8	45.00	20
			R: CGGTGGTGTAAGGCAAATG	63.9	45.00	20
11.	Primer 11	S11	F: ACCCATCTTTCACGTGTGTG	63.6	58.80	20
			R: TGCCTAATTAGCAATTTCCA	59.7	52.90	20
12.	Primer 12	S12	F:TGTTGCATTGACCCACTTGT	64.4	41.20	20
			R:TAAGCCAACATGGTATGCCA	63.4	41.20	20
13.	Primer 13	S13	F:TGTAATTGGGGAATGTTGCA	63.4	35.30	20
			R:TGGGAGACACAATGATCGAA	64.0	58.80	20
14.	Primer 14	S14	F:ATTGGAAGGGAAGGGAAATG	55.3	45.00	20
			R:CGCGCACATACGTAAATCAC	57.3	50.00	20
15.	Primer 15	S15	F:CAGTTTGTCCCACACCTCCT	59.4	55.00	20
			R:AACCCATGGTGAGAGTGAG	59.4	55.00	20
16.	Primer 16	S16	F:CATCAAAGCAAGCAATGGG	54.5	47.40	19
			R:CCATTGCTCTGTGATTGGG	52.6	52.60	20
17.	Primer 17	S17	F: CAAGCTTCCGTATTGGTGGT	57.3	50.00	20
			R: TATACCGCAAGCTCGCAAC	59.4	55.00	20
18.	Primer 18	S18	F:CTC GGT AAG CCA CAC CAA TT	57.3	50.00	20
			R:ACG GGC AGT GTA TGC ATG TA	57.3	50.00	20
19.	Primer 19	S19	F:TTA GTT AGC AAA CCC ACC CG	57.3	50.00	20
			R:AGA TGC ACA GAC CAA CCC TC	59.4	55.00	20
20.	Primer 20	S20	F: CACCGTCTTATGCCATCCTT	57.3	47.10	20
			R: GTGCACTGTGGACGAAGAGA	57.3	44.40	20
21.	Primer 21	S21	F:TGTGCTCTGATCTGCCTCC	58.8	57.90	19
			R:GGGTGGGTGAAAAGTAGCAA	57.3	50.00	20
22.	Primer 22	S22	F:CCC ATC TAC CGT TGC ACT TT	57.3	50.00	20
			R:GCT GGT GGT TCT ATC ATG GG	59.4	55.00	20
23.	Primer 23	S23	F:CTC ACT TTC TCG GCT CTT CC	59.4	55.00	20
			R:GGT CTT ATG TGG GCA GTC GT	59.4	55.00	20
24.	Primer 24	S24	F:TCCAATCGAAACTCCAAAGG	55.3	45.00	20
			R:TGTCCAAAGACGATGATGGA	55.3	45.00	20
25.	Primer 25	S25	F:GCCCTCAAAGTGATGAACGT	57.3	50.00	20
			R:TCATCCATATTTACCCCTTTCG	59.4	61.10	20
26	Primer 26	S26	F:GACGACGAAGGTGTACGGAT	57.3	50.00	20
			R:GACGACGAAGGTGTACGGAT	57.3	35.00	20
27.	Primer 27	S27	F: CAAACAAAATCCGACCGC	58.8	57.90	19
			R: AAACCTCGATGAGCGAAGAA	57.3	50.00	20
28.	Primer 28	S28	F: CTAGGCTTCCCTAGCCGTG	57.3	52.60	20
			R: GGCTCCTCTCGATCTCGAC	59.4	50.00	20
29.	Primer 29	S29	F: CCCTAGCCTGGCTTTTCTTT	59.4	50.00	20
			R: ATAGCCTCCACTGGTTGTGG	59.4	50.00	20

In accordance with the shapes suggested by UPOV (2000), the following classes were established: circular, triangular, broad ovate, ovate, short trapezoid, long trapezoid, broad elliptic, elliptic, and cordate. Additionally, nut shapes based on cross-sections perpendicular to the suture were categorized as circular, broad elliptic, and elliptic.

RESULTS AND DISCUSSION

Marker validation by PCR amplification

Among various molecular markers, SSR markers have been extensively utilized in numerous plant genetics and breeding studies, including genetic relationship evaluation, gene tagging, and QTL mapping. In the current study, a total of 29 genomic SSR primers were employed for genetic diversity analysis. The primer sequences used have been provided in Table 1. Out of these, only 19 primers successfully amplified the genomic DNA of walnut genotypes, while the remaining 10 primers showed either no amplification or produced unreadable gel smears. A total of 482 bands were generated by the 19 informative primer pairs across the 37 walnut genotypes, comprising 17 polymorphic and two monomorphic bands.

Previous studies have consistently demonstrated the efficacy of SSR markers in genetic diversity analysis and germplasm characterization in walnut (Pollegioni *et al.*, 2008; Mahmoodi *et al.*, 2013; Karimi *et al.*, 2014; Wang *et al.*, 2015; Ebrahimi *et al.*, 2016; Shah *et al.*, 2016; Vischi *et al.*, 2017). Among the primer pairs used, the maximum number of amplified bands (49) was observed with primers 'S20' and 'S24', both of which exhibited polymorphism. Conversely, primer 'S8' produced the minimum number of bands (nine). Ten primers (S4, S5, S9, S10, S15, S17, S26, S27, S28, S29, and S34) failed to produce any amplification and were excluded from further analysis.

The total number of amplified bands was counted for each Genomic SSR primer-genotype combination. The observed level of polymorphism provided by Genomic SSRs in this study is notably high for studying genetic diversity (Fig. 2).

Polymorphism Information Content (PIC value) of primers

The PIC value represents the polymorphism information content of a primer, with a higher value indicating a greater level of polymorphism and thus increased usefulness for conducting further molecular marker studies. In our study, we observed a high PIC range with Genomic SSR markers, along with high average values for individual primers, demonstrating the utility of these primers for DNA fingerprinting and marker studies. Previous studies by Varshney *et al.* (2007) and Vaidya (2014) have also reported high PIC values with Genomic SSRs. In particular, the PIC value of Genomic SSR primer 'S2' (0.652) stood out as highly informative compared to other Genomic SSR primers in our research.

Genomic SSR analysis

NTSYSpc ver. 2.02.h (Rohlf, 2000) was utilized to generate the Jaccard's similarity coefficient matrix (Nei and Li, 1979), based on DNA amplification using Genomic SSRs. The analysis revealed a wide range of genetic variability within the species, with similarity coefficient values ranging from 0.062 to 0.916. The highest similarity value of 0.916 was observed between genotypes G6 (PLACENTIA) and G3 (ZHONG LIN # 3), while the lowest similarity of 0.062 was found between genotypes G3 (ZHONG LIN # 3) and G25 (SOLDING SELECTION).

Cluster analysis of Genomic-SSR profile

A dendrogram illustrating the clustering of *J. regia* genotypes was constructed using NTSYSpc ver. 2.02h. The SAHN module of NTSYS utilized the pairwise coefficients obtained to construct the dendrograms. The 37 *J. regia* genotypes were classified into two main clusters, labeled 'C' and 'D'.

Cluster C was further divided into two main subclusters, C1 and C2. Subcluster C1 comprised genotypes including AKSU#71, JOGINDER NAGAR SELECTION 39, DAULAT RAM SELECTION, CHICO, CHAMBA SELECTION 60, JOGINDER NAGAR SELECTION 12, SCHARCH FRANQUETTE, HOWARD, PLANT NO. 46, RONDE DE MONTINAC, BLACKMORE, XIN ZHANG ZHU, ROOPA AKHROT, JOGINDER NAGAR SELECTION 61, JOGINDER NAGAR SELECTION 2, HARTLEY, GRAVES FRANQUETTE, SERR, ACO 38853, LUXMI AKHROT, LAKE ENGLISH, RAJGARH SELECTION 11, PLANT NO. 5, CHAMBA SELECTION 32, CISCO, MEYLANNAISE, PARSIENNE, ZHONG LIN #3, PLACENTIA, XIN ZAD FEN, and SHINREI, while Cluster C2 included genotypes such as CHAMBA SELECTION 20, INDER AKHROT, and NETAR AKHROT. Cluster D consisted of a single genotype, namely SOLDING SELECTION (Fig. 3).

Morphological studies

Foliage characters

All the walnut germplasm accessions included in the present study exhibited significant variation in most foliage characteristics. This observed variation can be attributed to the diverse genetic makeup of these accessions, originating from various sources.

Specifically, two accessions exhibited very early bud break, 27 exhibited early bud breaks, and seven exhibited mid bud break (Table 2). The categories of early, mid, and late bud breaks were classified according to Thakur (1993). Additionally, McGranahan *et al.* (1990) reported that 'CISCO' exhibited late leafing, similar to 'FRANQUETTE'. The observed variability in leafing out times may be attributed to the exceptionally diverse origins of the accessions.

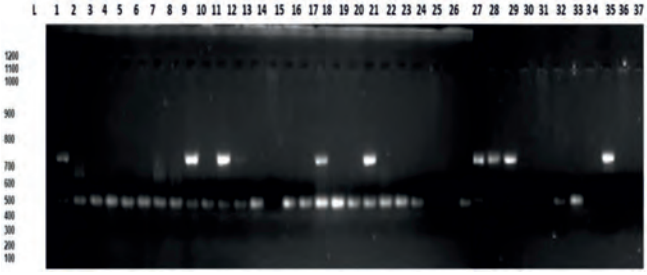

Fig. 2. Genomic SSR pattern of 37 *Juglans regia* genotypes

Floral Characters

Floral characteristics such as the abundance of male catkins and pistillate flowers, along with the degree of dichogamy, are essential criteria for the characterization and variation studies in walnut. Walnut genotypes exhibit a range of floral patterns, from nearly lateral-bearing to those where only terminal buds produce flowers (McGranahan and Leslie, 1991), with the former generally being more productive. The timing of floral events varied significantly among genotypes. For instance, 'HOWARD' exhibited the earliest male bloom on March 1st, while 'JOGINDER NAGAR SELECTION 39' was very late, with the first male bloom observed on March 30th, preceded by 'DAULAT RAM SELECTION', 'MEYLANNAISE', 'SHARSCH FRANQUETTE', and 'MAYETTE' on March 29th. The end of male flowering was earliest in 'HOWARD' on March 20th, while 'MEYLANNAISE' and 'PARISIENNE' were last to show the end of male flowering on April 25th. Similarly, Gupta (1999) reported that the period of male flowering extended from March 8th ('PLANT NO. 45' and 'LUXMI AKHROT') to April 1st ('SOLDING SELECTION').

Similarly, variations were observed in the timing of female flowering, with 'CHAMBA SELECTION 20' showing the earliest female bloom on March 20th, and 'MEYLANNAISE' and 'PARISIENNE' being the latest, with the first female bloom observed on April 19th. The end of female flowering ranged from March 28th ('PLANT NO. 5') to April 27th ('ZHONG LIN #3'). Previous reports from McGranahan *et al.* (1992) indicated that in the case of 'TULARE', the first female flower appeared on April 7th and the last on April 25th, while in the case of 'CHICO', the first female flower appeared on March 23rd and the last on April 12th. Similarly, Gupta (1999)

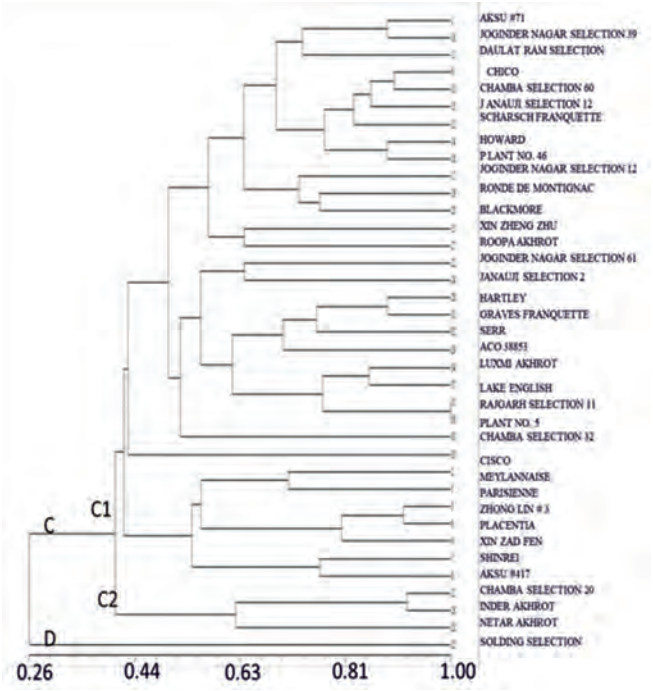

Fig. 3. UPGMA dendrogram of 37 *Juglans regia* genotypes based on genomic SSR analysis

reported the period of female flowering from March 21st (PLANT NO. 45) to April 13th (BLACKMORE).

Walnut is a heterodichogamous species, demonstrating protandry, protogyny, and homogamy in different genotypes, resulting in various degrees of dichogamy. In other words, there is complete overlapping to complete separation of pollen shedding and stigma receptivity periods. The extent of variation in the degree of dichogamy ranged from 27.27% ('CHAMBA SELECTION 60') to 100%. The data for nut shape through suture and nut shape perpendicular to suture are presented in Table 2. Nut shape through suture varied from ovate ('ROOPA AKHROT', 'CHAMBA SELECTION 32', 'JANAUJI SELECTION 12', 'ACO38853', 'PLACENTIA', 'BLACKMORE', 'XIN ZAD FEN', 'HOWARD', 'SHARSCH FRANQUETTE', 'MAYETTE'), CIRCULAR ('INDER AKHROT', 'SOLDING SELECTION', 'LUXMI AKHROT', 'RAJGARH SELECTION11', 'ZHONG LIN # 3', 'AKSU#417', 'CISCO', 'LAKE ENGLISH'), elliptic ('NETAR AKHROT', 'CHAMBA SELECTION 20', 'CHAMBA SELECTION 32', 'JANAUJI SELECTION 2', 'HARTLEY', 'AKSU#71'), broad elliptic ('PLANT NO. 5', 'CHICO'), trapezoid ('DAULAT RAM SELECTION' 'JOGINDER NAGAR SELECTION 61', 'SERR'), triangular ('JOGINDER NAGAR SELECTION 39') (Fig. 4).

Nut shape perpendicular to suture varied from elliptic ('NETAR AKHROT', 'CHAMBA SELECTION 20', 'CHAMBA SELECTION 32', 'AKSU#71',

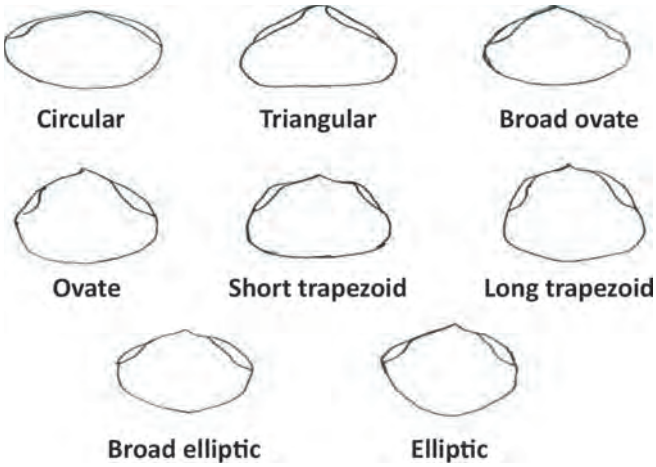

Fig. 4. Nut shape in longitudinal section through suture

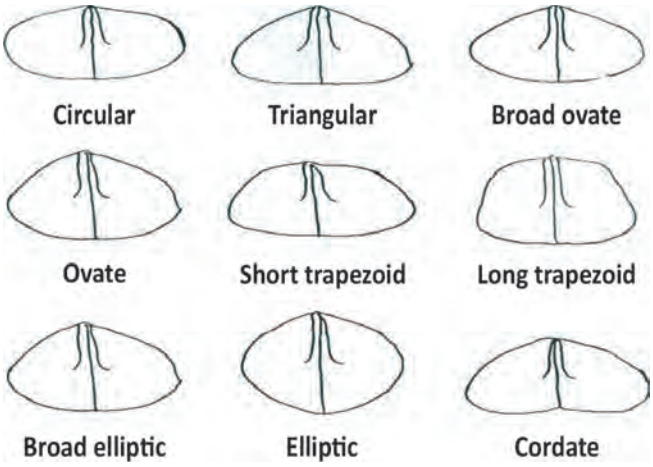

Fig. 5. Nut shape in longitudinal section perpendicular to suture

'SERR'), circular ('ROOPA AKHROT', 'INDER AKHROT','SOLDING SELECTION', 'LUXMI AKHROT', 'CHAMBA SELECTION 60', 'RAJGARH SELECTION 11', 'BLACKMORE', 'XIN ZAD FEN', 'AKSU#417', 'HOWARD', 'CISCO', 'LAKE ENGLISH'), obovate ('PLANT NO. 5'), cordate ('JANAUJI SELECTION 2', 'JOGINDER NAGAR SELECTION 39'), ovate ('JANAUJI SELECTION 12', 'DAULAT RAM SELECTION', 'PLACENTIA'), trapezoid ('JOGINDER NAGAR SELECTION 61', 'CHICO', 'GRAVES FRANQUETTE', 'ZHONG LIN #3', 'SHARSCH FRANQUETTE', 'MAYETTE'). These variations in nut shape have also been observed in previous studies (Chauhan and Sharma, 1979; UPOV, 2000; McGranahan and Leslie, 1991; Gupta, 1999) (Fig. 5).

Nut weight ranged from 4.5 g ('CHICO') to 26.0 g ('XIN ZAD FEN'). Mean and coefficient of variance were recorded as 15.25, and 143.75 per cent, respectively (Table 2). Shell hardness varied from hard ('NETAR AKHROT', 'LUXMI AKHROT', 'PLANT NO. 46', 'CHAMBA SELECTION 20', 'CHAMBA SELECTION 32', , 'JOGINDER NAGAR SELECTION 39', 'JOGINDER NAGAR SELECTION 61', 'RAJGARH SELECTION 11', 'ACO38853', 'BLACKMORE', 'XIN ZAD FEN', 'LAE ENGLISH'), intermediate ('ROOPA AKHROT', 'INDER AHROT', 'SOLDING SELECTION', 'HARTLEY', 'HOWARD'), very hard ('PLANT NO. 5', 'CHAMBA SELECTION 60', 'JANAUJI SELECTION 2', 'JANAUJI SELECTION 12'), soft ('DAULAT RAM SELECTION', 'GRAVES FRANQUETTE', 'ZHONG LIN #3', 'AKSU#71', 'AKSU#417', 'SERR', 'CISCO', ''SHARSCH FRANQUETTE', 'MAYETTE') very soft ('PLACENTIA', 'CHICO') (Table 2).

Kernel characters were recorded for those accessions which came into bearing at the time of study. Kernel having weight up to half of the nut weight is easy to remove (McGranahan and Leslie, 1991). Kernel weight varied from 2.5 g ('Blackmore') to 13.7 g ('Zhong Lin#3'). The mean and coefficient of variance were recorded as 5.45 and 139.93 per cent, respectively. Gupta (1999) reported kernel weight as low as 1.47 g in 'KX GIANT' to as high as 7.56 in 'INDER AKHROT'. Lonnie (1997) reported kernel weight between 5.1 g (FRANQUETTE) to 7.8 g (HOWARD) (Table 2).

Kernel characteristics, including weight and percentage, also displayed considerable variability among genotypes. Kernel weight ranged from 2.5 g ('BLACKMORE') to 13.7 g ('ZHONG LIN#3'), while kernel percentage ranged from 12.76% ('CHAMBA SELECTION 60') to 66.60% ('PLACENTIA'). Mean and coefficient of variance were recorded as 39.68 and 137.49 per cent, respectively, while previous reports indicate that kernel percentage ranged between 40.0 percent to 67.0 percent (Sen, 1983; Yarilgac and Koyuncu, 2001; He *et al.,* 2003).

However, in some cases, kernel percentage less than 25.0 per cent is reported (Gupta, 1999; Gautam, 2000). The use of morphological traits is important for breeding and selection programs, but most of the time, these traits can easily be affected by the development stage and environment. It has also been suggested that the type and number of morphological traits can affect the outcome of genetic analysis. Certain characters such as kernel and nut characters are considered to be more efficient markers for differentiating genotypes (Ebrahimi *et al.*, 2011; Wang *et al.*, 2015). In the present investigation, the morphological traits revealed genetic variation among walnut genotypes. These findings are similar to the results of Thakur (1993), McGranahan and Leslie (1991), and Gupta (1999). The value of the measured quantitative traits of 37 genotypes fell within the ranges reported in earlier studies (Christopolous *et al.*, 2010; Ebrahimi *et al.*, 2011; Karimi *et al.*, 2014; Wang *et al.*, 2015) (Table 2).

Table 2. Data showing morho-physical characters among 37 *Juglans regia* genotypes

Name of Germplasm	Time of Bud Burst		Flowering Time				Degree of Dichogamy (%)	Nut Shape		Shell hardness	Nut wt. (g)	Kernel wt. (g)	Kernel Recovery (%)
	Start	End	Male		Female			Through suture	Perpendicular suture				
			F	L	F	L							
NETAR AKHROT	18/3	3/4	10/3	22/3	27/4	8/4	100.00	Ovate	Circular	Hard	14.0	3.9	29.80
ROOPA AKHROT	26/2	10/3	12/3	22/3	26/3	5/4	100.00	Ovate	Circular	Intermediate	16.5	5.0	30.30
INDER AKHROT	16/3	31/3	14/3	22/3	24/3	3/4	100.00	Circular	Circular	Intermediate	13.0	4.5	34.60
SOLDING SELECTION	21/3	5/4	25/3	1/4	26/3	9/4	46.66	Circular	Circular	Intermediate	10.5	4.0	38.00
LUXMI AKHROT	18/3	4/4	8/3	18/3	1/4	11/4	100.00	Circular	Circular	Hard	12.0	3.0	25.00
PLANT NO.5	13/3	26/3	20/3	28/3	26/3	28/3	100.00	Broad	Elliptic obovate	Very hard	13.0	2.4	18.40
PLANT NO. 46	25/2	9/3	20/3	28/3	27/3	7/4	83.33	Ovate	Broad elliptic	Hard	14.5	3.3	22.70
CHAMBA SELECTION 20	9/3	9/4	12/3	29/3	20/3	30/3	100.00	Elliptic	Elliptic	Hard	12.5	3.2	25.76
CHAMBA SELECTION 32	13/3	28/4	15/3	30/3	26/3	4/4	60.00	Elliptic	Elliptic	Hard	16.5	4.5	27.20
CHAMBA SELECTION 60	12/3	5/4	15/3	¼	25/3	3/4	27.27	Ovate	Circular	Very hard	23.5	3.0	12.76
JAUNAJI SELECTION 2	17/3	22/3	20/3	29/3	26/3	¾	50.08	Elliptic	Cordate	Very hard	14.5	2.0	13.79
JAUNAJI SELECTION 12	7/3	28/3	22/3	31/3	29/3	6/4	33.33	Ovate	Ovate	Very hard	17.5	3.0	17.14
DAULAT RAM SELECTION	20/3	14/4	29/3	5/4	14/4	22/4	100.00	Trapezoid	Ovate	Soft	12.5	6.0	48.00
JOGINDER NAGAR SELECTION 39	24/3	0/4	30/3	6/4	10/4	18/4	100.00	Triangular	Cordate	Hard	18.5	3.0	25.00
JOGINDER NAGAR SELECTION 61	8/3	25/3	12/3	21/3	21/3	¾	92.30	Trapezoid	Trapezoid	Hard	17.5	4.5	25.70
RAJGARH SELECTION 11	12/3	30/3	18/3	30/3	25/3	5/4	54.54	Circular	Circular	Hard	14.0	3.0	21.40
CHICO	11/3	23/4	18/3	30/3	25/3	3/4	40.00	Broad	Trapezoid elliptic	Very soft	4.5	3.0	66.60
MEYLANNAISE	8/3	30/3	29/3	25/4	19/4	30/4	-	-		-	-	-	-
GRAVES FRANQUETTE	21/3	20/3	28/3	5/4	31/3	10/4	54.54	Elliptic	Elliptic	Soft	9.5	4.0	42.10
ACO38853	8/3	28/3	17/3	28/3	1/4	10/4	100.00	Ovate	Broad elliptic	Hard	12.5	3.5	28.00
PLACENTIA	6/3	26/3	20/3	5/4	30/3	24/4	-	Ovate	Ovate	Very soft	6.0	4.0	66.60
ZHONG LIN #3	12/3	8/4		1/4	27/4		100.00	Circular	Trapezoid	Soft	24.5	13.7	55.90
BLACKMORE	20/3	5/4	18/3	28/3	4/4	13/4	100.00	Broad elliptic	circular	Hard	9.0	2.5	27.70
HARTLEY	20/3	3/4	10/3	19/3	1/4	12/4	100.00	Ovate	Ovate	Intermediate	14.0	4.5	32.10
XIN ZADFEN	12/3	8/4	25/3	10/4	28/3	31/4	80.95	OVATE	Circular	Hard	26.0	6.0	23.00
AKSU#71	10/3	13/4	23/3	10/4	30/3	25/4	55.55	ELLIPTIC	Elliptic	Soft	17.0	8.5	50.00
AKSU#417	12/3	8/4	20/3	10/4	29/3	20/4	43.48	CIRCULAR	Circular	Soft	15.0	7.0	46.66
HOWARD	3/3	30/3	1/3	20/3	15/4	3/4	100.00	OVATE	Circular	Intermediate	15.0	5.4	36.10
RONDE DE MONTIGNAE	18/3	1/4	-	-	-	-	-	-	-	-	-	-	-
XIN ZHENG ZHU	14/3	8/4	-	-	-	-	-	-	-	-	-	-	-
SHINREI	12/3	15/4	-	-	-	-	-	-	-	-	-	-	-
PARISIENNE	12/3	20/4	25/3	25/4	19/4	5/5	56.25	-		-	-	-	-
SERR	12/3	3/4	25/3	19/4	4/4	25/4	66.66	TRAPEZOID	Elliptic	Soft	18.5	8.0	43.24
CISCO	4/3	20/3	25/3	5/4	1/4	25/4	80.00	CIRCULAR	Circular	Soft	14.0	8.0	57.10
SHARZSCH FRANQUETTE	12/3	1/4	29/3	10/4	15/4	24/4	100.00	OVATE	Trapezoid	Soft	10.0	4.5	45.00
MAYETTE	15/3	10/4	29/3	15/4	10/4	25/4	66.66	OVATE	Trapezoid	Soft	10.0	5.1	51.00
LAKE ENGLISH	20/3	1/4	25/3	10/4	6/4	20/4	50.00	CIRCULAR	Circular	Hard	18.5	4.0	21.60
Mean							50.00				15.25	05.45	39.68
SD							35.12				06.69	02.71	18.18
SE							09.79				13.46	12.25	09.01

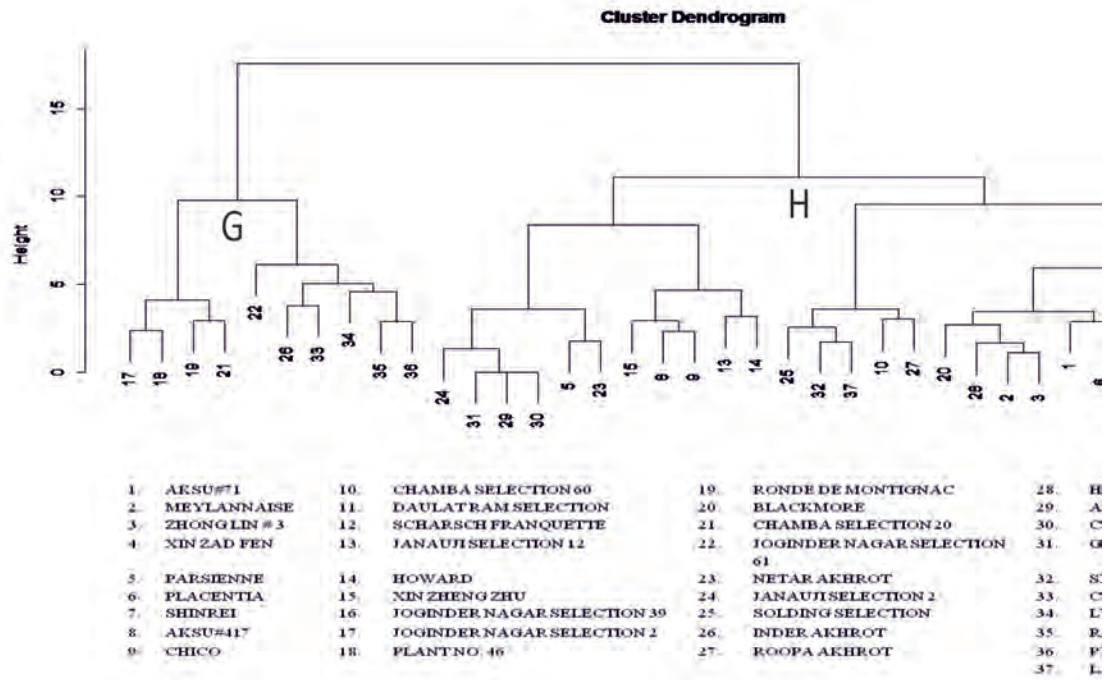

Fig. 6. Dendrogram of 37 *Juglans regia* genotypes based on morpho-physical characters

Dendrogram based on morphological characters

A dendrogram was constructed based on morphological traits using Eucledia analysis. The 37 genotypes of walnut were divided into two main clusters G and H. Cluster G was found to contain genotypes namely 'JOGINDER NAGAR SELECTION 2', 'PLANT NO. 46','RONDE DE MONTIGNAC', 'CHAMBA SELECTION 20', 'JOGINDER NAGAR SELECTION 61', 'HARTLEY', 'CISCO', 'LUXMI AKHROT', 'RAJGARH SELECTION 11' and 'PLANT NO. 5'. Whereas Cluster H was found to contain genotypes namely 'LAKE ENGLISH', 'SERR', 'CHAMBA SELECTION 32', 'GRAVES FRANQUETTE','ACO38853', 'ROOPA AKHROT', 'JANAUJI SELECTION 2', 'SOLDING SELECTION', 'INDER AKHROT', 'HOWARD', 'XIN ZHENG ZHU', 'NETAR AKHROT', 'BLACKMORE', 'AKSU#417', 'XIN ZAD FEN', 'PARISINNE', 'ZHONG LIN #3', 'SHINREI', 'AKSU#71', 'MEYLANNAISE','PLACENTIA', 'JANAUJI SELECTION 12', 'DAULAT RAM SELECTION', 'SCHARSCH FRANQUETTE', 'CHICO' and 'CHAMBA SELECTION 60' (Fig. 6).

Mantel Test

The relationship between two matrices, one representing morphological characters and the other molecular markers, was found to be significant (r=0.85), indicating a strong correlation as evidenced by the Mantel test. In this study, we compared the dendrograms generated from morphological and molecular marker data across 37 walnut genotypes. The results indicate that while both matrices depict genetic diversity in a similar manner, there are discrepancies between the analyses. Consequently, it is concluded that molecular markers should be prioritized for analyzing the genetic diversity of the 37 walnut genotypes due to their DNA-based nature, which offers greater stability compared to morphological traits.

Walnuts are an important horticultural crop cultivated for commercial purposes. In India, walnut cultivation is primarily concentrated in a few states. This report presents an efficient protocol for assessing the diversity of walnut germplasm. Our findings highlight that certain walnut genotypes, such as SOLDING SELECTION, NETAR AKHROT, INDER AKHROT, and CHAMBA SELECTION #20, exhibit the highest levels of diversity, making them valuable candidates for breeding programs and the conservation of walnut germplasm. Additionally, these markers can facilitate fingerprinting studies.

Authors' contribution

Conceptualization and design of experiments (RK and SS); Procurement of plant material (KK and SS); Conduct of experiments, data analysis and manuscript preparation (SS).

Declaration

The authors declare that there is no conflict of interest.

LITERATURE CITED

Anderson J A, Churchill G A, Autrique J E, Tanksley S D and Sorrells M E 1993. Optimizing parental selection for genetic linkage maps. *Genome* **36**: 181-86.

Atefi J 1997. Study on phenological and pomological characters on walnut promising clones in Iran. *Acta Hortic* **442**: 101-08.

Christopoulos M V, Rouskas D, Tsantili E and Bebeli P J 2010. Germplasm diversity and genetic relationships among walnut (*Juglans regia* L.) cultivars and Greek local selections revealed by inter-simple sequence repeat (ISSR) markers. *Sci Hortic* **125**: 584-92. https://doi.org/10.1016/j.scienta.2010.05.006

Chauhan J S and Sharma S D 1979. Phenotypic variability in walnuts of Kinnaur district of H.P. *Indian J Agric Sci* **49**: 420-23.

Dangl G S, Mendum M L, Prins B H, Walker M A, Meredith C P and Simon C J 2001. Simple sequence repeat analysis of a clonally propagated species: a tool for managing a grape germplasm collection. *Genome* **44**: 432-38.

Doyle J J and Doyle J L1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* **19**: 11-15.

Ebrahimi A, Fatahi R and Zamani Z 2011. Analysis of genetic diversity among some Persian walnut genotypes (*Juglans regia* L.) using morphological traits and SSR markers. *Sci Hortic* **130**: 146-51.

Ebrahimi A, Zarei A, Lawson S, Woeste K E and Smulders M J M 2016. Genetic diversity and genetic structure of Persian walnut (*Juglans regia*) accessions from 14 European, African, and Asian countries using SSR markers. *Tree Genet Genome* **12**: 2-14.

Food and Agriculture Organization (FAO) 2012. FAOSTAT http://faostat.fao.org

Gautam D R 2000. Selection of Persian walnut (*Juglans regia* L.) varieties in Shimla district of Himachal Pradesh. *IJPGR* **13**: 294-97.

Gupta S K 1999. Characterization and evaluation of walnut (*Juglans regia* L.) germplasm accessions. M.Sc. Thesis, Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, India.

He S L, Deng L, Xie R J, Wen L M, Yang Q and Yin X G 2003. The performance of the promising walnut varieties in the Daba mountain range. *South China Fruit* **32**: 35-38.

Itoo H, Shah A R, Qurat S, Jeelani A, Khursheed S, Bhat Z A, Mir M A, Rather G H, Zargar M S, Shah M D and Padder B A 2023. Genome-wide characterization and development of SSR markers for genetic diversity analysis in northwestern Himalayas Walnut (*Juglans regia* L.). *Biotech* **13**: 136.

Karimi R, Ershadi A, Ehtesham N A, Sharifani M, Rasouli M, Ebrahimi A and Vahdati K 2014. Morphological and molecular evaluation of Persian walnut populations in Northern and Western regions of Iran. *J Nutr* **5**: 21-31.

Laddha A P, Adki K M, Gaikwad A B and Kulkarni Y A 2020. Beneficial effects of nuts from India in cardiovascular disorders. **In**: *Nuts and Seeds in Health and Disease Prevention.* Academic Press, pp. 453-69.

Magige E A, Fan P Z, Wambulwa M C, Milne R, Wu Z Y, Luo Y H, Khan R, Wu H Y, Qi H L, Zhu G F and Maity D 2022. Genetic diversity and structure of Persian walnut (*Juglans regia* L.) in Pakistan: Implications for conservation. *Plants* **11**(13): 1652.

Mahmoodi R, Rahmani F and Rezaee R 2013. Genetic diversity among *Juglans regia* L. genotypes assessed by morphological traits and microsatellite markers. *Spanish J Agric Res* **11**: 431-37.

McGranahan G H, Forde H I , Synder D G, Sibbett G S, Reil W, Hasey J and Ramos D E 1992. 'Tulare' Persian walnut. *HortScience* **27**:186-187.

McGranahan G H, Ramos D E, Forde H I and Synder D G 1990. 'CISCO' Persian walnut. *HortScience* **25**: 371-72.

McGranahan G H and Leslie C 1991. Walnuts (*Juglans*). *Acta Hortic* **290**: 907-74. https://doi.org/10.17660/ActaHortic.1991.290.20

Mitchell S E, Kresovich C A, Jester C A, Hernandez and Szewc M A K 1997. Application of multiplex PCR and fluorescence-based, semi-automated sizing technology for genotyping plant genetic resources. *Crop Sci* **37**: 617-24.

Nei M and Li W H 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *PNAS* **74**: 5269-73.

Phippen W B, Kresovich S, Candelas F G and McFerson J R 1997. Molecular characterization can discriminate and partition variation among gene bank holdings: A case study with phenotypically similar interspecific comparisons. *Mol Ecol* **5**: 99-110.

Pollegioni P, Major A, Bartoli S, Ducci F, Proiett I and Malvolti M E 2008. Application of microsatellite and dominant molecular markers for the discrimination of species and interspecific hybrids in genus *Juglans*. *Acta Hortic* **705**: 191-97.

Rohlf F J 2000. *NTSYS-pc numerical taxonomy and multivariate analysis version 2.0*. Applied Biostatics. Inc, Port Jefferson, NY.

Ros E and Mataix J 2006. Fatty acid composition of nuts – implications for cardiovascular health. *Br J Nutr* **96**: S29-S35.

Sen S M 1983. Studies on the selection of walnut in Northeastern Anatolia and the eastern Black Sea region. *DoagabilimDergisi, D2 Tarin Ve Ormancilik* **7**:163-70.

Shah N U, Mir J I, Ahmed N and Fazili K M 2016. Assessment of germplasm diversity and genetic relationships among walnut (*Juglans regia* L.) genotypes through microsatellite markers. *J Saudi Soc Agric Sci* **17**(4): 339-50.

Thakur D 1993. Genetic variability in bearing seedlings walnuts (*Juglans regia* L.) in Kullu valley. M.Sc. Thesis, Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, India.

UPOV 2000. General information walnuts (*Juglans* sp.) International union for the protection of new varieties of plants/TG/125/1 (Prog.), Geneva, Switzerland.

Vaidya E 2014. Studies on QTL analysis for growth characteristics in apple *Malus domestica* Borkh. Ph D. Thesis, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, India, 136p.

Varshney K R, Chabane K, Hendre S P, Aggarwal K A and Graner A 2007. Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys. *Plant Sci* **173**: 638-49.

Vischi M, Chiabà C, Raranciuc S, Poggetti L, Messina R, Ermacora P, Cipriani G, Paffetti D, Vettori C and Testolin R 2017. Genetic diversity of walnut (*Juglans regia* L.) in the Eastern Italian Alps. *Forests* **8**:1-14.

Wang H, Wu W, Pan G and Pei D 2015. Analysis of genetic diversity and relationships among 86 Persian walnut (*Juglans regia* L.) genotypes in Tibet using morphological traits and SSR markers. *J Hortic Sci Biotechnol* **90**: 563-70.

Yarilgac T and Koyuncu M A 2001. Some promising walnut selections. *Acta Hortic* **544**: 93-96.