A Research on the Determination of Phenological and Molecular Characterization in Open-pollinated Genotypes in Walnut

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**A RESEARCH ON THE DETERMINATION OF PHENOLOGICAL AND
MOLECULAR CHARACTERIZATION IN OPEN-POLLINATED GENOTYPES
IN WALNUT**

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ABSTRACT

This research was carried out to reveal the phenological and genetic differences between the S-1/1 walnut genotype and 94 F1 genotypes obtained from this genotype with each other and with the maternal parent. In the phenological observations made, it was observed that bud burst in genotypes took 52 days, leafing 50 days, leaf yellowing 31 days, and defoliation date 27 days. When the maternal parent (S-1/1) and the genotypes were compared, it was found that there was a phenological variation of 75.54 % in budburst, 73.41 % in the leafing, 34.05 % in leaf yellowing, and 93.62 % in defoliation date, while the average variation was 69.15 %. In molecular genetic analyzes, 7 ISSR primers were used to determine genetic variations, as a result, 7 monomorphic and 45 polymorphic bands were obtained, and the rate of polymorphism was found to be 86.53 %. The average number of alleles was calculated to be 7.42. In genotypes, the polymorphism information content (PIC) value varied between 0.48 and 0.95, while the average PIC value was calculated to be 0.73. As a result of cluster analysis, it was seen that genotypes were divided into 2 main clusters and 2 subsets. At the end of the study, it was determined that the S-1/1 walnut genotype and F1 genotypes obtained from this genotype have a significant variation both phenologically and genetically.

Key words: *Juglans regia* L., molecular, phenology, ISSR, variation

INTRODUCTION

Since walnut is monoecious, has a tendency to dichogamy and has a heterozygous structure, seedlings do not show the same characteristics as the main variety when propagated by seed. This population is a great source of genes for breeders, while causes economic losses in domestic and foreign markets, as well as inability to provide standardization for breeders. A long period of time (10-15 years) is needed for characterization of a variety by selection and hybridization breeding in walnuts. Biotechnological methods recently were used to decrease the

time needed for characterization. The use of these methods enables the characterization of genetic populations grown in different ecologies in a shorter time. Molecular marker techniques allow measuring biotechnological DNA levels and tracking the desired gene. The marker techniques are used in many areas related to genes such as phylogenetic analysis, mapping, and new gene discoveries (Filiz and Koç 2011). Reliability and practical use of ISSR markers and the length of primers provide advantages. The use of ISSR primers, which will deliver the desired information, is preferred due to the less time, labor and cost. The ISSR primers recently have been widely used to determine the genetic diversity in many plant species, phylogenetic studies and reveal genome maps (Yorgancılar et al. 2015).

Very few studies have been carried out to determine the phenological and genetic variation of the seedling genotypes obtained from the walnut seed compared to the main variety (Sutyemez et al. 2018; Bukucu et al. 2020; Ozcan et al. 2020; Yıldırım and Sütyemez 2020). Therefore, the studies investigating the phenological and genetic variation in F1 genotypes obtained especially from varieties to be used as main parents, are important to provide valuable information for crossbreeding breeding studies.

The purpose of this study was to determine the phenological and genetic relationships between the F1 plants obtained from the open-pollinated seeds of the S-1/1 walnut genotype with very high fruit size and the main parent variety.

2. MATERIAL AND METHOD

2.1. Plant Material

In the study, S-1/1 walnut genotype selected by Sütyemez (2019) and 94 seedling genotypes (F1) obtained from open-pollinated seeds belonging to this genotype were used.

Important characteristics of S1/1 genotype are highly productive and early, fruit weight ranges from 23 and 24g, kernel ratio is between 50 and 52 %, kernel color is light, outer shell is clear and smooth.

2.2. Phenological observations

The phenological traits such as budburst, leafing, leaf yellowing and defoliation dates of the main genotype S-1/1 and other walnut genotypes were defined according to Walnut Descriptors (Table 1). The classification of phenological characteristics was given in Table 2.

Table 1. Definitions used to determine the phenological traits (IPGRI 1994)

Traits	Description
Budburst	When over 50% of terminal buds have enlarged and the bud scales have split to expose the inside green leaves
Leafing	When over 50% of terminal buds have enlarged and the bud scales have split exposing the green leaves
Leaf yellowing	When more than 50 % of the green leaves on the plant turn yellow
Defoliation	When all the leaves of the plant have fallen

Table 2. Classification of phenological characteristics of genotypes according to the main cultivar

Classification	Phenological similarity to main cultivar (in days)
Very close	±0-3
Close	±4-8
Far	±9-14
Very Far	≥ ±15

Source: Sütyemez (2018), + after, - before

2.3. Phenological data analysis

Descriptive statistics, cluster analyses, Principal Component Analyses (PCA) and correlation test were carried out using the JMP13 statistical software to reveal the information on genetic diversity. Phenological pair-wise distances of the walnut genotypes were clustered using Ward's method (Anderberg 1973).

2.4. Molecular analyzes

DNA isolation and extraction

The leaflets of S-1/1 genotype and leaflets 94 seedlings obtained from S-1/1 genotype were collected in the spring and brought to the laboratory in dry ice. The young and healthy leaves samples were passed through 70% alcohol and distilled water and kept at -80 °C until DNA isolation. The DNA isolation of leaf samples was performed using the CTAB protocol developed by Doyle Doyle (1987) and modified by Bardak (2012). In order to obtain gel images of the S-1/1 walnut genotypes; 3 % agarose gel was prepared and the DNAs were run in 1 x TBE buffer solution at 160 voltage electric current for 2 hours. After running, the gel was treated with ethidium bromide (1 liter distilled water + 300 µl ethidium bromide) for 15 minutes. Stained DNA bands were visualized under UV (ultraviolet) light.

ISSR-PCR amplification

Twelve ISSR primers were prepared using the ISSR technique developed by Zietkiewicz et al. (1994) and modified by Bardak and Bölek (2012) to determine the genetic similarity rates between 94 S-1/1 genotypes. The ISSR reading range of PCR analysis for 94 walnut genotypes is between 200 and 1000 base pairs. In the scoring, DNA bands of walnut genotypes and main variety were compared, and the bands of the same length were evaluated as '1', while bands of different lengths were evaluated as '0' (Zhang et al. 2005; Bardak and Bölek 2012).

Data analysis

The polymorphism information contents of the primers used in the study were calculated in Excel using the data obtained by scoring (Laborda et al. 2005). The sums of '1' and '0' allele scores from the polymorphic bands were obtained and the allele frequencies of each band were calculated using the following equation;

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

Where, f_i represents the frequency of i band.

Genetic distance was determined using POPGENE32 (Population Genetic Analysis; version 1.32) software according to Nei (1972). The phylogenetic tree was obtained in the MEGA 7 (Molecular Evolutionary Genetics

analysis) program by the 'Arithmetic Mean and Unweighted Pair Group Method' (UPGMA) method using genetic distance data.

Cluster analysis of S-1/1 walnut genotypes was performed using Structure 2.3.4 software. Each K value was run from 1 to 10 with 10 independent simulations to determine the ideal number of groups.

The permutation module was chosen between 10000 and 100000 and the ΔK value, which determines the number of groups, was calculated by 5 replications for each K value. The results of analysis were archived in a zip file and the ideal ΔK value was determined by uploading this file to the "structure harvester" web page (<http://taylor0.biology.ucla.edu/structureHarvester/>).

3. RESULTS AND DISCUSSION

Late spring and early autumn frosts cause the most damage in fruit growing (Ağaoğlu et al. 2019). Therefore, the first leafing and defoliation dates of fruit species are important. The walnut is adversely affected by late spring and early autumn frosts, therefore, obtaining genotypes with late leafing and early defoliation is one of the main objectives in breeding studies (Özcan et al. 2020).

3.1 Phenologic Observations

Phenological variation was determined between the S-1/1 main cultivar and the seedlings obtained from open-pollinated seeds of S-1/1 offspring genotypes (Table 3; Table 4).

The budburst date of the S-1/1 main cultivar occurred on March 16, while the budburst of F1 genotypes occurred in a 52-day period. Twenty three of F1 genotypes were classified (24.46 %) in the "very close" category to main cultivar, 44 genotypes (46.8 %) were in the "close" category, 24 genotypes (25.53 %) were in the "far" category, and 3 genotypes (3.19 %) were in the "very far" category (Table 4). Phenological variation between the F1 genotypes and the main cultivar as indicated by budburst date was 75.54 %. The mean number of days of variation ratio between S-1/1 and the other 94 genotypes was 4.52, and the period ranged from 0 to 41 days (Table 4).

The first leafing date in F1 genotypes was in a wide range of 50 days. The number of F1 genotypes classified in "very close" category in terms of first leafing was 25 (26.46 %), 26 (22.67 %) of F1 genotypes were classified in "close" category, 21 (22.34 %) of them were in "far" category, and 22 (23.4 %) of them were "very far" category. A phenological variation of 73.41 % was detected between the main cultivar and the F1 genotypes in terms of the first leafing.

The average variation in days between the main genotype and the F1 genotypes was 8.7, and the period was between 0-44 days (Table 4). The leaf yellowing date of main cultivar was November 4, and the leaf yellowing start time of the 9 F1 genotypes was spread over 26 days. The leaf yellowing dates of the 62 F1 genotypes (65.95 %) were "very close" to the main genotype, 18 genotypes (19.14 %) were "close", 10 genotypes (10.63 %) were "far" and 4 genotypes (4.25 %) were "very far". The phenological variation as indicated by the onset of leaf yellowing between the main cultivar and F1 genotypes (32 genotypes) was 34.05%. The mean number of days of variation between S-1/1 and 94 F1 genotypes was 1.52, and the date of onset of yellowing ranged from 0 to 24 days (Table 4).

133 The defoliation in the S-1/1 main genotype occurred on November 15, while the defoliation dates of 94
134 genotypes were spread over 43 days. The defoliation date of 6 (6.38 %) seedling genotypes was “very close” to
135 the main cultivar, 31 (32.97%) of genotypes were “close”, 31 (32.97 %) of them were “far” and 26 (27.65%) of
136 them were “very far” to main cultivar. The phenological variation between the main cultivar and the other
137 genotypes (with 88 genotypes) in terms of defoliation date was calculated as 93.62 %. The average number of
138 days of variation ratio between S-1/1 and 94 F1 genotypes was 11.21 %, and the date of defoliation ranged from
139 0 to 33 days (Table 4).

Table 3. Some phenological characteristics and classification of walnut genotypes (According to S-1/1)

Genotypes	Budburst	From S-1/1 (days)	Classification	First leafing	From S-1/1 (days)	Classification	Leaf yellowing	From S-1/1 (days)	Classification	Defoliation	From S-1/1 (days)	Classification
Genotype 1	22/03	+6	Close	31/03	+10	Far	06/11	+2	Very Close	13/12	+28	Very Far
Genotype 2	20/03	+4	Close	28/03	+7	Close	04/11	0	Very Close	13/12	+28	Very Far
Genotype 3	11/03	-5	Close	22/03	+1	Very Close	04/11	0	Very Close	17/12	+32	Very Far
Genotype 4	09/03	-7	Close	18/03	-3	Very Close	03/11	-1	Very Close	29/11	+14	Far
Genotype 5	15/03	-1	Very Close	23/03	+2	Very Close	05/11	+1	Very Close	28/11	+13	Far
Genotype 6	19/03	+3	Very Close	28/03	+7	Close	04/11	0	Very Close	13/12	+28	Very Far
Genotype 7	27/03	+11	Far	8/04	+18	Very Far	09/11	+5	Close	28/11	+13	Far
Genotype 8	08/03	-8	Close	17/03	-4	Close	31/10	-4	Close	29/11	+14	Far
Genotype 9	13/03	-3	Very Close	21/03	0	Very Close	12/11	+8	Close	02/12	+4	Close
Genotype 10	16/03	0	Very Close	23/03	+2	Very Close	03/11	-1	Very Close	14/12	+29	Very Far
Genotype 11	21/03	+5	Close	02/04	+12	Far	19/11	+15	Far	15/12	+30	Very Far
Genotype 12	26/03	+10	Far	06/04	+16	Very Far	08/11	-4	Close	03/12	+18	Very Far
Genotype 13	27/03	+11	Far	06/04	+16	Very Far	13/11	+9	Far	17/12	+32	Very Far
Genotype 14	23/03	+7	Close	01/04	+11	Far	07/11	+3	Very Close	18/12	+33	Very Far
Genotype 15	13/03	-3	Very Close	21/03	0	Very Close	05/11	+1	Very Close	28/11	+13	Far
Genotype 16	11/03	-5	Close	20/03	-1	Very Close	03/11	-1	Very Close	06/12	+21	Very Far
Genotype 17	26/03	+10	Far	02/04	+12	Far	02/11	-2	Very Close	23/11	+8	Close
Genotype 18	02/04	+17	Very Far	08/04	+18	Very Far	12/11	+8	Close	28/11	+13	Far
Genotype 19	28/03	+12	Far	07/04	+17	Very Far	31/10	-4	Close	13/11	-2	Very Close
Genotype 20	30/03	+14	Far	08/04	+18	Very Far	02/11	-2	Very Close	28/11	+13	Far
Genotype 21	26/04	+41	Very Far	03/04	+43	Very Far	30/10	-5	Close	28/11	+13	Far
Genotype 22	29/03	+13	Far	07/04	+17	Very Far	03/11	-1	Very Close	09/11	-6	Close
Genotype 23	28/03	+12	Far	08/04	+18	Very Far	03/11	-1	Very Close	18/11	+3	Very Close

Table 3. Continue

Genotypes	Budburst	From S-1/1 (days)	Classification	First leafing	From S-1/1 (days)	Classification	Leaf Yellowing	From S-1/1 (days)	Classification	Defoliation	From S-1/1 (days)	Classification
Genotype 24	12/03	-4	Close	20/03	-1	Very Close	05/11	+1	Very Close	21/11	+6	Close
Genotype 25	19/03	+3	Very Close	25/03	+4	Close	03/11	-1	Very Close	20/11	+5	Close
Genotype 26	5/03	-11	Far	17/03	-4	Close	31/10	-4	Close	28/11	+13	Far
Genotype 27	21/03	+5	Close	27/03	+6	Close	12/11	+8	Close	28/11	+13	Far
Genotype 28	21/03	+5	Close	29/03	+8	Close	05/11	+1	Very Close	21/11	-6	Close
Genotype 29	20/03	+4	Close	28/03	+7	Close	05/11	+1	Very Close	24/11	+9	Far
Genotype 30	21/03	+5	Close	29/03	+8	Close	04/11	0	Very Close	11/11	-4	Close
Genotype 31	20/03	+4	Close	26/04	+5	Close	01/11	+1	Very Close	25/11	+10	Far
Genotype 32	11/03	-5	Close	19/03	-2	Very Close	05/11	+1	Very Close	25/11	+10	Far
Genotype 33	17/03	+3	Very Close	26/03	+5	Close	05/11	+1	Very Close	23/11	+9	Far
Genotype 34	18/03	+2	Very Close	25/03	+4	Close	19/11	+15	Far	29/11	+14	Far
Genotype 35	24/03	+8	Close	02/04	+12	Far	02/11	-2	Very Close	10/11	-5	Close
Genotype 36	16/03	0	Very Close	22/03	+1	Very Close	29/10	-6	Close	10/11	-5	Close
Genotype 37	08/03	-8	Close	20/03	-1	Very Close	03/11	-1	Very Close	11/11	-6	Close
Genotype 38	22/03	+6	Close	01/04	+11	Far	02/11	-2	Very Close	20/11	+5	Close
Genotype 39	21/03	+5	Close	31/03	+10	Far	03/11	-1	Very Close	10/11	-5	Close
Genotype 40	30/03	+14	Far	10/04	+20	Very Far	31/10	-4	Close	29/11	+14	Far
Genotype 41	21/03	+5	Close	01/04	+11	Far	03/11	-1	Very Close	24/11	+9	Far
Genotype 42	21/03	+5	Close	30/03	+9	Far	05/11	+1	Very Close	08/11	-7	Close
Genotype 43	24/03	+8	Close	03/04	+13	Far	03/11	-1	Very Close	11/11	-4	Close
Genotype 44	23/03	+7	Close	01/04	+11	Far	04/11	0	Very Close	11/11	-4	Close
Genotype 45	22/03	+6	Close	30/03	+9	Far	02/11	-2	Very Close	3/12	+18	Very Far
Genotype 46	19/03	+3	Very Close	26/03	+5	Close	02/11	-2	Very Close	10/11	-5	Close
Genotype 47	19/03	+3	Very Close	27/03	+6	Close	01/11	-3	Very Close	18/11	+3	Very Close
Genotype 48	12/03	-4	Close	21/03	0	Very Close	31/10	-4	Close	25/11	+10	Far

Table 3. Continue

Genotype	Budburst	From S-1/I (days)	Classification	First leafing	From S-1/I (days)	Classification	Leaf Yellowing	From S-1/I (days)	Classification	Defoliation	From S-1/I (days)	Classification
Genotype 49	18/03	+2	Very Close	24/03	+3	Very Close	02/11	-2	Very Close	05/11	+10	Far
Genotype 50	24/03	+8	Close	02/04	+12	Far	04/11	0	Very Close	18/11	+13	Far
Genotype 51	21/04	+5	Close	04/05	+44	Very Far	03/11	-1	Very Close	24/11	+9	Far
Genotype 52	20/03	+4	Close	27/03	+6	Close	03/11	-1	Very Close	10/11	-5	Close
Genotype 53	12/03	-4	Close	21/03	0	Very Close	26/10	-9	Far	19/11	+4	Close
Genotype 54	26/03	+10	Far	05/04	+15	Far	02/11	-2	Very Close	24/11	+9	Far
Genotype 55	27/03	+11	Far	06/04	+16	Very Far	02/11	-2	Very Close	18/11	+3	Very Close
Genotype 56	30/03	+14	Far	06/04	+16	Very Far	03/11	-1	Very Close	19/11	+4	Close
Genotype 57	26/03	+10	Far	06/04	+16	Very Far	03/11	-1	Very Close	28/11	+13	Far
Genotype 58	27/03	+11	Far	06/04	+16	Very Far	02/11	-2	Very Close	13/11	-2	Very Close
Genotype 59	12/03	-4	Close	21/03	+0	Very Close	12/11	+8	Close	28/11	+13	Far
Genotype 60	11/03	-5	Close	20/03	-1	Very Close	05/11	+1	Very Close	10/11	-5	Close
Genotype 61	13/03	-3	Very Close	27/03	+6	Close	30/10	-5	Close	19/11	+4	Close
Genotype 62	30/03	+14	Far	08/04	+18	Very Far	08/11	+4	Close	28/11	+13	Far
Genotype 63	17/03	+1	Very Close	23/03	+2	Very Close	31/10	-4	Close	20/11	+5	Close
Genotype 64	21/03	+5	Close	29/03	+8	Close	05/11	+1	Very Close	21/11	+6	Close
Genotype 65	15/03	-1	Very Close	24/03	+3	Very Close	02/11	-2	Very Close	10/11	-5	Close
Genotype 66	26/03	+10	Far	02/04	+12	Far	03/11	+22	Very Far	03/12	+18	Very Far
Genotype 67	24/03	+8	Close	02/04	+12	Far	18/11	+24	Very Far	18/12	+33	Very Far
Genotype 68	30/03	+14	Far	08/04	+18	Very Far	01/11	-3	Very Close	19/11	+4	Close
Genotype 69	21/03	+5	Close	29/03	+8	Close	02/11	-2	Very Close	23/11	+8	Close
Genotype 70	21/03	+5	Close	29/03	+8	Close	03/11	-1	Very Close	03/12	+18	Very Far
Genotype 71	11/03	-5	Close	20/03	-1	Very Close	04/11	0	Very Close	28/11	+13	Far
Genotype 72	25/03	+9	Far	02/04	+12	Far	02/11	-2	Very Close	30/11	+15	Far
Genotype 73	31/03	+15	Far	08/04	+18	Very Far	05/11	+1	Very Close	28/11	+13	Far

Table 3. Continue

Genotypes	Bud burst	From S-1/1 (days)	Classification	First leafing	From S-1/1 (days)	Classification	Leaf Yellowing	From S-1/1 (days)	Classification	Defoliation	From S-1/1 (days)	Classification
Genotype 74	16/03	0	Very Close	23/03	+2	Very Close	13/11	+9	Far	13/12	+28	Very Far
Genotype 75	20/03	+4	Close	28/03	+7	Close	13/11	+9	Far	29/11	+14	Far
Genotype 76	21/03	+5	Close	30/03	+9	Far	02/11	-2	Very Close	19/11	+4	Close
Genotype 77	22/03	+6	Close	31/03	+10	Far	03/11	-1	Very Close	19/11	+4	Close
Genotype 78	21/03	+5	Close	29/03	+8	Close	04/11	0	Very Close	19/11	+4	Close
Genotype 79	14/03	-2	Very Close	23/03	+2	Very Close	04/11	0	Very Close	06/12	+21	Very Far
Genotype 80	27/03	+11	Far	06/04	+16	Very Far	04/11	0	Very Close	04/12	+19	Very Far
Genotype 81	16/03	0	Very Close	24/03	+3	Very Close	01/11	-3	Very Close	22/11	+7	Close
Genotype 82	19/03	+3	Very Close	26/03	+5	Close	04/11	0	Very Close	14/11	-1	Very Close
Genotype 83	24/03	+8	Close	01/04	+11	Far	05/11	+1	Very Close	15/12	+30	Very Far
Genotype 84	7/03	-9	Far	18/03	-3	Very Close	03/11	-1	Very Close	28/11	+13	Far
Genotype 85	12/03	-4	Close	22/03	+1	Very Close	02/11	-2	Very Close	19/11	+4	Close
Genotype 86	25/03	+9	Far	06/04	+16	Very Far	04/11	0	Very Close	28/11	+13	Far
Genotype 87	15/03	-1	Very Close	28/03	+7	Close	16/11	+12	Far	15/12	+30	Very Far
Genotype 88	22/03	+6	Close	02/04	+12	Far	12/11	+8	Close	12/12	+27	Very Far
Genotype 89	5/03	+11	Far	15/03	-6	Close	08/11	+4	Close	01/12	+16	Very Far
Genotype 90	4/04	+19	Very Far	12/04	+22	Very Far	13/11	+9	Far	15/12	+30	Very Far
Genotype 91	14/03	-2	Very Close	23/03	+2	Very Close	20/11	+16	Very Far	05/12	+20	Very Far
Genotype 92	15/03	-1	Very Close	29/03	+8	Close	18/11	+14	Far	04/12	+19	Very Far
Genotype 93	14/03	-2	Very Close	28/03	+7	Close	21/11	+17	Very Far	10/12	+25	Very Far
Genotype 94	23/03	+7	Close	08/04	+18	Very Far	14/11	+10	Far	12/12	+27	Very Far
S-1/1	16/03	0	-	21/03	0		04/11	0	-	15/11	0	-

Table 4. Phenological differences/similarities of S-1/1 walnut genotypes with the main cultivar

Phenologic Characteristic	Variation between S-1/1 and 94 genotypes (mean day)	Belong to S-1/1	First and last date of 94 genotypes	Period	Number of genotype (piece/%)				Mean variation (%)
					Very close	Close	Far	Very far	
Bud Burst	4.52 0-41	16.03	05.03 26.04	52	23 (24.46)	44 (46.8)	24 (25.53)	3 (3.19)	75.54
Leafing	8.7 0-44	21.03	15.03 04.05	50	25 (26.46)	26 (22.67)	21 (22.34)	22 (23.4)	73.41
Leaf Yellowing	1.52 0-24	4.11	26.10 21.11	27	62 (65.95)	18 (19.14)	10 (10.63)	4 (4.25)	34.05
Defoliation	11.21 0-33	15.11	05.11 18.12	43	6 (6.38)	31 (32.97)	31 (32.97)	26 (27.65)	93.62

Late spring and early autumn frosts cause significant economic losses in walnuts. Therefore, the walnut genotypes with early leafing and late defoliation are damaged by late spring and early autumn frosts and yield decreases significantly. This study was aimed to identify new genotypes with both of late leafing and early defoliation characteristics and to reveal the relationships between main cultivar and F1 genotypes. The budburst time of the S-1/1 walnut genotype occurred on March 16, while the budburst time of the F1 genotypes changed between March 5 (Genotype 26-89) - April 26 (Genotype 21). The first leafing in main cultivar occurred on March 21, while the first leafing among F1 genotypes changed between March 15 (Genotype 89) and May 4 (Genotype 51). Leaf yellowing occurred on 4 November in the main genotype (S-1/1), while the time for leaf yellowing in other genotypes ranged from October 26 (Genotype 53) to November 21 (Genotype 93). The defoliation date in the main cultivar (S-1/1) occurred on 15 November, while it ranged from November 5 (Genotype 49) to December 18 (Genotype 67) in F1 genotypes. The studies carried out in different ecological regions of Turkey reported that genotypes and cultivars have leafing and flowering characteristics at different times (Asma et al. 1999; Tosun and Akçay 2005; Hassankhah et al. 2017; Akca et al. 2018; Orman 2018; Ozcan et al. 2020). Similar to the previous studies, phenological differences were determined between S-1/1 walnut genotype and the F1 genotypes. The differences in phenology among the genotypes can be attributed to genetic characteristics of the genotypes.

3.2. Phenological data analysis

Phenological observations are important in obtaining new varieties in fruit species. The results indicated that the phenological diversity among walnut genotypes was significant (Table 3). Coefficient of variation values for budburst, leafing, leaf yellowing and defoliation were calculated as 10.52, 9.32, 1.71 and 3.36, respectively (Table 5). Descriptive statistics of the phenological traits for the walnut genotypes were given in Table 5. In a similar study, Bukucu et al. (2020) reported PC1 as 50.226 %, PC2 as 36.635 %, and PC3 as 12.413 % and PC4 as 0.726 % in 684 walnut genotype. The CV value for the leafing date in 540 walnut genotypes was reported as 77.78 % by Khadivi-Khub et al. (2015).

Table 5. Units, number of samples, maximum, minimum, mean, and standard deviation of phenological traits in the walnut genotypes.

Traits	Units	N	Min	Max	Mean±SD*	CV (%)*
Budburst	Day	95	63	115	78.55±8.26	10.52
Leafing	Day	95	73	123	87.62±8.17	9.32
Leaf Yellowing	Day	95	298	324	308.20±5.27	1.71
Defoliation	Day	95	308	351	329.05±11.07	3.36

*Standard Deviation (SD), Coefficient of Variance (CV%)

The results of the Pearson correlation carried out to determine the relationships between phenological traits were given in Figure 1. Significant positive correlation ($r = 0.8285$) was obtained between budburst and leafing, and a negative correlation was recorded between budburst and leaf yellowing and defoliation ($r = -0.0511$, $r = -0.0025$).

The leafing had a positive correlation with leaf yellowing and defoliation ($r = 0.0220$, $r = 0.0436$). The defoliation had a positive correlation with leaf yellowing ($r = 0.5430$). Similarly, Özcan et al. (2020) reported positive correlation between leafing and defoliation ($r = 0.0957$), and Amiri et al. (2010) indicated similar relationship between these two traits ($r = 0.298$). Significant correlations between leafing in walnut and other phonologic traits have been reported also by others (Ebrahimi et al. 2015; Khadivi-Khub et al. 2015; Abedi and Parvaneh 2016; Hassankhah et al. 2017; Bukucu et al. 2020).

Figure 1. Scatter plot matrix and heatmap of correlations between phenological traits

Principal component analysis (PCA) was carried out to determine overall differences among walnut genotypes. The first component (PC1) explains 45.739 % of the total variation in phenological traits, the PC2 explains 38.646 %, PC3 11.419 % and PC4 4.207 %. Bi-plot partially grouped walnut genotypes according to their parents (Table 6; Figure 2). Multivariate analysis is performed to summarize the variability of a complex data set and present the data in an interpretable form as in the principal components (Ribeiro et al. 2013). The purpose of PCA is to identify the main factors and influential parameters that distinguish participation (Khadivi-Khub et al. 2015). The PCA analyzes have been carried out for many plant traits in walnut, however, the number of PCA analyzes for phenological traits is limited (Cosmulescu and Trandafir 2011; Pop et al. 2013; Bou Abdallah et al. 2016; Arab et al. 2019).

Table 6. Eigenvectors of principal components (PC) for phenological traits in the walnut population

Traits	PC1	PC2	PC3	PC4
Budburst	0.706	-0.045	-0.015	0.706
Leafing	0.707	0.016	-0.051	-0.705
Leaf Yellowing	-0.003	0.707	0.704	0.063
Defoliation	0.032	0.707	0.704	-0.063
% of variance	45.739	38.646	11.419	4.207
Cumulative variance	45.739	84.375	95.789	100.00

Figure 2 A) Scatter plot for the first two principal components for the walnut accessions based on phenological traits. B) Principal component analysis bi-plot of phenological traits among 94 walnut genotypes

Cluster analysis of the S-1/1 walnut genotype and 94 genotypes, including the seedlings from this genotype, was performed to assess the relationship between walnut genotypes in a dendrogram based on 4 phenological traits (Fig. 3). Walnuts were mainly split into 2 main clusters and 10 sub-clusters. The main cultivar (S-1/1) was included in subset 5 of the first main cluster (Fig. 3).

The results of cluster analysis partially confirmed the genotype-related results obtained in PCA (Fig. 3). These findings suggest that the walnuts are heterozygous, however, dominant genes largely determine the traits such as leafing and defoliation dates. Morphological features have been used effectively to determine the genetic variation among walnut genotypes (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; Bukucu et al. 2020). This

3.2. Genetic Differences between Genotypes

ISSR marker technique was used to determine the variation between the S-1/1 walnut genotype and the seedlings obtained from this genotype. Twelve ISSR primers were used in the study and the bands could not obtain for 5 of the primers. Fifty two bands were obtained from 7 primers. Polymorphism was detected in 45 of the 52 bands, while 7 bands were monomorphic (Figure 4).

Figure 4. The appearance of the bands belonged to the ISP 5 primer combination

The polymorphism information content (PIC) of the primers used varied between 48 and 95 %. The polymorphism rate of the ISSR primers used was 86.53 %. The primer that produced the most alleles (9) was ISSR 5, and the average number of alleles was calculated as 7.42 (Table 7).

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

Number	Name of Primer	Base sequence	Number of Allele	PIC
1	ISSR1	CACACACACACAA	5	0,63
2	ISSR4	CACACACACACAGC	4	0,94
3	ISSR5	CACACACACACAG	9	0,74
4	ISSR7	ACACACACACACACCCG	6	0,82
5	ISSR8	ACACACACACACACACACC	6	0,79
6	ISSR12	GAGAGAGAGAGAGAGAC	7	0,66
7	ISSR13	AGAGAGAGAGAGAGAGC	8	0,47

Christopoulos et al. (2010) used the ISSR marker technique to determine the genetic differences and relationships among 56 walnut genotypes in Greece. Pedigrees of 56 genotypes were revealed and many international cultivars were grouped together with the walnut genotypes examined. Potter et al. (2002) investigated the genetic relationships among 48 walnut (*J. regia*) cultivars in California using the ISSR molecular marker technique. Eight polymorphic ISSR primers used in the study were selected after screening 47

primers in four cultivars. Amplification of eight primers in 48 walnut cultivars yielded 54 bands and 31 (57 %) of the bands indicated polymorphism. The number of bands per primer varied between 5 and 9, and the number of polymorphic bands varied between 1 and 7. The researchers have reported that the ISSR technique can be used to identify walnut cultivars and genotypes and to determine the genetic relationships between them. In addition, they stated that the ISSR technique has similar cost to RAPD but it has more polymorphism. Hu et al. (2015) screened the polymorphism of genetic variation in 27 individuals belonging to the *Juglans hopeiensis* Hu species from two different regions and identified 25 SSR primers. The number of alleles ranged from 2 to 9, heterozygosity observed ranged from 0.016 to 0.933 (mean 0.468), and heterozygosity expected ranged from 0.022 to 0.823 (mean 0.462). The researchers stated that the improvement of new SSR primers will be useful to investigate the genetic structure of endemic species *Juglans hopeiensis* Hu and other *Juglans* species populations.

3.4. Dendrogram of the Genotypes

The dendrogram of walnut genotypes was scanned with 7 ISSR markers, and the dendrogram genotypes calculated using the NTSYSpc version 2.2 program with the UPGMA (unweighted pair group method with arithmetic mean) method according to genetic distance matrix of Nei (1972) were divided into 2 main clusters. Genetic similarity rates of walnut genotypes ranged from 0.52 to 0.93. Seventy four genotypes were placed in the same cluster (A) with the main genotype and the other 20 genotypes were in a separate cluster (B). Each of two main clusters was divided into 2 intermediate clusters and 4 sub-clusters were formed. The results of cluster analysis revealed that genotypes 85 and 86 had the closest similarity with 93.15 % and genotype 22 with 90 %. In addition, 14 genotypes (genotypes 9, 24, 25, 28, 30, 36, 37, 46, 47, 52, 61, 62, 65, 83) had both phenologic and genetic similarities (Fig. 5).

Özcan et al. (2020) obtained 2 main and 2 subsets in the dendrogram formed using the UPGMA test for 91 genotypes obtained from Pedro variety. The similarity ratio was reported between 0.61 and 0.99. The similarity ratio of genotypes 44 and 64 was 100 % and genotype 6 was more than 95 %. The results of crossbreeding studies indicated that Pedro variety is an excellent parent that prioritizes late leafing.

Figure 5. Genetic similarity dendrogram according to UPGMA method using ISSR data

3.5. Genetic Structure Analysis of Population

Cluster analysis was performed using Structure 2.3.4 software based on the Bayesian method to obtain the optimum genetic group number of 94 walnut genotypes (Pritchard et al. 2000). The ideal group number of 94

genotypes was determined by running the ΔK values from 1 to 10 with 10 independent simulations for each ΔK value. The permutation module was scanned in the range of 10,000-100,000 and repeated 5 times for each K value to determine the appropriate ΔK value (Fig. 6). The results were stored in a zip file and uploaded to the 'Structure Harvester' program. The ideal ΔK value was determined as 2. (<http://taylor0.biology.ucla.edu/structureHarvester/>).

Figure 6. The ideal ΔK value obtained from the Structure program

The diagram in Figure 7 was obtained by calculating the ideal K value obtained from the Structure 2.3.4 program. Cluster analysis was carried out once more in Structure 2.3.4 program to determine the genetic difference between genotypes. The cluster analysis indicated that genotypes were divided into 2 main groups. Different colors in Figure 7 shows that the differences between genotypes. The genotypes close to the S-1/1 genotype were colored green and those farther away were colored red.

Figure 7. Cluster analysis obtained in Structure 2.3.4 program

CONCLUSION

The phenological and genetic similarity ratios between main parent genotype and 94 new genotypes obtained from 200 open-pollinated seeds of S-1/1 walnut genotype were investigated. Important phenological differences were recorded between the main genotype and F1 walnut genotypes. Average variation in phenological traits among genotypes was 69.15 %, and the variation ratio in budburst, leafing, leaf yellowing and defoliation was 75.54, 73.41, 34.05 and 93.62 %, respectively.

In molecular studies; 52 bands were obtained from 7 of 12 ISSR primers, and 45 of which were identified as polymorphic and 7 as monomorphic. Thus, the polymorphism was calculated as 86.53%. Allele number was in monomorphic bands was calculated as 7.42. The PIC value ranged between 0.48 and 0.95, and the average PIC value was calculated as 0.73.

The dendrogram used to determine genetic variation indicated 2 main and 2 subsets. Seventy four genotypes were in the same cluster with the parent, and 20 genotypes were in the second cluster. The genetic similarity of walnut genotypes was between 52 and 93 %. The highest similarity rates were determined as 93% in 85 and 86 genotypes, and 90 % in 15 and 22 genotypes. In addition, 14 genotypes (9, 24, 25, 28, 30, 36, 37, 46, 47, 52, 61, 62, 65, and 83) were both phenologically and genetically similar to the S-1/1 genotype.

Significant variation at both phenological (69.15%) and molecular level (52%) in the seedling genotypes obtained from the S-1/1 genotype has important consequences for the future breeding programs. The results showed that evaluating the phenological and molecular findings together in the management of gene resources will provide a better characterization in breeding studies.

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