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Genetic diversity assessment of alfalfa (*Medicago sativa* L.) populations using AFLP markers

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Abstract

In the present study, genetic diversity of 26 Iranian cultivated populations of alfalfa (*Medicago sativa* L.) was studied using eight AFLP selective primer combinations. The number of polymorphic fragments detected per primer combination rang from 3 to 6 bands with an average 4.9 bands. Average polymorphic information content (PIC) was 0.26 over all primer combinations. M-CAG/E-ACC primer combination showed the highest PIC which can be a good candidate primer combination to verify genetic diversity in alfalfa. Cluster analysis using Unweighted Pair-Group Method using arithmetic Average (UPGMA) and Jaccard's coefficient grouped the populations into four main clusters with no correlation between genetic and geographical diversity. Principle coordinates analysis (PCO) showed PC1 and PC2 explained 42.65 and 15.95 percent of total variance, respectively. The result of PCO was confirmed by cluster analysis. The lowest genetic distance was observed between Koohpaie and Gonabad populations and the highest between Dameghan1 (cluster 1) and Divandarreh (cluster 3) populations.

Keyword: *Medicago sativa*, genetic distance, genetic diversity, AFLP

Abbreviation: *AFLP,* Amplified Fragment Length Polymorphism. *PIC,* polymorphic information content. *UPGMA,* Unweighted Pair-Group Method using arithmetic Average. *PCO,* Principle coordinates analysis.

Introduction

Alfalfa (*Medicago sativa* L.) is a widely grown legume and one of the most important forage species throughout the world and is believed to have originated in the Caucasus region: northeastern Turkey, Turkmenistan and northwestern Iran (Michaud *et al*., 1988). Cultivated alfalfa is autotetraploid (2n=4x=32) (McCoy and Bingham, 1988), cross-pollinated (allogamous) and seed propagated. The analysis of genetic variability within and among populations of cultivated alfalfa can assess future risk of genetic erosion and help in the development of sustainable conservation and genetic improvement strategies (Stanford, 1951, Flajoulot *et al*., 2005). Genetic variation is the basis for breeding programs; therefore, it is important to identify genetically distinct plants for breeding purposes (Brummer *et al*., 1995). Identification based on morphological characters is time-consuming and requires extensive field trials and evaluation (Astarini *et al*., 2004), while morphological differences may be epigenetic or genetic based characters (Tahir, 2001; Mukhtar *et al*., 2002; Migdadi *et al*., 2004). During last three decades genetic diversity was studied in plants through isoenzymes (Hemrick & Godt, 1990). The development of molecular (DNA) marker provides new dimension, accuracy and perfection in the screening of germplasm (Tar'an *et al*., 2005). Efficient and quick screening of such genotypes speed up the process of varietal evaluation, thus molecular marker plays pivotal role in this regard. Different molecular marker types have been used to assess genetic diversity in alfalfa such as restriction fragment length polymorphism (RFLP) (Kidwell *et al*., 1999; Maureira *et al*., 2004), random amplified polymorphic DNA (RAPD) (Tucak *et al*., 2008; Crochemore *et al*., 1996; Gherardi *et al*., 1998; Musial *et al*., 2002), simple sequence repeat (SSR) (Falahati-anbaran *et al*., 2007; Touil *et al*., 2008, Flajoulot *et al*., 2005), sequence related amplified polymorphisms (SRAP) (Vandemark *et al*., 2005) and amplified fragment length polymorphism (AFLP) (Segovia-Lerma *et al*., 2003). AFLP markers are highly polymorphic and reproducible and thus represent a powerful technique for DNA analysis that has revolutionized fingerprinting and diversity studies (Vos *et al*., 1995). AFLP analysis detects genetic variation throughout the genome by using a pair of specific restriction enzymes and their corresponding adapters combined with 2 selective rounds of PCR. Because PCR primers are based on the sequences of the restriction enzyme and universal adapters to which they are ligated, the procedure requires no prior information about the nucleotide sequences under investigation. Polymorphism is detected by using a number of selective bases following the restriction site. Primers with one or no selective base are used in a round of pre-amplification. This reaction is diluted for use in a second round of PCR in which primer pairs with 2 or 3 selective bases are used (Bartolini *et al*., 1998). In this study, genetic variability of 26 alfafa populations which cultivated in different area of Iran was evaluated. Most of the populations are Iranian natural landraces and some of them

Table 1. List of populations used in this study

Number	Population name	Origin	Latitude, N	Longitude
2	Zarinshahr1	Iran	32° 23'	51° 22'
4	Unknown1	Iran	-	-
5	Dameghan1	Iran	36° 10'	54° 21'
6	Varamin	Iran	35° 19'	51° 38'
7	Unknown2	Iran	-	-
9	Faminche-Hamedan	Iran	34° 47'	48° 30'
10	Turkieh-Hamedan	Turkey	34° 46'	48° 29'
11	Dameghan2	Iran	36° 11'	54° 20'
13	Abanbar-Hamedan	Iran	34° 46'	48° 29'
14	Unknown3	Iran	-	-
15	Sabzevar	Iran	36° 12'	57° 40'
16	Fezveh	Iran	-	-
17	Unknown4	Iran	-	-
18	Zarinshahr2	Iran	32° 23'	51° 21'
19	Jolfa	Iran	32° 38'	51° 39'
21	Italia	Italy	41° 52'	12° 34'
24	Unknown5	Iran	-	-
25	Azar-sharghi	Iran	38° 04'	46° 17'
27	Golpayegan	Iran	33° 27'	50° 16'
28	Koohpaieh	Iran	32° 42'	52° 26'
29	Gonabad	Iran	34° 21'	58° 42'
30	Kashan	Iran	33° 59'	51° 26'
31	Gorgan	Iran	36° 50'	54° 25'
32	Divandarreh	Iran	35° 54'	47° 01'
33	Turkey	Turkey	38° 57'	35° 14'
34	Marand	Iran	38° 25'	45° 46'

were imported from Turkey and Italy which have cultivated for a long time in Iran. We tried to select at least one population from different province of Iran to cover the all around country diversity. AFLP marker was used, because of its high reproducibility and high power to detect polymerphism. The population with high genetic distance can be used to make synthetic variety and reach high heterosis.

Materials and methods

Plant material

The DNA of 8 individuals from each of 26 alfalfa germplasm sources (Table 1) was analyzed in this study. Each population was selected from different province of Iran to cover maximum diversity. 23 out of 26 populations were originated from Iran and the origin of two other populations was Turkey and one is Italy. But the 3 foreign originated populations were cultivated for a long time in Iran.

DNA preparation

Total genomic DNA of each plants representing each germplasm was extracted from young trifoliate leaves (n = 208), using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Saghai-maroof *et al*., (1984). The quantity and quality of DNA were determined by spectrophotometric analysis and agarose gel electrophoresis, respectively. 26 genomic bulked-DNA solutions were prepared by mixing equal quantities of DNA from each of 8 plants representing each germplasm. All samples were stored at –20°C.

AFLP analysis

The AFLP analysis was performed as described by Vos *et al*., (1995) with minor modifications. All reagents, restriction enzymes and their buffers were supplied by Fermentase (Germany). Restriction reaction for each germplasm were prepared in 15 µL volume reaction containing 50 ng of bulked genomic DNA, 4 µL Restriction-Ligation buffer, 5 U of *Eco*RI and 2 U of *Tru1*I enzyme. Tubes were leaved overnight in incubator at 37ºC. 5 µL of 4X ligation master mix were added to each digested reaction tube. 4X ligation master mix containing 1 µL of dd H_2O, 1 µL of 10x T4 DNA ligase buffer with ATP, 1 µL of each *Tru1*I and *Eco*RI adaptors and 1 U of T4 DNA ligase enzyme. Ligation reaction was performed at 16ºC for 2 hours followed by 20 h at room temperature. The digested and ligated DNA were then diluted by the addition of 50 µL of dd H_2O and pre-amplified using *Eco*RI and *Mse*I primers with one additional selective nucleotide (Table 2).

Pre-amplification was performed in a total volume of 25 µL containing 2.5 µL of 10x PCR buffer, 0.6 mM dNTP, 4mM $MgCl_2$, 800 nM of each *Eco*RI+A and *Mse*I+C primers, I U of Taq DNA polymerase and 4 µL of the diluted digested and ligated DNA. The temperature profile for pre-amplification was as follows; 2 min at 72ºC, 20 cycles: 30 sec at 94ºC for denaturing, 1 min at 60ºC for annealing and 2 min at 72ºC for extension and finally a hold 5 min at 72ºC before storing the sample at 4ºC. For selective amplification, the product of pre-amplification was diluted by addition of 120 µL dd H_2O.

Selective amplifications were performed with same protocol was used for pre-amplification except the selective primers were altered (Table 2). The *Eco*RI selective primer (ACC) which labeled fluorescently with TAMRA was used in combination with eight *Tru1*I selective primers. The PCR program for the selective amplification procedure conducted with the following cycle profile: 2 min at 94ºC for pre-heating, 15 cycles: 30 sec at 94ºC for denaturing, 30 sec at 63ºC (touchdown 1ºC per cycle to 54ºC) for annealing, 2min at 72ºC for extension, followed by 23 cycles: 30 sec at 94ºC for denaturing, 30 sec at 54 ºC for annealing and 2 min at 72ºC for extension and finally a hold 2 min at 72ºC before storing the sample at 4ºC.

Table 2. Oligonucleotide sequences for adaptors and primers used for AFLP analyses

Restricton enzyme	Sequence
Adaptors	
*Eco*RI	5ó -CTCGTAGACTGCGTACC-3ó 3ó -CATCTGACGCATGGTTAA-5ó
*Mse*I	5ó -GACGATGAGTCCTGAG-3ó 3ó -TACTCAGGACTCAT-5ó
Preselective primers	
*Eco*RI	5ó -GACTGCGTACCAATTCA-3ó
*Mse*I	5ó -GATGAGTCCTGAGTAAC-3ó
Selective primers	
*Eco*RI	5ó -GACTGCGTACCAATTCACC-3ó
*Mse*I	5ó -GATGAGTCCTGAGTAACNN-3ó *

*NN represents the selective dinucleotides AA, TG, AG, AT, AC, TT, TA, and TC

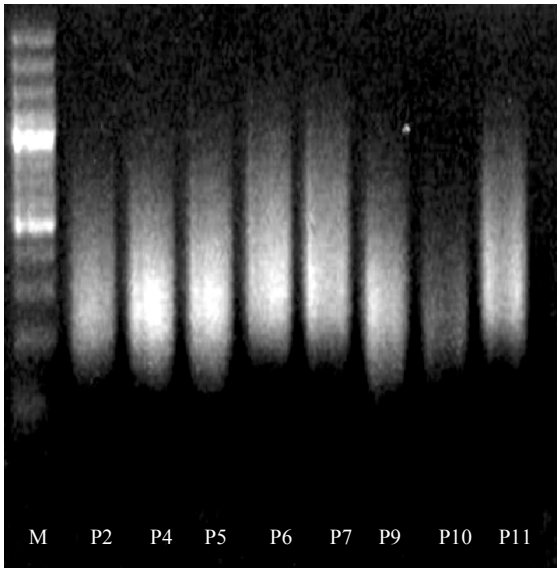

Fig 1. Eelectrophoresis pattern of population 2 by eight primer combinations. (M: size marker; P2…..P11: primer combination). Agarose gel was used before selective amplification to verify fidelity of preamplfied PCR products.

Gel analysis

PCR product is mixed with 25% (w/v) of denaturing loading dye and denatured at 94ºC for 3 min and cool on ice immediately. 2 µL of each reaction mix were used for electrophoresis on a 5% (w/v) denaturing polyacrylamide gel with 7 M urea in 20 cm length in Gelscan 2000 (Corbett Co., Australia) using manufacturer's recommendations. The output files were saved as Tiff format file for scoring and further analysis.

Data analysis

Electropherogram produced by the ONE-Dscan v2.03 software program, evaluated visually to distinguish polymorphic fragments. Only sharp and precise bands were scored as 1 for present and 0 for absent as a data matrix. NTSYSpc v.2.02e (Rohlf, 1998) was used to analyze binary matrix and pairwise genetic similarity was estimate. Calculation of similarity was performed using Jaccard's

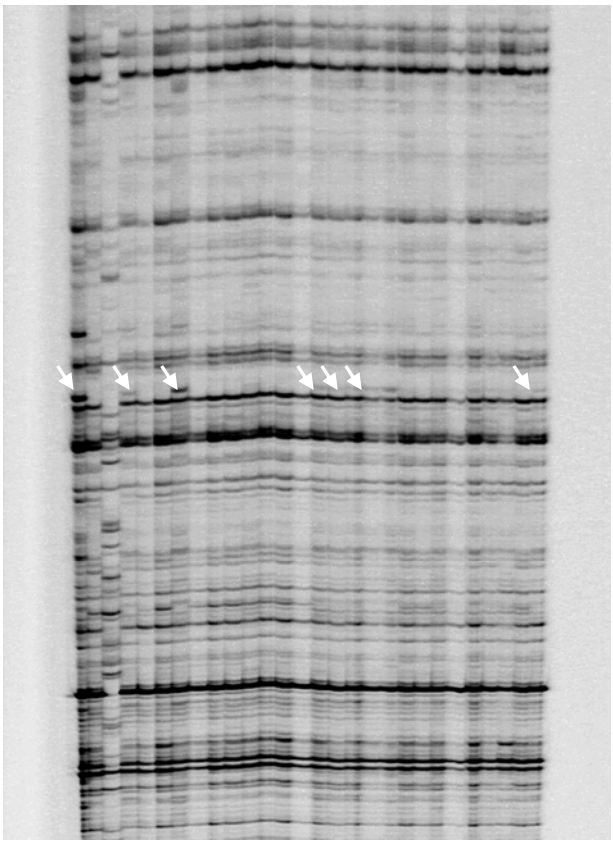

Fig 2. Electrophoresis pattern obtained in the AFLP combination primer M-CAC/E-ACC in 26 populations of alfalfa (*Medicago sativa* L.) [1……26: different populations]. Arrows show the one polymorphic band. The population which has band (arrow) got 1 and those without band (arrow) got 0 in scoring. The same scoring system was performed for other polymorphic bands.

Table 3. Selective primer sequence, number of scored polymorphic fragment and polymorphic information content (PIC)

Selective primer sequence	Number of scored polymorphic fragments	PIC
M-CAA/E-ACC	6	0.23
M-CTG/E-ACC	4	0.19
M-CAG/E-ACC	5	0.37
M-CAT/E-ACC	5	0.28
M-CAC/E-ACC	4	0.20
M-CTT/E-ACC	3	0.20
M-CTA/E-ACC	6	0.28
M-CTC/E-ACC	6	0.32
Average	4.87	0.26

coefficient of similarity (J_{ij}) where $J_{ij} = a/(n - d)$, and a is the number of fragments in common between two germplasms, i and j, n is the total number of fragments scored, and d is the number of fragments absent in both germplasms. Similarity estimates were converted to genetic distance (D) where $D = -\ln J$, according to Swofford and Olson (1990). The similarity matrix was run on Sequential, Agglomerative, Hierarchical and Nested (SAHN) clustering, (Sneath and Sokal, 1973) by

Table 4. Comparison of different methods for constructing dendrogram

Cophenetic coefficient (r)	Dice (Nie & Li)	Jaccard	Simple Matching
UPGMA	r = 0.85	r = 0.86*	r = 0.74
Complete Linkage	r = 0.82	r = 0.82	r = 0.75
Single linkage	r = 0.75	r = 0.70	r = 0.50

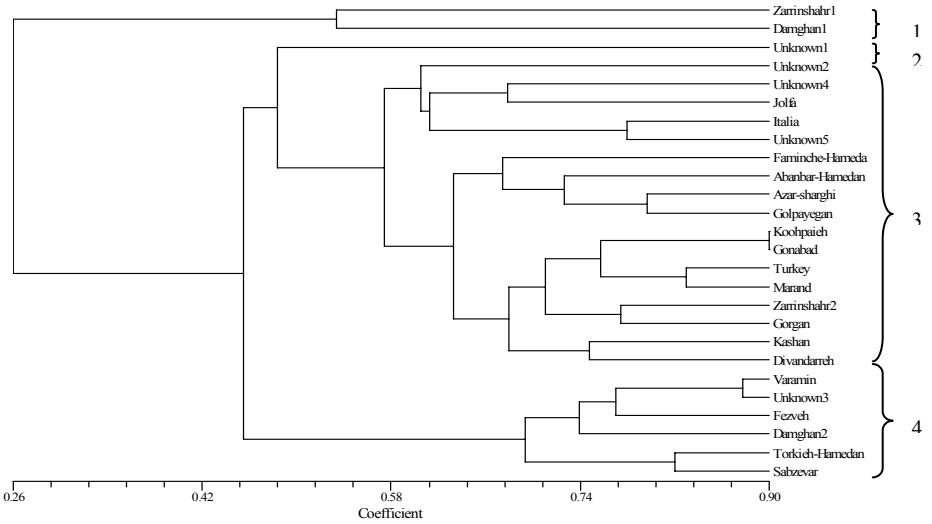

Fig 3. Dendrogram showing the genetic relationship among 26 populations of *Medicago sativa* based on AFLP data, UPGMA clustering method and Jaccard's coefficient. 26 populations were divided in 4 different clusters. There is no high correlation between genetic distance and geographical distance of population. The lowest genetic distance was recorded between Koohpaieh and Gonbad populations in cluster 3 and highest genetic distance was between Divandarreh from cluster 3 and Damghan1 from cluster 1 population.

using the Unweighted Pair Group Method with Arithmetic average (UPGMA) clustering algorithm (Sokal and Michener, 1958) to generate a dendrogram. The MXCOMP subroutine was used to calculate a cophenetic correlation matrix between the similarity matrix and original matrix to measure goodness-of-fit. SAS v.9.1 was used to distinguish the best cut line using CCC plot, pseudo F and T^2 parameters. Principal Coordinate Analysis (PCO) was conducted by using GenAlex v.6.2. This multivariate approach was chosen to complement the cluster analysis information, because cluster analysis is more sensitive to closely related individuals, whereas PCO is more informative regarding distances among major groups (Hauser and Crovello, 1982).

Allelic polymorphic information content (PIC) was calculated using the formula

$$PIC = 1 - \Sigma(P_{ij})^2,$$

where P_{ij} is the frequency of the *i*th allele in the *j*th population, for each locus (Botstein *et al*., 1980).

RESULTS

AFLP profiling of 26 alfalfa genotypes with eight primer combinations revealed a total 39 scorable and polymorphic band ranging in size from 100-800 nucleotides. The eight primer combinations were screened for their ability to generate AFLP polymorphic DNA bands using the accessions total cellular DNAs. Based on the band patterns, the AFLP primer combinations M-CAA/E-ACC, M-CTA/E-ACC and M-CTC/E-ACC generated the highest (6 fragments) number of polymorphic bands and the lowest (3 fragments) were generated by primer combinations M-CTT/E-ACC (Table 3). Based on the results (Table 3) the highest polymorphic information content (PIC) was related to primer combination M-CAG/E-ACC which introduces it as a most informative primer combination to study genetic diversity between alfalfa populations in next studies. Fig. 1 shows typical example of pre-amplification of PCR products on agarose gel with DNA stretching ranging from 100 – 1000 bp and Fig. 2 shows typical examples of the amplified AFLP banding pattern M-CAC/E-ACC primer combination on acrylamide gel. In order to distinguish the best clustering and similarity coefficient calculation methods, the cophenetic correlation, a measure of the correlation between the similarity represented on the dendrograms and the actual degree of similarity, was calculated for each method combination. Among different methods, the highest value (r =0.86) was observed for UPGMA clustering method based on Jaccard's similarity coefficient (Table 4). Therefore, the dendrogram constructed based on this method was used for depicting genetic diversity of genotypes (Fig. 3). Cluster analysis (Fig. 3) divided the 26 populations into four groups. Group 1 contains two populations as zarinshah1 and Dameghan1. Group 2 contains one populations named as Unknown1 which was placed in a separated cluster with very low similarity to other groups. All other alfalfa populations were placed in group 3 and 4. Group 3 contains 17 populations as Unknown 2, Unknown 4, Jolfa, Italia, Unknown5, Faminche-Hamedan, Abanbar-Hamedan, Azar-sharghi, Golpaygan, Koohpaieh, Gonabad, Turkey, Marand, Zarinshahr2, Gorgan, Kashan and Divandarreh. In group 3 the highest similarity value was observed between Koohpaie and Gonabad populations. Group 4 contains 6 populations as Varamin, Unknown 3, Fezveh, Dameghan2, Torkieh-Hamedan and Sabzevar. Principal coordinate analysis (PCO) based on genetic similarity metrics was used to visualize the genetic relationships among species. The first two eigenvectors accounted for 58.60% of the total molecular variation. Therefore, PCO results confirmed the results of cluster analysis (Fig. 4). The genetic distances between studied populations were represented in Table 5. The highest genetic distance was recorded between Divandareh and

Table 5. Genetic distances between different genotypes (The numbers represents the population number in Table 1)

	2	4	5	6	7	9	10	11	13	14	15	16	17	18	19	21	24	25	27	28	29	30	31	32	33	34
2	0.00																									
4	1.00	0.00																								
5	0.63	1.56	0.00																							
6	0.64	0.96	1.20	0.00																						
7	1.61	1.10	2.30	0.81	0.00																					
9	1.18	0.74	1.65	0.61	0.76	0.00																				
10	0.75	1.10	1.15	0.33	0.90	0.57	0.00																			
11	0.60	1.06	1.10	0.36	0.88	0.58	0.31	0.00																		
13	0.83	0.83	1.39	0.22	0.52	0.34	0.53	0.43	0.00																	
14	0.64	0.96	1.44	0.13	0.81	0.61	0.33	0.24	0.34	0.00																
15	0.96	1.15	1.44	0.38	0.93	0.50	0.19	0.48	0.57	0.38	0.00															
16	0.44	1.10	0.94	0.19	1.03	0.69	0.27	0.31	0.41	0.33	0.46	0.00														
17	1.42	0.82	1.72	0.81	0.44	0.46	0.65	0.77	0.52	0.81	0.69	0.90	0.00													
18	1.39	0.55	2.08	0.83	0.73	0.32	0.94	0.92	0.50	0.83	0.83	0.94	0.41	0.00												
19	1.10	0.65	1.57	0.65	0.59	0.62	0.74	0.86	0.36	0.78	0.78	0.74	0.39	0.34	0.00											
21	1.69	0.79	2.16	0.92	0.49	0.73	0.88	0.99	0.69	0.92	0.92	1.02	0.49	0.57	0.43	0.00										
24	1.79	0.92	2.27	1.03	0.50	0.84	0.99	1.10	0.80	1.03	1.03	1.14	0.50	0.69	0.55	0.25	0.00									
25	1.27	0.79	2.16	0.65	0.59	0.41	0.74	0.73	0.36	0.65	0.78	0.74	0.59	0.45	0.43	0.32	0.45	0.00								
27	1.01	0.69	1.75	0.43	0.73	0.43	0.65	0.65	0.27	0.56	0.69	0.51	0.62	0.48	0.45	0.57	0.69	0.22	0.00							
28	1.35	0.74	1.91	0.73	0.46	0.39	0.82	0.81	0.45	0.73	0.86	0.82	0.37	0.32	0.41	0.30	0.52	0.30	0.43	0.00						
29	1.47	0.79	2.16	0.78	0.59	0.30	0.88	0.86	0.47	0.78	0.78	0.88	0.49	0.22	0.43	0.43	0.55	0.32	0.45	0.10	0.00					
30	1.53	0.75	2.44	0.89	0.77	0.57	1.01	0.98	0.65	0.89	0.89	1.01	0.77	0.38	0.48	0.48	0.61	0.48	0.65	0.34	0.24	0.00				
31	1.30	0.44	1.99	0.89	0.65	0.57	1.01	0.98	0.53	0.89	1.06	1.01	0.54	0.25	0.36	0.48	0.61	0.36	0.38	0.34	0.36	0.41	0.00			
32	2.04	0.81	3.09	0.96	0.82	0.61	1.10	1.06	0.69	0.96	0.96	1.10	0.69	0.41	0.65	0.38	0.53	0.51	0.55	0.48	0.38	0.29	0.44	0.00		
33	1.06	0.60	1.79	0.48	0.66	0.36	0.56	0.57	0.43	0.48	0.61	0.56	0.55	0.41	0.61	0.50	0.62	0.38	0.29	0.26	0.27	0.43	0.43	0.46	0.00	
34	1.53	0.59	2.44	0.74	0.77	0.45	0.85	0.83	0.65	0.74	0.74	0.85	0.54	0.25	0.61	0.48	0.61	0.48	0.38	0.34	0.24	0.41	0.41	0.29	0.18	0.00

The number is based on Table 1 for each population.

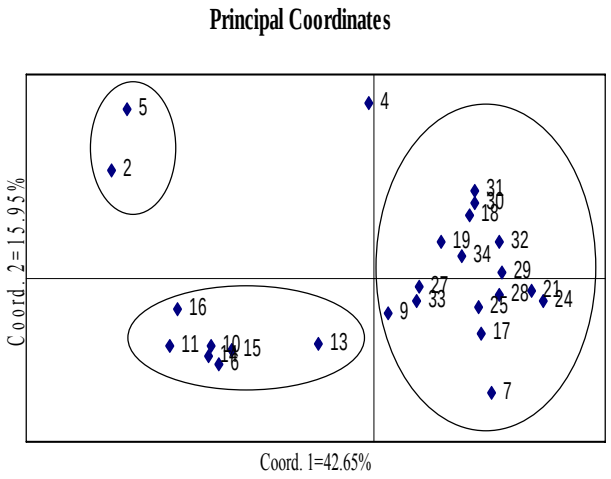

Fig 4. The scatter plot of the first and second principal coordinates analysis on 26 Alfalfa populations based on the AFLP obtained with eight primer combinations. The number is genotype name presented in Table 1. The grouping result by PCO completely revealed Cluster analysis results.

Damghan 1, and lowest one between Koohpaieh and Gonbad population. The average genetic distance is 0.63, 0.47 and 0.31 in cluster 1, cluster 3 and cluster 4, respectively.

Discussion

Since only a wide genetic base gives the opportunity to select genotypes with a trait of interest, it is essential to understand the extent and distribution of genetic variation. This information is particularly important in alfalfa which is an allogamous and self-incompatible species susceptible to severe inbreeding depression. Decreased heterozygosity and heterogeneity of populations will decrease vigor and productivity (Tucak *et al*., 2008). The ability of DNA-based markers such as AFLP as a reliable technique for assaying genetic variation among plant species has widely been reported (Blears *et al*., 1998). This technique is more informative and reproducible compared to previously used biochemical and molecular methods such as isozyme and RAPD markers in detecting genetic relationships of alfalfa genotypes (Tucak *et al*. 2008; Touil *et al*., 2008) In addition to the AFLP marker, several molecular markers are used to identification and study of genetic diversity of alfalfa, as SSR and RAPD markers are very much used for medicago genus. Julier *et al*., (2003) was used a set of 107 SSRs identified in the EST database of *Medicago truncatula* to map in *M. sativa* and can be used to perform genetic diversity analysis. The cultivated alfalfa (*Medicago sativa* L.) is characterized by a great genetic variability which makes it able to adapt to very contrast mediums of hottest to cold. Data on the genetic diversity of alfalfa population with different geographical origin are presented in this study. The measures of relative genetic distances among populations did not completely correlate with geographical distances of places of their origin. For instance, Jolfa and Italy with high geographical distances grouped in cluster 3 together, also Azar-sharghi and Gonbad populations with enough geographical distance grouped in cluster 3. The same results was obtained on alfalfa (Tucak *et al*., 2008; Touil *et al*., 2008), *Bunium persicum* (Pezhmanmehr *et al*., 2010), *Daucus carota* (Bradeen *et al*., 2002), *Phaseolus vulgaris* (Martins *et al*., 2006), *Matricaria chamomilla* (Solouki *et al*., 2008) and Grapevine (Theocharis *et al*., 2010). This study provides evidence that AFLP marker is an informative and suitable approach to evaluation of molecular polymorphism and polygenic relationships in cultivated alfalfa (*Medicago sativa* L.). The results showed that lowest genetic distance was recorded between Koohpaieh and Gonbad populations both in cluster 3 and highest genetic distance was calculated between Divandarreh from cluster 3 and Damghan1 from cluster 1 population. This information can be more useful to make synthetic variety in order to select population with higher genetic distance to reach more heterosis. Work is currently in progress to improve the primer combinations in order to have a deeper insight into the genetic diversity in molecular level and establish varietal identification key in this crop.

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