RESEARCH ARTICLE

Similarities of leafing and leaf fall date of ‘Pedro’ walnut variety with its progenies in breeding programs

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Abstract In this research, ‘Pedro’ walnut variety (maternal parent) was open pollinated. Offspring genotypes were compared with their maternal parent in terms of morphological and molecular features. Leafing and leaf fall dates were measured as two phenological traits. Genetic differences were identified by molecular fingerprinting using twelve Inter Simple Repetitive Sequence Polymorphism (ISSR) primers. In some progenies, leafing and leaf fall occurred later than the maternal parent (‘Pedro’). Progenies were grouped according to their similarities with the maternal parent regarding leafing date. Specifically, 14.44% of the progenies were ‘Very close’ to the parent, 16.66% were ‘Close’, 30.00% ‘Far’ and 38.89% ‘Very far’. Regarding the leaf fall date, 7.78% of the progenies were ‘Very close’ to the parent, 28.88% were ‘Close’, 36.66% ‘Far’ and 26.67% ‘Very far’. A total of 43 bands were

obtained in molecular analyses. These bands were largely polymorphic (95.34%). The average number of the alleles was 3.90, while the polymorphism information content varied between 0.14 and 0.91 among the genotypes. Genetic distances were tested according to the UPGMA (Unweighted Pair Group Method with Arithmetic Average) and by The Structure Analysis. The UPGMA dendrogram divided the genotypes into two main clusters and the similarity coefficient of the genotypes varied between 0.61 and 0.99. This study revealed that ‘Pedro’ can be used as an excellent parental variety in hybridization programs which prioritize late-leafing in walnut breeding. Furthermore, ISSR primers can be safely used for fingerprinting walnuts in general.

Keywords ISSR · *Juglans regia* L. · Molecular variance · Phenology · Walnut

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Introduction

Walnut is a deciduous tree and a diploid species with 32 chromosomes (Woodworth 1930). Dichogamous walnut genotypes are more common, while homogamous walnut genotypes are very infrequent. Therefore, walnuts are generally open-pollinated. The heterozygous nature of walnuts makes it difficult to reproduce progenies that resemble high degrees of similarity with their parents. Although this situation has a negative effect on production and growers propagate superior cultivars clonally (Ebrahimi and Vahdati 2006), it provides an important genetic variation for breeders (Vahdati et al. 2019). However, walnuts are characterized by long periods of juvenility which further restrict the breeding of this fruit tree (Haj-najari et al. 2012; Özcan and Sütyemez 2017; Vahdati et al.

2019). In recent years, biotechnological methods have contributed significantly to shortening the breeding period (Karimi et al. 2010; Arab et al. 2019a, b).

Morphological and molecular markers together are usually helpful in plant breeding programs (Hajnajari et al. 2012; Dogan et al. 2014). They are used in many plant species in order to determine genetic diversity, phylogenetic studies and in creating genome maps (Reddy et al. 2002; Golein et al. 2012; Arab et al. 2019b). ISSR markers are used for fingerprinting because of their low cost (Topçu et al. 2015; Ikhsan et al. 2016; Sadat Hosseini et al. 2019). ISSR markers can determine the genetic diversity of genotypes at the seedling stage of plants (Potter et al. 2002; Pollegioni et al. 2003; Christopoulos et al. 2010; Malvolti et al. 2010; Erdoğan and Aygün 2016; Eser et al. 2019).

Open pollination in heterozygous nut species such as walnut usually cause a high level of variation. The genetic variation of genotypes is very important to breeders. So far, however, no studies have examined phenological and genetic variation among walnut offspring when a known variety is open-pollinated with an unknown pollinator. ‘Pedro’ (‘Conway Mayette’ × ‘Payne’) is a high-yielding, lateral bearing walnut variety which originated in California. It is considered as the maternal parent of most commercial and important walnut varieties (‘Chandler’ and ‘Howard’), while also being the paternal parent of the most important walnut pollinizer in California (‘Cisco’). In fact, it is preferably used as a parental variety in many breeding programs in the world (Vahdati et al. 2019).

The available literature does not contain research on the phenology and genetic features of genotypes obtained from the ‘Pedro’ walnut variety and on the degree to which they are similar to the main parent variety. This study was carried out to determine the extent that plant phenology and features of the ‘Pedro’ walnut variety can be seen in offspring genotypes. In this study, the phenological and molecular characterization of offspring were studied after the seeds of open-pollinated Pedro trees grew into seedlings. Also, genetic relationships were described between genotypes and their maternal parent (Pedro). As a result, the current study bears important findings that can provide new information on walnut hybrids and on their breeding programs.

Materials and methods

Plants materials

‘Pedro’ is one of the most prevalent varieties used in hybridization breeding programs in the USA, and so it was chosen as the main material of this study.

In the current study, ninety genotypes were obtained from open-pollinated ‘Pedro’ cultivar as the maternal (main) parent. In the study, 200 seeds randomly taken from 5 open-pollinated walnut trees belonging to Pedro walnut cultivar, which is 10 years old and found in the same area plot, were used. From 200 seeds that germinated, 90 genotypes were randomly selected. In addition, 5 ‘Pedro’ saplings were used as the control.

In the research, phenological observations were made at the 2nd, 3rd and 4th Ages of the genotypes and control plants. In the first year (1.age), germination of seeds and herbal healthy development were provided.

Plants to be used as a control were obtained by grafting on 1-year-old seedlings (*Juglans regia* L.) obtained from the Pedro variety 1 year ago. Chip budding was used as the grafting method.

In the 4th year of the study, no suitable data were obtained as proplem was observed in the plants of “21, 37, 42, 43, 62” genotypes. Therefore, these genotypes were excluded from the study. In other words, data of 90 genotypes are presented in the study. Numbering remained between 1 and 95.

Phenological observations

Phenological differences such as leafing date and defoliation (leaf fall) date of walnut genotypes were determined according to descriptors that are specific for walnut (IPGRI 1994). In all plants used in the study, data for the first year were not obtained. Phenological observations in genotypes and control plants were made for 3 years at the 2nd, 3rd and 4th ages in a row. Average values are presented in the study (Table 3).

Phenological variation classification

Phenological characteristics of the plant materials were evaluated according to the IPGRI (1994) walnut descriptors (Table 1). The classification of the phenological differences according to the maternal parent was made according to Table 2.

Phenological data analysis

The data were analyzed statistically with descriptive statistics, cluster analyses, Principal Component Analyses (PCA) and correlation by using the JMP13 Statistical Package Program for genetic diversity based on phenological traits. Phenological pair-wise distances of the walnut genotypes were clustered using Ward’s Method (Anderberg 1973).

Table 1 Definitions used in the determination of phenological traits. Source: IPGRI (1994)

Traits	Description
Leafing date	Date when over 50% of terminal buds have enlarged and the bud scales have split exposing the green leaves inside
Defoliation date	When all the leaves of the plant have fallen

Table 2 Classification of the phenological data of the offspring of open pollinated ‘Pedro’ in comparison with the parent ‘Pedro’ cultivar. Source: Sütyemez 2018

Classification	Distance between genotypes and the maternal parent cultivar in terms of phenological features. Distance is expressed as number of days
Very close	± 0–3
Close	± 4–8
Far	± 9–15
Very far	± 16 and above

Molecular analysis

DNA isolation and extraction

Walnut DNA was isolated using the CTAB protocol (Doyle and Doyle 1987), modified by Bardak (2012). Young and healthy leaves were used for DNA extraction. The collected leaves were kept in dry ice, transferred to the laboratory and then stored in at -80 °C for DNA isolation. Genomic DNA was extracted from approximately 0.5 g of young, fresh walnut leaves by using the optimized Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Doyle. DNA concentration by spectrophotometry (SPECTROstar Omega) were standardized to 2.5 ng/μL and visualized on 1% agarose gels stained with ethidium bromide.

ISSR-PCR amplification

In order to determine the genetic differences between walnut genotypes, ISSR primers used by Bardak and Bölek (2012) were employed. PCR reactions were performed in a total 20 μL volume. The reaction mixture contained 2 μL 10X PCR buffer, 1 μL dNTP (25 mM), 1.5 μL MgCl₂, 1 μL ISSR primer (10 pmol), 2 μL genomic DNA (25 ng), 0.5 μL DNA Taq polimeraz (vivantis), 12 μL dH₂O. The PCR

temperature reaction was carried out as follows: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation of 1 min at 94 °C, annealing for 1 min at 55 °C (based on the used primer) and extension for 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. The gel images were then visualized by UV light and recorded.

Data analysis

The ISSR PCR product was run in an electrophoresis gel. The gel images were analyzed and data were scored as 1 for the presence and 0 for the absence of the products (Laborda et al. 2005). Polymorphism information contents of the primers were used in this study and calculated by the Excel software. Total polymorphic bands were calculated and allele frequencies of each band were calculated according to the following formula. Here, f_i denotes the frequency of the ‘i’ band.

$$PIC = 1 - \sum (f_i)^2$$

The coefficient of similarity between walnut genotypes was calculated in the POPGENE 3.2 version 1.2 package program (Nei 1972). The unweighted pair group with arithmetic means (UPGMA) entered the clustering method using NTSYSpc software (Ver. 2.02), (Rohlf 1998). Cluster analysis of walnut genotypes was done by the Structure 2.3.4 package program (Pritchard et al. 2000). To determine the ideal number of groups, each K value was run from 1 to 10 with 10 independent simulations. The permutation module was chosen between 10,000 and 100,000. Delta K value, which determines the number of groups, was calculated by making 5 repetitions for each K value (Evanno et al. 2005). The results of the analysis was uploaded to the ‘structure harvester’ (Earl and VonHoldt 2012) web page and the ideal ΔK value was found (Anonymous 2020).

Result and discussion

The results of classification of the phenological differences of the offspring genotypes of open pollinated ‘Pedro’ showed a wide range of variation in terms of leafing date and leaf fall date in comparison with their maternal parent (‘Pedro’). In the current research, very important phenological and molecular similarities were obtained between the maternal parent cultivar and the offspring genotypes (Tables 3, 4, 5, 6, and 7; Figs. 1, 2, 3, 4, 5 and 6).

Phenological observations (similarity-differences rate)

The leafing date is an important parameter for fruit species which are damaged by late spring frosts. Late leafing date is one of the main objectives of many walnut breeding programs. It has been determined that walnut plants in the population of this study have high degrees of variation in terms of the leafing date.

Phenological findings of ‘Pedro’ (main variety) and genotypes are given in Table 3. The leafing date of the maternal parent walnut (‘Pedro’) started on 29 March and the leaf fall date started on November 24. Leafing in genotypes started as early as March 19 and as late as May 9. Leaf fall started as early as November 6 and as late as December 30 (Table 3). The leafing dates of walnut genotypes varied in a span of 51 days. Among the 90 genotypes, 7 genotypes began leafing earlier and 83 genotypes began leafing later than the ‘Pedro’ parent. It was observed that leafing dates in genotypes, on average, were 12.9 days different compared to the parent variety (Table 4). The start of leaf-fall in walnut genotypes spanned for 54 days. Among the progenies, 7 genotypes started leaf fall earlier and 83 genotypes started leaf fall later than the parent cultivar. Leaf fall dates differed by an average 8.75 days, compared with the parent walnut variety (Table 4). The genotypes generally started leafing and leaf fall later than the main variety.

In this study, genotypes were categorized in comparison with the ‘Pedro’ parent (Table 3). The similarities of the leafing date of genotypes with the main parent were classified in different groups. In total, 14.44% of the genotypes were ‘Very close’ to their parent, 16.66% were ‘Close’, 30.00% ‘Far’ and 38.89% ‘Very far’. Regarding the leaf fall date, the similarities between progenies and the ‘Pedro’ parent were 7.78% ‘Very close’, 28.88% ‘Close’, 36.66% ‘Far’ and 26.67% ‘Very far’ (Table 4).

Leafing date and leaf fall date of genotypes were evaluated comparatively. There was a very important variation between the maternal parent and the next-generation progeny. Late leafing is one of the most important objectives in walnut breeding programs. In this study, most genotypes (83) had leafing later than the main variety. Furthermore, according to the main variety ‘Pedro’, 9 genotypes (4, 6, 25, 28, 30, 33, 38, 56, and 93) were observed to leafing later and leaf fall earlier (Table 3). If the ‘Pedro’ variety is used as a parent in walnut breeding programs, it is very likely that new genotypes with late leafing can be obtained.

Mahmoodi et al. (2016) compared 5 walnut genotypes with some varieties such as ‘Chandler’, ‘Hartley’, ‘Pedro’, ‘Ronde de Montignac’ and ‘Franquette’ in two consecutive years (2010–2011). In this research, the leafing date in ‘Pedro’ occurred on April 7 in the first year and on March

29 in the second year. The difference between the two years was attributed to inconsistencies in the weather of the location. Ünal (2011) adapted 12 local and foreign varieties to the Niksar (Tokat) ecology. According to their results, leaf fall in the ‘Pedro’ variety occurred on 3–10 December, whereas leaf fall in offspring occurred from November 6 to December 30. Sütyemez and Kaşka (2002) observed variations in leaf fall dates of 32 domestic and foreign walnut varieties under Kahramanmaraş ecological conditions. Among all walnut varieties, the latest leaf fall date was observed in ‘Payne’, ‘Chandler’ and ‘Pedro’ varieties.

Phenological data analysis

Phenological features are considerably essential in the breeding and introduction of fruit species. In this study, phenological diversity was substantial among walnut genotypes (Table 5). The leafing variation coefficient (CV = 9.92%) was determined to be greater than the leaf fall variation coefficient (CV = 2.30%) (Table 5). The summary findings pertaining to leafing and defoliation dates of genotypes are presented in Table 5. In this regard, a similar report indicated that the CV = 77.78% (date of leafing), in an experiment that involved 540 walnut trees selected in Iran (Khadivi-Khub et al. 2015).

Pearson’s Correlation Coefficient was used for associations between the phenological traits. Correlations of phenological traits are presented in Fig. 1. In the analysis, it is seen that there is a positive relationship between leafing and leaf fall ($r = 0.0957$). Amiri et al. (2010) found similar relationships associated with these two traits ($r = 0.298$). In walnut, significant correlations are reported between leafing and some phenological properties (Ebrahimi et al. 2015; Khadivi-Khub et al. 2015; Abedi and Parvaneh 2016; Hassankhah et al. 2017). However, studies on the relationship between the said parameters and the dates of leaf fall remain limited (Amiri et al. 2010).

PCA analysis was carried out to make an overall view of the differences in our walnut accession. PCA showed that the first component (PC1) of phenological traits explained 54.786% of the total variation. The second component (PC2) represented a total variance of 45.214% of the variation. The bi-plot segregated the walnut genotypes into groups based partially on their parents (Table 6; Fig. 2). Multivariate analysis summarized the variability of a complex dataset and presented it in an interpretable form, such as in principal components (Ribeiro et al. 2013). The aim of PCA is to determine the main factors and effective parameters that discriminate among accessions (Khadivi-Khub et al. 2015). Although the PCA analysis has been performed on many plant traits in walnut, results on such phenological traits have remained limited (Cosmulescu and

Table 3 Classification of the phenological data of the offspring of open-pollinated ‘Pedro’ in comparison with their maternal parent cultivar (‘Pedro’)

Genotypes	Leafing date differentiate from ‘Pedro’ (in days)	Leafing date differentiate from ‘Pedro’	Defoliation date differentiate from ‘Pedro’ (in days)	Defoliation date from ‘Pedro’	Genotypes	Leafing date differentiate from ‘Pedro’ (in days)	Leafing date differentiate from ‘Pedro’	Defoliation date differentiate from ‘Pedro’ (in days)	Defoliation date from ‘Pedro’
Genotype 36	– 1	Very close	+ 3	Very close	Genotype 82	+ 10	Far	+ 12	Far
Genotype 5	+ 3	Very close	+ 6	Close	Genotype 85	+ 14	Far	+ 12	Far
Genotype 32	+ 3	Very close	+ 4	Close	Genotype 87	+ 14	Far	+ 9	Far
Genotype 40	+ 3	Very close	+ 5	Close	Genotype 88	+ 13	Far	+ 11	Far
Genotype 41	– 1	Very close	+ 6	Close	Genotype 94	+ 11	Far	+ 12	Far
Genotype 53	+ 1	Very close	+ 4	Close	Genotype 4	+ 10	Far	– 18	Very far
Genotype 69	+ 2	Very close	+ 8	Close	Genotype 27	+ 14	Far	+ 20	Very far
Genotype 76	+ 2	Very close	+ 7	Close	Genotype 55	+ 10	Far	+ 23	Very far
Genotype 31	+ 3	Very close	+ 9	Far	Genotype 77	+ 13	Far	+ 36	Very far
Genotype 60	+ 3	Very close	+ 9	Far	Genotype 78	+ 13	Far	+ 16	Very far
Genotype 61	+ 3	Very close	+ 14	Far	Genotype 9	+ 10	Far	+ 4	Close
Genotype 45	+ 2	Very close	+ 9	Far	Genotype 26	+ 13	Far	+ 8	Close
Genotype 51	– 1	Very close	+ 12	Far	Genotype 35	+ 9	Far	+ 4	Close
Genotype 39	+ 8	Close	+ 4	Close	Genotype 46	+ 11	Far	+ 5	Close
Genotype 66	+ 8	Close	+ 7	Close	Genotype 48	+ 10	Far	+ 4	Close
Genotype 73	+ 6	Close	+ 7	Close	Genotype 50	+ 10	Far	+ 4	Close
Genotype 74	+ 6	Close	+ 6	Close	Genotype 57	+ 9	Far	+ 4	Close
Genotype 6	+ 8	Close	– 15	Far	Genotype 59	+ 9	Far	+ 4	Close
Genotype 10	– 4	Close	+ 9	Far	Genotype 65	– 10	Far	+ 4	Close

Table 3 continued

Genotypes	Leafing date differentiate from 'Pedro' (in days)	Leafing date differentiate from 'Pedro'	Defoliation date differentiate from 'Pedro' (in days)	Defoliation date differentiate from 'Pedro'	Leafing date differentiate from 'Pedro' (in days)	Leafing date differentiate from 'Pedro'	Defoliation date differentiate from 'Pedro' (in days)	Defoliation date differentiate from 'Pedro'
Genotype 16	− 4	Close	+ 9	Far	+ 10	Far	+ 6	Close
Genotype 47	+ 8	Close	+ 9	Far	+ 9	Far	− 3	Very close
Genotype 71	+ 6	Close	+ 14	Far	+ 14	Far	− 1	Very close
Genotype 75	+ 4	Close	+ 11	Far	− 14	Far	− 2	Very close
Genotype 28	+ 8	Close	− 18	Very far	+ 18	Very far	+ 17	Very far
Genotype 54	+ 8	Close	+ 19	Very far	+ 32	Very far	+ 17	Very far
Genotype 56	+ 7	Close	− 16	Very far	+ 23	Very far	+ 16	Very far
Genotype 63	+ 8	Close	+ 24	Very far	+ 32	Very far	+ 16	Very far
Genotype 72	+ 4	Close	+ 19	Very far	+ 20	Very far	+ 16	Very far
Genotype 14	+ 9	Far	+ 9	Far	+ 16	Very far	+ 24	Very far
Genotype 23	+ 14	Far	+ 15	Far	+ 28	Very far	+ 16	Very far
Genotype 58	+ 11	Far	+ 15	Far	+ 16	Very far	+ 17	Very far
Genotype 79	+ 11	Far	+ 10	Far	+ 18	Very far	+ 26	Very far
Genotype 67	+ 28	Very far	+ 20	Very far	+ 41	Very far	+ 9	Far
Genotype 70	+ 28	Very far	+ 18	Very far	+ 18	Very far	+ 11	Far
Genotype 80	+ 18	Very far	+ 24	Very far	+ 23	Very far	+ 11	Far
Genotype 84	+ 25	Very far	+ 16	Very far	+ 28	Very far	+ 9	Far
Genotype 92	+ 23	Very far	+ 17	Very far	+ 38	Very far	+ 4	Close
Genotype 1	+ 17	Very far	+ 9	Far	+ 32	Very far	− 4	Close

Table 3 continued

Genotypes	Leafing date differentiate from 'Pedro' (in days)	Leafing date differentiate from 'Pedro'	Defoliation date differentiate from 'Pedro' (in days)	Defoliation date differentiate from 'Pedro'	Genotypes	Leafing date differentiate from 'Pedro' (in days)	Leafing date differentiate from 'Pedro'	Defoliation date differentiate from 'Pedro' (in days)	Defoliation date differentiate from 'Pedro'
Genotype 7	+ 18	Very far	+ 9	Far	Genotype 68	+ 16	Very far	+ 8	Close
Genotype 12	+ 21	Very far	+ 9	Far	Genotype 81	+ 28	Very far	+ 4	Close
Genotype 13	+ 21	Very far	+ 9	Far	Genotype 86	+ 28	Very far	+ 7	Close
Genotype 15	+ 20	Very far	+ 9	Far	Genotype 33	+ 17	Very far	– 1	Very close
Genotype 17	+ 18	Very far	+ 9	Far	Genotype 34	+ 17	Very far	0	Very close
Genotype 18	+ 17	Very far	+ 15	Far	Genotype 91	+ 23	Very far	0	Very close
Genotype 20	+ 19	Very far	+ 10	Far	Pedro	0	–	0	–
Genotype 22	+ 16	Very far	– 15	Far					

Table 4 Phenological properties of walnut genotypes in comparison with their maternal parent cultivar ('Pedro')

Phenological properties	Being active (realization)	Variation in comparison with the 'Pedro' cultivar				Number of active genotypes by 'Pedro' cultivar		Number of genotypes in each category by comparison with the 'Pedro' cultivar			
	Dates	Duration days	Range (days)	Average duration (days)	Before	After	Very close	Close	Far	Very far	
Leafing	19/03–09/05	51	1–41	12.90	7 (7.78%)	83 (92.22%)	13 (14.44%)	15 (16.66%)	27 (30.00%)	35 (38.89%)	
Defoliation	06/11–30/12	54	0–36	8.75	10 (11.11%)	80 (88.89%)	7 (7.78%)	26 (28.88%)	33 (36.66%)	24 (26.67%)	

Table 5 Descriptive statistics for phenological traits in our walnut collection

Traits	Unit	Min.	Max.	Mean	SD ^a	CV ^b (%)
Leafing	Day	78	129	100.75	9.99	9.92
Defoliation	Day	310	364	336.02	7.74	2.30

^aSD standard deviation, ^bCV coefficient of variation = (SD/mean) × 100

Table 6 Eigenvectors of the principal components (PC) for the studied walnut accessions

Phenological traits	PC1	PC2
Leafing	0.71	− 0.71
Defoliation	0.71	0.71
% of variance	54.786	45.214
Cumulative variance	54.786	100.000

Principal component (PC) coefficients of leafing and defoliation

Trandafir 2011; Ercisli et al. 2012; Pop et al. 2013; Bou Abdallah et al. 2016; Arab et al. 2019a).

Cluster analysis of the 90 genotypes, including seedlings and their parent, was performed on the basis of 2 phenological traits and to estimate the relationships between the walnut genotypes in a dendrogram (Fig. 3). In this analysis, walnut participation was mainly classified into 2 main clusters and in 10 subset cluster groups. The parent cultivar (Pedro) was included in the 5th subcluster of the first main cluster (Fig. 3).

The results of the cluster analysis partially confirmed the results of PCA on the genotypes (Fig. 3). These findings show that although walnut is genetically heterozygote in most cases, dominant genes largely determine features such as leafing and leaf fall dates. Morphological traits have been used effectively in detecting genetic variation among walnuts in various studies (Arzani et al. 2008; Ebrahimi et al. 2010, 2011; Ghasemi et al. 2012; Norouzi et al. 2013; Hussain et al. 2016; Cosmulescu and Stefanescu 2018;

Rezaei et al. 2018). The conclusion from the present study clearly showed that morphological markers are able to differentiate partially between seedlings obtained from open pollinated walnut cultivars.

Molecular analysis

In current study, the ISSR marker technique was used for determining variations between walnut genotypes. Twelve ISSR primers were used. All eleven primers showed amplifications except one primer (ISSR15) which showed no amplification. Forty-three bands (alleles) were obtained from 11 primers. Among all 43 bands, 41 were polymorphic, while two of the amplified bands were monomorphic. Primers that showed monomorphic bands (ISSR4, ISSR11) were not included in the review. The polymorphism information content (PIC) of the primers varied from 14 to 91%. The polymorphism rate of ISSR primers was 95.3%. As a result, the ISSR12 primer had the highest number of bands (8). The average number of alleles was calculated as 3.90 (Table 7).

Potter et al. (2002) used the ISSR marker technique for determining the genetic relationships between 48 walnut genotypes in the Wolfskill research garden, California, USA. In amplifying 8 ISSR primers, 54 bands were obtained. From a total of 54 bands, 31 (57%) were polymorphic. The number of alleles in each primer varied from 5 to 9, while the number of polymorphic bands varied from 1 to 7. The researchers reported that ISSR primers showed more polymorphic bands than the RAPD technique in determining the genetic differences between walnut varieties and genotypes. Such molecular polymorphism in walnut varieties and genotypes are common by the ISSR technique and according to previous reports, the polymorphism varies between 43 and 72% (Potter et al. 2002; Akcan 2007; Pollegioni et al. 2008; Najafi et al. 2014; Doğan et al. 2014).

A wide range of similarity index between walnut genotypes is an expected situation. ‘Pedro’ is a dichogamous walnut variety and, therefore, it has high levels of

Table 7 Allele numbers and PIC (polymorphism information content) values of ISSR primers which were used in the current study

No.	Primer	Sequence	Number of alleles	PIC
1	ISSR1	CACACACACACAA	2	0.60
2	ISSR3	CACACACACACAGG	3	0.91
3	ISSR5	CACACACACACAG	3	0.16
4	ISSR6	CACACACACACACAGT	6	0.14
5	ISSR7	ACACACACACACACACCG	5	0.78
6	ISSR8	ACACACACACACACACACC	4	0.72
7	ISSR9	ACACACACACACACACTG	4	0.42
8	ISSR12	GAGAGAGAGAGAGAGAC	8	0.28
9	ISSR13	AGAGAGAGAGAGAGAGC	6	0.75

Fig. 1 Scatter plot matrix and heatmap of correlations in phenological traits

Fig. 2 A) Scatter plot for the first two principal components for the studied walnut accessions based on phenological traits. B) Principal component analysis bi-plot of phenological traits among 90 walnut genotypes

genetic variation due to cross-pollination with different genotypes.

Doğan (2006) used 25 ISSR and RAPD primers to determine genetic similarities between 62 walnut genotypes originating in Turkey and elsewhere. The genetic similarity coefficient of the ISSR markers was 0.62–0.94,

while it ranged from 0.63 to 0.95 when using the RAPD technique. Nonetheless, ISSR and RAPD marker techniques gave similar results in the genetic similarity between varieties. The ISSR technique is more efficient in terms of reproducibility.

Fig. 3 Phenotypic clustering of 90 walnut genotypes based on Ward's phenological pair-wise distance and phenological heat map. Cluster analysis of the genotypes is based on the traits with high heritability

Vahdati et al. (2015) analyzed 17 SSR primer pairs in 6 walnut varieties from the Kerman region of Iran and reported that heterozygosity ranged between 0.65 and 0.87.

Similarity indexes between genotypes in many genetic studies of walnut varied from 0.60 to 0.93 (Akcan 2007),

Fig. 4 Genetic similarity dendrogram according to UPGMA method using ISSR data

12 to 79% (Ahmed et al. 2012), 0.47 to 0.88 (Najafi et al. 2014) and 0.56 and 0.82 (Vahdati et al. 2015).

UPGMA (Unweighted Pair Group Method of Arithmetic Averages) analysis is used for determining the difference

Fig. 5 The ideal value obtained in the Structure program of the ISSR molecular marker technique of the main variety and in 90 genotypes

Fig. 6 Cluster analysis results of the main variety and in 90 genotypes created by the ISSR molecular marker technique ($\Delta K = 2$, $\Delta K = 4$)

between walnut genotypes. It is carried out by NTSYSpc. Furthermore, the Structure 2.3.4 programs are used for clustering the genotypes (Fig. 4). Genotypes were found to be genetically similar to 0.61–0.99 (Fig. 4). According to the results, 90 walnut genotypes were divided into 2 main groups. These two were subsequently clustered in different groups (Fig. 4).

The ‘Pedro’ parent and 78 of its progenies (genotypes) were placed in one group. The remaining 12 genotypes

were clustered in the other group. There are also 4 sub-groups within the large group. In cluster analysis, genotypes 44 and 64 were 100% similar. Furthermore, genotypes 60 and 78, genotypes 8 and 17, genotypes 26 and 63 were determined to be more than 95% similar to each other. In addition, no more than 12 genotypes (6, 40, 49, 60, 73, 74, 75, 78, 77, 80, 84, 83) were observed to be genetically similar to the main variety (Pedro).

In a relevant study, Ipek et al. (2019) selected eight genotypes out of 2500. These 8 genotypes had superior traits. The ISSR technique was used for determining the genetic similarities of these 8 walnut genotypes. They were genetically very close to important walnut varieties. Also, the UPGMA analysis revealed genetic similarities. The highest genetic similarity was 0.891 in the ‘Chandler’ variety and the lowest genetic similarity was 0.491 in the ‘Franquette’ variety. Moreover, ‘Pedro’ and ‘Fernor’ varieties had very close relatives.

The diagram is illustrated by calculating the ideal ΔK value via the Structure 2.3.4 Program, based on the genetic data being available in this study (Fig. 6). Clustering analysis was repeated in the Structure 2.3.4 program to determine genetic differences between the genotypes. The highest Delta K (ΔK) value occurred at $\Delta K = 2$ (Fig. 6), which corresponded to the most probable number of groups, and $\Delta K = 4$ corresponded to the number of subgroups in the study (Figs. 5, 6). Thus, all genotypes were categorized into two major clusters similar to results of the UPGMA analysis (Fig. 4). The dendrogram and clustering analysis yielded consistent results through statistical evaluation.

Conclusion

Classifying the phenological differences of offspring (genotypes) that resulted from open pollination of the ‘Pedro’ variety showed a wide range of variation in terms of leafing date and leaf fall date. The features of offspring had substantial differences compared to their maternal parent (Pedro). In the current research, important phenological and molecular differences were obtained between the maternal parent variety and the offspring genotypes.

In walnut hybrid breeding programs, obtaining a genotype with late leafing and early leaf fall is one of the most important issues. Highly (92.22), the leafing and leaf fall dates of the offspring genotypes were later than those of the parent cultivar (Pedro). In the research, it was determined that 9 genotypes (4, 6, 25, 28, 30, 33, 38, 56, 93) leaf later than the main variety and they leaf fall before. According to the parent variety, genotype 36 showed ‘very close’ characteristics both in the first leafing and in the leaf fall. In this terms of both features, it was determined that the genotypes 39, 66, 73, and 74 were ‘close’ to the main variety, while 9 genotypes (14, 23, 58, 79, 82, 85, 87, 88, and 94) were in the ‘far’ category. According to the main variety, 14 genotypes (2, 3, 8, 11, 19, 29, 44, 49, 64, 67, 70, 80, 84, 92) were found in a very distant category in terms of leafing and leaf fall.

This should be considered as an important finding when ‘Pedro’ is preferred as a parent in walnut hybridization

programs. Using it as a parent makes phenological features such as late leafing and late leaf fall more likely to be seen in offspring genotypes.

Hybridization programs using the ‘Pedro’ walnut variety can lead to new individual plants, in which most of them are late leafing but shed their leaves earlier than their parent does. Accordingly, hybridization breeding programs need to consider the paternal parent as a determining factor.

In this study, morphological and molecular markers were used for determining the gene resource, thereby providing a clear characterization. This study is not only important in revealing the phenological and genetic similarities between seedlings but is also important in providing genetic resources for future hybridization programs in walnut.

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