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Genetic Diversity Assessment of Alfalfa Populations Using AFLP Markers

[1]Mohammad Keivani, [2]S. Sanaz Ramezanpour, [2]Hassan Soltanloo,
[3]Rajab choukan, [4]Mohammadreza Naghavi and [4]Mojtaba Ranjbar

[1]Department of Agronomy and Plant Breeding,
Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Plant Breeding and Biotechnology,
Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[3]Department of Maize and Forage Crop, Seed and Plant Improvement Institute (SPII), Karaj, Iran
[4]Department of Agronomy and Plant Breeding, Tehran University, Karaj, Iran

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 combination. M-CAG/E-ACC primer combination showed the highest PIC which can be a candidate good 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 lowest genetic distance was observed between Koohpaie and Gonabad populations and the highest between Dameghan1 (cluster 1) and Divandarreh (cluster 3) populations.

Key word: *Medicago sativa* · Genetic distance · AFLP

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 [1]. Cultivated alfalfa is autotetraploid (2n = 4x = 32) [2], 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 [3, 4]. Genetic variation is the basis for breeding programs; therefore, it is important to identify genetically distinct plants for breeding purposes [5].

Identification based on morphological characters is time consuming and requires extensive field trials and evaluation [6], while morphological differences may be epigenetic or genetic based characters [7, 8, 9]. During last three decades genetic diversity was studied in plants through isoenzymes [10]. The development of molecular (DNA) marker provides new dimension, accuracy and perfection in the screening of germplasm [11]. Efficient and quick screening of such genotypes speedup the process of varietal evaluation, thus molecular marker plays pivotal role in this regard.

Different molecular markers have been used to assess genetic diversity in alfalfa such as restriction fragment length polymorphism (RFLP) [12, 13], random amplified polymorphic DNA (RAPD) [14-17], simple sequence repeat (SSR) [18, 19, 4], sequence related amplified polymorphisms (SRAP) [20] and amplified fragment length polymorphism (AFLP) [21].

AFLP markers are highly polymorphic and reproducible and thus represent a powerful technique for DNA analysis that has revolutionized fingerprinting and diversity studies [22]. AFLP analysis detects genetic variation throughout the genome by using a pair of specific restriction enzymes and their corresponding

Corresponding Author: Mohammad Keivani, Department of Agronomy and Plant Breeding,
Science and Research Branch, Islamic Azad University, Tehran, Iran.

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 [23].

In this study, genetic variability of 26 alfalfa populations which cultivated in different area of Iran was evaluated. AFLP marker was used, because of its high reproducibility and high power to detect polymorphism. The population with high genetic distance can be used to make synthetic variety and reach high heterosis.

MATERIALS AND METHODS

DNA Extraction and AFLP Analysis: Genomic DNA of each plant of 8 genotypes from each of 26 alfalfa germplasm sources (Table 1) was extracted from young trifoliate leaves, using (CTAB) method according to Saghai-maroof *et al*. [24].

AFLP analysis was performed as described by Vos *et al*. [22] with minor modifications. All reagents, restriction enzymes and their buffers were supplied from 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 37°C and 5 µl of 4X ligation master mix were added to each digested reaction tube. The 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 16°C for 2 h followed by 20 h at room temperature. The digested and ligated DNA were 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, 4 mM $MgCl_2$, 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 72°C, 20 cycles: 30 sec at 94°C for denaturing, 1 min at 60°C for annealing and 2 min at 72°C for extension and 5 min at 72°C. For selective amplification, the product of pre-amplification was diluted by addition of 120 µl dd H_2O.

Selective amplifications were performed with the same protocol and were 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

Table 1: List of populations used in this study

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

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

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

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

amplification procedure conducted with the following cycle profile: 2 min at 94°C for pre-heating, 15 cycles: 30 sec at 94°C for denaturing, 30 sec at 63°C (touchdown 1°C per cycle to 54°C) for annealing, 2min at 72°C for extension, followed by 23 cycles: 30 sec at 94°C for denaturing, 30 sec at 54 °C for annealing and 2 min at 72°C for extension and 2 min at 72°C.

Gel Analysis: PCR product is mixed with 25% (w/v) of denaturing loading dye and denatured at 94°C for 3 min and cool on ice immediately. A 2 µl of each reaction mix were used for electrophoresis on 5% (w/v) denaturing polyacrylamide gel with 7 M urea in 20 cm length in Gelscan 2000 (Corbett Co., Australia) using manufacturer's recommendations.

Data Analysis: Electropherogram produced by software ONE-Dscan v 2.03, 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 [25] was used to analyze binary matrix and pairwise genetic similarity was estimate. Similarity was calculated using Jaccard's 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 [26]. The similarity matrix was run on Sequential, Agglomerative, Hierarchical and Nested (SAHN) clustering, [27] by 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 [29].

Allelic polymorphic information content (PIC) was calculated using the formula $PIC = 1 - \Sigma(P_{ij})^2$, where P_{ij} is the frequency of the ith allele in the jth population, for each locus [30].

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).

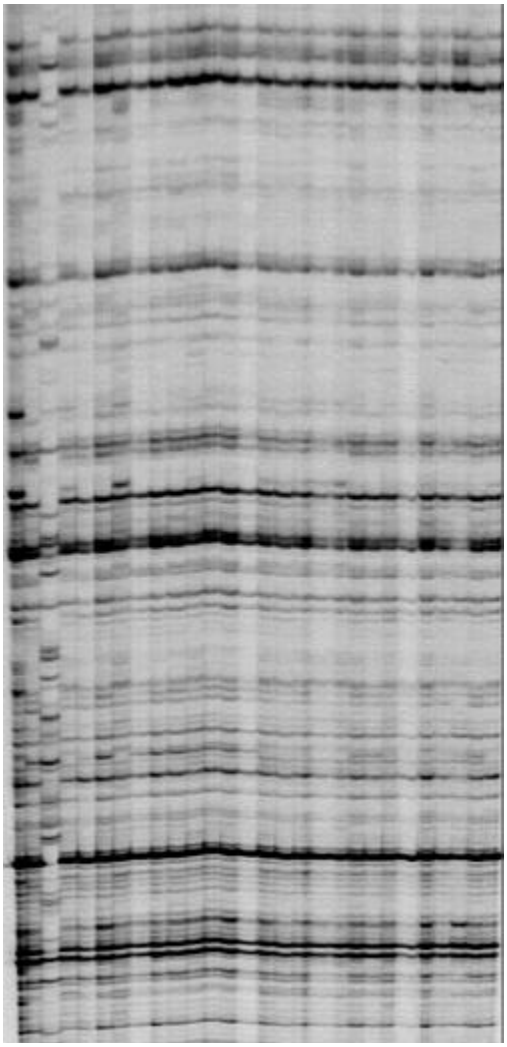

Fig. 1: Electrophoresis pattern obtained in the AFLP combination primer M-CAC/E-ACC in 26 populations of alfalfa (*Medicago sativa* L.) [1……26: different populations based on Table 1]

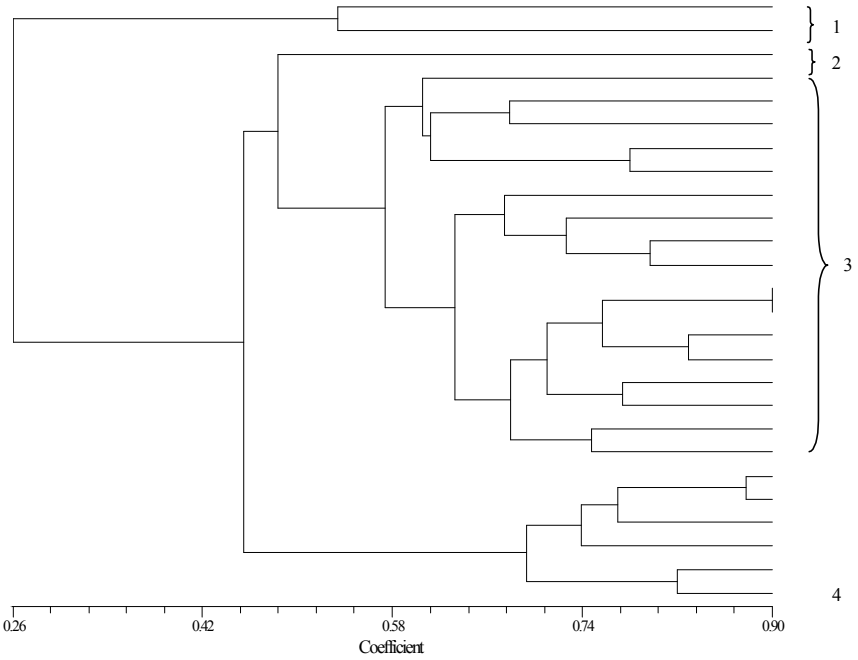

Fig. 2: Dendrogram showing the genetic relationship among 26 populations of Medicago sativa based on AFLP data, UPGMA clustering method and Jaccard's coefficient

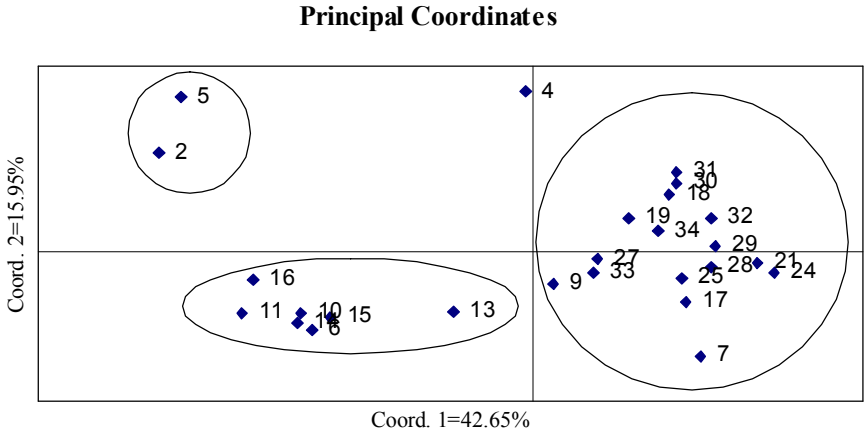

Fig. 3: Relationships among the alfalfa population revealed by principal coordinate analysis based on AFLP genetic similarity. The number is genotype name presented in Table 1

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.

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

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

Selective primer sequence	Number of scored polymorphic fragments
M-CAA/E-ACC	6
M-CTG/E-ACC	4
M-CAG/E-ACC	5
M-CAT/E-ACC	5
M-CAC/E-ACC	4
M-CTT/E-ACC	3
M-CTA/E-ACC	6
M-CTC/E-ACC	6
Average	4.87

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

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

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. 2).

Cluster analysis (Fig. 2) 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. 3).

The genetic distances between studied populations were represented in Table (5). The highest genetic distance was recorded between Divandareh and 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 [14].

The ability of DNA-based markers such as AFLP as a reliable technique for assaying genetic variation among plant species has widely been reported [31]. 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 [14, 19].

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*. [32] 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 [14, 19] *Bunium persicum* [33]*, Daucus carota* [34], *Phaseolus vulgaris* [35] *and Matricaria chamomilla* [36].

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 3and highest genetic distance was calculated between Divandarreh from cluster 3 and Damghan1 from cluster 1 populations. 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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