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Assessment of genetic diversity of iranian population of *Anethum graveolens* L. (dill) using AFLP molecular markers

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Abstract

Anethum graveolens L. (dill) is an important medicinal and industrial plant. The essential oil of this species is used in different industrial such as pharmacy, nutrition, perfume manufacturing and veterinary. The populations collected from different regions of the country were cultivated in order to investigate the genetic diversity of dill and were studied in aspect of genetic variation and its correlation with geographical distribution. 337 alleles were observed polymorphic and 108 alleles in monomorphic of total 455 scorable alleles. Percentage of polymorphic bands was equal to 74 and the number of alleles observed for each primer combination ranged from 18 to 60. The highest number of alleles was associated with M22-E2 and E11-M17 primer and the lowest value was observed in E2-M35 primer combination. The coefficient of genetic similarity was varied between genotypes from 56/0 to 88/0. The minimum of genetic similarity was between populations of Shahrekord, Ahvaz and Yasuj with

similarity coefficient 34%. The maximum of similarity was observed among samples of 17 and 18 that genetic difference was very little and almost zero between them. Cluster analysis of populations using UPGMA algorithm and SM indicated high genetic diversity among populations of dil and also no correlation between molecular diversity and geographic distribution. The existence of genetic diversity in these samples confirms that the phytochemical and morphological differences of the samples is not simply due to environmental impacts, but are controlled by genetic factors as well. The results of this study can be useful in the management of germplasm of dill.

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Introduction

The use of medicinal plants is very valuable as major and minor products in Iran and elsewhere in the

world. Clearly, determination of these plants genetic characteristics, morphological, physiological, bio-chemical and ecological of these plants in order to sustainable utilization of economic, health and sanitary goals and also maintaining diversity in natural areas and preventing the extinction of endangered plant is necessary. On the one hand the medicinal plants are increasingly important due to secondary metabolite components, but on the other hand nowadays it is essential to find alternative sources in order to maintain health and reduce the harmful effects of chemicals because of pathogen resistance against the existing drugs and antibiotics (Hariri Akbari *et al*., 2010; Karimi, 1998).

In the past two decades, biotechnology in agriculture has provided promising prospects. The increasing development of biotechnology science in the twenty-first century in any country has lead to the development and progress of science, industry and the economy of that country. In the meantime, molecular discussions have growing importance in biology studies.

Medicinal plants are the main storage resources of medicinal compounds which about 80% of the world population still need the resources to maintain their health and sanitation. Plant secondary metabolites have great economic importance as pharmaceuticals, aromatic compounds, pigments, food additives and etc. (Hariri Akbari *et al*., 2012).

Biotechnology tools are very useful and significant in selection, reproduction, analysis, increasing and improving the metabolite compounds of medicinal plants. Genetic engineering and genetic manipulation, particularly with the use of indirect methods (bacteria) and direct (gene transfer in plants), are powerful tools in order to produce new secondary metabolites (Hariri Akbari *et al*., 2010).
Functional and comparative genomics, have provided the efficient and convenient tools such as Expressed Sequence Tags (EST), micro-arrays (Microarray) and molecular markers that can examine expression patterns of several genes (hundreds of genes) and the mechanism regulating the expression of these genes in a short time, even at a time. Nowadays it has increased our understanding of the interaction between genes and biosynthesis pathway and production of biochemical compounds in plants. Also it has made more efficient breeding programs in the germplasm by determining the relationships and interval genetic (Kamalodini *et al*., 2006; Hariri Akbari *et al*., 2010).

Green leafy vegetables are good sources of minerals as well as vitamins. *Anethum graveolens* L. (dill) a green leafy, widespread vegetable belongs to the family Apiaceae (Umbelliferae) that has an attractive flavor (Cankur *et al*., 2006). From 1500 BC, dill was considered as analgesic and the only species of the genus which cultivated in Iran is *Anethum* that have been found in temperate regions of Europe (Mediterranean and West Asia) (Majnoon Hosseini and Davazdah Emami, 2007).

The origins of dill have been attributed to the eastern Mediterranean and Europe (Zohary and Hopf, 2000).This plant has an old long history in many countries and cultures. Earliest source obtained indicates that, this plant has been used as an analgesic in 5000 years ago in ancient Egypt (Omid Baigi, 2006; Zargari, 1996; Yazdani *et al*., 2005). The essential oils of this species has limonene (20 - 28%), Terpinene or α-Phellandrene, b- Phellandrene, Alpha-pinene, Parasmyn, myristicin (0.062 %), Carvone, Dill ether, myrcene (40 - 60%). Carvone is the main component of the essential oils in this plant among the others (Kamalodini *et al*., 2006; Yazdani *et al*., 2004). The composition of dill essential oil changes markedly through the growing season. The characteristic of dill oil depends largely on the ratio of carvone and a-phellandrene (Callan *et al*., 2007).
From old times dills have been used to treat some digestive disorders such as bloating, indigestion and ulcers of the stomach and intestines] (Kobayashi and Kamata, 1999; Haji sharify, 2005). Dills are inhibitors on the enzyme Lipoxyginese due to the presence of

flavonoids. That is why is used as a lipid- lower blood drug. Limonene in dill) is used in the perfume manufacturing, cosmetics, Colorful soaps, deodorant, spice, resins manufacturing and moisturizer (Barghamady, 2006; Haji sharify, 2005). Furocoumarins components of this plant cause genetic mutations. These components exist in some plant species, particularly in Family *Apiaceae* and interact in the presence of activated UV light and with vital macromolecules such as DNA and RNA (Zolala, 2008). It has been reported that it is a possible source of antioxidant and also has anti-microbial properties against *Rhodotorula glutinis, Aspergillus ochraceus* and *Fusarium moniliforme* (Cankur *et al.*, 2006; Nanasombat and Wimuttigosol, 2011).

Recently, molecular methods have been used for the identification and classification of different species of herbs and medicinal plants (Yu *et al.*, 2011). The study of genetic diversity is the first step of a breeding program and genetic resources that are very valuable for breeders (Papini *et al.*, 2007). Genetic variation might be evaluated by assessing morphological or biochemical traits but molecular markers In particular, DNA-based markers provide the best assessment of genetic variation because they are plentiful and are not dependent on environ-mental effects. This made this evaluation more efficient and reliable (Dongre *et al.*, 2007; Fu *et al.*, 2003).

After the development of polymerase chain reaction (PCR) technology (Etminan *et al.*, 2012), several PCR-based markers were developed and applied to assess the genetic variation among populations and genetic resources. These marker systems are different in technical principle, type of inheritance, reproducibility, amount of polymorphism and in their costs (Etminan *et al.*, 2012).

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The amplified fragment length polymorphism (AFLP) technique (Vos *et al.*, 1995) is one of the best marker systems that can be used to detect high levels of DNA polymorphism and is extremely promising for genetic diversity studies (Etminan *et al.*, 2012).

The objective of this study was to estimate the genetic diversity among the 42 Iranian populations of *Anethum graveolens* L. (dill) using the Amplified Fragment Length Polymorphism (AFLP).

Table 1. changes Comparison of five main components of *Anethum* at different stages of growth (percent) (Yazdani *et al.*, 2004)

compounds	Dill-1	Dill-2	Dill-3
□-phellandrene	19.2	6.49	5.63
Limonene	14.21	17.71	15.54
Dill ether	15.69	2.74	1.49
Carvone	48.82	64.86	64.62
trans-dihydrocarvone	2.22	6.64	10.60
Sum total	89.14	97.42	98.19

Dill-1: The flowering stage and Beginning of seed formation

Dill-2: seed formation before fully ripening

Dill-3: full ripening

Materials and methods

Plant material and collecting population of Anethum graveolens L. (dill)

42 populations were collected from different cities of the country. And cultivated in order to perform tests and take samples in the Research farm of Institute of Medicinal Plant, Karaj, Iran. In order to achieve accurate and comprehensive information regarding to Iranian Populations of dill, we have attempted that collected populations to be appropriate for the geographic distribution throughout the country (tabe 2).

Table 2. Origin of collecting Samples

Number of sample	Origin of collecting samples	Number of sample	Origin of collecting samples

1	Urmia	22	Abadeh
2	Ajab Shir	23	Bandar Abbas
3	Tabriz	24	Qeshm
4	Ardabil	25	Bojnord
5	Tehran	26	Arak
6	Lahijan	27	Quchan
7	Kermanshah	28	Nishapur
8	Eslamabad-e Gharb	29	Kashmar
9	Zanjan	30	Zoeram, Shirvan
10	Karaj	31	Gonabad
11	Arak	32	Birjand
12	Varamin	33	Zabol
13	Delijan	34	Baft
14	Kashan	35	Sari
15	Isfahan	36	Qaem Shahr
16	Najafabad	37	Dorud
17	Shahrekord	38	Semnan
18	Ahvaz	39	Yazd
19	Yasuj	40	Shiraz 2
20	Marvdasht	41	Rudehen
21	Shiraz 1	42	Tehran

Plant cultivation and production of plant material

First the seeds were collected from plants of natural habitats. The seeds were put in Petri dishes on wet filter paper and transferred to plastic pots after germination in order to grow these plants to a height of 10 to 15 cm and also leaves grow enough. After three weeks, fresh leaves from each pot were picked for genomic DNA extraction.

Genome extraction and purification

Total genomic DNA was extracted and the quality of the extracted DNA was tested on 1% agarose gel electrophoresis and quantity was measured with a spectrophotometer (Hariri Akbari *et al*., 2012). The fresh and young leaves of each sample produced in a mortar were ground to a fine powder in liquid nitrogen, and transferred to the tubes 1/5 ml. 900 µl of SDS 4% extraction buffer was added per tube and placed for 45 Min in water bath at 65 ° C. and then added 300 µl of potassium acetate per tube for 25 Min under ice container. The tubes were centrifuged at a temperature of 3 ° C for 15 Min and at 12000 rpm. 750 µl of the upper liquid transferred to a new tube and 750 µl of cold isopropanol added to it and placed 5 Min at laboratory temperature (25 ° C). Then the tubes were centrifuged at a temperature of 3 ° C for 15 Min and at 12000 rpm and after removing supernatant and tubes containing plates were incubated at 37 ° C for 20 to 30 Min. In order to purify, 700 µl of autoclaved deionized -distilled water (DDW) was added to each tube that has been kept for 1 h at 4 ° C. and then added the chloroform isoAmyl alcohol solution (24:1) volume of 700 µl and shaken well until a uniform emulsion was obtained. Centrifugation was performed for 10 Min at 13000 rpm, the lower phase is chloroform Isoamyl alcohol and upper phase is solution containing DNA. The upper phase are transferred to a new 2 ml tube and was added 0/1 volume of sodium acetate supernatant liquid (3 M, pH =8). Absolute alcohol (100%) that is 2/5 times volume of supernatant liquid was added and then dehydrated after 5 Min. centrifuge was performed for 10 Min at 13000 rpm and discarded the supernatant liquid and added 100 ml 70% alcohol to the sediment. The alcohol was removed and the samples incubated at 37 ° C for 20 to 30 Min to dry completely and then added TE buffer (100 µl) or deionized -distilled water (DDW). The quality of the extracted DNA was tested on 1% agarose gel electrophoresis and quantity was measured with a spectrophotometer.

AFLP assays

The AFLP procedure was performed with appropriate modifications of the method described by Vos *et al*., (1995).

Enzyme digestion and ligation of adapters

Genomic DNAs Concentration was reached to 250 Ng / µl by dilution. To perform double digestion with EcoRI and MseI restriction enzymes were used as follows: 2.5 unit of each restriction enzyme, 4 µl Tango buffer (10x), Template DNA: 10 µl of genomic DNA (250 ng) of each sample, DDW:10 µL] In the following, reaction components were placed in for 3 h at 37 ° C. and kept for 1 h at 65 ° C. respectively and

treated at laboratory temperature for one day (Etminan *et al*., 2012; Vos *et al*., 1995).

The digested fragments were ligated to double stranded adaptors appropriate with the EcoRI and MseI restriction sequences, the process of ligation of adapters were performed as follows: adding connection components to each tube that contain Buffer Ligase 10x: 1µL E.Adaptor (CTCGTAGACTGCGTACC): 1µL, M. Adaptor (GACGATGAGTCCTGAG): 1.5 unit T4 DNA ligase to materials derived from the digestion of the reaction components at 25 ° C for 4 h and then maintaining 24 h at 37 ° C. The ligated DNA fragments were diluted three times with sterile distilled water and stored at -20°C. (Vos *et al*., 1995).

Preamplification and selective amplification

Preamplification was carried out using non-selective primers E000 and M000 (Table 3) in a 25 µl reaction volume containing 3.75 µl of (1:3) diluted ligation product, 1 unit of Taq polymerase, 1X Taq polymerase buffer, 0.4 µM of each of the two primers, 150 µM of each of dATP, dCTP, dGTP and dTTP, and 2 mM MgCl2. This amplification was performed in a thermocycler programmed for 25 cycles, each consisting of 1 min at 94°C, 1 min at 60°C and 72°C for 2 min. The final extension was done at 72°C for 7 min. The pre-amplification product was diluted 1:9 in sterile double distilled water to prepare template DNA for selective amplification (Etminan *et al*., 2012; Vos *et al*., 1995).

Selective proliferation stage was performed using 15 different combinations (Table 3) of primers, Selective amplification was performed in a 25 µl reaction mixture volume containing 3.75 µl of diluted pre-amplification product, 1x Taq polymerase buffer, 2 mM MgCl2, 1 Unit of Taq polymerase, 150 µM of dNTPs, and 0.4 µM of each of the two primers with two or three additional nucleotides at the 3´end. For the selective amplification step, the following cycle profile was used: 5 min at 94°C for pre-denaturing, 35 consecutive cycles each consisting of 1 min at 94°C for denaturing, 1 min at 65°C for annealing and 2 min at 72°C for extension. After these 35 cycles, a final extension step was done at 72°C for 7 min. 15 primer combinations were used for diversity assessment. The PCR products were separated on denaturing 6% (w/v) polyacrylamide gel electrophoresis. For amplified fragment detection, silver staining method was used as described by Etminan *et al*., (2012) and Vos *et al*., (1995).

Table 3. primers names and sequence

Primer name	Primer sequence
E4	5′-GAC TGC GTA CCA ATT CGT C-3′
E11	5′-GAC TGC GTA CCA ATT CAG G-3′
E8	5′-GAC TGC GTA CCA ATT CAC T-3′
E2	5′-GAC TGC GTA CCA ATT CAA C-3′
ETG	5′-GAC TGC GTA CCA ATT CTG-3′
M35	5′-GAT GAG TCC TGA GTA AGA G-3′
M22	5′-GAT GAG TCC TGA GTA ACC C-3′
M20	5′-GAT GAG TCC TGA GTA ACA T-3′
M17	5′-GAT GAG TCC TGA GTA ACA A-3′
E000	5′-GAC TGC GTA CCA ATT C-3′
M000	5′-GAT GAG TCC TGA GTA A-3′

Band scoring and statistical analysis

Obtained gels were scored for each primer pair after recording the information. Presence of band was considered as 1 and absence of band as 0 respectively (Negi *et al*., 2006). Variables of each primer were placed in a row and name of genotypes in the column by using Microsoft Excel software. The similarity matrix and cluster analysis was performed using NTSYS-pc analytical software v.2. and dendrogram was constructed based on DICE similarity coefficient and the accessions were grouped by cluster analysis using the UPGMA algorithm method. To determine the correlation between similarity matrices and cophenetic, Mantel test was used that MAXCOMP coefficient obtained via COPH and SM coefficients was applied for the Mantel test (Powell *et al*., 1996).

Results

The results of AFLP reaction

Molecular markers, in particular DNA based markers provide reliable genetic information because of the in-

dependence of the confounding effects of environmental factors (Powell *et al*., 1996). 15 AFLP primer combinations were used to investigate genetic diversity of 42 populations of dill samples. In total 455 entirely specific and scorable bands were recorded that 337 polymorphic bands were observed. This amount includes 74/06% of all observed bands. The number of bands varied from 18 to 60 per primer. Maximum and minimum of observed bands was associated with primer E11-M17, M22-E2 with 60 bands and E2-M35 primer with an 18 bands respectively (Fig. 1). The average number of bands was 33/30 per primer. But maximum and minimum of polymorphism was the primers E11-M17 with 34 bands and E2-M35 with 18 observed bands respectively, and also the average of polymorphic bands were 46/22 (table 4).

Table 4. Primer combinations, the total number of bands for each primer, the number of polymorphic bands and percentage of polymorphism

Percentage of polymorphism	Number of polymorphic bands	total number of bands	Primer combination	Row
89/2	25	28	E11-M35	1
85/7	24	28	E46-M17	2
53/3	16	30	E46-M20	3
80/7	21	26	E46-M22	4
100	25	25	E8-M20	5
72/2	13	18	E46-M35	6
92	23	25	E11-M22	7
50	30	60	E2-M22	8
56/99	34	60	E11-M17	9
64	32	50	E8-M17	10
85	17	20	E11-M20	11
86/3	19	22	E2-M17	12
100	18	18	E2-M35	13
92	23	25	E2-M20	14
85	17	20	E8-M35	15
74/06	337	455	Et-Mt	Total

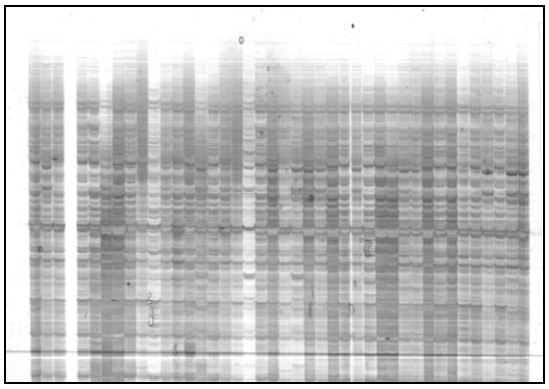

Fig. 1 Polymorphism Investigation of Iranian *Anethum graveolens* L. (dill) by using primers E2-M22. In this sample the total number of bands were 60, number of polymorphic bands were 30 and polymorphism percent was 50%.

Cluster analysis

These values were arranged in a symmetrical matrix of genetic tree similarity based on UPGMA algorithms for clustering in NTSYS-pc software and cluster diagram was drawn. Degree of genetic similarity between genotypes varied from 54% to 88%. The lowest similarity between sample numbers 17 (Shahrekord) and 18 (Ahvaz) with Yasuj (19) about 34% and the highest similarity about 88% were between samples 17 and 18 (Fig. 2).

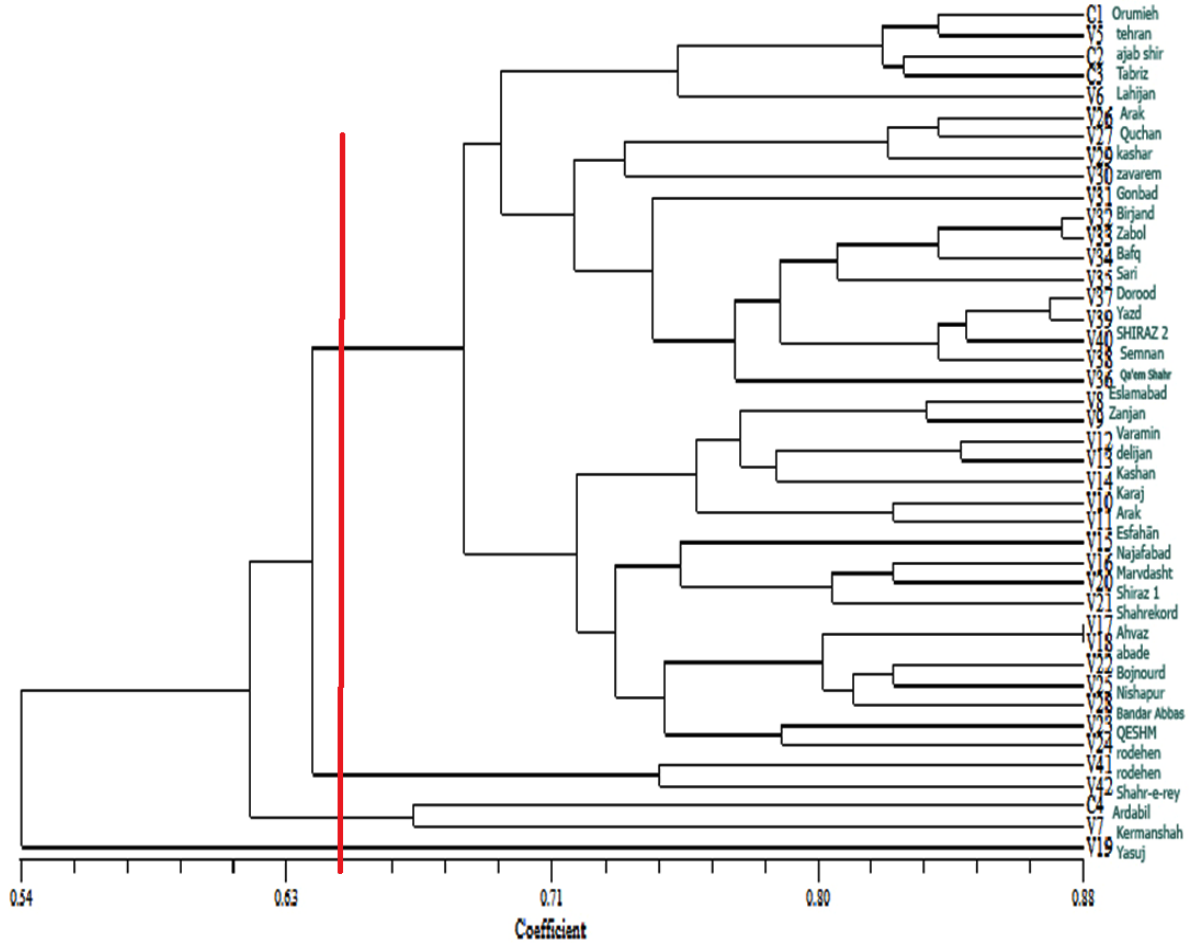

Fig. 2 Cluster diagram of 42 *Anethum graveolens* L. (dill) based on 15 different primer combinations with an Analytical method (UPGMA)

The results of Mantel test

In general, a cluster diagram doesn't indicate the accuracy of the obtained results, so Mantel test was used for determining the correlation between the similarity matrix and cophenetic correlation. Therefore the results of Mantel test was obtained from principal components analysis and the purpose of this calculation is to identify the parameters that firstly include all aspects of information that contained original data and secondly the numbers of data be fewer than the number of main variables (Martinez *et al*., 2003; Negi *et al*., 2006).

To this end, molecular and genetic similarity values and Individuals genealogy were arranged as corresponding in format of matrix and compared with software MAXCOMP (Negi *et al*., 2006).

Therefore cophenetic correlation coefficient (r) was used and converted to its equivalent for calculating the dendrogram, and then the cophenetic matrix was compared with the similarity matrix. The obtained clustering graph had cophenetic coefficient r=0/79. In molecular studies, high cophenetic coefficient is a factor for efficiency of obtained diagram. On the other hand low cophenetic coefficient could not be a reason for of inefficiency of obtained diagram, but low cophenetic correlation coefficient may be due to unusual circumstances in the data, in particular on molecular data (Khunani, 2008) (Fig. 3).

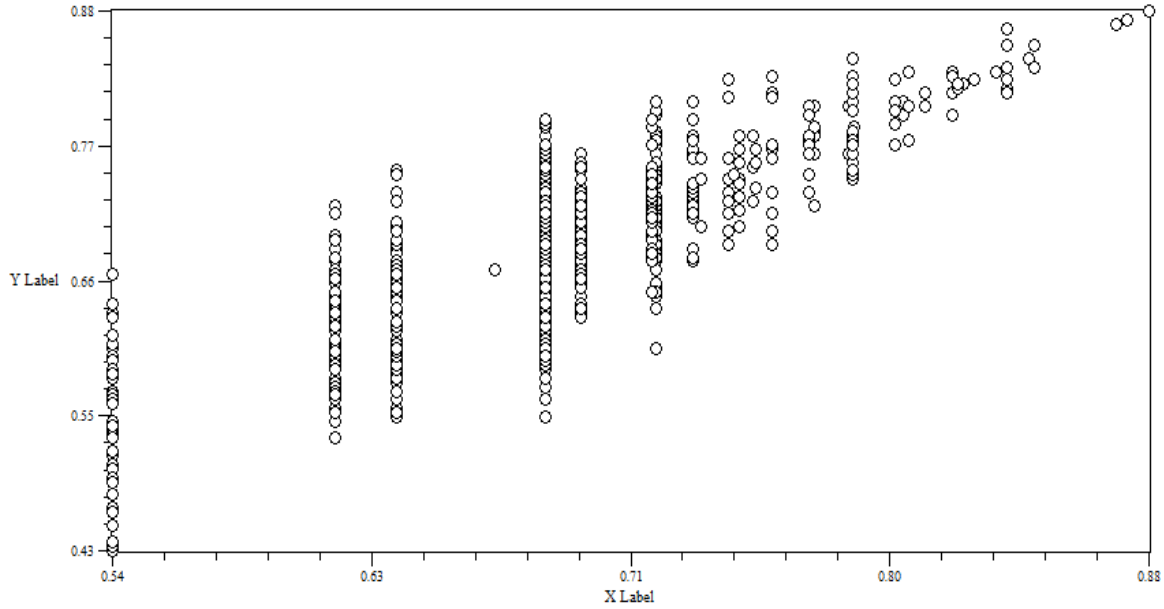

Fig. 3 graphics resulting from Mantel test were obtained using COPH and SM Pearson coefficients MAXCOMP and confirms Cluster analysis resulting from UPGMA cluster analysis.

Discussion

The results showed that AFLP molecular markers are useful tools for studying the genetic diversity of medicinal plants like dill. The results of other studies have revealed that several factors have affected estimation of genetic relationships among individuals that some of them include the number of markers have been used, distribution of the markers in the genome and the nature of evolution mechanisms that basis for causing diversity (Rezaei *et al.*, 2012; Kermani *et al.*, 2006).

It should be noted about the number of markers that the amount of information acquired from AFLP method highly depend on the number of used primer combinations but (Ellis *et al* 1977) showed that it is possible to explain 80% of the genetic relationships with choosing the 6 combinations of the best primers (Khunani, 2008).

Study of genetic diversity in the family *Apiaceae* using molecular markers has been reported in *Changium smyrnioides* (RAPD) by (32), *Carum* L. (Santos and Simon, 2002) and carrot (AFLP) by Negi *et al.*, (2006). As regard to this subject, in this study it has been used 15 AFLP primer combinations (table 4) and analysis of banding patterns of revealed 337 polymorphic bands that with an average of 22.467 fragments per each primer combination. The maximum and the minimum number of polymorphic bands per assay were 18 and 60 bands, respectively (Table 4).

Based on the results, maximum genetic similarity were observed between populations 17 (Shahrekord) and 18 (Ahvaz) that have been at a subgroup and most likely the origin of these two populations is similar. In the end, 42 populations were exposed to study with Cluster analysis of AFLP classified into 4 different main groups. Comparing the geographical distribution and genetic diversity showed that there is no correlation between them in some cases but it can not be sataed definitely that there is no relationship between the two of them. This lack of correlation have been also reported in assessment of genetic diversity in the samples of *Ferula gummosa* from Iran using AFLP and RAPD markers (Khunani, 2008; Talebi Kouyakhi *et al.*, 2008).

Castellini *et al.*, (1983) suggested that principal components analysis as a complementary method with cluster analysis lead to the optimum use of molecule data. The comparison of plot with clustering graph has

showed the appropriate distribution of populations (Castellini *et al*., 2006). Using of molecular markers and genetic diversity has been observed frequently in the family Apiaceae (Zolala, 2008).

Khunani *et al*., (2008) indicated in the study of genetic diversity *from Ferulla gummosa of Different regions of Iran with* 10 AFLP primer combinations that high genetic diversity between populations of *Ferulla gummosa* can be identified with 10 AFLP primers and also high genetic diversity of among the populations of dill was observed in this research with 15 AFLP primer combinations (Khunani, 2008).

Kermani *et al*., (2006) used AFLP molecular markers to study the genetic variation of 15 lines of Iranian cumin (*Cuminum cyminum* L.*)* and 3 population of *Cuminum* setifolium. In this study 149 scorable bands were detected by 6 AFLP primer combinations that 73 numbers of them were polymorphic (49%). The maximum number of polymorphic bands (20 bands) and minimum number of polymorphic bands (3 bands) were produced using the primer combinations (E-AGT/M-CCG) and primer combinations (E-ACT/M-CCG), respectively. Genetic diversity of *Cuminum cyminum* L. (0/150) was more than *Cuminum* setifolium (0.084), while the variation between these species (0.163) was more than the diversity within theirs. Drawn Dendrogram using the UPGMA could completely separate these two species in the genetic distance of 45٪. This study showed that genus of *Cuminum* has relatively low level of diversity due to being self-pollinated and it could be possible *Cuminum* setifolium has been used as a new source of genetic variation in crosses between species and transferring desirable genes to cumin (Kermani *et al*., 2006).

It has been reported that the numbers of amplification products in *Withania somenifera* varied from 53 to 101 in E+ACG/M+CAT and E+AAC/M+CAA primer combinations, respectively. The percentage of polymorphism ranged from 79 in the primer combinations of E+ACC/M+CTC and E+AAC/M+CAA to 89 which were illustrated by E+ACG/M+CAT with an average of 82% bands being polymorphic (Negi *et al*., 2006).

In the case of dill and using populations that have appropriate distribution throughout the country and also 15 primer AFLP combination It can be concluded with high confidence that this plant has a high level diversity in the country. It is essential to investigate phytochemical variation and the routes of metabolite synthesis among the samples in order to introduce appropriate varieties and perform breeding programs. In conclusion, AFLP data sets showed a high level of polymorphism among Iranian population of Iranian dill reflecting their efficiency in the assessment of the genetic diversity. These results can be used for germplasma management and breeding.

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