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Research Article/Araştırma Makalesi

A research on the determination of phenological and molecular characterization in open-pollinated genotypes in walnut

Cevizde açık tozlanmış tohumlardan elde edilen genotiplerde fenolojik ve moleküler karakterizasyonun belirlenmesi üzerine bir araştırma

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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 its 94 progenies. In the phenological observations made, it was observed that budburst in genotypes took 52 days, leafing 50 days, leaf yellowing 31 days, and defoliation date 27 days. When the mother plant (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 the dendrogram obtained using phenological data, 2 main and 5 subgroups were obtained. 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 and its progenies have a significant variation both phenologically and genetically.

Key Words: *Juglans regia* L., Molecular, Phenology, ISSR, Variation

ÖZ

Bu araştırma, S-1/1 ceviz genotipi ile bu genotipten elde edilmiş 94 farklı genotipin birbirleriyle ve ana bitki ile fenolojik ve genetik farklılık seviyelerini ortaya koymak amacıyla yürütülmüştür. Yapılan fenolojik gözlemlerde, genotiplerde tomurcuk patlamanın 52 gün, ilk yapraklanmanın 50 gün, yaprak sararmanın 31 gün ve yaprak dökümünün ise 27 gün gibi bir periyotta gerçekleştiği görülmüştür. Ana bitki (S-1/1) ile çöğür genotipler karşılaştırıldığında, tomurcuk patlamada %75.54, ilk yapraklanmada %73.41, yaprak sararmada %34.05 ve yaprak dökümünde ise %93.62 seviyesinde fenolojik varyasyon olduğu tespit edilirken, ortalama varyasyon %69.15 olarak belirlenmiştir. Moleküler analizlerde genetik varyasyonların belirlenmesi amacıyla 7 adet ISSR primeri kullanılmıştır. Bu analizlerde 7 monomorfik, 45 polimorfik bant elde edilirken, polimorfizm oranının %86.53, ortalama allel sayısının ise 7.42

olduğu belirlenmiştir. Genotiplerde polimorfizm bilgi içerik (PIC) değeri 0.48 ile 0.95 arasında değişirken, ortalama PIC değerinin ise 0.73 olduğu hesaplanmıştır. Kümeleme analizi sonucunda, genotiplerin 2 ana ve 2 alt kümeye ayrıldığı görülmüştür. Çalışma sonunda S-1/1 ceviz genotipi ile bu genotipten elde edilmiş F1 genotiplerin hem fenolojik hemde genetik olarak önemli bir varyasyona sahip olduğu tespit edilmiştir.

Anahtar Kelimeler: *Juglans regia* L.*,* Moleküler, Fenoloji, ISSR, Varyasyon

Introduction

Since walnut is monoecious, tends to dichogamy. Dichogamy is usually a mechanism that reduces self-fertilization. Therefore, walnut is an outcrossing species and is propagated by grafting due to its heterozygous structure (Unver and Sakar, 2011; You et al., 2012). While open-pollinated populations are a great source of genes for breeders, the market value of their products is low because there is no standard among trees. The traditional characterization of walnut populations is based on morphological and agronomic traits. In this way, a long period of time (8-15 years) is needed for the characterization of a genotype by selection and hybridization breeding in walnuts. Biotechnological methods recently were used to decrease the time needed for characterization.

To reveal the genetic variation between genotypes and to find the most suitable molecular marker technique, numerous studies have been conducted on different plants. As a result of these studies, it has been determined that SSR (Simple Sequence Repeat) and AFLP markers are advantageous in terms of polymorphism, RAPD and ISSR (Inter Simple Sequence Repeat) techniques are advantageous in terms of cost, and RFLP, SSR, ISSR and AFLP markers are advantageous in terms of repeatability. Besides these considering the laboratory possibilities to be studied, it has been reported that RAPD and ISSR methods are methods that can be easily used in laboratories where radioactive materials are not used, and conditions are limited (Doğan et al., 2006). 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 Koc, 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 (Yorgancilar et al., 2015).

To date, many techniques have been used to determine the genetic diversity of walnuts. Morphological markers have been used for a long time to determine the genetic diversity of walnut populations (Yarılgac et al., 1999; Zeneli et al., 2005; He-ping, 2010; Khadivi-Khub, 2014; Akhiani et al., 2017; Bukucu et al., 2020a). Morphological markers can vary according to the age of the plants, environmental and maintenance conditions. However, it has been used effectively to characterize plants until today. Besides, molecular markers have been used to determine genetic diversity in walnuts, such as Random Amplified Polymorphic DNA (RAPD) (Nicese et al., 1998; ; Sutyemez, 2006; Fatahi et al., 2010; Ahmed et al., 2012), Restriction Fragment Length Polymorphism (RFLP) (Fjellstrom et al., 1994; Potter et al., 2002; Kafkas et al., 2005), Inter-Simple Sequence Repeat (ISSR) (Potter et al., 2002; Pollegioni et al., 2003; Christopoulos et al., 2010; Li et al., 2011; Sutyemez et al., 2021), Amplified Fragment Length Polymorphism (AFLP) (Bayazit et al., 2007; Wang et al., 2011; Xu et al., 2012), Simple Sequence Repeats or microsatellites (SSR) (Dangl et al., 2005; Foroni et al., 2005; Victory et al., 2006; Wang et al., 2008; Chen et al., 2014; Vahdati et al., 2015; Bernard et al., 2018; Shah et al., 2020), and Single Nucleotide Polymorphism (SNP) (Bukucu et al., 2020a; Orman et al., 2020; Wang et al., 2020). With these techniques, walnut breeding studies have gained significant momentum. However, few

studies have been carried out to determine the phenological and genetic variation of the seedling genotypes obtained from a walnut cultivar seed compared to their parents (Sutyemez et al., 2018; Ozcan et al., 2020; Yıldırım and Sutyemez, 2020, Sütyemez et al., 2021). Therefore, the studies investigating the phenological and genetic variation in progeny 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 progenies obtained from the open-pollinated seeds of the S-1/1 walnut genotype and their mother plant. It is also to reveal the relationship between progenies and their mother plant. Thus, the level of phenological and genetic variation in progenies obtained by open pollination from a genotype with superior traits and their mother was revealed.

Material and Method

Plant material

In the study, S-1/1 walnut genotype selected by Sutyemez (2019) and 94 progenies obtained from open-pollinated seeds belonging to this genotype were used. Seeds of S 1/1 were planted in 2017, in pots in the greenhouse at Kahramanmaras Sutcu Imam University.

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

Phenological observations

The phenological observations were examined in 2019 and 2020. The phenological traits of the mother genotype S-1/1 and progenies such as budburst, leafing, leaf yellowing, and defoliation dates were recorded by observing every 2 days according to the Walnut Descriptors (Anonymous, 1994) (Table 1). The classification of phenological traits was given in Table 2.

Table 1. Definitions used to determine the phenological traits (Anonymous, 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 traits 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: Sutyemez, (2018), + after, - before

Phenological data analysis

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

Molecular analyzes

DNA isolation and extraction

The leaves of the S-1/1 genotype and leaves 94 progenies obtained from the S-1/1 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 ºC 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).

ISSR-PCR amplification

Extracted genomic DNA was PCR-amplified using 12 ISSR primer pairs (Table 3). PCR reactions were performed in a 20 µl volume. The reaction mixture contained 2 µL 10x PCR buffer, 5 mM dNTP

(Vivantis), 1 µL ISSR primer, 1.5 µL $MgCl_2$, 1 µL Taq DNA polymerase, 12 µL dH_2O, and 1 µL genomic DNA. The PCR-amplification program consisted of one cycle at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 10 min. Amplified PCR products were separated by gel electrophoresis using 3% agarose gel. Then, the genomic DNA was stained with a dyeing solution containing ethidium bromide (1 lt pure water and 300 µl ethidium bromide) for 15 minutes. The stained DNA bands were visualized under UV light. Fragment lengths were scored in the range of 200–1000 bp.

Table 3. Sequences of ISSR primer pairs used in the genetic diversity of walnut genotypes

No	Primer	Sequence
1	ISSR1	CACACACACACAA
2	ISSR3	CACACACACACAGG
3	ISSR4	CACACACACACAGC
4	ISSR5	CACACACACACAG
5	ISSR6	CACACACACACACAGT
6	ISSR7	ACACACACACACACACCG
7	ISSR8	ACACACACACACACACACC
8	ISSR9	ACACACACACACACACTG
9	ISSR11	GAGAGAGAGAGAGAGATC
10	ISSR12	GAGAGAGAGAGAGAGAGAC
11	ISSR13	AGAGAGAGAGAGAGAGC
12	ISSR15	ATATATATATATATAT

Data analysis

The products of ISSR were scored manually as present (1) or absent (0) and data recorded. The polymorphism information contents (PIC) 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 1;

$$PIC=1-\sum (fi)\ 2 \qquad (1)$$

Where *f* I represent 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 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 the 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/).

Results and Discussion

Late spring and early autumn frosts cause the most damage in fruit growing (Agaoglu 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 (Ozcan et al., 2020).

Phenologic observations

In this study, phenological variation was determined between the S-1/1 and the progenies obtained from open-pollinated seeds of S-1/1 and the results are presented in Table 4 and Table 5.

In the study, phenological differences between the mother plant and its progenies were revealed and categorized as Julian day. The budburst date of the S-1/1 occurred on March 16, while the budburst of progenies occurred in 52 days. Twenty-three of progenies were classified (24.46%) in the 'very close' category to S-1/1, 44 progenies (46.8%) were in the 'close' category, 24 progenies (25.53%) were in the 'far' category, and 3 progenies (3.19%) were in the 'very far' category (Table 5). Phenological variation between the progenies and S-1/1 as indicated by the budburst date was 75.54%. While the average day difference

between the budburst date between the S-1/1 and its progenies was determined as 4.53 days, this value varies between 0-40 days for each progeny (Table 5).

According to the first leafing date, 25 (26.46%) of the progenies were in the 'very close' category, 26 (22.67%) of them were in the 'close' category, 21 (22.34%) of them were in 'far' category and 22 (23.40%) of them were in the 'very far' category. A phenological variation of 73.41% was detected between the S-1/1 and the progenies in terms of the first leafing. The average variation in days between the mother genotype and the progenies was 8.7, and the period was between 0-44 days (Table 5).

The leaf yellowing date of S-1/1 was November 4, and the leaf yellowing date of the 9 progenies was spread over 26 days. The leaf yellowing dates of the 62 progenies (65.95%) were 'very close', 18 progenies (19.14%) were 'close', 10 progenies (10.63%) were 'far' and 4 progenies (4.25%) were 'very far'. The phenological variation between the S-1/1 and its progenies in terms of leaf yellowing date was determined as 34.05%. The mean number of days of variation between S-1/1 and 94 progenies was 1.52. In terms of these traits, the difference of the progenies from their mother genotype varied between 0 and 24 days (Table 5).

The defoliation in the S-1/1 occurred on November 15, while the defoliation dates of 94 progenies were spread over 43 days. The defoliation date of 6 (6.38%) progenies was 'very close', 31 (32.97%) of progenies were 'close', 31 (32.97%) of them were 'far' and 26 (27.65%) of them were 'very far' to their mother genotype. The phenological variation between the S-1/1 and the other genotypes (with 88 genotypes) in terms of defoliation date was calculated as 93.62%. The average number of days of variation ratio between S-1/1 and 94 progenies was 11.21%, and the date of defoliation ranged from 0 to 33 days (Table 5).

Table 4. Some phenological traits and classification of progenies 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	08/04	+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	26/03	+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
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	05/03	-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

Table 4. 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 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	03/12	+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
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

Table 4. Continue

Genotype	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 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
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	07/03	-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

Table 4. 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 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	05/03	+11	Far	15/03	-6	Close	08/11	+4	Close	01/12	+16	Very Far
Genotype 90	04/04	+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 5. Phenological differences/similarities of progenies with the S-1/1

Phenological 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	
Budburst	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 late leafing and early defoliation traits and to reveal the relationships between the mother genotype and its progenies. The budburst of the S-1/1 occurred on March 16, while the budburst of the progenies changed between March 5 (Genotype 26-89) - April 26 (Genotype 21). The first leafing in S-1/1 occurred on March 21, while the first leafing among progenies changed between March 15 (Genotype 89) and May 4 (Genotype 51). Leaf yellowing occurred on 4 November in the 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 S-1/1 occurred on 15 November, while it ranged from November 5 (Genotype 49) to December 18 (Genotype 67) in progenies. As a result of studies carried out in different ecological conditions, it is stated that there are important phenological differences between walnut genotypes (Asma et al., 1999; Tosun and Akçay, 2005; Akca et al., 2018; Orman, 2018; Ozcan et al., 2020). Similar to the previous studies, phenological differences were determined between the S-1/1 walnut genotype and the progenies. The differences in phenology among the genotypes can be attributed to the genetic characteristics of the genotypes.

Phenological differences among walnut genotypes and correlation between phenological traits

Phenological observations are important in obtaining new cultivars in fruit species. The results indicated that the phenological diversity among walnut genotypes was remarkable. The coefficient of variation values for budburst, leafing, leaf yellowing, and defoliation were calculated as 10.52, 9.32, 1.71, and 3.36, respectively. In a study conducted by Bukucu et al., (2020b) on similar phenological traits in 684 walnut genotypes, the coefficient of variation values was determined as 12.49% for budburst, 10.83% for leafing, 1.86% for leaf yellowing, and 2.22% for defoliation. Descriptive statistics of the phenological traits for our walnut genotypes were given in Table 6.

Table 6. 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. A significant positive correlation was obtained between budburst and leafing (r = 0.8285). In the study, a significant positive correlation was also determined between leaf yellowing and defoliation (r = 0.5430). Similarly, Ozcan et al., (2020) reported a positive correlation between leafing and defoliation (r = 0.0957), and Amiri et al., (2010) indicated a similar relationship between these two traits (r = 0.298). Significant correlations between leafing in walnut and other phenological traits have been reported also by other studies (Ebrahimi et al., 2015; Khadivi-Khub et al., 2015; Abedi and Parvaneh, 2016; Hassankhah et al., 2017; Bukucu et al., 2020a).

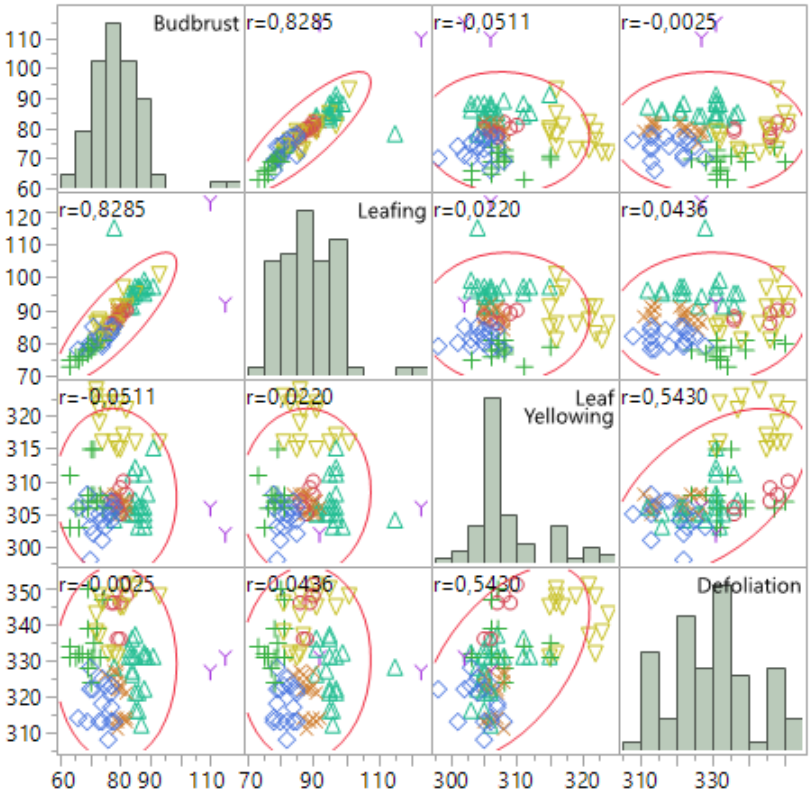

Figure 1. Scatter plot matrix and heatmap of correlations between phenological traits

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 7; Figure 2). The PCA analyzes have been carried out for many plants, however, the number of PCA analyzes for phenological traits in the walnut is limited (Cosmulescu and Trandafir, 2011; Pop et al., 2013; Bou Abdallah et al., 2016; Arab et al., 2019). In a similar study, Bukucu et al., (2020a) reported PC1 as 50.226%, PC2 as 36.635%, and PC3 as 12.413%, and PC4 as 0.726% in 684 walnut genotypes for our phenological traits (open-pollinated seeds of Bilecik, Chandler, Franquette, Howard, Kaman 1, Maraş 12, Pedro, Sütyemez 1 and Gimar).

Table 7. 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

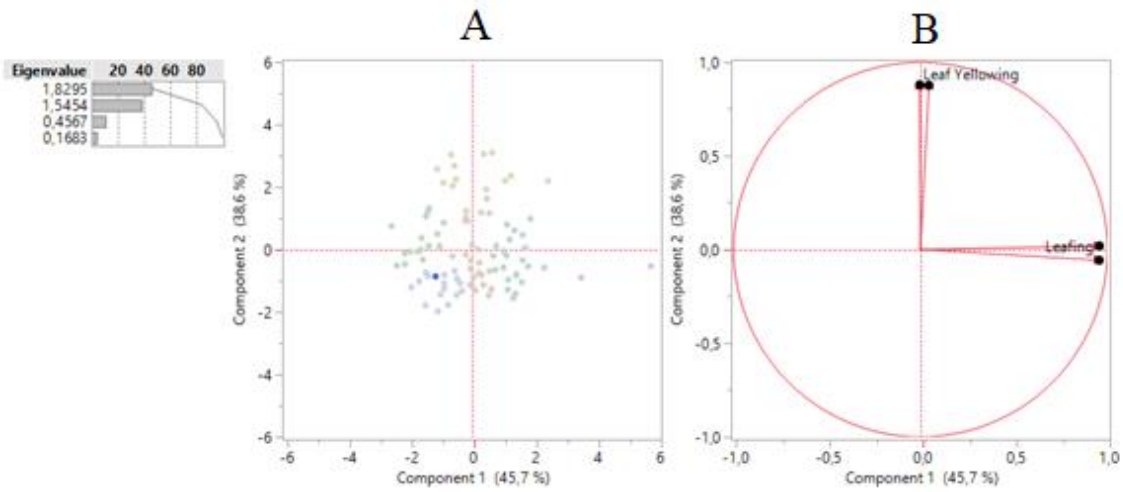

Figure 2. A) Scatter plot for the first two principal components for the walnut accessions based on phenological traits. B) Principal component analysis bi-plot of phenological traits among 95 walnut genotypes

Cluster analysis of the S-1/1 walnut genotype and its 94 progenies was performed to assess the relationship between walnut genotypes in a dendrogram based on 4 phenological traits. Walnuts phenologically were mainly split into 2 main clusters and 7 sub-clusters. S-1/1 took place in the A3 group, including sixteen progenies (Figure 3).

The results of cluster analysis partially confirmed the genotype-related results obtained in PCA (Figure 3). These findings suggest that the walnuts are heterozygous, however, dominant genes largely determine the traits such as leafing and defoliation dates. Phenotypical traits 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., 2020b). These results revealed that phenotypic markers could partially distinguish the seedlings obtained from open-pollinated walnut cultivars.

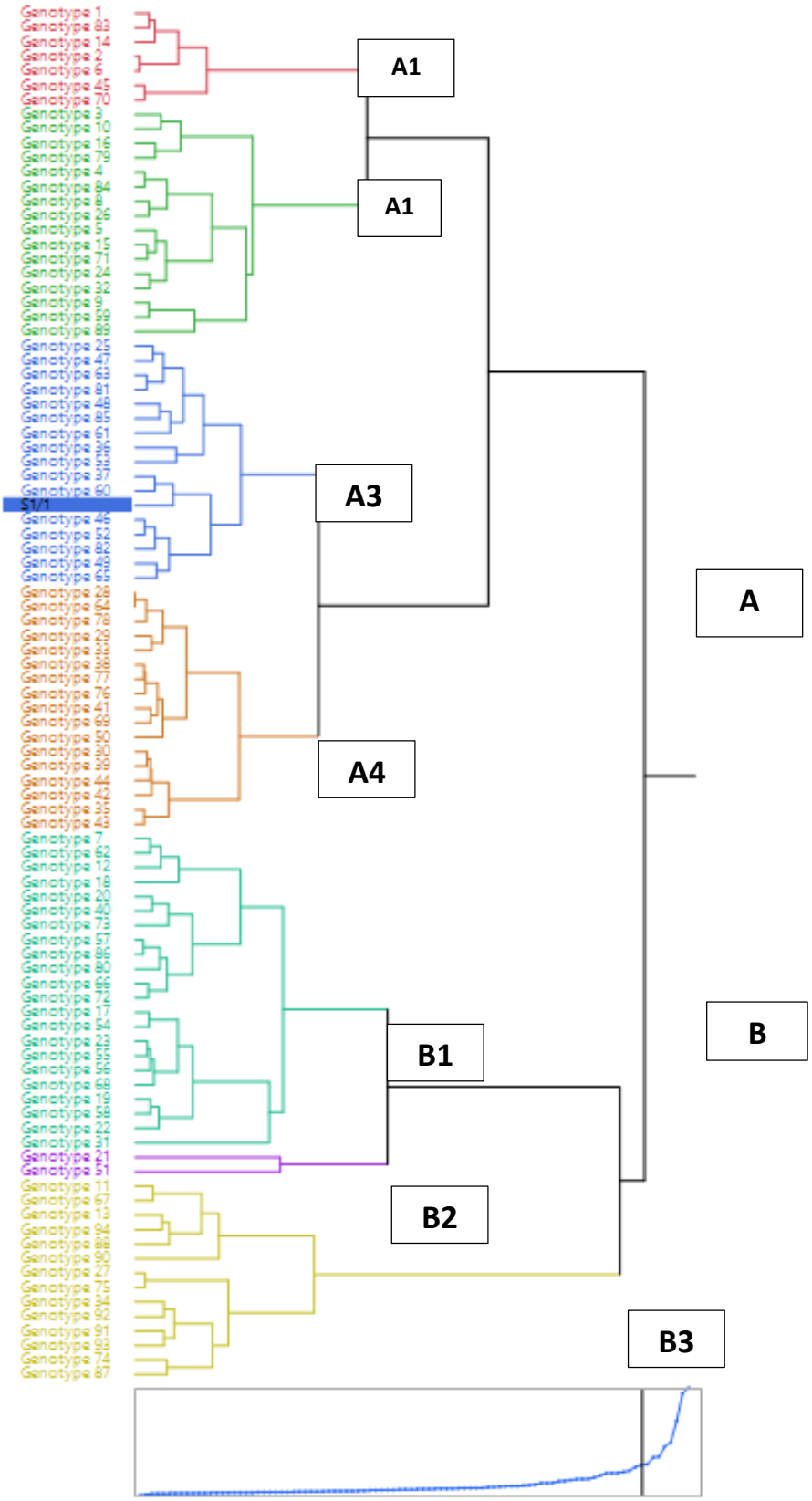

Figure 3. Phenotypic clustering of 94 walnut genotypes based on Ward's phenological pair-wise distance and phenological map. Cluster analysis of the genotypes is based on the traits with high heritability

Polymorphism levels of ISSR Loci

ISSR marker technique was used to determine the genetic variation between the S-1/1 walnut genotype and its progenies. Twelve ISSR primers were used in the study and the bands could not obtain for 5 of the primers (ISSR3, ISSR6, ISSR9, ISSR11, and ISSR15). Fifty-two bands were obtained from 7 primers. Polymorphism was detected in 45 of the 52 bands, while 7 bands were monomorphic (Figure 4).

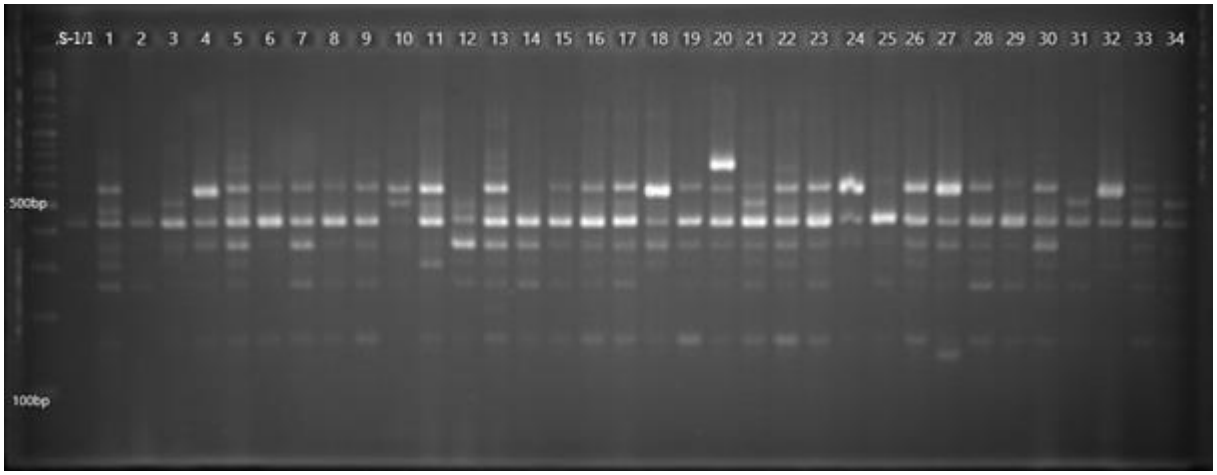

Figure 4. Amplification products from S-1/1 and its 34 progenies with primer ISSR5

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 bands (9) was ISSR5, and the average number of alleles was calculated as 7.42 (Table 8).

Table 8. 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	ACACACACACACACACCG	6	0,82
5	ISSR8	ACACACACACACACACACC	6	0,79
6	ISSR12	GAGAGAGAGAGAGAGAGAC	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. In this study, 82.8% of the polymorphism was detected with 7 primers and the number of polymorphic bands per primer ranged between 3 and 20, with an average of 11 polymorphic bands. Potter et al., (2002) investigated the genetic relationships among 48 walnuts (*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 mean polymorphism revealed by the ISSR markers varies between different studies depending on the genetic material, the primers, and the discriminant analysis method used. However, we obtained a higher polymorphism rate from these studies.

Genetic differences among walnut genotypes

The dendrogram of walnut genotypes was scanned with 7 ISSR markers, and the dendrogram for genotypes was calculated using the NTSYSpc version 2.2 program with the UPGMA (unweighted pair group method with arithmetic mean) method according to the 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 the 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 (Figure 5). These findings reveal that the combined use of both morphological and genetic markers is more effective in distinguishing genotypes from each other.

Structural genetic analysis was also performed in S-1/1 and its 94 genotypes using 7 ISSR markers by Structure and Structure Harvester programs. The highest value of Delta K (ΔK) was obtained at ΔK = 2. Thus, the cluster analysis indicated that genotypes were divided into 2 main groups similar to the UPGMA analysis results. Different colors in Figure 6 show the differences between genotypes. The genotypes close to the S-1/1 genotype were colored green and those farther away were colored red (Figure 6).

Ozcan et al., (2020) obtained 2 main and 2 subsets in the dendrogram formed using the UPGMA test for 91 genotypes obtained from Pedro. The similarity ratio was reported between 0.61 and 0.99. The similarity ratio of Genotype 44 and Genotype 64 was 100% and Genotype 6 was more than 95%. They suggested that it was attributed to gene flow. Similarly, in this study, a high similarity was found between the mother plant and its progenies.

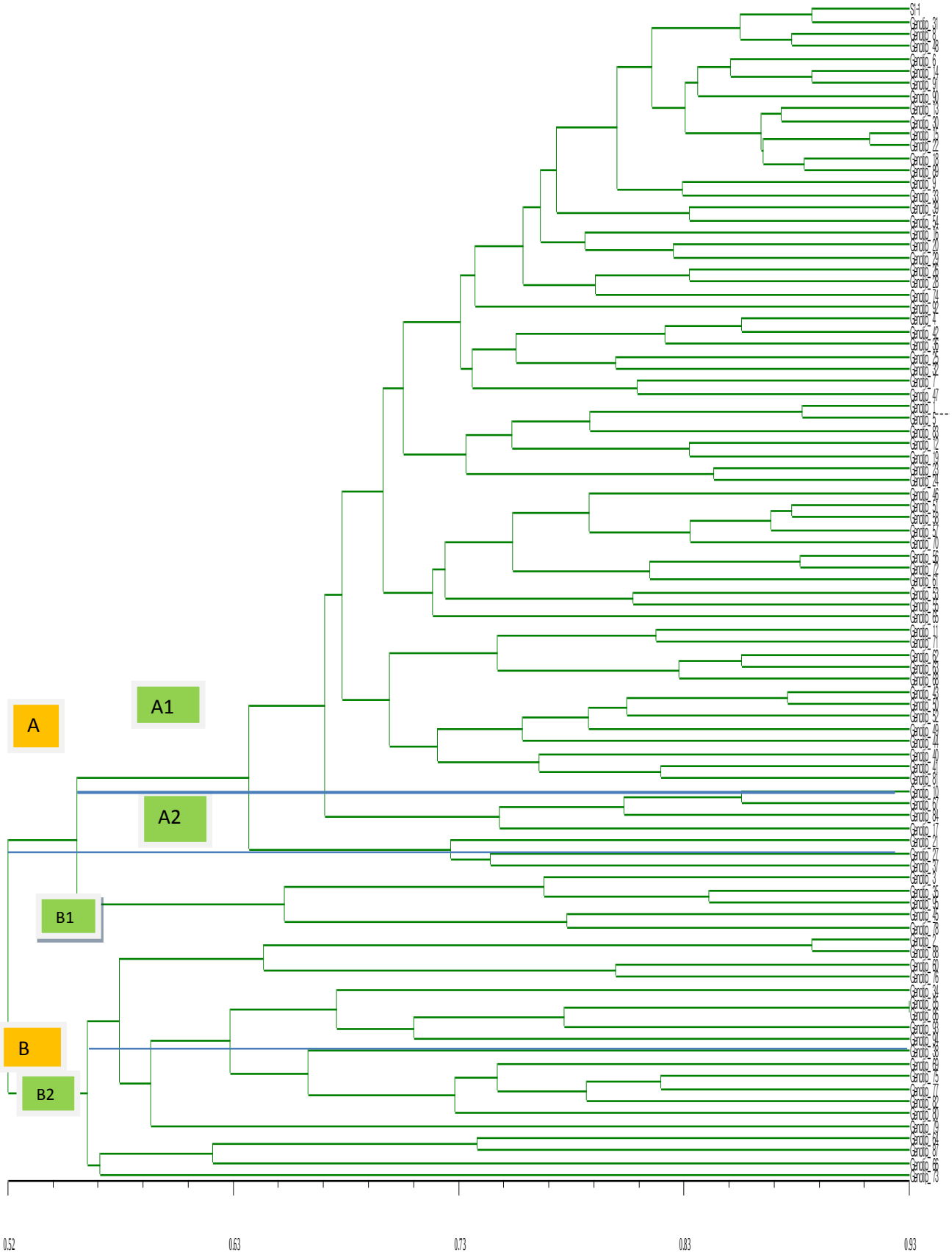

Figure 5. Genetic similarity dendrogram according to UPGMA method using ISSR data

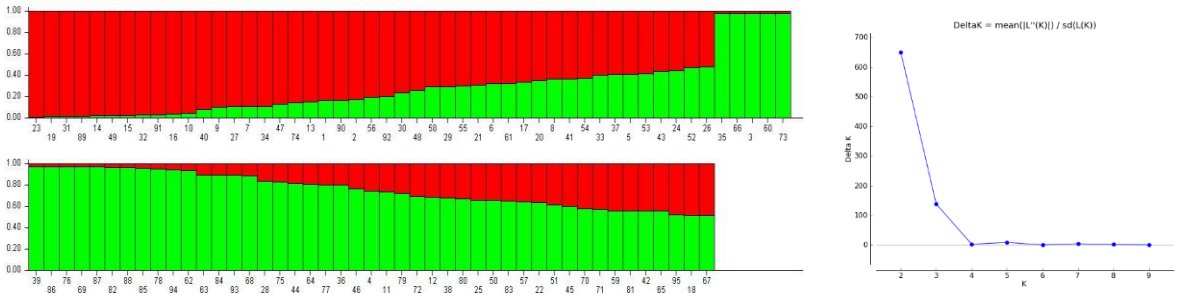

Figure 6. Population structure and ideal ΔK values for walnut accession

Conclusion

The phenological and genetic similarity ratios between mother genotype and 94 progenies obtained from 200 open-pollinated seeds of S-1/1 were investigated. Important phenological differences were recorded between the mother genotype and its progenies. The mean 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 this study, 52 bands were obtained from 7 of 12 ISSR primers, and 45 of which were identified as polymorphic and 7 as monomorphic. 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 S-1/1, and twenty 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% between Genotype 85 and Genotype 86, and 90% between Genotype 15 and Genotype 22. In addition, 14 genotypes (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.

Significant variation at both phenological (69.15%) and molecular levels (52%) in the seedling genotypes obtained from the S-1/1 genotype has important consequences for 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.

Conflicts of interest: The authors declare no conflict of interest

Authors contributions: Conceptualization, M.S. and S.B.; writing—original draft preparation, M.S., S.B., N.K., A.O., E.Y., B.S.B. A.B.; writing—review and editing, M.S., S.B., N.K., A.O., E.Y., B.S.B. A.B.; all authors contributed sections to the manuscript and approved the final version of the text.

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