Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating: 3.53
JMPS 2018; 6(4): 94-99

Received: 14-05-2018
Accepted: 15-06-2018

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Genetic diversity investigation of bitter melon (*Citrullus colocynthis* L.) populations using morphological and molecular markers

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Abstract
Citrullus colocynthis L. is growing as a weed in Iran, has much medicinal application. Fifteen different populations of this plant were collected from different regions and genetic diversity among them evaluated using morphological and molecular markers. First, cluster analysis based on nine evaluated morphological traits divided populations into four distinct groups. Principle component analysis based on morphologic traits showed that first three components were explained 78.04 percent of variation among populations. Second, ten primers were applied on whole genome extract to disclose DNA polymorphism among populations. Many bands were appeared and display a 99.16 percent polymorphism. Cluster analysis categorized populations into four groups. The least and the most genetic similarity were observed between Dezful and Bandan and Dezful and Lendeh populations, respectively. Finally, it was proved that random amplified polymorphic DNA markers could be a proper tool for genetic diversity investigation in Iranian populations of Bitter melon.

Keywords: Cluster analysis, principle component analysis, medicinal plant

Introduction
Bitter melon (*Citrullus colocynthis* L.) is a medicinal plant of Cucurbitaceae family. It is an herbaceous, perennial and drought-tolerant species with runner or climbing stems covered with enormous hairs (Mozaffarian *et al*. 2012; Zargari 1990) [15, 26]. This plant has remarkable anti-diabetic, antiviral, antibacterial and anticancer properties (Samsam-Shariat 1989) [18]. Since plant breeding is based on genetic variation and selection, the wide range of genetic resources are necessary. Molecular markers are more powerful tool to reveal genetic diversity while morphological markers and relevant field experiments comparisons are time-consuming and labor-intensive. Esquinas-Alcazar and Gulick (1983) [7] listed 250 groups of melon and explained how to assess their genetic diversity by applying morphological traits. Zeinali *et al*. (2000) [27] collected 14 populations of German chamomile (*Matricaria recutita* L.) from different areas to study their diversity in some morphological traits as well as essential oils. Their results showed that the observed diversity did not correspond with geographic distribution. Lorz and Wenzel (2005) [13] suggested that use of DNA markers for mapping and targeting loci and genomic regions in breeding programs would become more prevalent. Therefore, breeding programs require the development of different markers to evaluate plant genotypes, populations, and genetic diversity quickly and accurately. PCR-based molecular markers, especially random amplified polymorphic DNA (RAPD), are being extensively used and preferred to investigate genetic diversity since they are time-saving, cost-effective and needless of sequence information. RAPD is a proper technique for investigation about genetic structure and relationships, hybrid creation and parent identity; although it has a low reproducibility and its result may not be same in different laboratories (Naghavi *et al*. 2008) [16]. Zamiad *et al*. (2006) [25] studied genetic diversity of 46 *Cucumis melo* populations from Khorasan province (Iran) applying morphological and RAPD molecular markers. They showed 20 percent polymorphism while the level of correlation between RAPD and morphological markers was 50 percent. Razmi (2006) [17] observed high diversity among 22 landraces of *C. melo* using morphological and RAPD molecular markers and Thi Lang *et al*. (2007) [20] also examined genetic diversity using RAPD markers in the *Cucumis* genus. Feyzian *et al*. (2007) [9] used morphological and RAPD molecular markers to study the genetic diversity of collected melons from the central (Tehran, Qazvin, Semnan, Isfahan and Yazd)

and northern (Gilan, Mazandaran and Golestan) provinces of Iran. 38 selected accessions were divided into different groups after evaluation of diversity and relationships. Cluster analysis using molecular markers could not separate populations into different groups which implied that the genome of this group was very close. Levi *et al*. (2004) [12] used ISSR and RFLP markers for investigating genetic diversity among watermelon (*Citrullus lanatus*) cultivars. Used primers displayed polymorphism and high variability among heirloom cultivars. ISSR and RFLP markers identified 80.2% and 97.8% genetic similarity among heirloom cultivars, respectively. Enayat Avval (2017) [6] assessed genetic diversity of local species of C*itrullus colocynthis* in Sistan-Balochestan province using SSR markers and observed that 7 out of 10 used primers showed polymorphism, and its PIC value was been between 0 to 68.75. Zhang *et al*. (2016) [28] studied genetic diversity of 1197 *Citrullus* accessions using SSR markers. The average of PIC value was 0.49 and Nei index was 0.56, and observed heterozygosity was 0.10. Kumar *et al*. (2017) [11] studied genetic diversity of *Citrullus colocynthis* (L.) schard using RAPD and ISSR markers. The percent polymorphism in RAPD markers ranged from 22.2% to 83.3% with 55.14% polymorphism, but the percent polymorphism in ISSR markers ranged from 45.4% to 73.3% with 65.05% polymorphism. Approx. 50% RAPD and ISSR markers showed PIC value and heterozygosity ≥0.50 indicating markers as informative.

As it explained, despite the medicinal importance of bitter melon, no systemic study has been done about its morphological and genetic diversity in Iranian populations (except local species of *citrullus colocynthis* in Sistan-Balochestan province). On the other hand, there was no sequence information regarding genome of this plant. Thereby, it was decided to investigate the diversity of some Iranian populations through morphological and RAPD markers. This study is the first report about describing the Iranian bitter melon.

Materials and Methods

Fifteen different populations were collected from nine provinces of the Iran including Sabzevar (from Razavi Khorasan province, No.1), Bandan and Milnader (from Sistan-Balochestan province, No.2), Kerman (from Kerman province, No.3), Bandar Abbas (from Hormozgan province, No.4), Kazeron (from Fars province, No.5), Ahram and Daylam (from Bushehr province, No.6), Abrigun, Imamzadeh-Jafar and Lendeh (from Kohgiluyeh and BoyerAhmad province, No.7), Dezful, Shushtar and Hendijan (from Khozestan province, No.8), Kashan (from Isfahan province, No.9) (Figure 1).

Nine morphological traits such as leaf length and width stem and fruit diameter, seed length and diameter, fruit weight, stem length and 100 seed weight were measured. Data were analyzed using the SAS software (version 9.1), cluster analysis performed by Ward method and STATGRAPHICS software (version 16.1.11). Since seeds did not germinate in pots, seed kernels were isolated and cultured on Murashige and Skoog (MS) medium. In the early stage of vegetative growth, young leaves were picked up, and DNA was extracted from leaf samples from individual plants (Dellaporta *et al*. 1983). The quality and quantity of extracted DNA were evaluated through spectrophotometry and on the agarose gel (1%). Ten RAPD primers (Table 1) based on Feyzian *et al*. (2007) [9] investigation used and PCR done (Table 2).

The PCR reaction containing 2 µL template DNA (10 ng/L), 1.5 µL $MgCl_2$ (50 mM), 0.5 µL dNTP (10 mM), 0.4 µL Taq DNA polymerase (5 unit/ µL), 1.7 µL primer (10 ppm), 2.5 µL buffer PCR (1x) and 16.4 µL distilled water using a thermocycler (BIORAD, Germany) were conducted. Amplified fragments were separated on 1.5 percent agarose gel. The bands were scored and analyzed using NTSYS-pc (version 2.02e) and GENALEX (version 6.5) software. To draw the dendrogram, the STATGRAPHICS software and to principle component analysis SAS (9.1) software used.

Results

Mean comparison using LSD method show differences between populations for most morphological traits, Sabzevar population has high value in most of the traits (Table 3). Cluster analysis based on morphological traits categorized populations in four groups (Figure 2). In the first group, Sabzevar was given alone. The second group was divided in two subgroups. In this group, except Shushtar and Hendijan, other populations belong to different provinces. It is important to note that all of these populations belong to the south of Iran and if populations belong to neighbor provinces, there is also the possibility of gene flow between provinces. The third group includes Kashan, Daylam and, Kerman populations those who belong to three different provinces. Finally, in the fourth group, populations belong to three neighbor provinces (No. 8, 7 and 6 in Figure 1).

Based on principle components analysis, the first three components explained 78.04% of the variation (Table 4). The first component had a high positive correlation with seed width and length, and seed and fruit weight. The second component had the positive correlation with stem diameter and leaf length and the third component showed the highest correlation with stem length. Thus, it can consider as the component of fruits and seeds in the next selections.

Ten primers produced 138 bands and showed 99.16 % polymorphism that reflecting the high percentage of polymorphism among evaluated populations (Figure 3). The maximum and minimum numbers of bands belong to Ah6 and Ah1 primer, respectively. Ah6 primer produces the maximum number of polymorphic bands, expected heterozygosity and, probably it indicates more diversity. The highest Shannon index, indicating diversity within the population, belongs to Ah6 locus (Table 5).

According to Cophenetic correlation coefficient (r=0.93) and UPGMA method, created the most suitable cluster. Cluster analysis, categorized populations into four groups (Figure 4).

The first group divided into A and B subgroups. Only Hendijan population put in subgroup B, but six populations put in subgroup A, among them can observe populations from No. 8, 2, 3, 7 and 1 province. The subgroup A includes populations belong to south, northeast, and center of Iran, but Milnader and Bandan populations belong to Hormozgan province. The second group is also divided into A and B subgroups, all populations of this group belong to the south neighboring provinces in Iran. In the third group, Shushtar and Kazeron populations belong to two neighboring southern provinces. Bandar Abbas and Lendeh populations that belong to tropical and southern provinces of Iran put in Group 4. Interestingly, the populations of province No. 7 put in three different groups. This grouping is likely to show high diversity among populations of province No. 7. In cluster analysis based on morphological and molecular data, province No. 8 populations were divided into three groups. Clustering of province No. 7 populations based on morphological data

was better than classification based on molecular data. This may be due to high genetic diversity among the populations surveyed in each region. Abrigun and Imamzadeh-Jafar populations are both from province No. 7, but in RAPD dendrogram put in separate groups (Figure 4). The principle component analysis a multivariate statistical technique that has much application in analysis of genetic diversity. Based on results, the first four components explained 61.02 % of total variance (Table 6), meaning the used markers have useful distribution throughout the genome.

Distribution of populations in Figure 5 in agreement with the distribution of them in the obtained dendrogram. In the dendrogram, Dezful and Bandan populations have the shortest distance and the most genetic similarity and the most populations of group 1 put in the neighboring situation in the plot component analysis that confirmed grouping based on molecular data. The plot classification based on component analysis, separated Ahram, Daylam and Abrigun populations from other populations. Also Kashan, Kazeron, and Shushtar put in one group that shows these populations have genetic similarity.

In fact, the two-dimensional graph showing the distribution of populations for components and the other tool that can be considered as a complementary method to cluster analysis.

Table 1: Sequence of primers

Primer code	Primer sequence
Ah1	5'-GAG GAT CCC T-3'
Ah2	5'-ACG GTA CCA G-3'
Ah3	5'-GGA CCC TTA C-3'
Ah4	5'-CGA CAG TCC C-3'
Ah5	5'-GCC CGC GAG T-3'
Ah6	5'-GGT GGC GGG A-3'
Ah7	5'-TGT AGC TGG G-3'
Ah8	5'-CCA GTT CGC C-3'
Ah9	5'-GCA CCG AGA G-3'
Ah10	5'-CCT GGG CCT A-3'

Table 2: Termal program of PCR

Reaction step	Temperature (C°)	Time (s)	Numbers of Cycle
Denaturation of genomic DNA	94	240	1
Denaturation of DNA	94	30	1
Annealing of primers to template strand	44	25	1
Extension of new strand	72	75	1
Replication of 2 to 4 steps			44
Final extension	72	240	1

Table 3: Mean comparison for morphological traits

Trait Population	Stem length (cm)	Stem diameter (cm)	Leaf length (cm)	Leaf width (cm)	Seed length (cm)	Seed diameter (cm)	Fruit diameter (cm)	Fruit weight (gr)	100 seed weight (gr)
Dezful	86.67	0.583	4.823	3.830	0.710	0.473	3.820	18.370	4.413
Bandan	455.00	0.570	5.110	3.636	0.846	0.526	3.673	20.937	4.390
Sabzevar	320.00	0.646	6.080	4.446	1.283	0.686	6.863	28.543	7.720
Kerman	121.00	0.536	4.020	3.013	0.716	0.370	4.046	12.887	2.766
Imamzadeh Jafar	124.33	1.073	4.533	3.533	0.786	0.480	5.096	12.943	1.196
Milnader	108.33	0.516	3.726	2.726	0.630	0.356	4.450	5.637	2.120
Hendijan	236.67	0.623	4.783	3.296	0.700	0.450	5.736	17.683	4.240
Ahram	102.33	0.463	5.346	4.306	0.693	0.446	4.916	10.470	3.653
Daylam	89.17	0.366	4.290	3.543	0.740	0.483	6.006	20.177	5.723
Abrigun	123.67	0.406	4.040	3.086	0.773	0.436	4.363	12.157	3.943
Kashan	166.67	0.526	3.376	3.053	0.630	0.406	3.710	8.530	3.136
Kazeron	133.33	0.476	4.003	3.220	0.790	0.466	4.793	17.830	4.776
Shushtar	110.33	0.383	3.973	3.403	0.770	0.460	4.113	13.377	5.423
Bandar Abbas	189.67	0.596	4.553	3.166	0.746	0.476	3.890	19.407	3.956
Lendeh	237.33	0.71	5.023	3.723	0.730	0.443	1.970	6.280	3.206
LSD (0.05)	43.684	0.127	1.140	1.054	0.085	0.055	1.064	4.912	0.038
Mean	173.63	0.565	4.536	3.465	0.769	0.464	4.497	15.015	4.092

Table 4: Principle components amount for morphological traits

Traits	First component	Second component	Third component
Stem length (Stem length)	0.55	0.29	0.46
Stem diameter	0.11	0.74	0.38
Leaf length	0.71	0.53	-0.39
Leaf width	0.63	0.45	-0.60
Seed length	0.86	-0.08	0.28
Seed width	0.88	-0.05	0.29
Fruit diameter	0.56	-0.39	-0.15
Fruit weight	0.82	-0.24	0.006
100 seed weight	0.81	-0.48	-0.07
Eigen value	4.39	1.56	1.07
Comparative variance (%)	48.77	17.38	11.88
Accumulative variance (%)	48.77	66.16	78.04

Table 5: Data obtained from analysis of RAPD marker

Primer	Total numbers of bands	Percent of polymorphic bands	Numbers of polymorphic bands	Expected Heterozygosity (He)	Shanon`s Index (SI)
Ah1	11	100	11	0.359	0.53
Ah2	12	91.67	11	0.247	0.38
Ah3	13	100	13	0.320	0.49
Ah4	14	100	14	0.296	0.46
Ah5	12	100	12	0.296	0.47
Ah6	17	100	17	0.396	0.58
Ah7	16	100	16	0.277	0.44
Ah8	15	100	15	0.323	0.49
Ah9	12	100	12	0.324	0.49
Ah10	16	100	16	0.258	0.41

Table 6: Principle component for molecular data

Component	Eigen value	Explained variance (%)	Accumulative variance (%)
1	5.50	36.68	36.68
2	1.60	10.67	47.35
3	1.07	7.18	54.54
4	0.97	6.47	61.02

Fig 1: Display the collected, evaluated populations of Bitter melon on Iran map

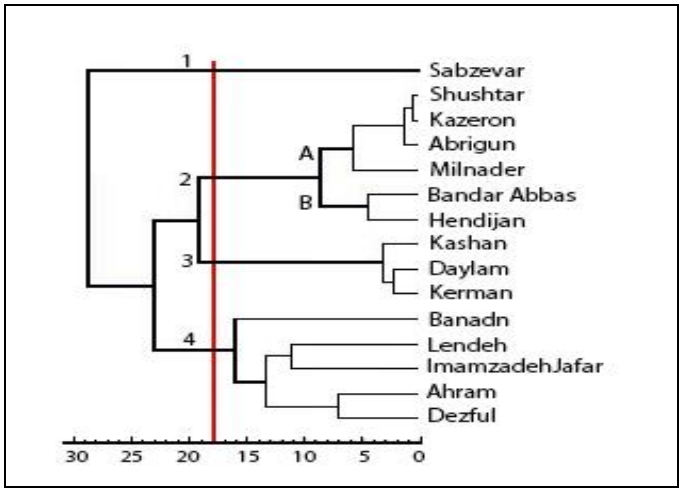

Fig 2: Dendrogram obtained from cluster analysis of evaluated populations based on morphological traits by Ward method

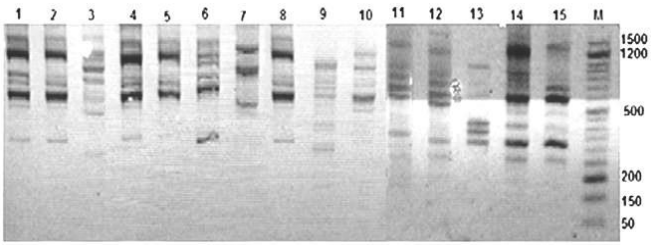

Fig 3: Banding pattern for 15 Bitter melon (1: Dezful, 2: Bandan, 3: Sabzevar, 4: Kerman, 5: ImamzadehJafar, 6: Milnader, 7: Hendijan, 8: Ahram, 9: Daylam, 10: Abrigun, 11: Kashan, 12: Kazeron, 13: Shushtar, 14: Bandar Abbas, 15: Lendeh populations) by Ah7 primer on 1.2% agarose gel, M: ladder (50-1500 bp, Cinnagen

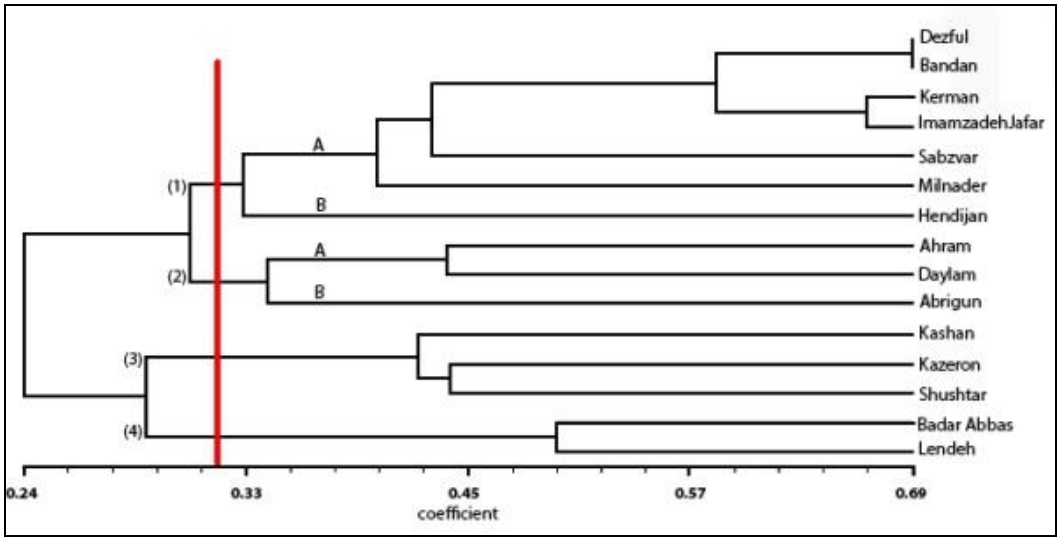

Fig 4: Dendrogram obtained from RAPD data using Jaccard similarity coefficient and UPGMA cluster analysis

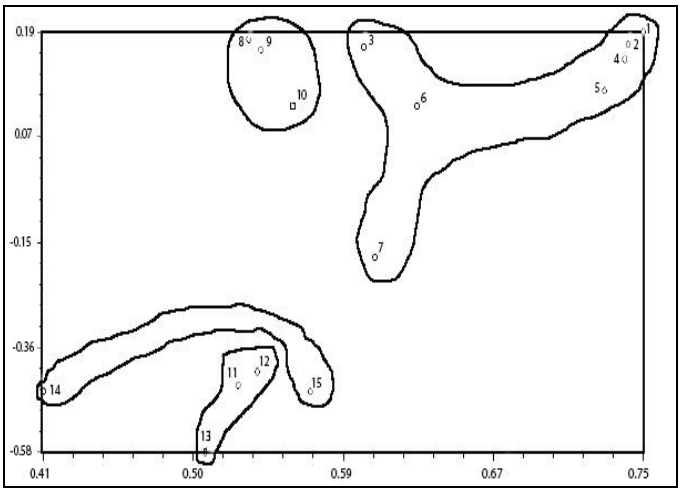

Fig 5: Principle component analysis plot for RAPD marker on studied Bitter melons (1: Dezful, 2: Bandan, 3: Sabzevar, 4: Kerman, 5: Imamzadeh Jafar, 6: Milnader, 7: Hendijan, 8: Ahram, 9: Daylam, 10: Abrigun, 11: Kashan, 12: Kazeron, 13: Shushtar, 14: Bandar Abbas, 15: Lendeh populations).

Discussion

According to these results, it can be said that morphological traits and geographical distribution are correlated, so the most southern provinces populations put in two and four groups, but Daylam population was separated from this groups. Also, Daylam and Hendijan populations, which are close to each other geographically and climate, were divided into two groups. Although principal components analysis reduce the number of variables to some hidden factors, and classification of traits based on inter-relationships used to identify the major components of genetic diversity, but its results were related to traits, populations and environmental conditions(Majidi and Arzani 1991). Southern provinces including Bushehr (No. 6 in Figure 1), Sistan-Baluchestan (No. 2), Kerman (No. 3) and Hormozgan (No. 4) have hot and dry climate, while Khorasan Razavi (No. 1), southern region of Kohgiluyeh and BoyerAhmad (No. 7) and Fars (No. 5) have mild semi-desert climate, Khuzestan (No. 8) and Isfahan (No. 9) have semi-desert and mild dry climate, respectively. Overall it could be concluded that some genotypes had a reasonable relation with climate, although some did not show a clear relation with climate conditions. However, it is obvious that bitter melon is exclusively growing in subtropical regions with hot and dry weather (www.irimo.ir). In this research, we collected 15 populations from 9 provinces which have the dry and hot climate. For example, we collect three populations from Kohgiluyeh and Boyer Ahmad province, but collect only one population from Isfahan province, because this plant grows in specific conditions.

Molecular markers in the classification of populations could succeed in spite of its defects, although it is necessary to ensure by using reproducible and strong markers such as SSR and ISSR. According to results, clustering based on RAPD and geographical distribution of populations has more or less relationship. These results are evidence of the genetic diversity among populations and match with the results of Fabriki-Orang *et al*. (2009) [8] on Iranian populations of melon, but do not match with results of Feyzian *et al*. (2007) [9] who investigated the diversity of Iranian melons. Enayat Avval (2017) [6] assessed the only genetic diversity of local species of *Citrullus colocynthis* in Sistan-Balochestan province using SSR markers, but in this research, we investigated 15 populations collected from 9 provinces of Iran using RAPD markers. According to the report of Agrama and Tuinstra (2003) [1] a large number of bands per marker, shows there is high diversity among studied cultivars.

Hitherto, RAPD markers were used in genetic diversity studies by Vyas *et al*. (2009) [21], Kumar *et al*. (2017) [11] and Ahmad *et al*.(2010) [2], Botanga *et al*. (2002) [4] and Yang *et al*. (2007) [24] have reported matched the genetic diversity and geographic diversity in their studies. Soorni *et al*. (2013) [19] also used RAPD markers to study genetic diversity of some Iranian populations of *Leonurus cardiac* and showed its ability to evaluate genetic diversity in different populations. In 47 evaluated populations in this research, in the most cases, in cases that collection locations of two populations were near, more genetic similarity observed. Ismaili *et al*. (2014) [10] by evaluating genetic diversity in populations of *Thymus kotschyanus* using RAPD markers showed that these markers are useful to study the genetic diversity of this plant and can also reveal geographical differences and *T. kotschyanus* showed a high percentage of polymorphism (77.57 %). This study showed that RAPD marker provided useful and adequate information about evaluate the genetic diversity of *T. kotschyanus*. Therefore, RAPD markers were the useful tool for evaluation of germplasm. Obtained polymorphism percentage in this study was more than the polymorphism percentage obtained for *T. kotschyanus* by Ismaili *et al*. (2014) [10], which may be due to the high diversity between different populations of Bitter melon. Ismaili *et al*. (2014) [10] evaluated a species of *T. kotschyanus* collected from different regions in northwest of Iran, but in this study, bitter melon collected from more regions of Iran (south, center and northeastern) and thus showed higher diversity. Also, in this study suitable range of Shannon index (0.38 to 0.58) revealed that genetic diversity among the studied populations was noticeable. Identification of genetic diversity within population is a prerequisite for the analysis of the genetic diversity. Similar results also were obtained by Babalar *et al*. (2013) [3]. Wilikie *et al*. (1993) [22] showed that species gathered from the wide geographical area have generally high genetic diversity and RAPD markers demonstrated high and low genetic diversity in populations grown in different or same environments, respectively.

Levi *et al*. (2004) [12] used ISSR and RFLP markers for studying genetic diversity of C*itrullus lanatus*. Zhang *et al*. (2016) [28] used SSR markers for studying genetic diversity of *Citrullus* accessions. Kumar *et al*. (2017) [11] also reported 55.14% polymorphism with RAPD and 65.05% polymorphism with ISSR markers for *Citrullus colocynthis* L., but Enayat Avval (2017) [6] only examined the native species of a province of Iran (Sistan-Baluchestan), Kumar *et al*. (2017) [11] studied Indian populations of bitter melon. Levi *et al*. (2004) [12] studied watermelon not bitter melon. Zhang *et al*. (2016) [28] studied some wild watermelon populations that are not related to the Iranian population. Therefore present study showed a high percentage of genetic polymorphism (99.16 %) in the bitter melon and showed that RAPD marker provides useful and sufficient information about the genetic diversity of this plant. In summary, this research provides valuable information for Iranian populations of this plant and is the first report about this plant from Iran.

Conclusion

It's the first report about the genetic diversity of bitter melon in Iran and it was shown that RAPD markers could be

successful tools for investigating the diversity of this plant. Iranian bitter melon was shown high genetic diversity. However, the least and most genetic similarity was observed between the populations belong to the south of Iran.

Acknowledgement
We are thankful to Yasouj University for financial assistance and, Mrs. Hajizadeh for technical helps in central Laboratory of Agriculture Faculty, Yasouj University.

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