

Morphometric Traits and iPBS Based Molecular Characterizations of Walnut (*Juglans regia* L.) Genotypes

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Abstract

In this study, walnut genotypes that selected during two growing seasons among thousands of seedlings were analyzed in terms of detailed morphometric, phenological, and chemical traits. A multivariate analysis was conducted with valuable traits for breeding and selection such as morphometric traits, chemical composition, and phenological characteristics. Also, genotypes were characterized by a retrotransposon-based iPBS marker system. The correlation analysis showed significant positive and negative correlations between agro-morphological characters. The principal component analysis explained 71.44% of the total variance into five main components. Principal component and hierarchical cluster analysis divided genotypes into three groups, whose subgroups were identified based on both agro-morphological characters and iPBS marker systems. A high level of polymorphism ratio was observed for tested markers. Mantel's test demonstrated relatively low correlations between molecular and morphological traits ($r=0.04$). The genetic similarities among all individuals ranged from 0.39 (between 018 and 015 or 045 genotypes) to 0.98 (between 090 and 094 genotypes) with a mean similarity of 0.67. Remarkable phenotypic and molecular variations were observed among the genotypes. The features of some investigated genotypes were above the acceptable thresholds for walnut selection in breeding programs and our study indicated that iPBS markers can be beneficial in walnut breeding programs, allowing the evaluation of the genetic relationship between genotypes, helping to differentiate and select the best genotypes to improve agronomic properties.

1. Introduction

The *Juglans* genus includes about 20 walnut species of deciduous trees of the family *Juglandaceae*. The economically and commercially cultivated species in this genus is Persian walnut (*Juglans regia* L.) which is a monoecious and self-compatible species. Walnuts are pollinated by windy dichogamy, which provides open-pollination and is an important source of genetic diversity for suitable opportunities in the walnut breeding to obtain new cultivars (Cosmulescu and Botu, 2012). Because of the open pollination, Turkish walnut populations have high genetic variability consisting of millions of natural hybrids grown on their own roots, which are important sources of genetic diversity for *J. regia* (Akça, 2016). This genetic variation in the native walnut populations presents many opportunities for walnut breeding (Karadağ and Akça, 2011). Thus, studies of phenological and morphological traits in natural walnut populations have remained for some time, while morphological characteristics often do not indicate clear relationships between genotypes, as the results can be affected by changing environmental conditions (Kumar, 1999). Therefore, several techniques have been developed to estimate genetic diversity in walnut genotypes (Sharma and Sharma, 2001). Walnuts trees have long-term infertility in adolescents, and breeding studies take long-time periods, thus, molecular markers have been used in walnut breeding to overcome these difficulties. Molecular markers are one of the most important methods for the characterization of genotypes and determining genetic resources (Badenes and Parfitt, 1998; Li and Quiros, 2000). Several marker techniques have been applied to reduce the duration of breeding programs, examine relationships between genotypes, and perform genotype selections with greater precision. To identify phylogenetic

relationships and characterize germplasm, molecular techniques including RFLP (Restriction Fragment Length Polymorphism: Fjellstrom et al., 1994), RAPD (Randomly Amplified Polymorphic DNA: Nicese et al., 1998; Doğan et al., 2014), ISSR (Inter Simple Sequence Repeat: Christopoulos et al., 2010; İpek et al., 2019), AFLP (Amplified Fragment Length Polymorphism: Kafkas et al., 2005), and SSR (Simple Sequence Repeats: Orhan et al., 2020) have been employed in recent years.

Retrotransposons are repetitive and mobile DNA fragments that can copy themselves to another region of the genome where they are located. In this way, the genome size can be increased, and variations can be created in the genome, making retrotransposons the best tools for molecular markers (Kalendar et al. 2010). For this reason, it is considered an excellent source of markers since it causes mutations, increased genome size, and ultimately genetic variations (Schulman et al., 2004). Retrotransposon is abundant in the entire genome of eukaryotic cells, particularly plant species (Finnegan, 1989). Plant genomes vary in their retrotransposon content and often make up 50 to 90% of the plant genome (San Miguel et al., 1996). In recent years, various retrotransposon marker systems have been used extensively in many studies on evolutionary and genetic diversity due to their general applicability, ease of use, and providing high genotype resolution among eukaryotic organisms (Schulman et al., 2004; Kalendar and Schulman, 2006). Because of the high potential for marker generation, retrotransposon markers such as inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) have been used for characterization and gene mapping of many eukaryotes as well as plants (Castro et al., 2012; Kaya and Yılmaz-Gokdoğan, 2016; El Zayat et al., 2021). These marker systems, however, have some bottlenecks such as the size of various PCR products and the sequence information requirements for designing primers that match neighboring genomic DNA at any given location, preventing the widespread use of them (Özer et al., 2016).

The inter Primer Binding Site (iPBS) retrotransposons-based amplification technology was declared by Kalendar et al. (2010) as a universal DNA labeling method that can be used in plants and animals based on the primer binding site for the reverse transcription enzyme of LTR retrotransposon. The iPBS marker system has proven to be a powerful DNA fingerprinting technique that does not require sequence information and is the preferred universal marker system for the genetic differentiation of several eukaryotic organisms at both an intraspecific and interspecific level (Özer et al., 2016; Milovanov et al., 2019; Aydın et al., 2020; Pérez-Vargas et al., 2020; Erper et al., 2021; Ouyang et al., 2021). Molecular markers are one of the most important methods for characterization of genotypes and determining genetic resources (Badenes and Parfitt, 1998; Li and Quiros, 2000).

Molecular markers including SSR, ISSR, AFLP, and RAPD have been used for generating genetic polymorphisms among walnut genotypes, however, no record of determination of genetic differences in walnut genotypes using iPBS markers exists. This study is the first characterization study of walnut genotypes using the iPBS retrotransposon marker system. Furthermore, relationships between nut agromorphological traits and genotypes were determined by multivariate analysis for the selection of desired features which are essential for breeding programs.

2. Materials And Methods

2.1. Plant material and fruit measurements

The study was conducted on seedling walnut trees from the Şanlıurfa region in two consecutive growing seasons, 2015-2016 and 2016-2017, in Turkey (Fig. 1). Among thousands of seedling walnut genotypes in the investigated population, according to UPOV criteria, 121 genotypes which the mature, healthy, and had full crop were labeled at optimal ripening time.

The walnuts were harvested when 50% of their husks were opened. After the husk was removed, walnuts were dried to reduce their moisture content to 10-12% immediately. The nut characteristics including nut width (mm), nut length (mm), nut height (mm), nut weight (g), kernel weight (g), and shell thickness (mm) were determined according to Union for the Protection of New Varieties of Plants (UPOV) walnut descriptors (UPOV, 2017). A digital caliper was used to measure the dimensions of nuts, and an electronic scale with an accuracy of 0.01 g was used for nut weights with 30 nuts in the replications for each measurement. Kernel percentage was calculated using the formula 'kernel weight/nut weight × 100'. The shell color was classified into three categories as "light", "amber", and "dark" and the kernel color was evaluated using the design for assembly (DFA) guidelines (United States Standards for Grades of Shelled Walnuts, 2017). The DFA is based on a chart for color evaluation that classified kernels into one of four categories as "extra light", "light", "light amber", and "amber".

In the last of the first evolutions, 20 genotypes with the highest value according to the UPOV descriptors from 121 genotypes were determined as "promising". The further nut and phenological characters [first leafing, the receptive period in male and female flowers, dichogamy, % of fruitful laterals (FL), harvest date, and chemical compositions] were estimated for the promising genotypes.

2.2. Chemical compositions

The kernel of the walnut was milled and moisture (MS) determinations were made (AOAC, 1995). The protein (PRT) amount was determined by the modified kjeldahl method for protein analysis were use (AOAC, 1990). The total fat (OIL) content was obtained by extracting 10 g of ground crushed walnut kernel petroleum ether at 45-50°C for 8-9 hours on a soxhlet device and weighing the pre-and post-extraction weights (AOAC, 1995).

2.3. Molecular characterizations

In promising genotypes, the genetic variation was determined using the iPBS molecular markers. The leave samples from promising genotypes were taken into sterile 50 ml tubes, labeled, and stored in an icebox to transfer to the laboratory. A leaf of each sample was frozen with liquid nitrogen and homogenized with a mortar and a pestle under liquid nitrogen. To extract genomic DNA, approximately 100 mg grounded tissue was put into a 1.5 ml microcentrifuge tube, and treated with preheated (65°C) extraction/lysis buffer according to "Plant DNA Extraction Protocol for DArT" (<https://www.diversityarrays.com>), followed by application of twice chloroform: isoamyl alcohol (24:1)

mixture, and a precipitation stage was conducted with ice-cold isopropanol. The final quantity of the resultant DNA was determined by the DS-11 FX+ spectrophotometer (Denovix Inc., Wilmington, DE, USA) and diluted to 10 ng/μl with sterile ultra-pure water. Template DNA samples were stored at -20°C till used.

Seven iPBS markers (Table 3) designated by Kalendar et al. (2010) were selected for molecular characterization of walnut genotypes collected in the Şanlıurfa region. PCR amplifications was conducted in a 25 μL reaction mix containing 1× Dream Taq Buffer, 0.2 mM of dNTPs, 1 μM of primer, 1.2-unit of Dream Taq DNA polymerase (Thermo Fischer Scientific, Waltham, MA, USA), 0.04-unit of *Pfu* DNA polymerase (Thermo Fischer Scientific, Waltham, MA, USA) and 20 ng template DNA. PCR reactions were carried out in a T100 thermocycler (Bio-Rad, Hercules, CA, USA) as follows: 35 cycles of 30 s at 94°C, 30 s at 52-60°C (depending on primers), and 2 min at 72°C. All reactions included an initial denaturation step for 3 min at 94°C and an ending step for 10 min at 72°C. The amplified fragments were separated on a 1.6% (w/v) agarose gel with 1× TAE buffer for over 2 hours and stained with ethidium bromide before visualizing via a UV transilluminator (G: BOX F3, Syngene, UK).

Table 3
The information of iPBS markers was used to evaluate walnut genotypes.

iPBS ID	Primer sequences (5'–3')	T _m (°C)	GC (%)	TB	PB	PPB (%)	PIC	RP
2081	GCAACGGCGCCA	56	75.0	13	10	76.92	0.22	3.90
2387	GCGCAATACCCA	52	58.3	14	10	71.43	0.20	3.80
2219	GAACTTATGCCGATACCA	52	44.4	11	8	72.73	0.24	3.70
2078	GCGGAGTCGCCA	54	75.0	12	8	66.67	0.21	3.80
2395	TCCCCAGCGGAGTCGCCA	60	72.2	14	11	78.57	0.23	4.40
2077	CTCACGATGCCA	50	58.3	10	6	60.00	0.15	2.20
2242	GCCCCATGGTGGGCGCCA	58	77.8	12	9	75.00	0.16	2.54
<i>Total</i>				<i>86</i>	<i>62</i>			
<i>Avg./primer</i>				<i>12.29</i>	<i>8.86</i>	<i>72.09</i>	<i>0.20</i>	<i>3.48</i>
T _m (°C), annealing temperature; GC (%), percentage of guanine-cytosine content; TB, total band; PB, polymorphic band; PPB (%) percentage of the polymorphic band; PIC, polymorphism information content; RP, resolving power.								

All the PCR amplifications were repeated at least two times and only reproducible bands were evaluated. All bands from iPBS analysis was scored as present (1) or absent (0) at positions to construct a binary data matrix. The data matrix from marker systems was combined and converted into a genetic similarity matrix using Jaccard's similarity coefficient and the Numerical Taxonomy System, NTSYS-pc version 2.10 program (Rohlf, 2000) was used to conduct an unweighted pair group method using arithmetic average (UPGMA). The resolving power (RP), and the polymorphic information content (PIC) values were

calculated for the markers as proposed by Prevost and Wilkinson (1999) and Roldàn-Ruiz et al. (2000), respectively.

2.4. Statistical analysis:

Data were subject to analysis of variance (ANOVA) using R Studio software (Allaire, 2012; R core team, 2017). The minimum and maximum values with standard deviation, and the coefficient of variation (CV%; $SD/mean \times 100$) were calculated for the measured characters. The coefficient of variation was used as a variability index. Pearson's correlation was performed to determine relationships among agro-morphological characters using R Studio software by the package of 'corrplot' (Wei et al., 2017). The Mantel test between agro-morphological trait distance matrix and Nei's genetic distance matrix was conducted with GenAlEx (Peakall and Smouse, 2012). The relationships of the studied characters and genotypes with each other were determined by principal component analysis (PCA) with the package of 'ggplot2' of R software (Wickham, 2016).

3. Results And Discussion

3.1 Morphometric and phenological characters

The genotypes showed significant differences in agro-morphological features (ANOVA, $P < 0.05$). The minimum, maximum, mean, and coefficient variations (CV) values of studied characters are presented in Table 1. The highest CV was observed in the shell color (51.29%) followed by kernel color (45.48%) and leafing date (45.16%), while the lowest CV was observed in the kernel ratio (6.47%) and nut length (6.77%). In total, the CV in 8 out of 17 measured characters was smaller than 10.00% that indicating low variations among the genotypes, thus these characters may be considered as more stable characters. Similarly, Khadivi et al., (2019) reported that CV for nut diameter, nut length, nut weight, and kernel weight were 11.03, 12.89, 23.34, and 25.99%, respectively. Moreover, Poggetti et al., (2017), reported 32.0% CV for nut weight, 28.7% for kernel weight, 25.0% for shell thickness and 48.0% for kernel skin color.

Table 1
Descriptive statistics for used characters utilized in the promising walnut genotypes

Characters	Abbreviation	Unit/code	Minimum	Maximum	Mean	SD	CV
Nut width	NW	mm	26.78	35.99	31.42	0.55	7.80
Nut length	NL	mm	28.38	35.73	32.69	0.49	6.77
Nut height	NH	mm	34.35	45.14	39.59	0.71	8.05
Nut weight	NWT	g	7.90	15.52	12.17	0.43	15.93
Kernel weight	KWT	g	4.15	7.55	6.05	0.20	14.67
Shell thickness	ST	mm	1.04	1.59	1.36	0.03	9.58
Kernel ration	KR	%	45.25	56.12	50.53	0.73	6.47
Moisture	MS	%	2.41	3.20	2.80	0.06	8.77
Total oil	OIL	%	50.49	62.50	55.72	1.04	8.36
Protein	PRT	%	15.40	20.74	17.96	0.34	8.41
Fruitful laterals	FL	%	40.00	75.00	57.75	1.76	13.61
Leafing date	LD	Date	1.00	4.00	1.80	0.20	49.69
Kernel color	KC	Code	1.00	3.00	1.95	0.19	45.48
Shell color	SC	Code	1.00	3.00	1.20	0.13	51.29
Receptive period in male flowers	RPMF	Date	1.00	4.00	2.95	0.16	25.73
Receptive period in female flowers	RPPF	Date	1.00	4.00	3.05	0.24	36.03
Altitude	Alt	m	527	600	550.9	6.52	5.29
Harvest date	HT	Date	1.00	2.00	1.90	0.06	16.19

As shown in Table 2, fruitful laterals, leafing date, kernel color, shell color, the receptive period in male and female flowers, and harvest date showed more than 15.0% CV, indicating a high level of variation, and these vegetative growth parameters affected by genetical and ecological conditions.

Table 2
Frequency distribution for agronomic characters utilized in the promising walnut genotypes

Characters	Abbreviation	Unit/code	Frequency %			
Leafing date	FFL	Date	Very Early (45.00)	Early (35.00)	Medium (15.00)	Late (5.00)
Kernel color	KC	Code	Extra light (40.00)	Light (25.00)	Light amber (35.00)	
Shell color	SC	Code	Light (90.00)	Dark (10.00)		
Receptive period in male flowers	RPMF	Date	Very Early (5.00)	Early (15.00)	Medium (60.00)	Late (20.00)
Receptive period in female flowers	RPPF	Date	Very Early (10.00)	Early (15.00)	Medium (30.00)	Late (45.00)
Dichogamy	DC	Code	Protandry (100.00)			
Harvest date	HT	Date		Early (10.00)	Medium (90.00)	

In walnut breeding, fruit characteristics are an important parameter for domestic and foreign markets. Nut weight, kernel weight, shell thickness, and kernel ratio are important quality parameters. These parameters in our study varied between 7.90–15.52 g, 4.15–7.55 g, 1.04–1.59 mm, and 45.25–56.12%, respectively (Table 1; Supp. Data 1). In walnut breeding, the acceptable value should be 6-8 g for nut weight, 50–70% for kernel ratio, and 0.7-1.5 mm for shell thickness (Zhadan and Strokov, 1977; Akça, 2016). All of our samples were above these critical thresholds. Nut width ranged from 26.78 to 35.99 mm, while nut length varied from 28.38 to 35.73 mm. Fruit height varied from 34.35 to 45.14 mm. These values are consistent with Mahmoodi et al., (2019), but are lower than Khadivi et al., (2019). Nut width is worthy for growers who accept a minimum grade of 32.0 mm, and bigger nut diameter means more income for growers. More than half of our samples yielded above the base limit.

Skin and kernel color are very important for walnut breeding while commercially acceptable walnut skin and kernel color should be 'light' for skin and 'extra light' or 'light' for the kernel. A total of 18 genotypes (90%) had 'extra light' or 'light' kernel skin color, and only 2 genotypes were 'dark'. Kernel color of 8 genotypes (40%) were observed as 'extra light', 5 (25%) as 'light', and 7 (35%) as 'light amber'. The 90% and 60% of genotypes were acceptable for commercial cultivation in terms of skin color and kernel color, respectively.

Significant differences ($P < 0.05$) were observed among genotypes in terms of the chemical compositions of kernels. Total oil, the predominant component, varied from 50.49 to 62.50%, followed by protein ranging from 15.40 to 20.74%, and moisture from 2.41 to 3.20%. Oil and protein consisted of approximately 70-80% of kernel weight (Table 1; Supp. Data 2). These results are in accordance with previously reported values in several commercial varieties studied by researchers who found walnut kernel as compensating 40–60% of walnut weight, while about 60-70% of the kernel weight was oil and 24% was protein (Muradoğlu et al. 2010; Kabiri et al. 2019; Verma et al., 2020). Genotypes with a kernel that has 60% or above oil and 12% or above protein are more desirable in walnut breeding programs. The genotypes that yielded higher oil and protein ratios were considered as precious materials for future breeding programs. Furthermore, the walnut chemical composition varies depending on many factors such as genetic characteristics, maturity, environmental factors, harvest date, and soil characteristics.

The late spring frosts are an important factor limiting walnut cultivation. For this reason, flowering date of secondary branches is the main requisition of walnut breeding programs. Agronomic characters including leafing date are evaluated relatively very early, early, medium, and late within this period. In our study, leafing date of one genotype was 'late' and three genotypes were 'medium' (Table 2; Supp. Data 3). The receptive periods in males were from 20-22 March to 28-30 March and from 25-27 March to 1-3 April in females that are relative to very early, early, late, and very late flowering. The flowering and female flower formation are important factors affecting the productivity of walnut. In our study, the receptive period of male flowers was determined 'medium' in eight genotypes and 'late' in twelve genotypes. In addition, the female flowers were 'medium' in six genotypes and 'late' in nine genotypes in terms of flowering dates.

Walnut cultivars or genotypes are generally tending dichogamy, especially protandry is seen more than the protogyny, and homogamy that provides advantage in terms of pollination and productivity. The blooming types of all promising genotypes were protandry (Table 2; Supp. Data 3). Generally, *J. regia* has dominant characteristics of protandry in dichogamy and it is affected by climatic conditions at the flowering time. Development of catkins (male flower) is highly affected by the temperature compared to female flowers (Akça, 2016; Kumar and Sharma, 2013). Persian walnut has two bearing habit. Fruit set can occur two way both only from the terminal buds of new branches or from both terminal and lateral buds, a very crucial characteristics in the breeding programs, that are affected by phytohormones such as IAA, and zeatin, and plant nutrition (Muradoğlu et al., 2009; Amiri et al., 2010). In our study, the rate of fruiting in the sub-branches was between 40% and 75%. The harvest dates of the genotypes took place on 1-7 October (Table 2; Supp. Data 3).

3.2. Molecular characters

The seven iPBS primers yielded 86 scorable and reproducible fragments to evaluate the extent of genetic variation among 20 promising genotypes. Sixty-two of those fragments (72.09%) were polymorphic. The number of fragments generated with the primers ranged from 10 (2077) to 14 (2387/2395), with a mean of 12.29 fragments per iPBS primer. The values of the PIC and RP index calculated for these primers are shown in Table 3. The range of PIC was 0.15 (2077)–0.24 (2219), averaging 0.20 The mean of the RP

values, a parameter that indicates the discriminatory potential of the primers selected, was 3.48 for all primers. The highest RP value of 4.40 was recorded for 2395, while the lowest RP value was observed at 2.20 for 2077. The band profile obtained with iPBS markers was shown in Figure 2.

3.3. Correlations and multivariate analysis among the characters

Pearson's pairwise correlation revealed the relationships among characteristics (Figure 3). The correlations with highly significant values were observed among most of the studied characters. Significant positive correlations were detected among nut variables such as nut width, nut length, nut weight, and kernel weight. The kernel ratio was negatively correlated with nut width, nut height, nut weight, and shell thickness. The results were in the line with previous reports by Cosmulescu and Botu (2012), Paggetti et al. (2017), and Khavidi et al. (2019). Having the knowledge of the relationships among phenotypic characters can lead breeders in breeding studies. In addition, a linear correlation among different properties indicates that an improvement of a property may evolve the other related traits (Yucel et al., 2009). Leafing date was positively correlated with shell color and was not significantly correlated with lateral branch fruitfulness, harvest date, and altitude. This situation proves that the nut characteristics and the phenology change parallelly in the similar altitude and ecological conditions.

PCA is used a descriptive method to evaluate prominent features in the data. PCA provides the opportunity of minimization of effective factors according to individuals. These superior features made it an essential part of breeding and population genetics researches and was frequently used in recent years (Hashemi and Khavidi, 2020). The PCA analysis was performed based on nut characterizations with selected walnut genotypes. As a result, PCA indicated that 71.44% of the observed variability was described by the first five components. The first two components described nearly 44% of total variability. The relationships among genotypes presented in Figure 4. Nut weight, kernel weight, nut width, nut height, and nut length had the highest effects on PC1, and they comprised 28.6% of the total variability. PC2 explained 14.99% of total variation and was strongly related to nut and agronomic traits including FL, SC, RPFF, RPFM. PC3 showed crucial positive variability with oil and negative variability with LD and HD (Supp. Data 4). In our study, the first two PCs were significant at the level of 99%, while the third PC was significant at 95%. Principal components with a statistical significance, the first three PCs, explained 54.77% of the total variance and indicated a high phenotypic diversity in agro-morphological characters for studied genotypes. Similar to our study, previous studies revealed a high phenotypic diversity in pomological characters of walnut genotypes (Khavidi et al., 2019; Farrokh Toolir and Mozaffari 2020; Skender et al., 2020).

Hierarchical cluster analysis based on agro-morphological characters classified the genotypes into three major groups as supported by biplot (Figure 4 and 5B). The first group, BI bifurcated into two subgroups: BI-I included 008, 018, and 026 genotypes while BI-II consisted of 015, 031, 29, 48, and 103 genotypes. The second group, BII, bifurcated two sub-groups and the primarily group (BII-I) partitioned also into two sub-groups consisting of 030, 036, 076 genotypes one group and 061, 094 and 090 genotypes

respectively. The second subgroup (BIII-II) formed of two genotypes 045 and 073 genotypes. The third group (BIII) bifurcated two sub-groups and both two groups (BIII-I) and (BIII-II) formed of 027, 118 genotypes, and 075, 089 genotypes, respectively. The highest similarity was between 061 and 094 genotypes, while the farthest distance was observed between 008 and 027 genotypes. (Figure 4B). The distribution of the genotypes depends on agro-morphological characters on the biplot supported the cluster analysis (Figure 4 and 5).

The UPGMA dendrogram produced using the Jaccard's similarity coefficient for iPBS profiling methods clustered distinctly 20 walnut genotypes into three major groups (Figure 5A). From selected walnut genotypes, two genotypes (030 and 075) unite in the first group and the first branch (AI-I) and five genotypes (094, 090, 045, 015 and 020) combine the second branching (AI-II). Within this group, the highest genetic similarity rate was determined as 0.98% between 090 and 094 genotypes. In the second group, one genotype (018) was brought to the first branching. In the third group, the genotypes 026 and 027 formed the first branch (AIII-I) and the second (AIII-II) branch consisted two sub-groups having 008, 031, 073, 118, 048, 061, 036, and 076 genotypes, and 089 and 103 genotypes in other groups. The highest similarity rate in this group was determined between 118, 048 and 061 genotypes with a 0.95% Jaccard's similarity coefficient.

In the study, the rate of polymorphism obtained from iPBS (72.9%) markers was higher than previously reported with the RAPD (69.1%) molecular marker technique by Doğan et al. (2014), with the ISSR (61.08%) technique by İpek et al. (2019), and with the ISSR (71.1%) by Doğan et al., (2014). It is known that iPBS-retrotransposon markers are more informative than other molecular marker systems such as IRAP, RAPD, ISSR, and SRAP (Boronnikova et al 2010). These results showed the iPBS-retrotransposon technique can be used to determine genetic variation in walnut breeding.

The Mantel test was performed to determine correlations between genetic and morphological distances of genotypes. As shown in Figure 6, the mantel test resulted in a very low correlation ($r=0.04$). The low correlation between morphology and genetic indicates that molecular markers do not separate genotypes only with the morphological locus but also with a whole genome. We previously stated that iPBS retrotransposon markers are universal markers and use whole genome. Similar results were observed between morphological and genetic (SRAP) distances ($r = 0.03$) by Doğan et al. (2014).

The genetic variation between genotypes were determined for the first time using iPBS markers based on retrotransposons which is a newer DNA marker system than the ISSR and RAPD methods. The phenological and morphological characters are rather affected by environmental conditions. So, during recent decades many studies have focused on morphological traits with molecular techniques, such as DNA-based markers SSR, ISSR, AFLP, RAPD, and SNP (Single-nucleotide polymorphism) to reveal genetic diversity, relationships among cultivars or genotypes, explain phylogenetically and origin in walnut (Fjellstrom et al., 1994; Nicese et al., 1998; Kafkas et al., 2005; Christopoulos et al., 2010; Orhan et al., 2020; Houmanata et al., 2021). The percentage polymorphism and the average polymorphic band per primer detected in our present results were higher than previously found by İpek et al. (2018) and Doğan

et al. (2010). İpek et al. (2018) reported that 61.08% DNA polymorphism was generated by 17 primers; 51 of these markers were polymorphic in walnut cultivars and genotypes. The report deals with the results of Doğan et al. (2014) who used three molecular markers for characterizations of 59 walnut genotypes and detected 181 well-resolved and clear bands were generated by 25 primers, and 129 total polymorphic bands with 71.1% polymorphism percentage in RAPD, 117 total polymorphic bands from 118 with 69.1% polymorphism percentage in ISSR, and by 16 primer and 129 total polymorphic bands with 99.1% polymorphism percentage by 16 primers in SSR marker system. In our study using the iPBS primers, 72.09% polymorphism percentages were observed in walnut genotypes. Previous studies also confirmed that retrotransposon marker system could be successfully used for molecular characterizations of different fruits such as grapevine (Castro et al., 2012), apple (Kuras et al., 2013), hawthorn (Rahmani et al., 2015), *Pictasia* species (Kırdök and Çiftçi, 2016), olive (Kaya and Yılmaz-Gokdoğan, 2016), *Citrus* species (El Zayat et al., 2021), peach, and nectarine (Naeem et al., 2021).

Conclusions

In this study, the iPBS retrotransposon markers were first time used in walnut and resulted high polymorphism rates that make it an excellent tool to use in future breeding programs. Moreover, our results clearly proved that morphological variability and relationships are related to agro-ecological conditions. According to morphological properties 008, 029, 061, 075, and 076 genotypes were superior having greater values than the base limit of walnut breeding criteria. These genotypes stood out as an important variety candidate with their lateral branch productivity, fruit weight, kernel weight, kernel ratio, protein, and oil content.

Declarations

Acknowledgments: Data of manuscript were received from Ph.D. thesis of İbrahim Başak

Supplementary data: Supplementary data associated with this article were given in the attachment

Conflict of Interest: The authors declare there is not any known financial or personal competing of interest.

Authors Contribution: İB performed field experiments. İB and FM carried out the laboratory studies. FM performed statistical analysis and data visualization. GÖ performed molecular studies. FM, GÖ, and İB wrote and reviewed the original draft.

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Figures

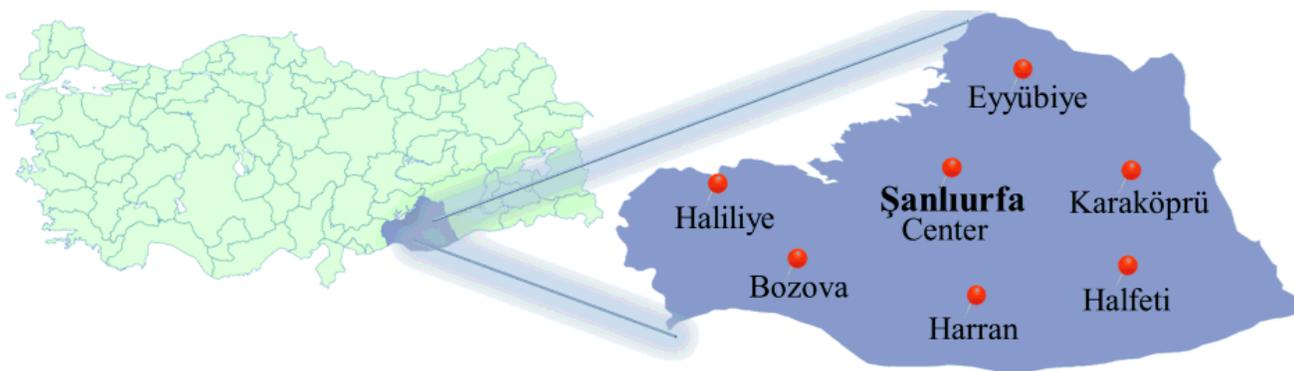


Figure 1

The region of Şanlıurfa where the study was conducted.

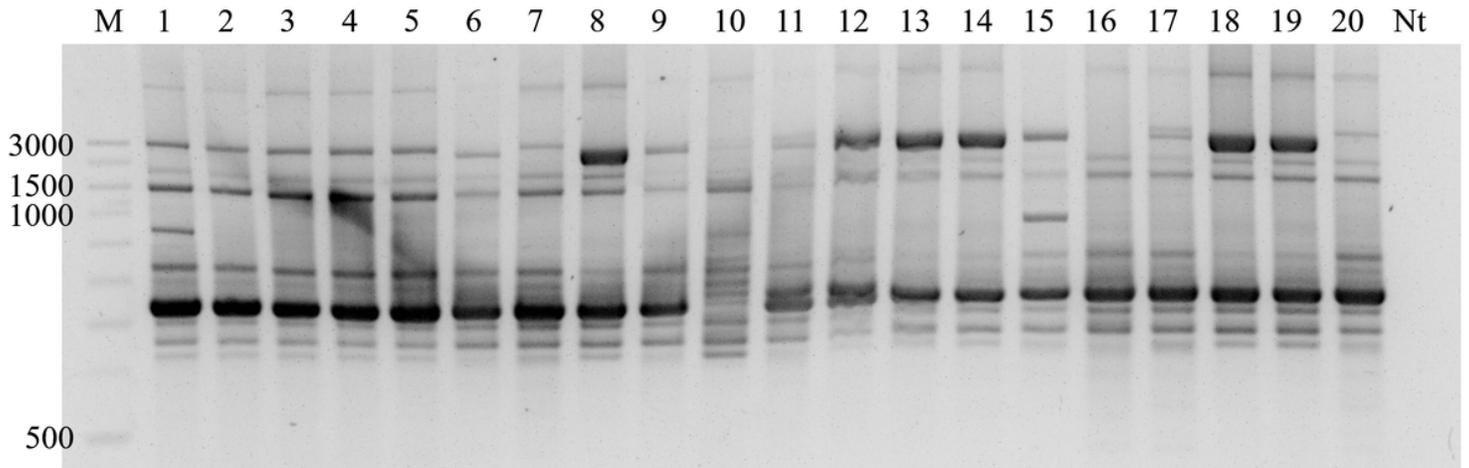


Figure 2

The band profiles with iPBS primer (2387) for walnut genotypes. M: 100 bp DNA Ladder (Solis BioDyne, Tartu, Estonia). Nt: Non-template DNA.

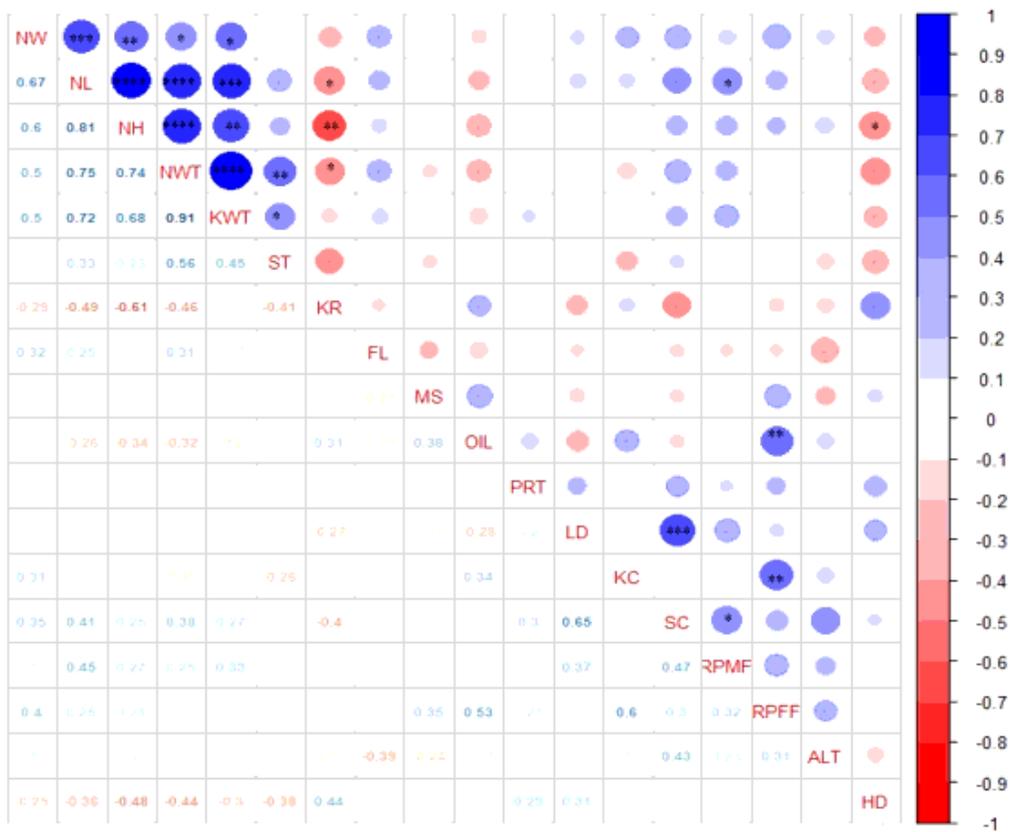


Figure 3

Correlation coefficients between major nut characteristics in walnut genotypes

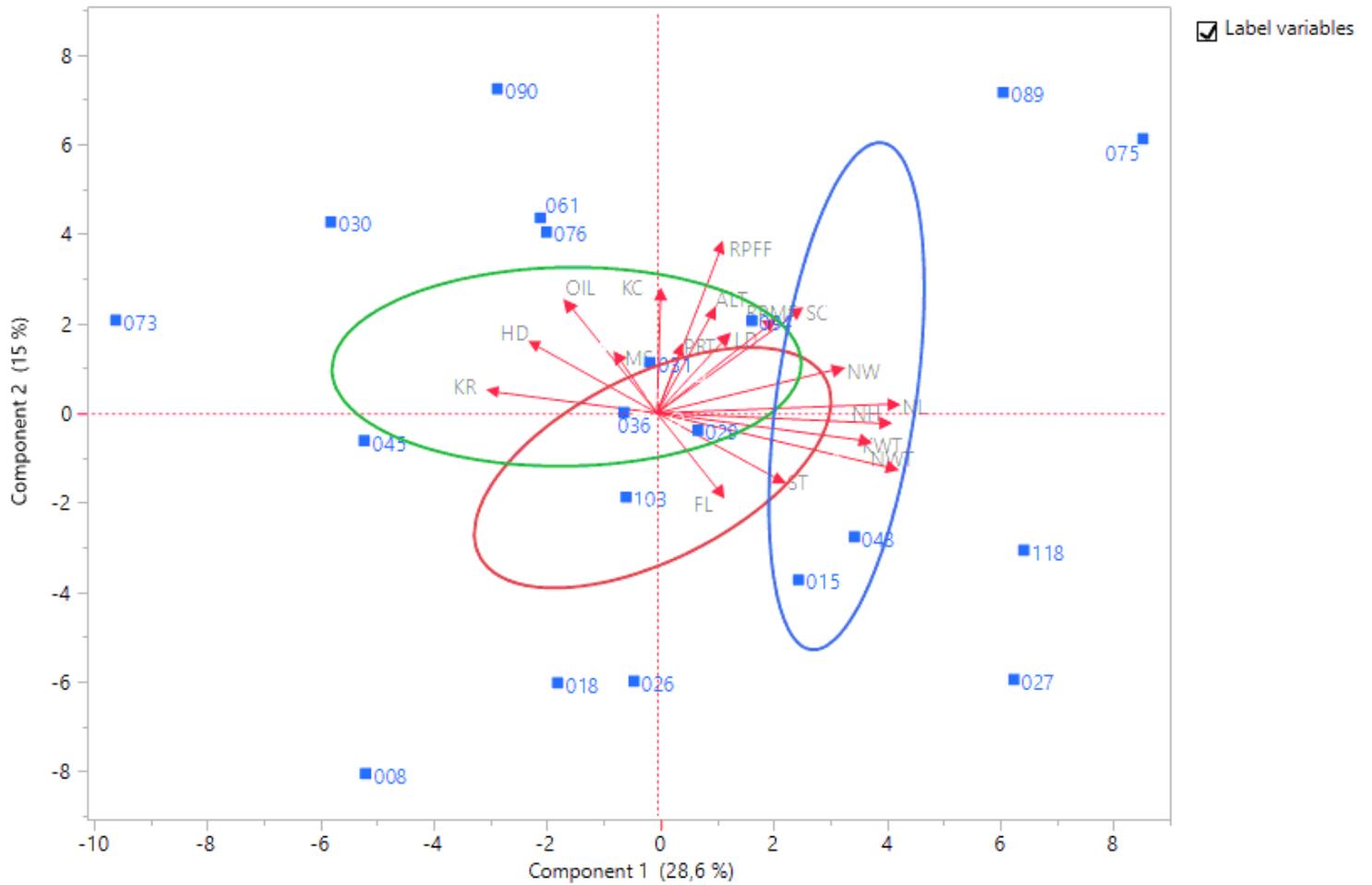


Figure 4

Biplot visualized by PC1 and PC2 using nut characters of the walnut genotypes

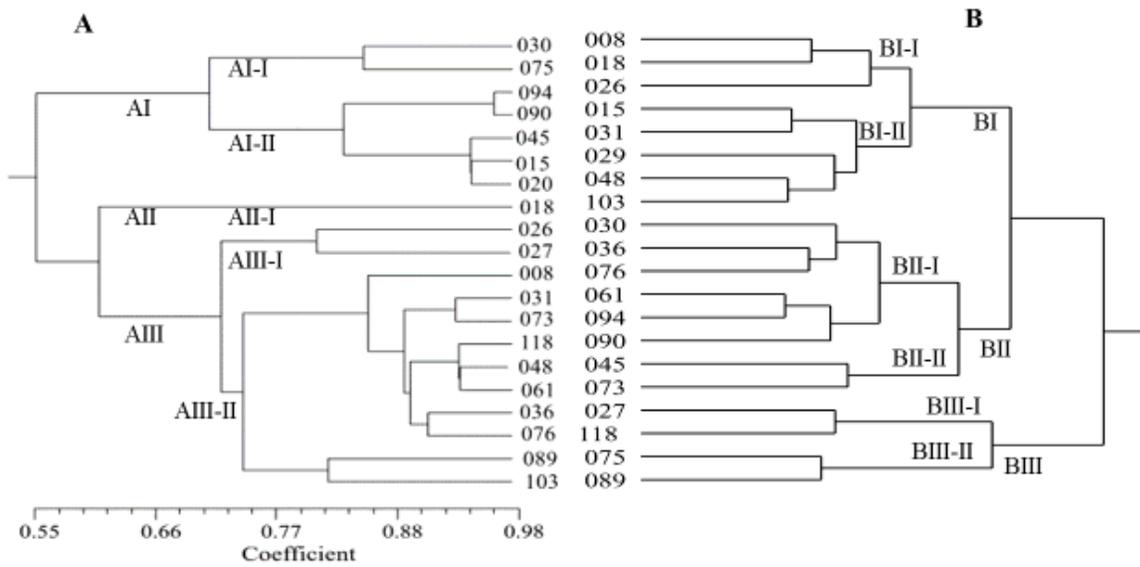


Figure 5

Dendrogram of the 20 selected walnut genotypes based on A: from iPBS molecular markers using UPGMA, B morphological traits using ward method similarity coefficients.

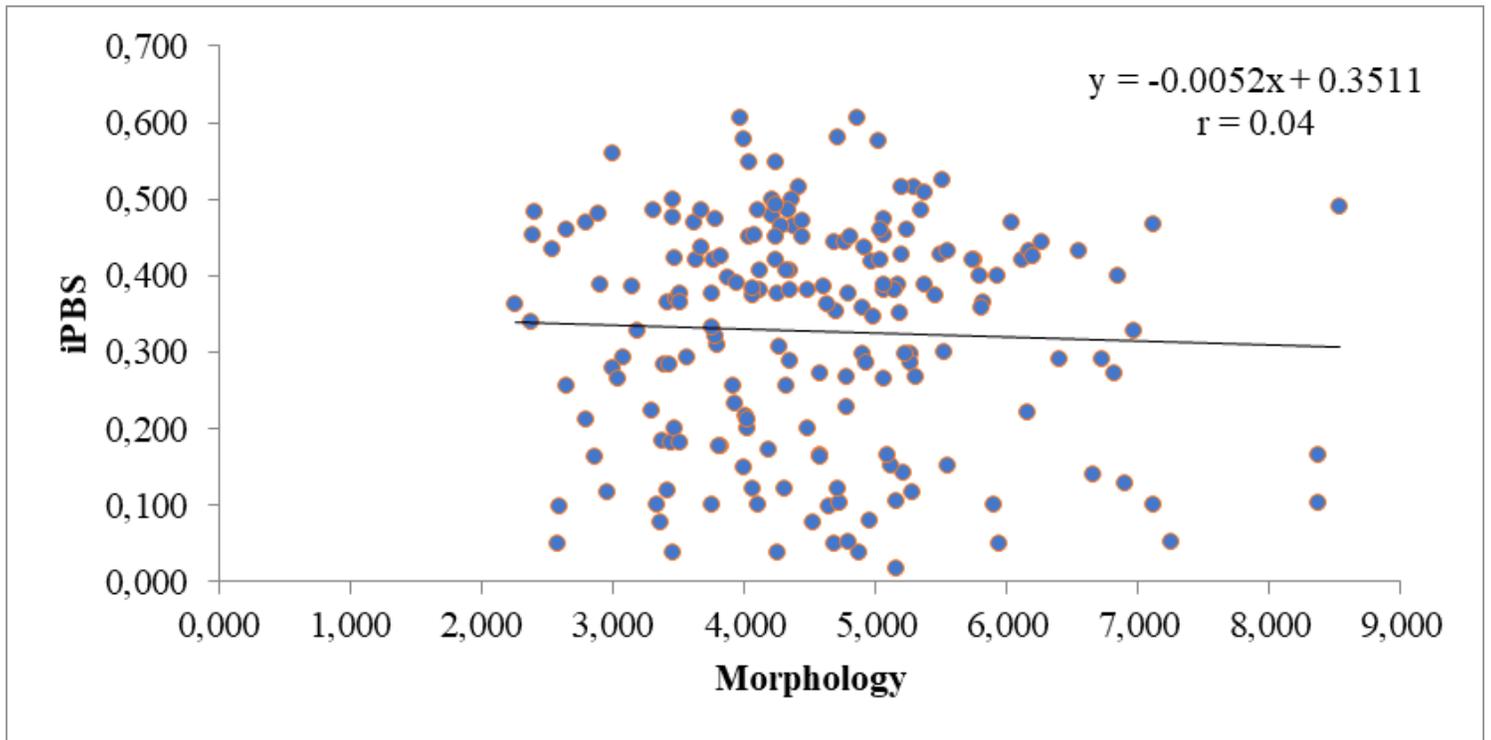


Figure 6

Mantel test correlation matrixes between walnut characters and combined molecular markers

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