J. Agr. Sci. Tech. (2021) Vol. 23(1): 187-200

Walnut Genetic Diversity Investigation Using Phenological and Morphological Characteristics and ISSR Markers

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ABSTRACT

There is little information about the genetic resources of Persian walnut (*Juglans regia* L.), an important edible and one of the most important plants in pomology and economy in Iran. Germplasm identification and conservation are important and fundamental issues in breeding programs. Six regions in BoyerAhmad and Dena Counties were chosen, and 82 walnut genotypes were selected randomly. Annual growth period was short in Setangun and Ganjegun Regions (190-200 days), but in other investigated regions was long and more than 230 days. Maximum fruit and kernel weights were 63.44 and 8.35 g, respectively. The number of observed alleles ranged from seven (ISSR7A) to 14 (ISSR3A) for primers, and the Number of effective alleles (Ne) in the primers used was between 1.19 to 1.45. The highest Ne alleles belonged to the ISSR1A primer (1.445). Walnut genotypes showed the highest genetic diversity based on the information derived from ISSR1A and ISSR2A with PIC index of 0.44 and 0.41, respectively. The Nei's gene diversity ranged from 0.13 to 0.24, and the Shannon's index was from 0.23 to 0.44. In general, increase in the altitude and decrease in the temperature delayed phenological characteristics.Suitable genetic diversity was found among the genotypes, based on the morphological and phenological characteristics and the ISSR markers. Overall, the results are useful in walnut breeding and conservation programs, and by choosing late leafing and flowering genotypes from Setangun and Ganjegun Regions, it would be possible to reduce the risks of late spring frost.

Keywords: Nei's diversity, Shannon's index, Walnut genotypes conservation.

INTRODUCTION

The main goals in walnut (*Juglans regia* L.) breeding are to increase yield, quality, and the range of harvest dates. In numerous regions, walnuts growth is restricted by early autumn chilling and late spring frosts, which can be relatively overcome by using late leafing cultivars. Walnut culture can also be limited by inadequate winter chilling and extremely high summer temperatures (McGranahan and Leslie, 2012). Persian walnut is known as a valuable herbal resource all around the world, especially in Iran. The center of diversity and origin of the Persian walnut species (Ehteshamnia *et al*., 2009), and its oil has a desirable quality (Safari and Alizadeh, 2007). Identification of the genetic diversity is an important issue in breeding programs, in the management of genetic resources, and selection of the superior walnut genotypes from different areas in Iran (Malvolti *et al*., 2010, Davoodi *et al*., 2019). There are various approaches for evaluating the genetic diversity of walnuts. The first one is the application of the morphological characteristics. Nut and kernel weight, shell thickness, and the strength

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[DOR: 20.1001.1.16807073.2021.23.1.2.5]

of separating the kernel halves are the most important characteristics, determining the kernel percentage (Ehteshamnia *et al*., 2009; Sarikhani Khorami *et al*., 2014); as the economic production index of walnuts (Ehteshamnia *et al*., 2009). The nut length, thickness and width, as well as the shell thickness play an important role in determining the size of the nut and kernel weight, and the thickness of the shell has a significant negative relationship with the kernel percentage (Ehteshamnia *et al*., 2009; Sarikhani Khorami *et al*., 2014). The shell weight has been reported to be between 0.4 and 1.4 g (Arzani *et al*., 2008; Mousavi *et al*., 2015). The walnut fruit weight (nut with green hull) has been reported to be between 14.9 to 55.9 g, and nut weight to be between 6 to 20.55 g (Sharma and Sharma, 2001; Yarilgac *et al*., 2001; Arzani *et al*., 2008; Ehteshamnia *et al*., 2009; Ebrahimi *et al*., 2010; Mousavi *et al*., 2015). The kernel weight of walnut has been reported to be between 2.15 and 14.1 g (Sharma and Sharma, 2001; Yarilgac *et al*., 2001; Arzani *et al*., 2008; Ehteshamnia *et al*., 2009; Ebrahimi *et al*., 2010; Mousavi *et al*., 2015). The maximum kernel percentage among the studied walnuts was reported to be between 12 to 79.6% (Sharma and Sharma, 2001; Yarilgac *et al*., 2001; Arzani *et al*., 2008, Ehteshamnia *et al*., 2009; Mousavi *et al*., 2015). As it is seen, the diversity among the genotypes based on the morphological characteristics mentioned above is obvious and different.

The morphological markers depend on the growth stage and environmental conditions of the plants, and this may lead to miss evaluation and identification of the genotypes. Thus, to have a more accurate identification of the genotypes, there is a need for methods that are less affected by environmental factors (Solar *et al*., 1994), and the solution is the usage of molecular markers, due to their capacity for attributing a sample of particular individuals with a negligible mistake, which has led to extensive practical applications. The most commonly used markers in genomic analysis (Jones *et al*., 2009) and in walnut genotypes are RFLP, SRAP, RAPD, AFLP, SSR and ISSR markers (Wani *et al*., 2010; Maghsoodi *et al*., 2018, Davoodi *et al*., 2019). ISSR markers are fast, relatively inexpensive, highly reproducible, and suitable for populations genetic diversity studies (Wani *et al*., 2010; McGranahan and Leslie, 2012; Ashraf *et al*., 2016; Dilsat Yegenoglu and Sesli 2017). The relationships of 21 Persian walnut genotypes from Urmia city, Iran, were investigated using 13 ISSR primers with 87.5% polymorphism. Genetic similarity among genotypes ranged from 0.39 to 0.67 (Mahmoodi *et al*., 2012).Walnut pomological traits of 21 genotypes cultivated at Shahrood research center originated from Tuyserkan, Urmia, Karaj and Khorasan province, Iran, were studied by using 10 ISSR primers. Among them, the highest weight of nut was 17.5 g, and of kernel was 0.3 g (Davoodi *et al*., 2019). In Greece, 27 genotypes of local Persian walnuts and 29 international varieties were compared by using 47 ISSR primers, from which 93 DNA bands (alleles) were observed. The results showed more diversity among the Greek local genotypes than that of the international cultivars, and the within genetic diversity (89%) based on the molecular variance analysis was greater than between that (11%) (Christopoulos *et al*., 2010). Eight ISSR primers were used for evaluating the genetic diversity of 48 walnut genotypes, among which, 15 genotypes were distinguished by one primer, 31 genotypes by at least 2 primers, and the other 2 genotypes by three primers (Potter *et al*., 2002). In Turkey, 59 walnut genotypes were evaluated by using 25 RAPD, 25 ISSR, and 16 SSR primers, among which, the RAPD and ISSR markers with a lower polymorphism (69.1 and 71.1%, respectively) than the SSR markers, showed relatively similar results (Dogan *et al*., 2014). Within population, diversity represents the high gene flow, which helps to reduce genetic drift within the populations. The genetic diversity observed among the populations may be not related to the studied geographic regions (Vahdati *et al*., 2015).

No information is available on Persian walnut genotypes in cold regions of Kohgiluyeh and BoyerAhmad province, the 4th

[DOR: 20.1001.1.16807073.2021.23.1.2.5]

province in walnut production in Iran, and therefore, this research was conducted to study and identify the genetic diversity of local Persian walnuts in this region, and the results can have wide applications in walnut breeding programs.

MATERIALS AND METHODS

Locations Studied

Six regions of the BoyerAhmad and Dena Counties, Kohgiluyeh and BoyerAhmad province, Iran, were selected (maximum distance up to 45 km between regions) and 82 genotypes (each tree considered as one genotype) were considered randomly from the selected regions (Figure 1, Table 1).

Measurement of Phenological and Morphological Characteristics

In late March to early April 2014, geographical information about the locations was collected for sampling (Table 1). Sampling of the selected walnut trees from each region (Table 1) was carried out to measure the nut traits in September, 2014. Phenological traits such as leafing, flowering, harvesting, and leaf loss (fall) time, and nut characteristics were calculated and evaluated (Table 2), according to the "Descriptors for Walnut (*Juglans spp*.)" instruction published by the International Plant Genetic Resources Institute (IPGRI, 1994). For each characteristic, 20 nuts or fruits were used.

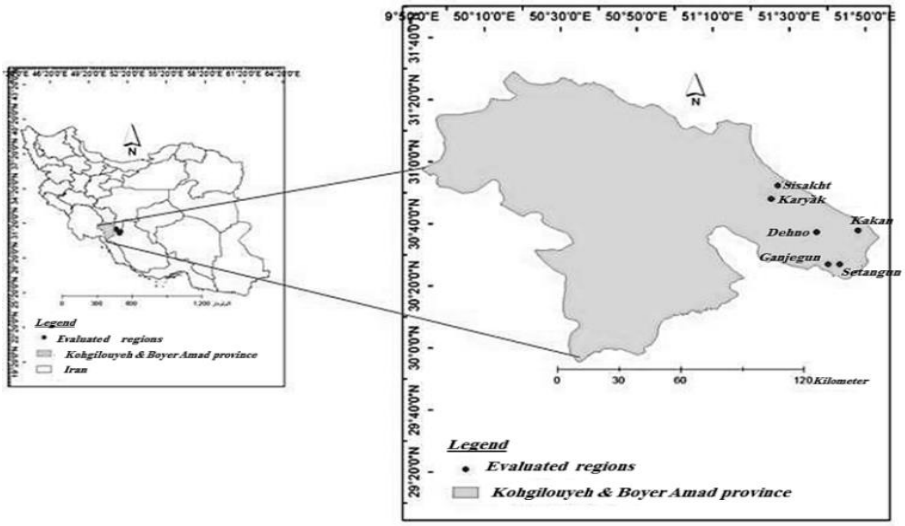

Figure 1. Location of the selected regions in this study.

Table 1. The code of trees and geographical location of the studied regions.

Region	Region code	Number of selected samples in region	Genotypes code in dendrograms	Longitude (Eastern)	Latitude (Northern)	Altitude (m)
Dehno	D	7	1-7	05137E	03037N	1872
Karyak	K	15	8-22	05148E	03038N	2373
Sisakht	S	15	23-37	05127E	03052N	2321
Kakan	E	15	38-52	05125E	03048N	1763
Ganjegun	G	6	53-58	05143E	03027N	2062
Setangun	CT	24	59-82	05140E	03027N	2283

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Table 2. The Recorded characteristics of walnut genotypes and their measurement method.

Characteristics	Measurement method
Nut weight with hull (g), nut weight with shell (g), and kernel weight (g)	Digital balance
fruit moisture(g)	Nut weight with fresh and dry hull
Kernel/Fruit weight (%)	Kernel weight/Fruit weight×100
Kernel percentage (%)	Kernel weight/Nut weight ×100
Budbreak, blooming (male and female), and harvesting	Very early (1), early (2), relatively early (3), intermediate (4), relatively late (5), late (6), very late (7), too late (8)
Leaf fall (Leaf drop)	Early (3), intermediate (5), late (7)

DNA Extraction, Quality and Quantity Evaluation

For DNA extraction, about 6 to 8 young leaves from selected trees in each region were collected. DNA extraction was carried out by using the CTAB method (Doyle and Doyle, 1987), with some modifications (AmiriFahliani *et al*., 2011) on a small scale (Mini preparation). The quality of the extracted DNA was evaluated by agarose gel (0.6% concentration) electrophoresis stained with ethidium bromide, and UV light visualization.

Polymerase Chain Reaction (PCR)

PCR reaction volume was 20 µL, containing the DNA template with a final concentration of 50 ng, 1x PCR Master Mix Buffer (Cinnagen, Iran), primer (2µM), and autoclaved double distilled water (AmiriFahliani *et al*., 2011). Ten ISSR primers were selected (Table 5), and applied based on Nunes *et al*. (2013) information reported. PCR reactions were run with a temperature pattern of initial DNA denaturation at 94°C for 5 minutes, followed by 35 cycles of DNA denaturation at 94°C temperature for 30 seconds, primer annealing temperature (proposed by company) for 35 seconds, and the extension stage with 72°C for 2 minutes, and finally, the extension of the DNA strands were performed at 72°C for 10 minutes in one cycle (AmiriFahliani *et al*., 2011). PCR products were evaluated by using 2% agarose gel electrophoresis prepared in TBE 1x buffer to observe the amplified DNA and the banding pattern and genetic diversity of different genotypes for ISSR Primers used. Based on the molecular data, cluster analysis of genotypes was performed by using a Simple Matching Coefficient (SMC) matrix with the highest correlation coefficient and UPGMA method, by using the NTSYS 2.02 software. The Number of observed alleles (Na), of effective alleles (Ne), the Shannon's genetic diversity index (I), and Nei's gene diversity (H) were calculated by using PopGene 1.32 software for each primer. Analysis Of Molecular Variance (AMOVA) was also performed by using the Genalex 6.4 software. The Polymorphism Information Content (PIC) was calculated from the following equation:

$$PIC_i = 1 - \sum P_{ij}^2$$

Where, P_{ij} is the frequency of the j^{th} allele (band) of the i^{th} marker (Botestien *et al*., 1980).

RESULTS AND DISCUSSION

Phenological Characteristics

Dates of some phenological stages such as leafing out, male and female blooming, harvesting, and leaf fall (fall season) for the

[DOR: 20.1001.1.16807073.2021.23.1.2.5]

genotypes (trees) of each region are presented in Table 3.

Flowering time in each region was almost the same for the studied phenological traits. Male and female flowering date in Dehno and Karyak Regions was earlier than the other regions, and the flowering date in the Setangun and Ganjegun Regions was later than the other regions. In relation to the leafing out date, the trees in Dehno Region, and after that, those in Karyak Region were earlier than the other regions, and the leaf out date in Setangun Region was the latest. In general, it can be said that with increasing altitude, and therefore decrease in the temperature, the phenological characteristics in the Setangun and Ganjegun Regions showed up later than the other regions with lower altitude (Table 3). However, there was no general rule regarding the relationship between altitude and the expected date of observation of phenological characteristics. Given the number of days and the length of the annual growth period of walnuts, Setangun and Ganjegun, with an altitude near to Sisakht and Karyak, showed the shortest growth period (between 190 and 200 days from budbreak to leaf loss stages). The results of other research have shown that, as the altitude increases, the flowering time is delayed in comparison to the regions with lower altitudes (Ehteshamnia *et al*., 2009). The risk of spring frost in all the investigated regions is a threatening phenomenon. However, it can be expected that by choosing late leafing and/or late flowering genotypes from Setangunand Ganjegun Regions, and with the improvement of walnut trees with the ability of delaying leafing out and flowering time, the risks caused by spring frost could be prevented to some extent.

Morphological Characteristics

The information of each morphological character among the genotypes surveyed in each region is presented in Table 4. The genotypes of the Sisakht Region showed the highest average fruit weight (52 g), and those of the Kakan and Dehno Regions had the lowest average (Ca. 38 g) (Data not shown).

The genotypes of Sisakht Region had the highest average nut weight. The highest average of kernel weight (6 g), fruit moisture (39 g), Kernel/Fruit weight ratio (14%), and the kernel percentage (53.42%) belonged to the genotypes of Karyak (the superior genotype with kernel percentage (58.78% for K112) also was in this region) (Table 4). These results showed that the genotypes of Dehno and Karyak Regions with a high average kernel weight, kernel/fruit weight ratio, and kernel percentage are high-yielding genotypes.

Table 3.Phenological traits of 82 walnut genotypes in 6 regions of BoyrAhmad and Dena Counties.

Region	Budbreak	Blooming		Harvest	Fall (Leaf fall)
		Male	Female		
Sisakht	Mid-April	Late April	Late April	Early to mid-September	Late November
Karyak	Late March	Mid-April	Mid-April	Late August	Mid to late November
Kakan	Mid-April	Late April	Early May	Early to mid-September	Early to mid-November
Dehno	Mid-March	Early April	Mid-April	Mid to late August	Late October
Setangun	Late April to early May	Early May	Early to mid-May	Mid to late September	Early to mid-November
Gangegun	Late April	Late April	Early May	Mid-September	Mid-November

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Table 4. Average of fruit and kernel characteristics for walnut genotypes. [a]

Genotypes	Tree code	Fruit weight (g)	Nut weight (g)	Kernel weight (g)	Fruit moisture (g)	Kernel/Fruit weight (%)	Kernel/nut weight (%)	Genotypes	Tree code	Fruit weight (g)	Nut weight (g)	Kernel weight (g)	Fruit moisture (g)	Kernel/Fruit weight (%)	Kernel/nut weight (%)
1	D101	40.39	9.61	5.44	30.78	13.47	56.61	42	E55	35.53	9.06	4.40	26.47	9.91	38.85
2	D102	49.77	13.05	7.00	36.72	14.07	53.64	43	E56	39.15	9.47	4.56	29.68	9.32	38.54
3	D103	31.41	9.57	4.81	21.84	15.33	50.30	44	E57	35.28	9.94	4.51	25.34	12.78	45.37
4	D104	39.24	11.62	5.40	27.62	13.76	46.47	45	E58	38.27	11.99	4.57	26.28	11.94	38.12
5	D105	39.52	9.30	4.62	30.22	11.69	49.68	46	E59	28.61	8.36	3.90	20.25	13.63	46.65
6	D106	41.05	11.11	5.08	29.94	12.38	45.73	47	E60	32.25	9.66	4.16	22.59	12.9	43.06
7	D107	29.46	10.63	4.86	18.83	16.5	45.71	48	E61	42.47	10.52	5.18	31.95	11.00	44.39
8	K101	54.43	10.71	5.47	43.72	10.05	51.07	49	E62	36.25	9.89	4.31	26.36	11.89	43.58
9	K102	50.34	15.71	**8.35**	34.63	16.59	53.15	50	E63	56.61	15.39	5.53	41.22	9.77	35.93
10	K103	28.66	9.87	5.64	18.79	**19.68**	57.14	51	E64	51.45	13.53	4.78	37.92	9.29	35.33
11	K104	40.84	11.80	5.12	29.04	12.54	43.39	52	E65	40.59	13.53	5.54	27.06	13.65	40.95
12	K105	45.3	13.47	6.56	31.83	14.48	48.7	53	G101	44.05	10.89	5.44	33.16	11.12	45
13	K106	49.07	10.35	5.73	38.72	11.68	55.36	54	G102	38.24	11.59	5.15	26.65	13.47	44.44
14	K107	33.91	8.54	4.95	25.37	14.60	57.96	55	G103	27.49	7.01	**3.02**	20.48	10.99	43.08
15	K108	42.42	11.12	6.30	31.3	14.85	56.66	56	G104	37.17	10.89	5.82	26.28	15.66	53.44
16	K109	48.13	11.42	6.18	36.71	12.84	54.12	57	G105	49.31	12.28	5.49	37.03	11.13	44.71
17	K110	44.17	10.82	5.94	33.35	13.45	54.9	58	G106	40.02	11.21	5.11	28.81	12.77	45.58
18	K111	53.55	12.49	6.76	41.06	12.62	54.12	59	CT1	33.39	9.83	4.42	23.56	13.24	44.96
19	K112	41.12	10.37	6.09	30.75	14.81	**58.78**	60	CT2	33.11	9.67	5.73	23.44	15.58	53.36
20	K113	50.61	11.27	5.53	39.34	10.93	49.07	61	CT3	41.84	10.55	5.63	31.29	12.12	48.06
21	K114	54.58	11.60	7.42	42.98	10.88	51.21	62	CT4	44.72	13.22	6.39	31.5	14.29	48.34
22	K115	**23.46**	7.09	3.95	**16.37**	16.84	55.71	63	CT5	61.07	13.69	6.68	47.38	10.94	48.8
23	S101	54.94	13.18	5.78	41.76	10.52	43.85	64	CT10	42.13	11.33	4.95	30.8	11.75	43.69
24	S102	57.97	12.86	6.05	45.11	10.44	47.05	65	CT11	38.15	9.59	4.86	28.56	10.20	40.56
25	S201	56.29	12.56	6.48	43.73	11.51	50.39	66	CT12	39.04	8.75	3.97	30.29	8.15	36.34
26	S202	51.02	**16.67**	6.43	34.35	12.6	38.57	67	CT13	36.12	9.14	3.81	26.98	10.55	41.69
27	S203	60.12	13.08	6.38	47.04	10.61	48.78	68	CT14	35.69	9.12	4.02	26.57	11.27	44.08
28	S204	57.95	14.05	6.56	43.90	11.32	46.69	69	CT15	42.31	12.68	5.91	29.63	13.97	46.61
29	S205	43.08	10.05	5.34	33.03	12.40	53.13	70	CT16	47.73	11.87	5.88	35.86	12.32	49.54
30	S206	57.23	13.60	6.06	43.63	10.59	44.56	71	CT17	29.74	6.30	4.35	23.44	11.70	55.24
31	S207	48.35	10.24	5.78	38.11	11.95	56.45	72	CT18	46.25	9.45	4.80	36.80	9.34	45.71
32	S208	56.26	13.06	4.34	43.20	7.71	**33.23**	73	CT19	49.32	9.74	5.31	39.58	10.77	54.52
33	S209	45.31	11.4	5.74	33.91	12.67	50.35	74	CT20	38.51	9.13	5.00	29.38	12.98	54.77
34	S210	41.71	10.21	5.70	31.50	13.67	55.83	75	CT21	39.80	10.33	5.23	29.47	13.14	50.63
35	S211	52.34	12.56	5.46	39.78	10.43	43.47	76	CT22	43.25	10.91	4.84	32.34	11.19	44.36
36	S212	48.00	11.47	5.18	36.53	10.79	45.16	77	CT23	**63.04**	13.09	5.23	**49.95**	8.30	39.95
37	S213	48.02	12.97	5.12	35.05	10.6	39.47	78	CT24	36.63	9.01	4.44	27.62	10.92	44.4
38	E51	24.58	6.93	3.04	17.65	12.37	43.87	79	CT25	27.55	**5.26**	3.55	22.29	12.91	47.34
39	E52	30.49	8.92	3.25	21.57	10.66	36.44	80	CT26	43.19	8.21	3.46	34.98	**7.22**	38
40	E53	42.96	12.42	4.65	30.54	10.82	37.44	81	CT27	45.49	11.04	4.34	34.45	9.54	39.31
41	E54	42.79	13.23	5.44	29.56	12.71	41.12	82	CT28	47.10	11.75	5.76	35.35	11.02	44.17

[a] The maximum and minimum values for each character are presented in bold face.

According to the Student's t-test, all the traits showed a significant difference ($P < 0.01$), indicating the difference of the regions based on these traits (data not shown). The evaluated fruit weight has been reported previously from 14.9 to 55.9 g, and of the nut weight about 5.7 to 25.2 g (Ehteshamnia *et al*., 2009), while in our

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research, we obtained a higher and better fruit weight among the studied genotypes. The fruit weight among the examined genotypes was from 10.38 to 17.04 g (Yarilgac *et al*., 2001), 6.4 to 20.55 g (Sharma and Sharma, 2001), and 6 to 15.2 g (Arzani *et al*., 2008).

The average walnut fruit weight was 7.52 to 17.7 g (Ebrahimi *et al*., 2010), and the highest fruit weight was reported to be 17.28 g (Mousavi *et al*., 2015). Results of other research on 48 walnut genotypes in Eqlid County, Iran, showed that the fruit weight varied between 8.58 and 19.8 g, and the kernel percentage varied from 17.57 to 62.6% (Sarikhani Khorami *et al*., 2014). Also, results of a research on 58 walnut genotypes in the Himachal Region, India, showed that the fruit weight varied between 6.4 and 20.55 g, and that of the kernel percentage varied from 12 to 62.5% (Sharma and Sharma, 2001).

Regarding the average of each genotype (Table 4), the minimum and maximum fruit weight belonged to K115 genotype from Karyak Region (23.46 g), and CT23 genotype from Setangun Region (63.04 g), respectively. The minimum nut weight belonged to CT25 from Setangun Region (5.26 g), and maximum belonged to S202 (16.67 g) from Sisakht Region; and minimum and maximum kernel weight belonged to G103 genotype (3.02 g) from Ganjegun Region, and K102 genotype (8.35 g) from Karyak Region, respectively.

Kernel weight was reported by other researchers to be between 1.5 and 14.1 g (Sharma and Sharma, 2001; Yarilgac *et al*., 2001; Arzani *et al*., 2008; Ehteshamnia *et al*., 2009; Sarikhani Khorami *et al*., 2014). The highest kernel weight was reported to be 10.11 (Mousavi *et al*., 2015), 9.8 (Ebrahimi *et al*., 2010), and 7.1 g (Sharma and Sharma, 2001) among the genotypes under study in different research. The minimum and the maximum moisture content of the fruits (Table 4) belonged to the K115 genotype from the Karyak Region (16.77 g), and CT23 genotype from the Setangun Region(49.95 g). The minimum and maximum kernel/fruit weight ratio belonged to the genotype CT26 from the Setangun Region (22.27%), and K103 genotype from the Karyak Region (19.68%); and the minimum and maximum kernel percentage belonged to the S208 genotype from the Sisakat Region (33.23%), and K112 genotype from the Karyak Region (58.78%), respectively. The highest kernel percentage reported in the studied walnut genotypes varied among genotypes between 12 and 79.6% (Sharma and Sharma, 2001; Yarilgac *et al*., 2001; Arzani *et al*., 2008; Ehteshamnia *et al*., 2009; Mousavi *et al*. 2015).

Matching the conditions obtained from the mean comparison of regions and genotypes based on the studied characteristics and considering the important characters of walnut fruit, only the highest mean of the kernel belonged to the best genotype in the Karyak Region, which had the highest average kernel weight. In terms of other characteristics, the highest genotype was not in the regions with the highest average of each attribute. In other words, it is not necessarily possible to find the top genotypes in the region with the highest average in terms of the desired attribute, although it is possible to find better, but not essentially the best, genotypes in the best regions. Such reports have already been reported on walnuts (Christopoulos *et al*., 2010; Vahdati *et al*., 2015). However, for the selection of genotypes in regions with the highest mean for the desired characteristics, based on the normal distribution, it can be hoped that half of the selected genotypes have a value equal to or greater than the mean of that characteristic in the population studied. Based on the evaluated morphological characteristics of walnut populations, the studied regions were divided into two main groups (clusters) (data not shown). The Sisakht Region was located in a separate cluster, and the other regions were in another cluster. The Sisakht Region has, on average, walnuts with more fruit and kernel weight than other regions. On the other hand, Dehno and Ganjegun Regions

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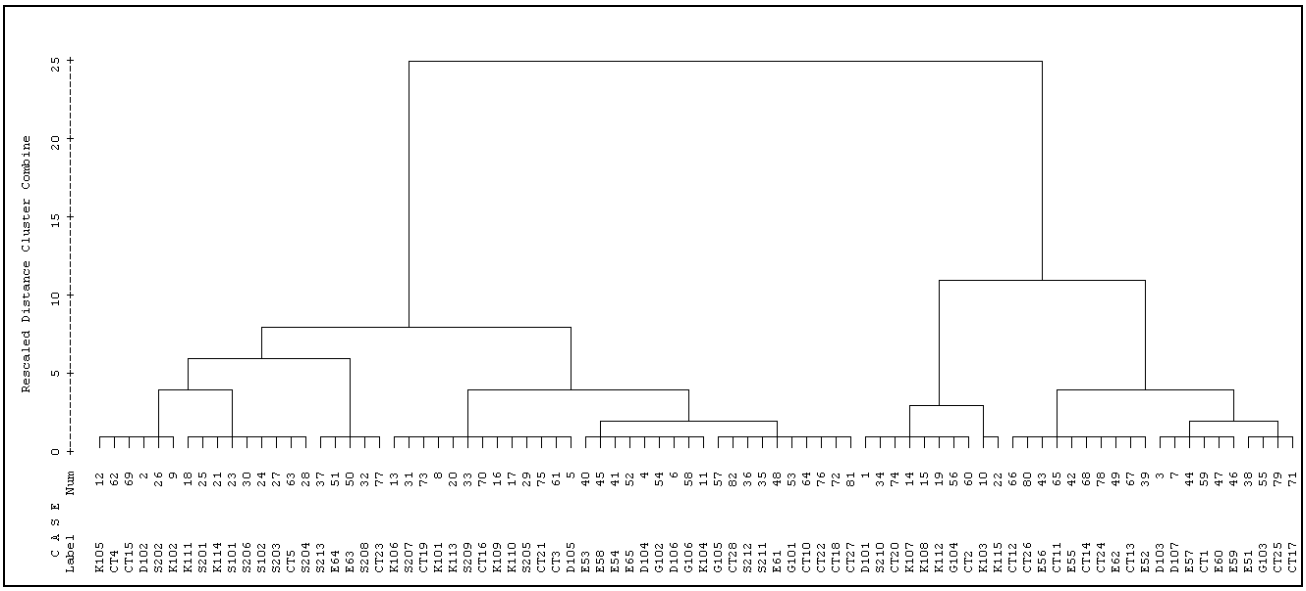

Figure 2. Dendrogram of the evaluated walnut genotypes based on the morphological characteristics.

have the most similarity among other regions, based on the morphological characteristics of the walnut fruit.

The dendrogram derived from cluster analysis based on genotypes' morphological traits is shown in Figure 2. As it is seen, genotypes of different regions are located in the same cluster, in other words, there is a similarity among the genotypes of different regions on the basis of morphological traits. Thus, this may be the most important reason that the genetic diversity among the regions is low and there is an active gene flow among the regions.

Genetic Diversity Evaluation Using the ISSR Marker

Eight of the 10 used ISSR primers in this study were amplified in the walnut samples, and a total of 82 bands (alleles) were obtained (Table 5).

By producing 14 bands, the ISSR3A primer had the highest number of alleles, and the ISSR7A primer, by producing 7 bands, had the lowest allele number. All the bands amplified from 8 primers showed 100% polymorphisms. In all genotypes, the Polymorphism Information Content (PIC) of primers with an average of 0.349 varied from 0.246 to 0.444, among which the highest polymorphism (0.444) belonged to the ISSR1A primer, and the lowest PIC value (0.246) related to the ISSR3A primers. In this study, from the 8 polymorphic primers, 5 primers had AC, AG, CT, TC, and GA repetitions of dinucleotide, and 3 other primers had four (tetra) nucleotide repeats. The results indicate that the ISSR1A and ISSR2A primers, which have the highest polymorphisms, have AC and AG repeats, respectively. The minimum polymorphism content was obtained from the ISSR3A primer, with CT dinucleotide repeat. ISSR primers with repetitions of AG, GA, CT, TC, AC, and CA are more polymorphic than other di, tri, and tetra nucleotide repeats (PradeepReddy *et al*., 2002). In our study, primers with AC and AG repeated sequence showed the most polymorphism among the studied genotypes, and similar results were obtained by Pradeep Reddy *et al*. (2002). The results of the research on 56 genotypes of walnuts in Greece showed that, among the 7 ISSR primers, the primer 827 with AC nucleotide repeat had the highest

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Polymorphism Information Content (PIC) (Christopoulos *et al*., 2010).

The Number of observed alleles (Na) for all the primers (locus) used in this study was 2. The reason for this is that, in all the amplified bands (locus), for each primer, numbers 1 and zero are used for presence or absence of the band, respectively. The Number of effective alleles (Ne) in the primers (loci) used ranged from 1.19 to 1.45, with a mean of 1.306 and a standard deviation of about 0.25. The highest Ne alleles belonged to the ISSR1A primer (1.445), and the lowest value was for the ISSR3A primer (1.191) (Table 5).

The highest Shannon's diversity index (I) was related to the ISSR1A primer (0.44), followed by the ISSR2A primer (0.41), and the lowest value beloned to the ISSR3A primer (0.23). Also, the highest value of the Nei's gene diversity index (H) belonged to the ISSR1A primer (0.28), and the lowest value was related to the ISSR3A primer (0.13) (Tab. 5). According to the results, ISSR1A and ISSR2A primers with the highest number of effective alleles, Nei's gene diversity index, and the Shannon's genetic diversity index showed the highest diversity among the 82 evaluated genotypes. These results indicate that there are significant and considerable genetic variations among the 82 studied Persian walnut genotypes in 6 different regions of Boyer Ahmad County, and this can lead to the planning of programs for the conservation, maintenance, and application of known gene reserves. Genetic variation among walnut genotypes in mountainous regions of north China was also observed by using the ISSR primers (Ji *et al*., 2014).

Table 5. The amplified ISSR primers, their sequence, annealing temperature, the genetic diversity indices in 82 walnut genotypes and the polymorphism information derived.

Primer name	Sequence	Annealing temperature (°C)	Number of total bands	Polymorphic band (%)	Effective Number of alleles (Ne)	Nei's gene diversity (H)	Shannon's diversity index (I)	PIC
ISSR1A	GTCACACACACACAC	52	8	100	1.45	0.28	0.441	0.444
ISSR2A	TGTAGAGAGAGAGAG	50	8	100	1.41	0.26	0.412	0.410
ISSR3A	AGCCTCTCTCTCTCT	52	14	100	1.19	0.13	0.231	0.246
ISSR4A	GCATCTCTCTCTCTC	52	10	100	1.31	0.22	0.352	0.348
ISSR7A	GCAAGCAAGCAAGCAA	55	7	100	1.35	0.23	0.359	0.359
ISSR8A	AGACACACACACACA	48	10	100	1.36	0.24	0.386	0.358
ISSR9A	GAAGGAAGGAAGGAA	51	13	100	1.22	0.17	0.293	0.281
ISSR10A	CTCGTGTGTGTGTGT	51	12	100	1.29	0.22	0.363	0.348
Mean		-	82	100	1.31	0.22	0.343	0.350
Standard deviation		-	-	-	0.25	0.14	0.187	-

Analysis Of Molecular Variance (AMOVA)

According to the analysis of molecular variance, the diversity within the populations (regions) was greater (83%) than the diversity among them (17%) (Data not shown), indicating a high level of

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diversity within populations (6 regions) and, on the other hand, showing lack of uniformity of walnut gardens in each region. In a study conducted in Greece on 56 genotypes of walnuts, the genetic variation within populations (89%) was more than the genetic variation among populations (11%), and it concluded that the examined genotypes could not be classified based on the collection regions (Christopoulos *et al*., 2010). High genetic diversity within the walnut populations has also been reported by other researchers, and it has been claimed that the population classification based on genetic diversity is not related to geographic regions. A greater variance within the populations than that among the populations may be caused by the gene flow (Vahdati *et al*., 2015); something like the transfer of pollen grains in plants from one population to another (migration). The second reason for the low diversity between populations may also be attributed to the accessibility of gardeners to similar sources of seedlings.

Grouping of Genotypes Based on the Information Obtained from Evaluated Genotypes Using the ISSR Marker

The dendrogram obtained from the Simple Matching (SM) Simulation by the UPGMA method was chosen due to having a higher correlation coefficient (77%) than Jaccard and Dice coefficients. The coefficient of convergence of this dendrogram varied from 0.67 to 0.94. Regarding the obtained dendrogram (Figure 3) and sign mark line, genotypes were divided into 4 groups (clusters). In the first group (numbers 1 to 14 from Dehno and Karyak and numbers 69 to 82 from the Setangun Region), genotypes CT15, CT16, and CT17 (respectively, with numbers 69, 70, and 71) from the Setangun region have the least similarity to the other genotypes in this experiment, and in the dendrogram, they are in this cluster with the least correlation coefficient. All of the genotypes in the second cluster belong to the Karyak Region, and only one genotype from the Sisakht Region (No. 23) is located in this group, which shows the similarity of this genotype with the genotypes of the Karyak Region, based on the molecular information. Most of the genotypes of Sisakht and Ganjegun Regions and all the genotypes of the Kakan Region are in the third cluster. According to the correlation coefficient and the sign mark line, these genotypes are located in one cluster, with a correlation coefficient of about 72%. These results show the similarity of the genotypes of the three regions of Sisakht, Kakan, and Ganjegun. In the fourth cluster, 10 genotypes from the Setangun Region (numbers 59 to 68) and 1 genotype from the Ganjegun Region (No. 58) were classified into the fourth cluster, indicating the similarity of the genotype with the number 58 with the genotypes of the Setangun Region.

Finally, with respect to this grouping, we find that the genotypes of Dehno, Karyak, and Setangun Regions are not highly similar to the genotypes in the Sisakht, Kakan, and Ganjegun Regions of genetic variation based on the ISSR marker, which indicates the diversity of genotypes in these regions, at least regarding the studied ISSR primers in our study.

There is a difference between the results derived from genotypes clustering based on morphological traits (Figure 2) and molecular profiling. Morphological traits can be influenced by environmental factors, but the molecular data could not be influenced by them, and it could be the reason that the clustering of the genotypes based on the morphological data is different than molecular data. Certainly, because of the lower effects of environmental factors on molecular data, the results derived from that could be more precise.

As the results were also obtained from the analysis of the molecular variance of genotypes (data not shown), the diversity between regions in the dendrogram; therefore, the classification cannot be attributed to a specific feature that separates the regions. Such a conclusion has already been attributed to the gene flow between

[DOR: 20.1001.1.16807073.2021.23.1.2.5]

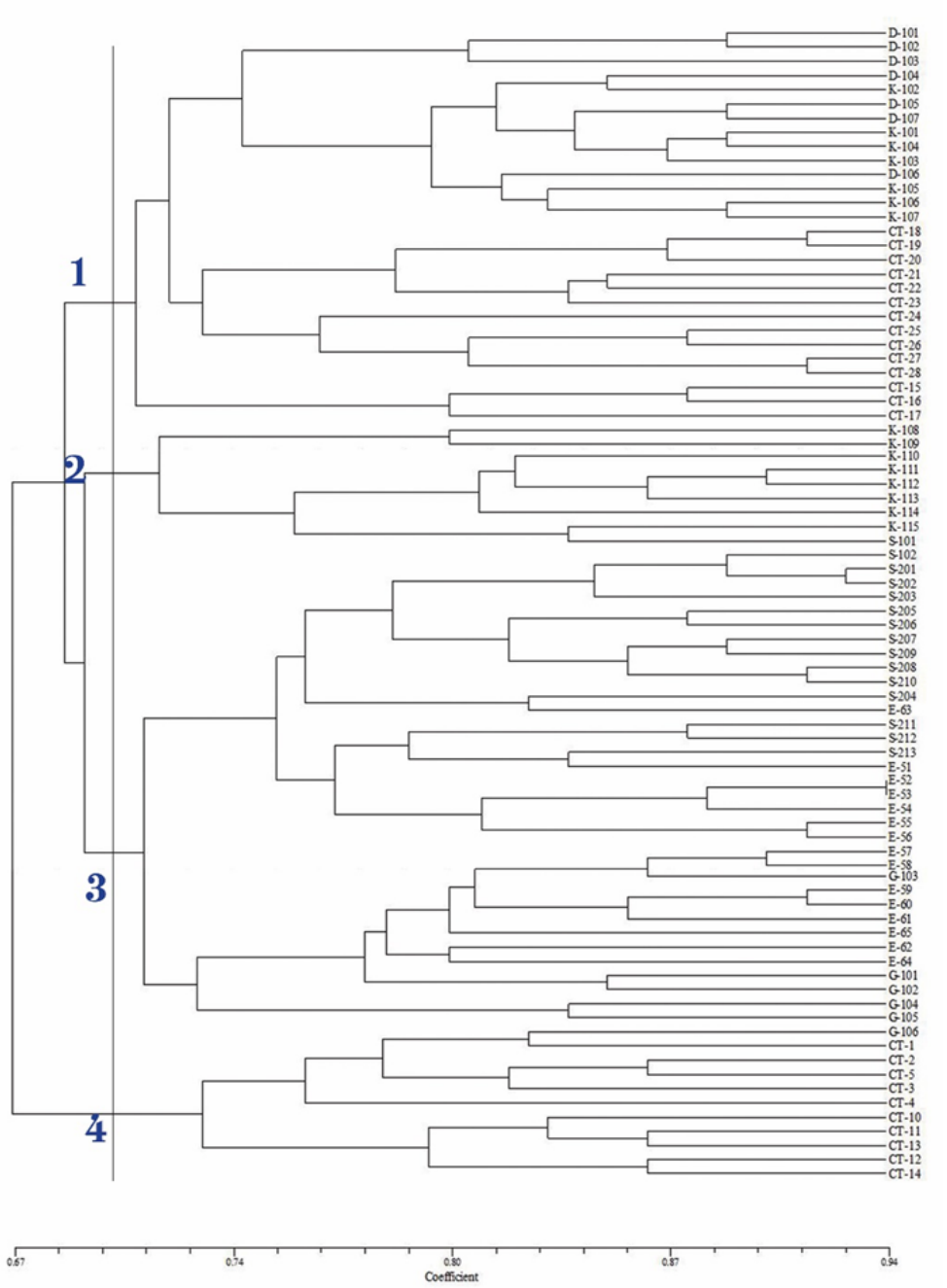

Figure 3. Dendrogram of walnut genotypes based on ISSR markers by UPGMA.

populations (Vahdati *et al*., 2015). Another reason for the mismatch of regions' grouping according to the morphological characteristics and the molecular properties may be the inadequate consideration of morphological characteristics.

CONCLUSIONS

The results indicate that the genotypes vary in terms of fruit traits. Among the six

[DOR: 20.1001.1.16807073.2021.23.1.2.5]

studied regions, Dehno and Karyak Regions with the highest kernel weight and the kernel percentage are the most suitable and promising regions for selecting trees with good kernel production. Also, the study of genetic diversity using the ISSR marker showed a good diversity among the evaluated genotypes, indicating the proper capability of the ISSR marker. ISSR1A and ISSR2A primers showed the highest diversity among genotypes with the highest amount of PIC, Shannon's diversity index (I), and Nei's gene diversity (H). Ultimately, the results of this research can be used in the breeding programs to identify desirable high yielding genotypes.

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بررسی تنوع ژنتیکی گردو با استفاده از ویژگی‌های فنولوژیکی و مورفولوژیکی و نشانگرهای مولکولی

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چکیده

اطلاعات کمی در مورد منابع ژنتیکی گردو ایرانی (*Juglans regia* L.)؛ گیاه خوراکی مهم و یکی از مهم‌ترین گیاهان در باغبانی و اقتصاد، در ایران وجود دارد. شناسایی و حفاظت از ژرم‌پلاسم از موضوعات مهم و اساسی در برنامه‌های به‌نژادی است. شش منطقه در شهرستان‌های بویراحمد و دنا انتخاب و ۸۲ ژنوتیپ گردو بطور تصادفی گزینش شدند. طول دوره رشد سالانه در مناطق ستنگان و گنجگان کوتاه (۱۹۰ تا ۲۰۰ روز) بود، اما در دیگر مناطق مورد بررسی طولانی و بیشتر از ۲۳۰ روز بود.

[DOR: 20.1001.1.16807073.2021.23.1.2.5]

حداکثر وزن میوه ۶۳/۴۴ گرم و حداکثر وزن مغز ۸/۳۵ گرم بود. تعداد آلل‌های مشاهده شده از ۷ (ISSR7A) تا ۱۴ (ISSR3A) برای پرایمرها متغیر بوده و تعداد آلل‌های موثر (Ne) در پرایمرهای مورد استفاده بین ۱/۱۹ تا ۱/۴۵ و بیشترین Ne متعلق به پرایمر ISSR1A (۱/۴۴۵) بود. بر اساس اطلاعات حاصل از پرایمرهای ISSR1A و ISSR2A به ترتیب با شاخص PIC برابر با ۰/۴۴ و ۰/۴۱ ژنوتیپ‌های گردو بیشترین تنوع ژنتیکی را داشتند. تنوع ژنی Nei دامنه‌ای از ۰/۱۳ تا ۰/۲۴ و شاخص شانون دامنه‌ای از ۰/۲۳ تا ۰/۴۴ را نشان دادند. بطور کلی، افزایش ارتفاع از سطح دریا و کاهش درجه حرارت سبب تاخیر در ویژگی‌های فنولوژیکی شد و بر اساس ویژگی‌های مورفولوژیکی و فنولوژیکی و نشانگرهای مولکولی تنوع ژنتیکی مناسبی در میان ژنوتیپ‌های مورد بررسی دیده شد. در پایان، نتایج می‌تواند در برنامه‌های به‌نژادی و حفاظتی گردو مفید باشد و با انتخاب ژنوتیپ‌های دیر برگ‌ده و دیر گل‌ده از مناطق ستنگان و گنجگان، می‌توان از ریسک سرمای آخر بهار کاست.

[DOR: 20.1001.1.16807073.2021.23.1.2.5]