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Genetic diversity of Iranian melon cultigens revealed by AFLP markers

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Abstract
Iran is a part of the secondary centre of origin of melons, and therefore, there is a wealth of genetic variation of these species in this country. The Amplified Fragment Length Polymorphism (AFLP) marker was applied to investigate the genetic variations among five major Iranian melon cultigens (*Cucumis melo*) and 'Ananasi' as a general well-known cultivar. Ten primer pairs were used on 90 individuals producing 318 polymorphic fragments, with an average of 31.8 fragments per primer combination. The polymorphism rates ranged from 80 to 100%. The genetic similarities among accessions were calculated according to Dice's Similarity Index and used to construct a dendrogram based on the Unweighted Pair Group Method with Arithmetic Average (UPGMA). The genetic distance estimates based on AFLPs ranged from 0.29 to 0.63, with a mean of 47±0.3. Iranian melon genotypes and the 'Ananasi' cultivar were considered as two separate groups on the cluster analysis. The principal coordinate analysis showed a separate allocation of the melon cultivar groups. The results demonstrated a wide diversity of Iranian melon cultigens. The high number of alleles and the high expected genetic diversity detected with the AFLP marker indicated that the Iranian melon cultigens had distinctive characteristics and were an important genetic diversity pool, which made them a valuable source of breeding materials.

Keywords: Amplified fragment length polymorphis, cucumis melo, genetic distance, molecular markers.

Abbreviations: **AFLP**, amplified fragment length polymorphism; **RAPD**, randomly amplified polymorphic DNA; **SSR**, simple sequence repeats; **RFLP**, restriction fragment length polymorphism; **UPGMA**, Unweighted pair group method with the arithmetic average; **PCA**, principal coordinate analysis; **FAO**, food and agriculture organization.

Introduction

Assessment of genetic variability in the germplasm of different species is of interest, not only for the organization and conservation of genetic resources, but also for practical applications such as broadening the genetic base of the species and exploitation of heterosis (Gvozdenović, 2009; Jagosz, 2011; Luan *et al*., 2011). Genetic diversity information will help breeders in selecting desirable parents in hybrid and new cultivar production and in maintaining population polymorphism. Increasing the genetic diversity is a major concern in species where inbreeding practices have resulted in the loss of genetic diversity (Ramanatha and Hodgkin, 2002), a process that could

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be responsible for the unsuccessful development of new combinations. On the other hand, because the performance of hybrids seems to be related to the genetic divergence of parental lines, information on the genetic similarity between genotypes may also facilitate the prediction of crosses that will produce hybrids with a higher performance (Luan *et al.* 2010; Sekhon and Gupta, 1995).

Iran is one among the centres of origin of melons. and therefore. there is a wealth of genetic variation of this species in the country (Raghami *et al.*, 2014). Iran ranks third in melon production after China and Turkey. More than 85,000 ha are under the cultivation of melons in Iran (FAO, 2014), a majority of which is included in five selected local cultivars belonging to two cultivar groups characterized in this article. These commercial cultivars, which are well adapted to dry climate and bear desirable fruits, are generally different from western types and have not been described in other countries.

Different types of markers have been used to assess the genetic diversity in melon (*Cucumis melo*). Thus far, the morphological characters of some Iranian melon accessions have been used in order to classify them at the National Plant Gene Bank of Iran (Naroui Rad *et al.* 2010). However, the morphological markers have limited importance, as they are generally influenced by environmental factors and the developmental stage of the plant; although, in some species, adequate levels of phenotypic polymorphism are not available (Fufa *et al.*, 2005). Molecular genetic variation among some Iranian melon accessions has also been characterized using different markers such as RAPDs (Zamyad *et al*., 2005; Feyzian *et al*. 2007; Soltani *et al.*, 2010), SSRs (Kohpayegani and Behbahani, 2008; Moaiedi nejad *et al*., 2010) and inter simple sequence repeats (ISSRs) (Fabriki-Orang *et al.*, 2008). In comparison with other marker types, these DNA-based marker techniques can detect the genetic diversity of a species in all tissues and at all stages of development, without being affected by the environmental conditions (Xu *et al.*, 2012). These studies reported a wide genetic base and high heterogeneity in Iranian melons. Moreover, studies comparing the RAPD, AFLP, and RFLP markers on five melon genotypes with different origins (Spain, South Korea, India, Zimbabwe, and Israel) suggest that while all three types of markers are equally informative, AFLPs show the highest efficiency in detecting polymorphism (Garcia-Mas *et al*., 2000). AFLP fingerprints are reliable and reproducible molecular markers, making them a more appropriate technique for detecting genetic variation among melon genotypes (Reddy *et al.*, 2005; Yashiro, 2005). In a study on identification of sweet cherry cultivars using AFLP and SSR markers, the probability of the correct clustering of cultivars in the dendrogram was reported to be higher for AFLP markers than for SSR markers (Stanys *et al.*, 2012). Wang *et al.* (1997) also, despite a relatively low level of polymorphism in the studied melon species, found AFLP markers to be more efficient in mapping the melon genome than RAPD or microsatellite markers.

The objective of this study was to determine the distribution of genetic relationships between the most popular and commercial Iranian melon cultivars (*Cucumis melo*) belonging to the Cantalupensis and Inodorus groups and assessing their association along with an introduced common cultivar using amplified fragment length polymorphism (AFLP).

Materials and Methods

Plant materials

Fifteen individuals from each of the five well known and popular Iranian local melon (*Cucumis melo*) cultivars were examined in this study. ‘Saveh’ and ‘Samsoori’ as juicy melons, belong to

Cantalupensis cultivar group, 'Khatooni', 'Sooski-Zard,' and 'Sooski-Sabz' as crispy melons, which are resemble Inodorous, but do not accord with the current grouping and are believed to belong to the Iranian cultivar group (Lotfi and Kashi, 1999), and 'Ananasi', an introduced open-pollinated common cultivar, were selected (Table 1). Both Inodorus and Cantalupensis are dessert-type melons. Accessions with long shelf-life were classified as Inodorus and Cantalupensis, their local names, 'Kharbozeh' (Inodorus) and 'Talebi' (Cantalupensis).

Table 1. List of melon cultigens and area of origin used for genetic analysis in this study

No	Cultigens name	Abbreviation	area of origin
1	'Sooski-Sabz'	SS	Ivanaki
2	'Sooski-Zard'	SZ	Ivanaki
3	'Khatooni'	KH	Mashhad
4	'Saveh'	TS	Saveh
5	'Samsoori'	SV	Varamin
6	'Ananasi'	A	Company

DNA extraction

Seed samples of each cultivar were germinated in vermiculite at 20 to 24ºC, under fluorescent light (300 µmol m^{-2} s^{-1}) and a 16-hour photoperiod in a greenhouse. A fungicide was used to avoid from any fungal disease contamination. About 0.1 g of leaf samples were collected from 15 individuals in each cultivar and transferred to -80°C freezer. Genomic DNA was extracted from young leaves following the procedure described by Doyle and Doyle (1987) and modified according to Staub *et al.* (1996). The integrity and quantity of the DNA was estimated on 1% agarose gel and a UV-Spectrophotometer. Next, diluted DNA in sterile water, to a concentration of 500 ng μl^{-1}, was used in the amplification reactions.

AFLP analysis

The AFLP analysis was performed according to Vos *et al*. (1995), with minor modifications. The restriction enzymes, adapters, and primers used are listed in Table 2. Total genomic DNA (500 ng) was digested with 2.5 units of *Eco*RI and *Mse*I restriction enzymes and incubated at 37ºC for three hours. The *Eco*RI and *Mse*I enzyme restriction sites of the genomic DNA fragments were ligated to a 50 µM *Mse*I-adaptor and a 5 µM *Eco*RI-adaptor (MWG Biotech Ebersberg, Germany) using two units of T4 DNA ligase (Boehringer Mannheim) and 1 mM Adenosine triphosphate (ATP) per reaction. The volume of the reaction mixtures was adjusted to 50 µl and incubated at 4ºC, overnight. The resulting reaction product was diluted with 75 µL of distilled water (3:1).

Pre-amplifications were performed with the *Eco*RI / *Mse*I primer combinations having no selective nucleotide at the 3' end (MWG Biotech Ebersberg, Germany). The pre-amplification products were used as templates for selective amplification, using 10 primer combinations with two and three selective nucleotides at the 3' end (Table 2). The amplified fragments were detected by silver staining as described by Bassam *et al.* (1991). The resulting gels were scored manually.

Data analysis

Each polymorphic band from 50 bp to 500 bp was scored as either present (1) or absent (0) for all genotypes, resulting in a binary data matrix. Fragments longer than 400 bp were rarely detected. Dice similarity coefficients were calculated based on the study by Nei and Li (1979). The binary matrix was used to generate the corresponding matrix showing the genetic

distance for each pair of genotypes. Cluster analysis was performed on the genetic distance matrix using the NTSY Spc version 1.80 (Rohlf, 1997). A dendrogram was constructed based on similarity data applying the unweighted pair-group method using the arithmetic average (UPGMA). Factors representing the original variables were extracted by the Principal Coordinate Analysis (PCA).

Table 2. Adaptors and primers used for AFLP pre-amplification and selective amplification

Sequence (5'-3')	Adapters/Primers names
*Eco*RI adaptor	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
*Mse*I adaptor	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
primers	Sequences
5'-GATGAGTCCTGAG TAAAT	MseI 5
5'-GATGAGTCCTGAG TAACAA	MseI 2
5'-GATGAGTCCTGAG TAACAG	MseI 7
5'-GATGAGTCCTGAG TAAATA	MseI 8
5'-GACTGCGTACC AATTCAAC	EcoRI 2
5'-GACTGCGTACC AATTCAGC	EcoRI 4
5'-GACTGCGTACC AATTCGAC	EcoRI 7
5'-GACTGCGTACC AATTCGTC	EcoRI 8
5'-GACTGCGTACC AATTCGTT	EcoRI 9

Results

Genetic diversity analysis

Ten AFLP primer combinations (*Mse*I and *Eco*RI primers) were used to assess the genetic diversity among 90 melon accessions and all primer combinations were polymorphic (Table 3). Ten primer pairs provided a total of 341 bands, of which 318 were polymorphic. These polymorphic bands accounted for 93.25% of the total amplified fragments, showing that genetic polymorphism using AFLP was rich among Iranian melon genotypes. However, there were differences, between the primer combinations, in the number of fragments and percentages of the polymorphisms.The number of polymorphic amplified fragments for each AFLP primer pair varied from 22 to 45, with an average of 34.1 across all genotypes. For each primer pair the number of polymorphic fragments varied from 22 for M-AT/E-GAC to 40 for M-CAA/E-AAC, averaging 31.8 polymorphic fragments per combination. The primer combination of M-AT/E-GAC gave the fewest number of fragments (22 fragments) while all other primer combinations detected 27 fragments. The M-AT/E-GAC, M-CAG/E-AGC, and M-ATA/E-GAC primer combinations generated the highest numbers of polymorphic fragments. The polymorphic information content ranged from 0.33 (M-ATA/E-GTC) to 0.43 (M-ATA/E-GAC), with an average of 0.38. The percent of polymorphic fragments varied from 80% for M-AT/E-GTC to 100% for MAT/E-GAC, M-CAG/E-AGC, and M-ATA/E-GAC (Table 3), with an average of 92.66%.

Cluster analysis

A dendrogram was established for the 90 melon genotypes using the UPGMA cluster analysis based on the SDs from the AFLP data presented in Figure 1. Results showed that the genetic similarity of evaluated

Table 3. Analysis of the level of polymorphism with AFLP primer combinations among 90 melon accessions

Primer combination	Total number of fragments (a)	Polymorphic fragments		PIC†
		Number (b)	Polymorphism (%) (=b/a×100)	
M-AT/E-GAC	22	22	100	0.42
M- AT/E-GTC	36	32	80	0.38
M-CAG/E-GAC	27	24	88	0.35
M-CAG/ E-AGC	33	33	100	0.41
M-ATA/E-GTC	45	37	82	0.33
M-AT/E-AGC	27	25	92.5	0.39
M-CAA/E-AAC	43	40	93	0.37
M-CAA/E-GTT	33	31	93.9	0.37
M-ATA/E-AGC	37	36	97.2	0.37
M-ATA/E-GAC	38	38	100	0.43
Total	341	318	93.25	
Average	34.1	31.8		0.38

† polymorphic information content

melon materials varied from 0.49 to 0.98. The highest similarity was detected between Inodorus accessions 339 and 3310 (1.00). The dendrogram of the genetic relationship separated melon cultigens into two major clusters, which diverged at a similarity index of 0.49. The first group (A) contained 15 genotypes of 'Ananasi' cultivars and revealed the lowest similarity with the Iranian melon accessions. Second group (B), consisted of 75 genotypes of Iranian sweet-type melon groups Inodorus and Cantalupensis, which showed more similarity with each other rather than with 'Ananasi'. This group further divided into two distinct sub-clusters. The first sub-cluster (C) consisted of 'Saveh', 'Sooski-Sabz', 'Sooski-Zard,' and 'Khatooni' cultivars, and the second sub-cluster (D) consisted of 15 genotypes of 'Samsoori' cultivars related to the Cantalupensis group. Sub-cluster C divided into two further clusters at a similarity index of 0.65. The first sub cluster (E) contained all the genotypes of the 'Saveh' cultivars and the other sub-cluster (F) was related to the Inodorus group, and later formed two distinct categories. The first category (G) contained all genotypes of the 'Khatooni' cultivars and second one (H) comprised of 30 genotypes of the 'Sooski-Sabz' and 'Sooski-Zard' cultivars.

The Nei's genetic distance of melon cultigens was in the range of 0.29 to 0.64 (Table 4), of which the highest genetic distance was seen between the cultivars of 'Samsoori' and 'Khatooni' (0.64) followed by 'Annanasi' and 'Sooski Zard' (0.635), and the lowest genetic distance was observed between 'Sooski-Sabz' with 'Sooski-Zard' cultivars (0.29). These remarkable genetic distances revealed the abundant genetic variance among melon cultigens in Iran.

The diagram of distances between two the Cartesian axes based on the principal coordinate analysis (PCA) is shown in Figure 2. On the basis of the results, 'Ananasi' and 'Samsoori' were located far from the other four cultivars. A summary of the genetic parameters of the melon cultivars are presented in Table 5. The number of polymorphic bands generated by primers ranged between 19.22 and 44.71. According to the DNA bands generated by all 10 primers, the least polymorphic was the "Sooski-Sabz" cultivar (19.22%) and the most polymorphic (44.71%) was the "Saveh" cultivar. For all populations, the mean value of polymorphic DNA

percentage was 32.62%. The highest Nei's gene diversity (0.15) was characteristic of "Saveh" and the lowest of "Sooski-Sabz" (0.07), and as a mean for all populations, it was 0.112. Extremes of the Shannon's index (0.22 and 0.11) were characteristic of the same populations as in the Nei's gene diversity case.

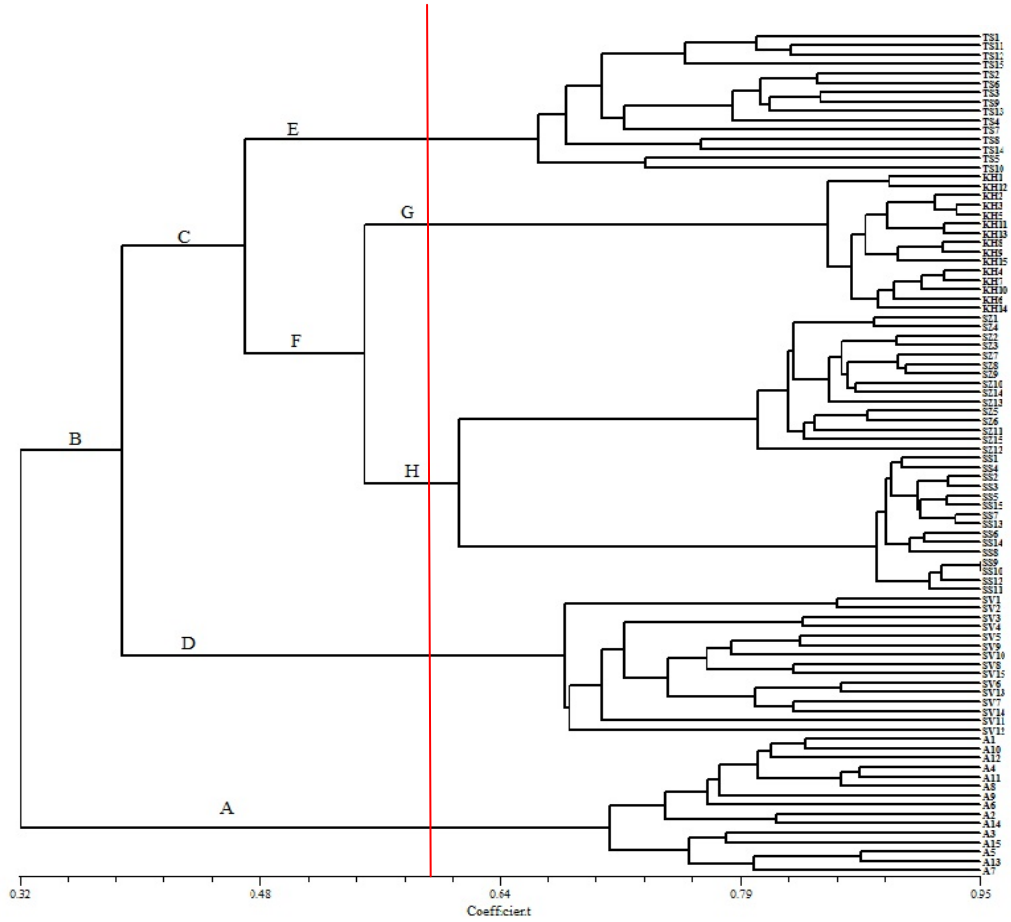

Fig. 1. Dendrogram generated based on UPGMA clustering algorithm depicting genetic relationships among melon cultigens, based on AFLP data

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among six cultigen of *C. melo*

Cultigens	'Khatooni'	'Sooski-Zard'	'Sooski-Sabz'	'Samsoori'	'Saveh'	'Ananasi'
'Khatooni'	********	0.6400	0.6751	0.5286	0.6470	0.6649
'Sooski-Zard'	0.4463	*******	0.7466	0.6606	0.6193	0.5302
'Sooski-Sabz'	0.3928	0.2922	******	0.6348	0.6861	0.5579
'Samsoori'	0.6394	0.4145	0.4544	*******	0.4719	0.6068
'Saveh'	0.4354	0.4792	0.3767	0.4719	*******	0.6649
'Ananasi'	0.6164	0.6345	0.5836	0.4996	0.4081	*******

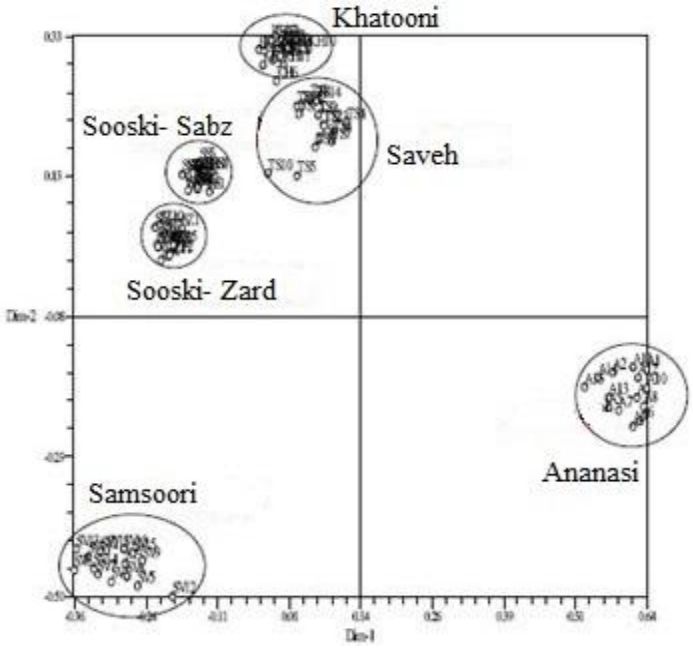

Fig. 2. Dispersion diagram of six melons cultigens by the principal coordinate analysis (PCA) method

Table 5. Genetic parameters for six cultigens of melon

Cultigen	PPB (%)	ne	H nei	I shanon
'Khatooni'	23.14	1.15	0.09	0.13
'Sooski-Zard'	34.51	1.23	0.13	0.19
'Sooski-Sabz'	19.22	1.14	0.07	0.11
'Samsoori'	39.22	1.21	0.12	0.19
'Saveh'	44.71	1.25	0.15	0.22
'Ananasi'	34.90	1.19	0.11	0.17

Note: PPB, Percentage of polymorphic bands; ne, Effective number of alleles; H nei, Nei's gene diversity; I, Shannon's information index

The results showed that the genetic diversity of 'Saveh' was the richest among the six cultivars and it was the lowest in 'Sooski-Sabz'. The concordance with the results was also verified by the UPGMA method. The coefficient of genetic differentiation between the populations (G_{ST}), estimated by partitioning of the total gene diversity was 0.11. In addition, the extent of gene-flow between the populations (Nm) was estimated to be 0.21 individuals per generation, indicating a rather low migration rate between these populations (Table 6).

Table 6. Coefficient of gene differentiation

groups	H_T	H_S	G_{ST}	N_m
Cantalopensis type	0.34	0.13	0.62	0.30
Indorous type	**0.28**	0.10	0.65	0.26
Overall	0.39	0.11	0.70	0.21

H_T: Total genetic diversity of populations
H_S: Average genetic diversity within population
G_{ST}: Average genetic diversity between populations N_m: Gene flow

Discussion

AFLP procedure

The study of the Iranian melon genotypes has been so far based on morphological traits, and SSR and RAPD markers (Zamyad *et al*., 2005; Feyzian *et al*., 2007; Fabriki-Orang *et al*., 2008; Kohpayegani and Behbahani, 2008; Moaiedi Nejad *et al*. 2010; Soltani *et al.*, 2010), while in spite of the abundant melon germplasm in Iran, very little progress has been made in AFLP research. Therefore, in this AFLP pattern study, we assessed the genetic diversity of Iranian germplasm- including Inodorus and Cantalupensis and a general common cultivar. All Iranian accessions used in this study were assessed for the first time. The 90 AFLPs used herein were sufficient to distinguish all the tested cultivars, indicating the usefulness of the chosen marker set to study the genetic variability among the analyzed cultivars. A number of recent studies have also shown the capacity of AFLP to be highly discriminating between the genotypes in a range of crops (Lombard *et al*., 2000; Behera *et al*., 2008; Maras *et al*., 2008).

The percent average of polymorphic fragments for each primer pair was 92.66% indicating the efficiency of AFLP markers in analyzing the genetic diversity of melon genotypes and the accuracy of this procedure. The efficiency of the AFLP marker in melon genetic diversity was shown by Frary *et al*. (2013), which was similar to our results. Other researchers also indicated that AFLP markers were highly efficient compared to other markers (Garcia-Mas *et al*., 2000; Yashiro *et al*., 2005; Xu and Zhang, 2012), as AFLP markers were reproducible and displayed intraspecific homology. The percentage of polymorphic fragments that Sheng *et al*. (2011) obtained using seven AFLP primers on eight genotypes was 57%.

The mean number of polymorphic amplified fragments for AFLP primer pairs for the studied cultivars were 34.1, while Yashiro *et al*. (2005) found 27.8 average bands in 99 East and South Asian melon accessions. Garcis-Mas *et al*. (2000) studied genetic diversity among six Spanish melons and found 15.08 polymorphic bands per total number of primer combinations, with 12 AFLP primer combinations. These results demonstrate that the Iranian melon is diversified and supports the idea of their origin in Asia (Renner *et al.*, 2007). In addition, it indicates the importance of Iranian accessions for the study of origin and diversification.

Genetic diversity and similarity of Iranian melon

On the basis of the UPGMA dendrogram of the six melon cultivars, the overall mean of the genetic distance for AFLP was 0.4658. The highest genetic distances were mostly obtained from pairs of exotic (Ananasi) and local collections (Garcia-Mas *et al*., 2000), indicating their wide dissimilarity. Genetic diversity analysis, genetic distance, and cluster analysis, all showed that the Iranian melon varieties had significant genetic differences from the 'Ananasi' melon cultivar. This showed that the range of genetic diversity was the greatest among the accessions contained in these clusters and cross-hybridizing between them might increase the genetic variation in the breeding population. This genetic distance also showed the importance of Iranian accessions for conservation and use in breeding programs. These results confirmed the usefulness of AFLP technology in cultivar fingerprinting, in melon cultivars.

Clusters were observed to contain all accessions assembled from a particular collection area, indicating the correspondence between the accessions and their geographical origin. It was evident from the AFLP cluster (Fig. 1) that two local accessions (Sooski-Sabz and Sooski-Zard) were most closely related to the remaining accessions. As 'Sooski-Sabz' and 'Sooski-Zard' were cultivated in

the same region, these resources had a close relationship. The present results showed that accessions from the same botanical groups (i.e., Inodorus) clustered together. This result was in accordance with the study of Raghami (2014) on Iranian melon, Lopez-Sese *et al.* (2003) on Spanish melon, Tzitzikas *et al.* (2009) on Greek and Cypriot melons, and Monforte *et al.* (2003) on a broad range of wild and cultivated melons.

However, our results showed that low gene diversity values were observed among some Iranian accessions, indicating a lack of intercrossing between them or high levels of inbreeding. 'Sooski-e-Sabz' and 'Khatouni' parameter of gene diversity were 0.07 and 0.09, respectively. This result was similar to the results of Raghami *et al.*, (2014), who assumed that, if outcrossing occurred, the farmers had made efforts to maintain the genetic originality of the accessions, probably to keep the original fruit traits, because of regional consumer preferences. It was likely that the selection for agronomic characters had been practised by farmers and this could partially explain the relatively low degree of gene diversity within some accessions observed in this study.

The PCA recommended a clearer and simpler way of visualizing the cultigen groups in the collection. The dispersion diagram (Fig. 2) showed that the greatest distribution area of 'Saveh', evidenced a higher genetic variability in this cultigen than in the others, while the concentrated area of 'Samsoori' cultigens was rather distant from the other Iranian genotypes. The accessions of 'Ananasi' were scattered in a different area than the other cultigens. Hence, clearly six different melon cultigens were separated in this order. The verified genetic variability indicated a wide diversity of Iranian melon cultigens. The results clearly indicated that the Iranian melon genotypes had a distinct genetic basis, which made them good sources of diversity and breeding materials. Although, some cultigens, such as 'Sooski-Sabz' and 'Khatooni,' had a narrow genetic basis and higher homogeneity.

Consequently in melon breeding, Iranian melon resources could be taken advantage of, to broaden the genetic background of new cultivars. This result was consistent with the results of related researches (Lotfi and Kashi, 1999; Feyzian *et al.*, 2007; Kohpayegani and Behbahani, 2008), where the unique characteristics of the Iranian melon were mentioned. The results of this study also revealed that the Cantalopensis groups were genetically more divergent, while the Inodorous groups had a narrow genetic base. In recent times, analysis of a *microsatellite* polymorphism also showed that the Inodorous group of Iranian melons had a low polymorphism (Kohpayegani and Behbahani, 2008; Moaiedi Nejad *et al.*, 2010). In summary, a knowledge of the diversity patterns and specific genetic distance estimates, could improve the efficiency of melon genetics in Iran.

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