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Phenological and Pomological Evaluation Reveals High Diversity among Walnut Populations in Southwestern Iran

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ABSTRACT

High genetic diversity guarantees success in a breeding program. Therefore, it is important to study the genetic diversity in plant populations. Because Iran considers as one of the primary walnut origin centers, this study was conducted to evaluate the genetic diversity of the walnut population in the Bavanat region in Southwestern Iran during the 2010-2015 growing season. For this purpose, 349 seed-originated walnut trees from seven different regions of Bavanat were pre-selected based on the local information. After primary evaluation, 92 walnut genotypes were selected for further studies. The phenological and pomological characteristics of the genotypes were evaluated based on IPGRI descriptor. The results revealed high genetic diversity in the studied germplasm in terms of important horticultural traits, especially nut and kernel weight, kernel percentage, nut size, shell thickness, lateral bearing, leafing date, and harvest date. The nut weight, kernel percentage, kernel weight, and shell thickness varied between 6.75-16.33 g, 40.15-67.22%, 3.30-8.15 g, and 0.58-2.30 mm, respectively. Cluster analysis classified the studied genotypes into six main groups. Furthermore, correlations among some horticultural traits indicated that kernel weight, nut weight, difficulty of removal of kernel halves, and shell thickness are main traits in determining kernel percentage. Also, lateral bearing, leafing date, nut weight, and nut size determine the final yield of walnut trees. In conclusion, high biodiversity in the walnut population of Bavanat region provides unique plant genetic resources for walnut genetic improvement programs to achieve desirable commercial cultivars and rootstocks.

Introduction

Genetic diversity is not only ensuring the survival of a plant species, but also, is the foundation of any plant breeding program (Bhandari *et al.*, 2017). Genetic diversity usually represents the intensity of genetic changes between individuals or a population of the same genus. The differences in DNA sequences and biochemical characteristics lead to genetic diversity, which in many cases manifests as the phenotype of individuals (Rauf *et al.*, 2010). The

phenotypic evaluation of genetic diversity based on descriptors is the foremost step for evaluating genetic biodiversity to establish core collections and exploit for breeding programs (Sarikhani *et al.*, 2021; Kaur *et al.*, 2022).

Persian walnut (*Juglans regia* L.) with a unique flavor and high nutritional values is known as a worldwide nutrient-dense food to manage obesity and some diseases such as heart attacks, Alzheimer's, and

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hypertension (Hassankhah *et al.*, 2017; Jahanbani *et al.*, 2018; Yang *et al.*, 2020; Sarikhani *et al.*, 2021; Akca and Sahin, 2022; Habibi *et al.*, 2023). Therefore, the global demand for its consumption has increased. The increase in demand for walnut consumption, along with production challenges caused by climate change, has made it necessary to accelerate walnut improvement programs. The presence of high genetic diversity gives walnut breeders the chance to advance their breeding program faster and more successfully (Kouhi *et al.*, 2021; Fallah *et al.*, 2022a). Iran as one of the primary walnut origin centers, with a share of 11% of the world's production is the third main producer of this crop in the world. Also, Iran is the fifth leading country in the world in terms of the walnut area harvested (Farsi *et al.*, 2020; FAO, 2021; Sarikhani *et al.*, 2021). Walnut trees have two propagation methods that divided into vegetative and sexual propagation (Thapa *et al.*, 2021). Walnut has been sexually propagated in Iran for many years; thus, a wide range of phenotypic and genetic variation is observed in the walnut populations in Iran (Arzani *et al.*, 2008; Sarikhani *et al.*, 2021). In addition to the origin center and sexual production, walnut is a wind-pollinated species which results a great variation in trees propagated through seeds (Sharma and Sharma, 2001). To exploit genetic diversity in the walnut breeding programs, it is necessary to assess this genetic diversity which not only provides the opportunity for introducing promising and superior genotypes, but also allows breeders to conserve walnut genetic resources as an international capital (Akça *et al.*, 2020; Mirmahdi and Khadivi, 2021). Evaluation and exploitation of genetic diversity have been conducted in many primary and secondary walnut-origin centers (Bernard *et al.*, 2018b; Vahdati *et al.*, 2019; Akça *et al.*, 2020; Vahdati and Khorami, 2021). Numerous researchers investigated walnut genetic diversity based on phenotypic and molecular characteristics to introduce commercial cultivars and rootstocks (Rezaee *et al.*, 2009; Karimi *et al.*, 2010; Bernard *et al.*, 2018a; Guney *et al.*, 2021; Vahdati *et*

al., 2021; Akça *et al.*, 2022; Sütyemez *et al.*, 2022). Evaluation of genetic diversity and its exploitation is the common walnut breeding strategy in Iran which is started by J. Atefi in 1983 (Atefi, 1990; Atefi, 1993) and continued by other researchers (Arzani *et al.*, 2008; Ebrahimi *et al.*, 2011; Sarikhani Khorami *et al.*, 2012; Ghanbari *et al.*, 2019; Guney *et al.*, 2021; Hajinia *et al.*, 2021). This breeding strategy in Iran led to introduce 6 commercial cultivars including 'Jamal', 'Damavad', 'Caspian', 'Persia', 'Alvand', and 'Chaldoran'. In addition, some walnut superior genotypes have been identified as being in the complementary evaluation stage and not only can be used as a parent in breeding programs based on hybridization, but also some of them can be introduced as cultivars (Vahdati *et al.*, 2019; Hassani *et al.*, 2020).

Considering the necessity of evaluating genetic diversity as the fundamental step in breeding programs, this study was conducted to evaluate walnut germplasm in the Bavanat region, Fars province to prepare an evaluated population for the future cultivar breeding program.

Material and Methods

Plant materials

To evaluate the walnut germplasm in the Bavanat region, Fars province, 349 walnuts seed-originated trees from seven different regions of Bavanat (Table 1) were pre-selected based on interviews with a local grower and relied on the information obtained from Agricultural Services of Fars province. The studied plant materials had different ages and were mainly cultivated on the boarder of traditional orchards. Generally, these regions have been classified in the semiarid climates with long and cold winters, and short and cool summers with annual precipitation averaging 175 mm. After primary observations, some of these trees were excluded because they showed symptoms of walnut blight (*Xanthomonas arboricola* *pv.* *Juglandis*) and anthracnose, or the average nut

weight was lower than 6.5 g (Zeneli *et al.*, 2005). Ultimately, 92 trees were selected and

morphologically evaluated for four consecutive years (2010-2015).

Table 1. Geographical coordinates of the studied areas in the Bavanat city, Fars province, Iran.

Area	Latitude	Longitude	Altitude (m)
Jian	30° 29' 31.30" N	53° 35' 23.85" E	2278
Ghale Snagi	30° 23' 42.99" N	50° 33' 35.71" E	2528
Dorah	30° 23' 42.48" N	53° 30' 00.51" E	2431
Fenjan	30° 23' 24.58" N	52° 29' 17.10" E	2363
Meseh	30° 25' 08.05" N	53° 27' 14.66" E	2409
Simakan	30° 25' 11.41" N	53° 26' 35.26" E	2390
Sardab	30° 25' 47.45" N	53° 29' 11.47" E	2489

The studied traits

Phenological (bud break date, harvest date, pollen shed and female receptivity date), morphological (growth habit, width, and length of leaf, and leaflet, and leaflet number), and pomological traits (bearing, bearing habit, nut weight, nut thickness, nut width, and length, kernel weight, kernel percentage, form index, roundness index, shell thickness, packing tissue thickness, the difficulty of removal of kernel halves, shell texture, shell seal, and kernel color) were evaluated based on IPGRI descriptor.

Phenological and general traits

Phenological traits are strongly influenced by environmental factors which are evaluated by reference standards (McGranahan and Forde, 1985). On this basis, the number of days after the earliest leafing genotype(s) as a reference standard was considered (Arzani *et al.*, 2008). For harvesting date, the first genotype whose was harvestable was considered as the reference standard. The bud break date was recorded in early spring, when more than 50% of terminal buds has enlarged and bud scales had split to expose the inside green leaves (IPGRI, 1994). Pollen shedding and female receptivity date and catkin appearance were recorded based on the IPGRI descriptor. In this study, the first pollen receptivity date was recorded when two stigma lobes reached at 35° angle (Janick and Paul, 2008). Also, the last pollen

receptivity date was recorded when stigma lobes were completely open and dry (Szentiványi and Szücs, 2001). In addition, the harvest date was determined when almost all (~95%) of the hulls come loose easily and completely separated from the shells. Trunk diameter, tree vigor, and growth habit of genotypes were determined based on the IPGRI descriptor (IPGRI, 1994). Leaflet number was estimated by computing leaflets in ten sampled leaves. Furthermore, leaf length and width were measured using a digital caliper (Mitutoyo, Japan).

Pomological traits

To evaluate pomological traits, 20 nuts from each genotype were harvested (IPGRI, 1994) and after removing hulls, samples were kept in a dark place for one month (Zeneli *et al.*, 2005). Nuts and kernels were weighed by a digital scale (KERN-1200, Balingen, Germany). In addition, kernel percentage was calculated by dividing kernels by nuts. The lateral bearing habit was calculated based on the method reported by Arzani *et al.* (2008). Shell thickness, nut size, and packing tissue thickness were measured by a digital caliper (Mitutoyo, Japan). The nut roundness index (RI) and form index (FI) were calculated by the following formula. Nuts with spheroid, ovoid, and ellipsoidal-elongated shape have the form index ≤ 110 , $111 \leq FI \leq 125$, and $125 < FI$, respectively (Arzani *et al.*,

2008):

$$FI = 200L / W + D$$

$$RI = W + D / 2L$$

L: Nut length; W: Nut width; D: Nut diameter

Shell texture, shell seal, nut shape, kernel color, the difficulty of removal of kernel halves, spring cold damage, and bearing were determined according to Table 2 (IPGRI, 1994).

Table 2. Some of the horticultural traits and their scaling for evaluating walnut.

The traits	Scaling
Nut shape	Round (1), triangular (2), broadly ovate (3), ovate (4), short trapezoid (5), long trapezoid (6), broad elliptic (7), elliptic (8), cordate (9)
Shell texture	very smooth (1) to very rough (9)
Shell Seal	Open or very weak (1), weak (3), intermediate (5), strong (7), very strong (9)
The difficulty of removal of kernel halves	Very easy (1) to very difficult (9)
Kernel color	Brown (1), amber (3), light amber (5), light (7), and extra light (9)
Spring frost damage	Low damage (1), moderate damage (3), extensive damage (5)
Bearing	No crop (1) to high yield (9)
Growth habit	Erect (1), semi-erect (2), spreading (3)

Statistical analysis

The Statistical Package for Social Science (SPSS) software (version 18) used to analysis the obtained data. High heritability traits including nut weight, form index, roundness index, nut width, thickness, and length, and kernel weight, kernel percentage, shell thickness, harvest date, bud break date, pollen shedding, and female receptivity date were used to cluster analysis. Cluster analysis was conducted according to the method of Average Linkage between Groups (Romesburg, 1984). The Pearson correlation coefficient was used to determine the correlation among the studied traits. Cluster and correlation analysis was conducted by SPSS software.

Results

Phenological and general traits

A study of general traits showed that the lowest (12.55 cm) and highest (243.30 cm) trunk diameters were related to A85 and A87 genotypes, respectively. These results confirm the fact that genotypes grown in the Bavanat region were too old. So that some trees were over 100 years old. In the case of growth habit, 64.13% of genotypes had spreading growth habit, while 8.70% of them were vertical (data not shown).

The obtained results indicated that the A92 genotype with 34 days delay compared with the standard genotype, and the A2, and A62 genotypes had the latest and earliest leafing date, respectively (Table 3). Furthermore, leafing dates of the A63, A65, and A92 genotypes occurred after the last spring frosts.

Harvest and bud break date, as high heritability phenological traits, are very important traits to select superior genotypes (Arzani *et al.*, 2008). The study of harvesting date of genotypes demonstrated that the harvesting date was from 25 August to 24 September. A15 genotype and A1, A25, A74, and A77 genotypes were known as early mature and late mature genotypes, respectively (Table 3).

According to the obtained results, the average of the first and last pollen-shedding dates were on 25 March and 18 May in the A84, and A92 genotypes, respectively (Table 3). Moreover, the longest and shortest pollen shedding period were related to A84 genotype (18 days) and A12 and A80 genotypes (7 days), respectively (data not shown). In this study, pollen receptivity date started from 2 April to 6 May in the Bavanat region. Female flowers in A12 and

A22 genotypes had the longest pollen receptivity period (15 days) while A9 and A79 genotypes had the shortest period (6 days). In the present study, the genotypes which had a minimum of 6 days overlapping in their pistil receptivity and pollen-shedding period were considered to be homogamous

(Arzani *et al.*, 2008). Dichogamy and homogamy were observed in 63.04% and 36.96% of the genotypes, respectively. In addition, 3.45% of dichogamy genotypes were protogynous (data not shown).

Table 3. Average of phenological traits in the studied walnut genotypes in Fars province, Iran during 2010-2015.

Traits		Date	Genotype
Bud break date	Earliest	12-Mar	A ₂ , A ₆₂
	Latest	14-Apr	A ₉₂
	Period*	33	
Pollen shedding date	Earliest	25-Mar	A ₈₄
	Latest	18-May	A ₉₂
	Period*	54	
female receptivity date	Earliest	2-Apr	A ₈₀
	Latest	6-May	A ₇₄
	Period*	34	
Harvest date	Earliest	25-Aug	A ₁₅
	Latest	24-Sep	A ₁ , A ₂₅ , A ₇₄ , A ₇₇
	Period*	30	

* The number of days between late and first of bud break, pollen shed, pistil receptivity, and harvesting

Pomological traits

According to the obtained results, lateral bearing varied from 0 to 80%. A51 and A86 genotypes had the highest lateral bearing percentage compared with other genotypes (Table 4). Excepting for these two genotypes, the lateral bearing percentage in A27, A29, A34, A49, A649 and A79 genotypes was more than 50%. Notably, A44, A71, A74, A76, and A77 genotypes were known as the terminal-bearing genotypes. One of the most important traits related to nut shape is the form index. The results showed that nut form averaged 114.13 among walnut genotypes in this study. Based on the nut form index, 47.83% of the studied walnut genotypes were ovoid. In addition, among genotypes, the roundness index varied from 0.75 to 1.04 (Table 4). Studies on the nut shape of genotypes revealed that the width and thickness average of the nuts were at 32.01 and 33.03 mm, respectively. The highest and lowest nut width was related to A12 (37.22 mm) and A58 (25.83 mm) genotypes, respectively. On the other hand, A52 and A30 genotypes had the lowest and highest nut thickness, respectively. Among genotypes, the

minimum and maximum nut length was recorded 31.21 mm in A82 and 43.17 mm in A12.

A study on shell seals showed that 7.61% of genotypes had very weak and open shell seals. The very strong shell seal was observed in 14.13% of genotypes (data are not shown). In addition, 15.22% of genotypes had “paper-shell” which is a desirable trait in walnut characteristics. In this study, the lowest and highest shell thickness was obtained from A79 (0.58 mm) and A86 (2.30 mm) genotypes, respectively (Table 4). The results showed that in 26.08% of genotypes, removal of kernel halves was difficult, while in A9, A16, and A64, removal of kernel halves was extremely easy (data are not shown). Our results indicated that the lowest, highest, and average nut weights were 6.75, 16.33, and 10.73 g, respectively (Table 4). The lowest and highest nut weight was related to A50 and A2 genotypes. The average kernel weight was 5.57, so the highest weight (8.15 g) was obtained from the A2 genotype. A50 genotype after A44 showed the lowest kernel weight (Table 4).

Kernel percentage is an important kernel quality-related trait. In this study, the average kernel percentage was 52.15%. The A9 genotype which can be considered a superior genotype had a 67.22% kernel percentage. The kernel percentage in A16, A31, A38, A40, A57, and A79 was registered more

than 60%. Kernel percentage in the A2 genotype was 49.91%. In most genotypes, kernels had light color. The other word, 60.87% of the genotypes had light to extra light color. There was no dark kernel among genotypes.

Table 4. the variation range of some pomological characters of 92 selected walnut genotypes originated from Fars province, Iran during 2010-2014.

Traits	Min	Max	Mean	SD	CV (%)
Nut weight (gr)	67.54	163.33	107.37	24.86	23.15
Kernel Weight (gr)	33.00	81.52	55.70	13.61	24.43
Kernel percentage (%)	40.15	67.22	52.15	15.94	30.57
Nut length (mm)	31.21	43.17	37.02	12.04	32.52
Nut width (mm)	25.83	37.22	32.01	12.32	38.49
Nut thickness (mm)	26.58	38.41	33.03	12.47	37.75
Shell thickness (mm)	0.58	2.30	1.28	0.33	25.78
Form index	95.87	134.12	114.13	18.86	16.53
Roundness index	0.75	1.04	0.88	0.17	19.32
Lateral bearing (%)	0.00	80.00	26.16	16.17	61.81

Cluster analysis

Highly heritable traits were used to cluster analysis (Khorami *et al.*, 2018). Cluster analysis classified the selected walnut genotypes into six cluster groups. Based on the results, the related genotypes to the same regions located in the same cluster. Since Dorah and Fenjan are so close to each other with the same climate, most genotypes of these regions have located in one cluster (Cluster 1). In addition, A79 and A84 genotypes that belonged to Meseh, have located in the same cluster (Cluster 3). A92 genotype as the most late-leafing genotype has located in one cluster alone (Cluster 1). Furthermore, A14 and A17 genotypes have classified in the same group (Fig. 1). Whereas these genotypes are native to a certain region (Jian region) so it seems that both of them had the same parents.

Correlation

Based on results, kernel weight had a positive correlation with growth habit, leaf and leaflet length, and leaflet number ($P < 0.05$). In other word, kernel weight in spreading growth habit genotypes was more than erect or semi-erect growth habits. Also, there is a positive and significant correlation ($P <$

0.05) between harvest and pollen receptivity date. Moreover, a negative correlation was found between kernel percentage and trunk diameter (Table 5). It is worth mentioning that there was no significant correlation between shell thickness, kernel color, and lateral bearing habit with phenological traits. Harvest date showed a positive correlation with nut width and thickness, while it had a negative correlation with kernel percentage at 5% probability level.

The results of this study indicated that kernel and nut weight have a positive correlation with nut width, length, and thickness. In other words, the genotypes with larger nut sizes had a higher nut and kernel weights. A positive and significant ($P < 0.01$) correlation found between nut weight and shell thickness and also between nut weight and kernel weight (Table 5). Unlike nut weight which had a negative correlation with kernel percentage, kernel weight had a positive correlation with kernel percentage ($P < 0.01$). The results showed that there is a negative correlation between shell thickness and kernel percentage. Generally, genotypes with paper shell thickness and high kernel weight had high kernel percentage (Table 5).

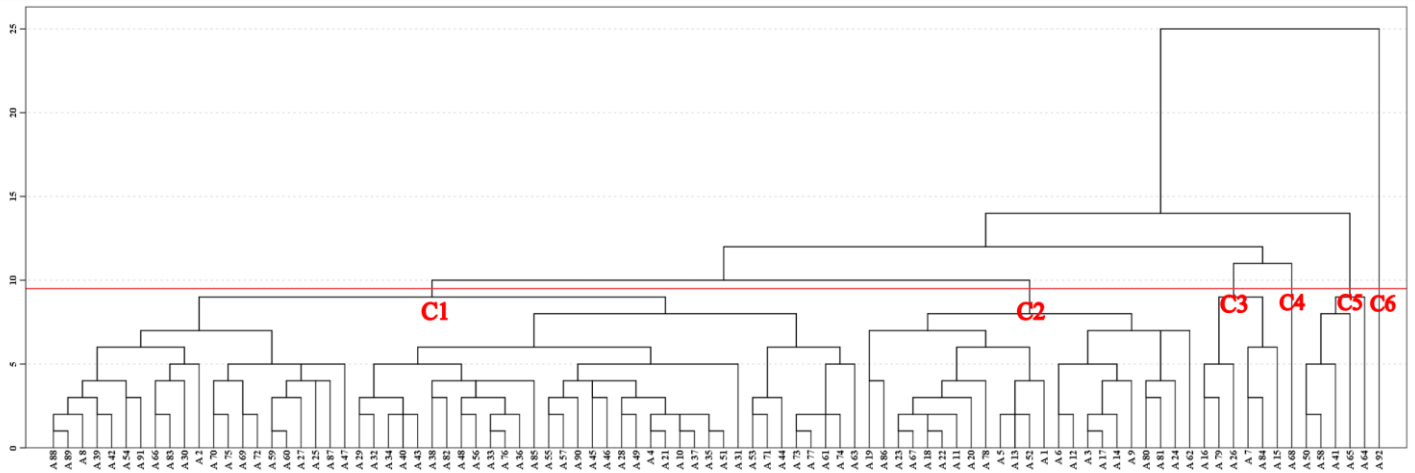


Fig. 1. Cluster analysis of 92 selected walnut genotypes originated from Fars province, Iran

Table 5. Correlation analysis between some high-heritability traits of walnut in the Bavanat region, Fars province, Iran.

Traits	1	2	3	4	5	6	7	8	9	10	11	12
Nut Weight	1											
Kernel weight	0.81**	1										
Kernel percentage	-0.25*	0.36**	1									
Lateral bearing	-0.17	-0.03	0.25*	1								
Trunk diameter	0.18	0.04	-0.21*	0.03	1							
Nut width	0.65**	0.59**	-0.04	-0.06	0.17	1						
Nut thickness	0.71**	0.60**	-0.12	-0.02	0.08	0.84**	1					
Nut length	0.53**	0.46**	-0.06	0.05	0.15	0.50**	0.47**	1				
Form index	-0.09	-0.08	0.02	0.09	0.04	-0.3**	-0.4**	0.6**	1			
Roundness index	0.08	0.09	0.00	-0.1	-0.06	0.33**	0.37**	-0.6**	-0.99**	1		
Shell thickness	0.19	0.09	-0.13	-0.08	-0.05	0.03	0.00	0.07	0.07	-0.06	1	
date of bud break	-0.06	-0.20	-0.27*	-0.07	0.09	0.01	0.09	0.13	0.09	-0.11	-0.05	1
Harvesting date	0.19	0.03	-0.25*	-0.27**	0.09	0.26*	0.25*	0.16	-0.08	0.08	-0.12	0.29**

*and** significant at 5 and 1% probability levels, respectively.

Discussion

Due to the difficulty of asexual propagation of walnut (Hassankhah et al., 2014; Farsi et al., 2016), historically, most walnut trees in Iran were propagated by seed; thus, the walnut population in the country possesses a wide range of morphological and genetic variations (Arzani et al., 2008; Sarikhani et al., 2021). This study was conducted to evaluate phenological and morphological traits of walnut genotypes in the Bavanat which is known as one of the most important walnut origins in the southwestern Iran. Today, late-leaving, early maturity, high yield, and quality are the most important goals in the walnut breeding programs (Vahdati et al., 2019).

The wide range of bud break between different genotypes is due to different chilling requirements and the sensitivity of each variety to high temperatures which is known as the heat requirement (Hassankhah et al., 2017; Hassankhah et al., 2020). In climates with wet springs and dry summers, late-leaving individuals escape the environmental conditions conducive to blight infection as well as late-spring frost (Khadiji et al., 2019). Later emergence of leaves in spring (even for a few days) plays a critical role in decreasing chilling injury. Late-leaving walnut genotypes can be planted in mountainous regions even under continuous cold-climates (Akca and Ozongun,

2004). The obtained results indicated that A92 and A632 genotypes were late-leafing genotypes with desirable nut and kernel traits. Therefore, these genotypes not only can be used as the parent in the future walnut breeding program based on hybridization, but also have the potential to introduce as superior genotypes. Based on four years of evaluation, we found that the order of leafing of studied genotypes was constant in different years, which could be due to the high heritability of this trait (Fallah *et al.*, 2022b).

Flowering habit is an important trait in the walnut orchard management, because of overlapping the pollen shedding and female receptivity date. In the current study, 36.96% of the genotypes were homogamous. Among the studied walnut genotypes, protandrous was more than protogynous due to cold and semi-humid weather during the flowering stage. These conditions stimulate the male flower to appear earlier than the female flower. Flowering and other phenological traits are strongly affected by environmental conditions (McGranahan and Forde, 1985).

Pomological traits are the most important traits in the walnut breeding programs because these traits are not affected by environmental conditions or age. Evaluation of walnut germplasm in India showed that the average nut and kernel weights were 12.55 and 4.80 g, respectively. Also, the highest kernel percentage was 62.5% (Sharma and Sharma, 2001). Our results showed that nut weight varied between 6.75 to 16.33 g. Also, the average kernel weight was recorded at 5.57 g (Table 4). These results were significantly higher than the results obtained by Sharma and Sharma (2001) and Simsek *et al.*, (2010). Shell thickness with more than 80% heritability is classified as a high heritable trait (Hansche *et al.*, 1972; Aslantas, 2006). Shell thickness should be between 0.7 and 1.5 mm for superior genotypes (Akca and Ozongun, 2004). The obtained results indicated that shell thickness ranged from 0.58 to 2.30 mm. Moreover, the shell thickness of 72.82% of the

genotypes was less than 1.5 mm; While 67.39% of the genotypes had a shell thickness between 0.7 and 1.5 mm.

The correlation study is a suitable guide for walnut breeding programs (Amiri *et al.*, 2010; Sarikhani Khorami *et al.*, 2014). Our results showed that there is a strong and significant correlation between lateral bearing habit and yield (Khorami *et al.*, 2018). Trees with lateral bearing habit were early-leafing and had better yield potential than trees with the terminal bearing habit (Solar *et al.*, 2001; Sarikhani Khorami *et al.*, 2014). The obtained results confirmed that lateral bearing habit had a positive correlation with bearing and kernel percentage, so the increase in yield was parallel with the increase in lateral bearing percentage (Khorami *et al.*, 2018). In the current study, we found that lateral bearing habit has no correlation with tree growth habit. Nonetheless, there is a positive correlation between early maturity and lateral bearing habit. So that the most of genotypes with lateral bearing habits were early harvest. In this study, nut weight had a positive correlation with kernel weight and shell thickness while nut weight negatively correlated with kernel percentage. These results are in agreement with Amiri *et al.* (2010) and Sharma and Sharma (2001).

In general, high genetic diversity was observed in the walnut germplasm in the Bavanat region, Fars province, Iran in terms of phenological and pomological characteristics. Also, we found that nut length, thickness, width, and also shell thickness play an important role in determining nut and kernel weight.

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Conflict of interests

The authors have no conflicts of interest to declare.

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