Article ID :ijbt150814103

Int. J. Biol. Technology, (2014) :5(2):21-30

OPEN ACCESS

ISSN 0946 - 4313

Evaluation of morphological diversity among some Persian walnut accessions (*Juglans regia* L.) in Guilan, northern Iran

Ebrahim Alinia Ahandani[1*], Hadi Darzi Ramandi[2], Jannat Sarmad[3], Majid Asadi samani[4], Alimohammad yavari[5], Reza Alinia Ahandani[6]

Received: 24, April, 2014 / Accepted: 12 August 2014 / Published Online: 15 August, 2014

Research Article

Abstract : In this study, the genetic relatedness of 15 walnut accessions adapted to the Lahijan region of Guilan province, Iran was analyzed by morphological characters. The traits like growth habit, leafing date, content of male flower, nut yield, nut weight, kernel weight, kernel percent, hard shell thickness, nut length, and nut thickness. The coefficient of genetic diversity obtained for nut yield was high, indicating high diversity of the samples under study for this trait. The association and correlation between the traits in most cases were significant at α=0.01 and α=0.05. Simple correlation analysis showed that nut yield had significant and positive correlation with weight of the nut, the weight and the length of the kernel and the thickness of hard shell (r= 0.762, 0.758, 0.558, respectively). Also, the association between nut yield and hard shell thickness was negative and significant (r=-0.741). Genetic diversity was assessed between and within accessions using statistical analyses including coefficients of variation (CV), principal component analysis (PCA) and hierarchical cluster analysis (HCA). The results obtained from cluster analysis placed the accessions in three separate groups. Principal component analysis indicated that the first and second components explained 69.6% of all variance. This grouping was completely consistent with cluster analysis. In addition, the results indicated high diversity based on the morphologic indices, which requires greater, closer attention to the region's native walnuts for subsequent breeding programmes.

Keywords: *Juglans regia*, walnut, morphological characteristics, PCA and HCA.

Citation: Ebrahim Alinia Ahandani, Hadi Darzi Ramandi, Jannat Sarmad, Majid Asadi Samani, Alimohammad Yavari,Reza Alinia Ahandani. 2014. Evaluation of morphological diversity among some Persian walnut accessions (*Juglans regia* L.) in Guilan, Northern Iran. *Int.J.Biol.Technology*,5(2):21-30.

Address for Communication

[1]Faculty of Biology, Payamnoor University, Roodsar, Iran.

[2]Faculty of Agriculture, University of Agricultural Sciences and Natural Resources, Sari, Iran

[3]Plant Biology Department, Faculty of Biology, University of Guilan, Rasht,Iran.

[4]Shahrekord University of Medical Science-Shahrekord -Iran.

[5]Horticulture Department, Faculty of Agriculture Sciences, Urmia University, Urmia, Iran

[6]Faculty of Agriculture Sciences, Guilan University, rasht, Iran.

[*]Corresponding Author Email: Ebi.alinia@gmail.com

Manuscript Type : **Research Article**

Received Manuscript : **Via Email**

Approved Letter : **Received** or Non Received

Funding Source: Support or **Nil**

Conflict of Interest : **Nil**

Manuscript Full Responses: **Authors**

Submission of manuscripts info:

biotekeditor@yahoo.com

1.Introduction

Walnut is a monoic, fully heterosexual, and from *Juglandaceae* family, which pollinates through wind (Chanandha *et al*. 2003) and its most important species is *Juglans regia* (2n=32). Walnut has 60 species, of which 21 genera are categorized as *Juglans* that is probably originated from Iran and some regions in Afghanistan and then distributed to China, Russia, and southern Europe. Most walnuts cultivated in the world are from this species and according to some resources, its initial distribution to other parts of the world has launched from Iran, for this, it has also been referred to as Persian walnut (Ducci *et al*., 1997). Iran is an internationally important walnut producer with a production of 485,000 tons in 2011, representing 14.2% of the total world market (FAO, 2013). In Iran, due to high internal consumption, low genetic corrections on different walnut genotypes and unavailability of suitable machinery for walnut harvesting and post-harvesting operations such as shelling, dehulling, sorting, sizing, separating nut and kernels and packing, the production of exportable walnut kernels is low. In addition, Vahdati (2001), citing Lesley and Granahan, mentioned that walnut wild genotypes originated from northern forests of Iran, particularly Guilan, which is the main origin of walnut in these areas. Identifying and assessing native walnuts is the first step of advancing corrective practices in this field (Rezaee *et al*. 200). Of the techniques under study for assessing diversity and determining genetic relationship is morphologic traits assessment, which has been already done in different regions of Iran (Jafari-Sayyadi, 2006).

Breeding programs on walnut have been initiated since the second half of 20th century through conducting crossing aiming to produce highly productive varieties via selecting the genotypes with high peripheral yield. In the USA a corrective project was commenced since 1984 in Davies University and continued to 1988. The important traits considered in the project were high yield, last budbreak, high quality shell and kernel, and high percentage and light color of kernel (Joolka and Sherman, 2004). Mamadjanov (2001) investigated genetic diversity of Kyrgyzstan walnuts and finally, depending on his purpose, selected spherical, papery and cluster forms. Farhatoglu (Farhatoglu, 1993) has selected nine walnut genotypes of sixteen in Turkey based on important qualitative and quantitative traits. In Spain, Aleta *et al*. (2003) have selected and presented 67 superior genotypes among seed populations in Spain according to quantitative and qualitative traits. Forde and Mc Granahan (1996), in the USA, have divided *Juglans* genus into 21 species of South America, North America, Central America, Europe, and Asia and remarked that of *Juglans* genus species Persian walnut, *J. regia*, thanks to its edible fruit and black walnut, *J. nigra*, thanks to its wood and, to some extent, fruit's kernel are economically significant. Other species of walnut as a basis could indirectly have economic importance. A profound genetic diversity of this crop is seen in Iran, particularly northern Iran, which represents rich, valuable potential of walnut in Iran; these genetic resources could be applied in the plants correction plans and selection of the varieties with high potential for Iran gardens (Jafari-Syadi, 2006). Germarin (1993) declared that since Iran is one of the walnut's origins in the world, there is an important, genetic variety in its species. Investigation of walnut genetic diversity based on morphologic traits in Iran according to Guidelines for tree assessment developed by *International Plant Genetic Resources Institute* and translated by Vazvaee *et al*. (2003) has been implemented in many regions of Iran. Mohsenipour (2007) examined the walnuts of Kerman province and classified them into four categories per qualitative morphological traits. Mansouri-ardakani (2001) studied a number of superior walnut genotypes using some morphological traits in some regions of Yazd province and finally identified superior genotypes in terms of the traits under study. Haghjoyan et al. identified and assessed the genotypes in Touyserkan, Hamadan per morphological traits. In this line, Ehteshamnia *et al*., (2009) examined some walnut populations in Golestan, Iran and identified 96 trees from five populations in heights and woodlands, investigating the association between some quantitative traits and the altitude. In view of the importance and value which is nowadays attributed to expansion of walnut gardens in Iran, usage of the amended genotypes with high potential is highly required. Regarding the area under walnut cultivation in Iran, this study is mainly aimed to identify and select desired native accessions in Lahijan region, Guilan; the next step is to apply them throughout eugenic plan, to select, and finally to offer promising clones. Identification and selection of superior genotypes have been previously conducted to some extent in some regions, but in view of high diversity of walnut in Guilan, as one of potential walnut origins, complementary studies to identify exemplary native varieties is more required than ever for their findings to be used in future walnut correction plans in case of confirming adequate morphologic diversity in trees' and seeds' traits.

2. Materials and Methods

2.1. Plant material and study area

In this study conducted from 2011 to 2012, fifteen native accessions from different regions of Lahijan, Guilan province were gathered after they were identified and finally screened. Native walnuts of the region were selected for each region according to high yield, and using field studies, the information elicited from Lahijan county agriculture experts and offered by Jafari-Sayadi et al. (2006), routine questioning and advice by local gardeners. Also, unfortunately no systematic and analyzed data on forest and local walnuts of the county was found in Agriculture Organization and Agriculture and Natural Resources Research Centers of Guilan province. In addition, according to the research by Malvolti *et al*., (1993) the trees with maximally 15 km distance from each other were referred to as a population. The trees under study were randomly selected for sampling, comprising five population and 88 selected trees for labeling. Of these, six native accessions (of 29 accessions) were gathered from Ahandan region, three (of 17 accessions) from Soostan village, two (of 15 accessions) from Lialestan village, two (of 16 accessions) from Bazkia-gourab, and two (of 11 accessions) from Flower and Decorative Plants Research Center. Totally, 15 accessions as superior native accessions, after being screened, were examined.

2.2. Sampling and morphological characterization

Identification and labeling of the accessions were done one year prior to study and at the time of fruit harvesting. Annual visits were arranged in these stages including: the time of leaves' opening and flowering from middle of March to early April (appropriate with region's conditions); the time of complete fertility and the time of harvesting (late summer and early autumn), during which a full assessment of each region's accessions was made per relevant guideline. Assessment of phenotypic, phonologic, and trading traits of the region's selected accessions was made per walnut descriptor for 15 traits (Akca, 2005; Aleta *et al*., 2033). For final selection, each superior genotype demonstrated relative and superficial superiority among the populations under study in view of at least one of the following trading traits. These traits include: late leafing date which was assessed in the first visit in March or April, when sprouts grow to one cm and/or the leaves of the genotype open; nut yield, which should be relatively higher than mean yield of the population under study and this assessment was made according to the age or diameter of trunk and expansion of tree crown through relative estimate of the nut yield; lateral percentage which is directly related to nut yield and according to projections most lateral accessions also had high yield. In this assessment the accessions higher than 50% lateral were selected; fruit weight and size which were assessed in the third visit in which those accessions with a dry fruit heavier than 10 gr (kernel moisture should be less than 78%) and fruit size larger than 30 mm were selected for examination; and kernel color and percentage. At least 10 samples were determined for assessment these two traits. Kernel percentage of higher than 48% was decided to be the index for selection. In addition, it should be mentioned that the accessions with more than one or a number of superior traits had priority for selection. Excellent accessions based on desired trading, phonologic, and pomological traits were identified and labeled and then 15 superior accessions were detected. Since all walnut trees in the regions' gardens were obtained from plant a seed, a great genetic difference existed among them. Of the accessions under study, a great diversity in the time of male and female flowers emergence, male flowers rate, late leafing date, etc. was observed.

2.3. Data analysis

Descriptive statistics such as averages and coefficients of variation (CV) were used to detect diversity between and within the studied accessions. Following correlation analysis, using Pearson correlation coefficient ($\alpha = 0.01$), was used to estimate the relationships between studied variables. Principle component analysis (PCA) was used to detect the characters that are most relevant to distinguish between the accessions. Clusters of accessions were formed on the basis of these factors using Ward's method; the graph representing classification is a dendrogram of dissimilitude with standardized Euclidean Distances representing the closest accessions in homogeneous groups. Statistical analyses are done using the software XLSTAT.

3. Results and Discussion

3.1. Contents variability of morphological traits

The specifications of measured traits and coefficient of each trait's diversity in different accessions have been shown in Table 3. In most traits under study, there is a good diversity in this set of accessions. Germarin (1993) has also reported a high genetic diversity in Iran's walnut genotypes. The traits under study include tree morphology, the time of opening of the leaves, male flower rate, flowering type, nut yield, fruit weight, kernel weight, kernel percentage, shell thickness, fruit length, and fruit width. Based on the data registered the time of leaves' opening was found to be form 13March to 26April (Table 1). In this study, the accessions JR8 and JR15 were identified as respectively early budbreak and last budbreak. Since one of the important purposes in walnut breeding is achieving some accessions with leaves appearing more lately in spring, last budbreak was addressed in these accessions, as well. Early budbreak accessions are more exposed to risk of spring training during flowering era, which could disturb their pollination; in addition, early budbreak accessions are predisposed to blight (Forde, 1975). Kernel percentage has been considered as the most important index of economic yield in tress, which should be further concentrated on and the effective factors on its quantity should be further investigated. The mean rate obtained for this trait was 53.7 and the rate of diversity index 6.87. Kernel percentage in accession JR14 with 59% was the highest and in JR13 with 48% the lowest. According to the results of the study by Ence *et al*., (1972) kernel percentage in walnut has a high inheritability.

The factors influencing kernel percentage include kernel size, kernel meatiness, the shell filled with kernel, and shell thickness; of these the three first factors are directly related to kernel percentage and the fourth one is inversely associated. As reported by Kazankaya *et al*., (2002) in Turkey, nut weight, kernel weight and kernel ratio of superior walnut genotypes were 11-12g, 6-7 g and more than 45%, respectively. It has been reported that fruit characteristics are not affected by tree age (Sharma and Sharma, 1998). In the accessions under study, JR12 and JR5 had the highest shell thickness and JR15 the least thick shell among these accessions, which causes increase in kernel percentage in accession JR15 compared to the accessions JR12 and JR5. In view of nut weight accession JR15 with mean weight of 13.60 gr had the highest mean weight of the fruit among the accessions and the accessions JR9, JR10, and JR11 with 10.10 gr had the least (Table- 3). Here, it is clarified that the only factor which probably has the greatest effect on kernel percentage in these accessions with mean fruit weight equal to hard shell is the weight and thickness of each accession's shell. Accession JR15 in view of the traits like yield, nut weight, and kernel weight had a higher mean compared to other accessions, which causes it to have greater nut volume with hard shell and ranked higher in nut yield compared to other accessions. On the other hand, the accessions JR2, JR3, JR4, JR5, and JR6 had a higher mean after accession JR15 in nut yield, fruit weight, and kernel weight compared to other accessions and these accessions overall had a higher kernel percentage due to less shell thickness compared to other accessions (Table -5). Accessions JR10 and JR13 had the least kernel percentage compared to other accessions due to having less dimensions, weight, and volume and thicker shell.

3.2. Correlation between traits

The results of correlations among the traits under study, of which some are significant, are shown in Table 4. Of these traits fruits weight, kernel weight, and fruit length could be mentioned; these are positively, significantly correlated with nut yield, which is in agreement with the results obtained by Eskandari *et al*., (2005) and Ebrahimi, (1388). The time of leaves' opening was highly correlated with nut yield, fruit weight, and kernel weight. There was a positive association and correlation between kernel weight and kernel percentage, shell thickness, and skin tissue, and also between

Tab 1. Phonological characteristics of Persian walnut (*Juglans regia* L.) accessions

No.	Accession	Growth habit	Score of leafing date	Content of male flower
1	JR1	semi upright	7	low
2	JR2	spreading	8	high
3	JR3	semi spreading	11	intermediate
4	JR4	spreading	10	high
5	JR5	spreading	9	high
6	JR6	spreading	8	very high
7	JR7	semi spreading	5	low
8	JR8	spreading	1	intermediate
9	JR9	spreading	6	high
10	JR10	semi spreading	4	high
11	JR11	spreading	3	high
12	JR12	spreading	2	very high
13	JR13	semi upright	7	low
14	JR14	spreading	10	high
15	JR15	spreading	12	very high

Tab 2. Morphological characteristics of Persian walnut (*Juglans regia* L.) accessions

No.	Acces-sion	Nut yield (kg/ha)	Nut weight (gr)	Kernel weight (gr)	Kernel percent (%)	Hard shell thickness (mm)	Nut length (mm)	Nut width (mm)	Nut thickness (mm)
1	JR1	39.00	30.00	39.00	1.23	57.00	6.40	11.30	94.05
2	JR2	34.00	31.66	42.00	1.24	58.00	6.10	10.40	108.90
3	JR3	41.00	32.06	42.00	1.25	58.00	7.50	12.90	115.50
4	JR4	36.00	34.00	36.00	1.27	56.00	6.86	12.30	122.10
5	JR5	40.00	34.00	44.00	1.57	50.00	6.50	13.00	99.00
6	JR6	35.00	32.06	37.00	1.34	52.00	6.40	12.30	113.85
7	JR7	38.00	32.06	37.00	1.39	49.00	4.95	10.10	72.60
8	JR8	33.00	30.00	35.00	1.49	55.00	5.70	10.40	49.50
9	JR9	33.00	31.00	34.00	1.29	52.00	5.25	10.10	99.00
10	JR10	32.00	32.00	35.00	1.46	49.00	4.94	10.10	54.45
11	JR11	32.00	35.00	35.00	1.26	52.00	5.80	11.10	82.50
12	JR12	29.00	29.00	33.00	1.66	55.00	5.66	10.30	68.80
13	JR13	31.00	30.00	34.00	1.27	48.00	5.18	10.80	52.30
14	JR14	32.00	29.00	35.00	1.40	59.00	6.40	10.90	59.40
15	JR15	29.00	31.00	40.00	0.78	55.00	7.50	13.60	180.51

Table- 3: Descriptive statistics for morphological characteristics in 15 Persian walnut (*Juglans regia*) accessions.

Characteristics	Unit	Max	Min	Mean	Std Dev	CV (%)
Nut yield	Kg/ha	180.51	49.50	91.50	24.86	28.10
Nut weight	gr	13.60	10.10	11.31	1.20	10.61
Kernel weight	gr	7.50	4.94	6.08	0.83	13.63
Kernel percent	%	59.00	48.00	53.67	3.64	6.78
Hard shell thickness	mm	1.66	0.78	1.33	0.20	15.05
Nut length	mm	44.00	33.00	37.20	3.41	9.16
Nut width	mm	35.00	29.00	31.52	1.81	5.73
Nut thickness	mm	41.00	29.00	34.27	3.81	11.11

Tab 4. Correlation coefficients between the morphological and phonological characteristics

Morphological characteristics	1	2	3	4	5	6	7	8
Nut yield								
Nut weight	0.762**							
Kernel weight	0.758**	0.877**						
Kernel percent	0.305	0.200	0.643**					
Hard shell thickness	-0.741**	-0.475	-0.500	-0.214				
Nut length	0.558*	0.626*	0.610*	0.242	-0.268			
Nut width	0.306	0.367	0.152	-0.293	-0.090	0.349		
Nut thickness	0.113	0.317	0.284	0.079	0.134	0.635*	0.391	
Leafing date	0.720**	0.735**	0.804**	0.431	-0.482	0.620*	0.181	0.279

*, **Significant and highly significant correlation at 5% and 1% probability level, respectively

Tab 5. Correlation matrix between PCA axes and analyzed characters

Morphological characteristics	Component and explanation coefficient						$\sum r^2$
	First component	r^2	Second component	r^2	Third component	r^2	
Nut yield	**0.772**	**0.878**	0.037	-0.193	0.091	-0.302	0.900
Nut weight	**0.801**	**0.895**	0.003	0.058	0.015	-0.123	0.820
Kernel weight	**0.848**	**0.921**	0.049	-0.221	0.037	0.191	0.934
Kernel percent	0.192	0.439	0.289	-0.537	**0.388**	**0.623**	0.869
Hard shell thickness	**0.385**	**-0.620**	0.188	0.434	0.228	0.478	0.801
Nut length	**0.609**	**0.780**	0.124	0.352	0.054	0.232	0.787
Nut width	0.142	0.377	**0.472**	**0.687**	0.163	-0.404	0.778
Nut thickness	0.179	0.423	**0.477**	**0.690**	0.208	0.456	0.863

Numbers in bold indicate the characters that are most relevant to distinguish between accessions on the respective axe. F_i: principal component representing a PCA axis.

the time of leaves' opening and nut yield, fruit weight, and kernel weight.

3.2. Correlation between traits

The results of correlations among the traits under study, of which some are significant, are shown in Table 4. Of these traits fruits weight, kernel weight, and fruit length could be mentioned; these are positively, significantly correlated with nut yield, which is in agreement with the results obtained by Eskandari *et al*., (2005) and Ebrahimi (1388). The time of leaves' opening was highly correlated with nut yield, fruit weight, and kernel weight. There was a positive association and correlation between kernel weight and kernel percentage, shell thickness, and skin tissue, and also between the time of leaves' opening and nut yield, fruit weight, and kernel weight. The rate of correlation among these traits is so high that we could find out the changes in relevant trait through measuring another trait; therefore, measurement of a trait could be indirectly done more rapidly and more economically.

3.3. Principle component analysis (PCA)

In multivariate statistics of principle component analysis (PCA) to decrease the number of initial variables, description and explanation of total diversity in a population and determination of each trait's contribution to total variation are used (Pearson, 1981). In view of the diversity of walnut accessions under study, the analysis into main components was done to determine contribution to and effect rate of each trait under study in the present diversity. The results of analysis into main components in the eight measured traits are shown in Table 5. To develop the component's matrix coefficients, those components whose specification root was above one were selected. In analysis into main components, the aggregation of Eigen values is equal to data's total variance and a main component's value represented the contribution of component to total variance. Eigen values obtained from the components 1, 2, and 3 explained respectively 49.9%, 20.5, and 14.8%, overall 84.4%, of the variables' total variance (Fig.1). The coefficient of Eigen vectors in the first component indicated that nut yield, fruit weight, kernel weight, and fruit length had the greatest contribution to this component development. Therefore, any increase in these traits could lead to the enhancement of yield. In the first component, shell thickness contributes negatively and other traits do positively. In the second component, fruit width had higher Eigen vector coefficients compared to other traits. In the third component, kernel percentage had the highest significance in explaining this component in positive direction. Accession JR15 explaining 25% of the total variance had the highest significance in the first component obtained from analysis into main ones, followed by JR3, JR12, JR10, and JR13. In the second component development, the accessions JR5, JR15, JR14, and JR7 had the highest significance (Table -5). The second and third components were used to draw the accessions' bi plot diagram (Fig. 2), in which the accessions were classified into three independent categories. In the first group, eight accessions consisting of JR7 to 14, in the second group six accessions JR1 to 6, and in the third group JR15 alone were placed. Accessions classification using the first two main components confirmed cluster analysis-based grouping and the three groups were placed in independent categories. As shown in figure 2, the accessions in the groups 2 and 3 are superior over other accessions in two main components. The relations among the morphologic trait under study based on the first and second components of the analysis into main components for 15 walnut accessions are shown in figure 3.

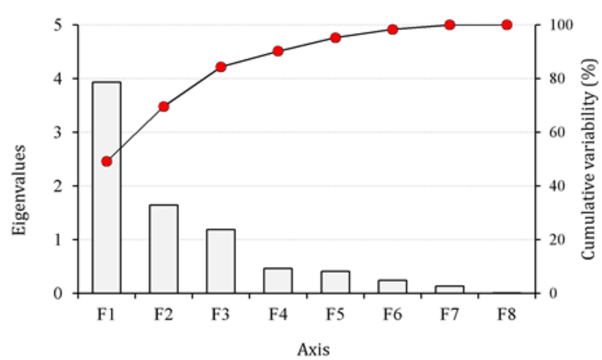

Fig. 1. Eigenvalues and cumulative variability based on principal component analysis of the morphological characteristics in 15 Persian walnut (*Juglans regia* L.) accessions.

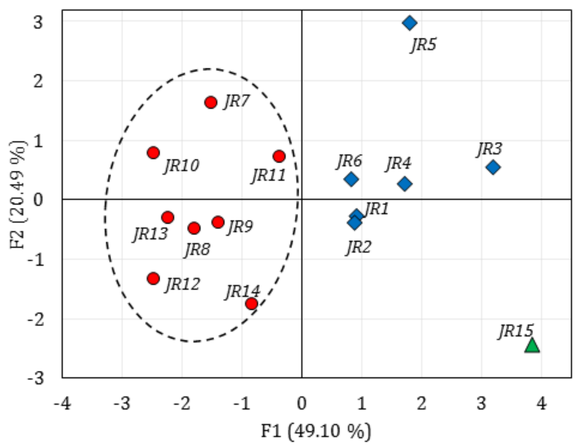

Fig. 2. Two dimensional principal component analysis representing the axes F1 and F2 illustrating the variation of the morphological characters of 15 Persian walnut (*Juglans regia* L.) accessions. The variability shown accounts for 79.6% of total variability.

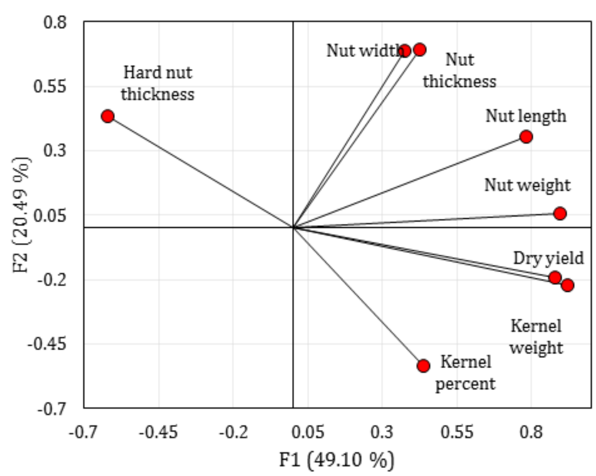

Fig. 3. Relationships among the eight morphological characters used for 15 Persian walnut (*Juglans regia* L.) accessions based on principal component analysis.

3.4. Hierarchical cluster analysis (HCA)

To achieve maximum heterosis, the researchers searched for the varieties or accessions which were far, in genetic traits, from each other for finding the best parents in each crossing. This could be achieved through examining the existing genetic distance among the accessions per morphologic traits using cluster analysis. While the morphological traits are used, the accessions which are placed in the groups far from each other as a result of grouping are used as parents in conducting crossings in corrective plans, so that they will have a higher genetic generation (Danesh-Gilvaei et al. 1390). Cluster analysis of the accessions per morphologic traits identified three distinct groups. In the distance 102 based on UPGMA algorithm the accessions were divided into three main groups (Figure 4).

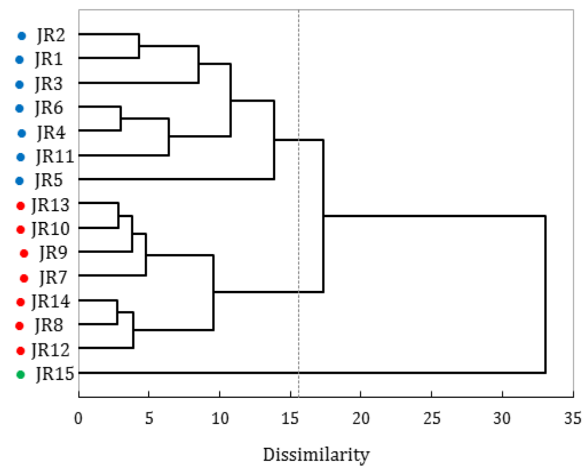

Fig. 4. Dissimilarity dendrogram generated by Ward‘s method using XLSTAT Software showing three major clusters on the basis of morphological a characters of 15 Persian walnut (*Juglans regia* L.) accessions

In the first group, the accessions JR7, JR8, JR9, JR10, JR12, JR13, and JR13 had traits like high width and shell thickness. The first group consisted of the accessions JR1, JR2, JR3, JR4, JR5, JR6, and JR11, most of which had traits like high nut yield, fruit weight, kernel weight, and fruit length and hence are useful. Accession JR15 was placed alone in the third group and was distinct due to nut yield being higher compared to other accessions and had traits like thinner shell. The Early budbreak accessions often are more exposed to winter cold and due to high moisture of the air in early season of this accessions' growth, they are sooner predisposed to blight disease. In the present study a wide range of variation was observed in nut and kernel characters from different locations. Similar variation for these characters was obtained by various workers with different cultivars and seedling trees in different walnut growing areas (Pandey and Sinha, 1984; Rathore, 1984; Thakur, 1993; Sharma and Sharma, 1997, 1998). Presently, one of the important objectives of walnut breeding is to achieve the varieties with traits like late leafing date, earlier fruiting, high yield, and optimal seed quality. Regarding late coldness of spring in recent years in Iran, selection of last bud break accessions with supreme quality fruit seems vital for the country. Breeding of new walnut varieties is characterized by earlier fruiting, higher yield, lateral bearing, good adaptability to different ecological conditions and good fruit quality (Germain, 1988; Akça, 2009). Depending on corrective purposes in question for the province's native walnuts, a suitable accession could be selected based on the traits in question. The results indicate that overall, the accessions under investigation enjoy high diversity of fruit traits and the selection should be done in view of the traits in question. Since the samples were selected from different regions of Guilan province with different environmental conditions, it is desirable to select suitable trees in view of the traits under study and it is necessary to gather all these trees as a whole and then to completely examine their desired traits for close comparison and assessment of their capabilities and then to select suitable ones. Finally, after complementary study of the selected trees, boding can be prepared and crossbred on seed saplings and then required investigations on breeding and selection of varieties can be done.

Acknowledgment
The authors of this article gratefully thank late Jafar Alinia-Ahandani for their technical help and support while doing this research.

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