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Determination of Isoenzyme Variation in Some *Medicago sativa* Ecotypes

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This study was carried out in order to determine variation of *Medicago sativa* ecotypes, using Polyacrylamide Slab Gel Electrophoresis (PAGE) on the 5 enzyme systems (PRX, MDH, PPO, SOD, GDH). Differences between ecotypes were analyzed statistically. The highest genetic distance was between Dilburnu, Diableverde and the least was between Dilburnu, Çayirbasi. The results from isoenzyme analysis correlated with some morphological characteristics. This study of *Medicago sativa* ecotypes suggests that the geographical and ecological distribution of the plants contributes to higher genetic similarity.

Key Words: *Medicago sativa*, Ecotypes, Isoenzymes variation.

INTRODUCTION

Information about alfalfa germplasm diversity in cultivated plant species is relevant for plant breeding. In particular, it is often necessary to distinguish between germplasm sources of different origin, especially when they are individually registered by a seed certification board. The knowledge of the extent of genomic variability and relationship in alfalfa cultivars and ecotypes bearing desirable agronomic traits is an area that could benefit from detailed molecular genetic analyses[1,2].

Genetic system is under genetic control may be modified by selection so that different populations of the same species may vary in genetic system and structure. Breeders are more concerned with specific adaptations which can be incorporated into existing adaptability of the crops. It is also expensive to incorporate extensive collection of exotic plant germplasm into breeding programs directed towards the improvement of quantitatively inherited agronomic traits. A pooling of accessions based on similarity of geographic origin or on a high degree of genotypic resemblance in early screening trials would reduce these expenses[3].

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Alfalfa ecotypes (Land races) may cover large geographical regions and the problem arises as to whether they still conform to the definition of ecotype. This situation requires the development of a method for the unambiguous and reproducible identification of alfalfa ecotypes. Numerous studies[2,4-9] have been conducted to culture *Medicago sativa* L.

Bulking DNA of fresh tissue from several plants allows one to identify markers linked to any specific trait in a heterogeneous population segregating for the trait of interest[10]. The genetic variations are crucial for the success of selection programs. However, these variations could be genetically regulated. Different methods such as DNA isoenzyme and protein electrophoresis should be applied to determine how much the observed differences stem from the gene's structure. Isoenzymes have been used in evaluation studies[11-14]. Protein benefiting, each variety have their own biochemical properties. Separation of proteins by electrophoresis is a common method as a genetic pointer or indicator. Determination of genetic variety in the gene pool is used for characterization of breeding lines which have different biochemical features but of the same morphological properties[15-17]. Because of allogamy and tetraploidy, a high degree of genetic diversity is expected among individual plants in alfalfa populations. It could also be relevant to assess the extent of this diversity and to elaborate on how it could supplement studies of population genetics in this species. However, studies on isoenzymes variation *Medicago sativa* ecotypes have not been carried out.

The aims of the study were: (1) Description of electrophoresis isoenzyme phenotypes and their variation patterns among some *Medicago sativa* ecotypes and (2) Evaluation of possibilities to use isoenzymes as molecular characters to discriminate between *Medicago sativa* ecotypes.

EXPERIMENTAL

Plant materials in this research were used 14 ecotypes (genotypes) *i.e.*, Adigüzel, Ahlat, Alaköy, Burcu, Çayirbasi, Dilburnu, Dönemeç, Gülgören, Gülsinberk, Hidirköy, Köprüler, Mahmudiye, Mollakasim, Otluca and four cultivars were Arrow, Kayseri, Diableverde and L-1312 (Table-1). This material was cultivated at the experimental field of the Field Crops Department, Faculty of Agriculture, Atatürk Üniversity, Erzurum, Turkey (39º55′N lat. 41º16′E long and 1950 m above see level). The trial site had a silty clay loam soil type. The experimental location is arid characterized by dry cool temperature summers and 187 mm rainfall during April-August, 46.5 % of the annual average rainfall (Table-2). All plots were established spring 2002 in randomized complete block with three replicates. Analyses were carried out on mature plant leaves from field conditions[18].

TABLE-1
DESCRIPTION OF SEED SOURCES USED IN PRESENT STUDIES

Acces. No.	Region	Designation	Source	Altitude*
1	Eastern Anatolia	Mahmudiye	Agr. Res, St. Erzurum	1650
2	Cultivar	Arrow	USA	–
3	Eastern Anatolia	Dilburnu	Agr. Res, St. Erzurum	1700
4	Eastern Anatolia	Cayirbasi	Agr. Res, St. Erzurum	1680
5	Eastern Anatolia	Gulsinberk	Agr. Res, St. Erzurum	1570
6	Eastern Anatolia	Otluca	Agr. Res, St. Erzurum	1600
7	Eastern Anatolia	Hidirkoy	Agr. Res, St. Erzurum	1800
8	Eastern Anatolia	Adiguzel	Agr. Res, St. Erzurum	1805
9	Eastern Anatolia	Donemec	Agr. Res, St. Erzurum	1820
10	Cultivar	Kayseri	Turkey	–
11	Eastern Anatolia	Mollakasim	Agr. Res, St. Erzurum	1780
12	Eastern Anatolia	Ahlat	Agr. Res, St. Erzurum	1710
13	Eastern Anatolia	Kopruler	Agr. Res, St. Erzurum	1750
14	Cultivar	Diyabre verde	USA	–
15	Eastern Anatolia	Gulgoren	Agr. Res, St. Erzurum	1800
16	Eastern Anatolia	Alakoy	Agr. Res, St. Erzurum	1670
17	Cultivar	L-1312	USA	–
18	Eastern Anatolia	Burcu	Agr. Res, St. Erzurum	1850

*Where seed collected area.

TABLE-2
CLIMATIC DATA OF THE RESEARCH LOCATION,
LONG-TERM AVERAGE (LTA) (75 YEARS, 1929-2004)

Months	Mean temp. (º C)				Relative humidity (%)				Rainfall (mm)			
	2002	2003	2004	LTA	2002	2003	2004	LTA	2002	2003	2004	LTA
Jan.	-16.1	-7.7	-9.0	-8.8	72.4	77.6	76.9	76	14.0	17.7	14.3	22.5
Feb.	-3.4	-8.2	-8.7	-7.6	72.6	73.3	77.8	76	8.9	30.7	90.0	26.5
Mar.	-1.0	-6.6	-1.7	-2.8	71.3	75.8	69.7	74	37.4	32.9	33.7	35.0
Apr.	4.2	4.4	4.0	5.3	67.1	65.2	58.0	64	81.2	81.4	36.0	51.9
May	9.8	11.6	9.7	10.6	55.8	52.0	63.5	60	73.1	29.9	121.7	70.5
Jun.	14.3	14.5	14.5	14.9	57.0	50.6	52.8	56	74.0	45.7	40.7	47.9
Jul.	18.3	18.9	17.9	19.3	53.0	49.3	41.9	50	39.1	18.5	2.4	27.4
Aug.	16.6	20.0	19.6	19.4	53.6	42.7	41.1	47	54.6	5.1	1.3	17.1
Sep.	13.6	13.8	13.8	14.7	52.9	46.3	40.9	50	52.9	19.3	6.0	24.4
Oct.	8.9	8.8	17.2	8.1	61.9	64.1	59.2	61	61.9	90.9	27.4	44.6
Nov.	1.3	-0.7	-0.5	1.1	69.4	74.5	71.9	72	69.4	36.1	43.6	33.9
Dec.	-12.0	-6.6	-14.1	-5.6	73.5	71.3	78.0	76	73.5	16.1	8.2	22.9

Protein extraction: Individual samples were extracted in 0.05 M sodium phosphate (pH 6.5) buffer for peroxidase (PRX) and polyphenol oxidase (PPO). Malate dehydrogenase (MDH), superoxide dismutase (SOD), glutamate (GDH) was extracted with 0.2 M *Tris*-HCl buffer (pH 7.5)[19]. Extractions were made at 4 ºC for 1 h period. Samples were filtered and centrifuged at 22.075 g for 15 min at ± 0 ºC. The supernatant was stored at 4 ºC for electrophoresis.

Gel electrophoresis: The polyacrylamide gel electrophoresis (PAGE) process was conducted in an OWL dual vertical slab gel apparatus. Resulting gels were 12.5 (h) × 20 (w) × 0.75 (t) cm and contained 7 % acrylamide, while the stacking gels were 2.5 (h) × 20 (w) × 0.75 (t) cm and contained 2.5 % acrylamide. Reservoir buffer contained 0.025 M *Tris* and 0.133 M glycine at a pH 8.3. Protein supernatant was placed in the stacking gel sample wells, followed by 20 µL of reservoir buffer containing bromophenol blue, which served as the tracking dye electrophoresis was done at 2-4 ºC for 0.5 h at a constant voltage of 80 V and followed by 4.5-5.0 h at a voltage of 150 V until the tracking dye was *ca.* 1 cm from the gel bottom[20].

Staining procedure: The staining solution for MDH contained: 100 mL 0.2 M *Tris*-HCl (pH 7.5), 3 mL 1.0 M D-L malate (pH 7.5), l2 mg β-nicotine with adenine dinucleotide (NAD), 15 mg 3-(4-5-dimethylhiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), 2 mg phenozin metasulfate (PMS). Gels were stained at 37 ºC in the dark until the bands developed. After the bands became visible, the gel was rinsed with distilled water and stored in 3 % acetic acid solution[19].

The staining solution for PRX enzyme consisted of 20 mL 0.6 % hydrogen peroxide, 20 mL stock benzidine (2 g benzidine in 18 mL acetic acid was solved by heating slowly. Then 72 mL dd H_2O was added), 70.4 mg ascorbic acid and 60 mL distilled water. The gels were stained at room temperature until the bands developed. After the bands become visible the reaction was halted with 10 % acetic acid solution and left in 3 % acetic acid solution.

The staining solution for PPO contained 0.3 M dihydroxyphenyl alanine (DOPA) (DOPA resolved 1 % KOH) and 0.1 M sodium phosphate buffer. Gels were stained at 37 ºC in the dark until the bands developed[20].

The staining solution for MDH contained GDH/SOD 75 mL 0.1 M *Tris*-HCl (pH 8), L-glutamic acid 2 g, l2 mg β-nicotine with adenine dinucleotide (NAD), 15 mg 3-(4-5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), 2 mg phenozin metasulfate (PMS). Gels were stained at 37 ºC in the dark until the bands developed. SOD bands are white against the blue background[19].

Statistical analyses: Band frequencies were calculated for each ecotype and for each isoenzyme system. The intra-ecotype variation was estimated

by Jaccard diversity index (1908). Based on the matrix of band frequencies the variation among ecotype was examined by cluster analysis, and UPGMA method using program package NTSYS-PC version 2.02 k (Applied Biostatistics Inc. 1986-1998) which was utilized based on Euclidean distances. All of the experiments in this study are repeated at least twice.

RESULTS AND DISCUSSION

Peroxidase (PRX): The observation band pattern for PRX enzyme revealed fifth bands on the gel. The first band was observed at Mahmudiye, Dönemeç, Kayseri, Mollkasim, Ahlat, Diableverde, Gülgören, Alaköy, L-1312 and Burcu ectypes. Second band exist only Gülsinberk, Dönemeç, Ahlat and Köprüler ecotypes. Third band have seen most of the varieties except, Dilburnu, Çayirbasi, Gülsinberk, Otluca ecotypes, Arrow and L-1312 cultivars. On the other hand the fourth band did not existed Dilburnu, Çayirbasi ecotypes, Arrow and Kayseri cultivars. Fifth band was observed almost all ecotypes except with Dönemeç, Mollakasim, and Alaköy ecotypes. Arrow, Dilburnu, and Çayirbasi ecotypes had only one band on the gel observed with this site.

Polyphenol oxidase (PPO): The result of analysis of electrophoresis showed that PPO enzyme in anode and cathode regions on gel formed total of six bands R_f values of these bands were calculated as 0.15, 0.22, 0.38, 0.49, 0.65 and 0.73, respectively. Arrow and Alaköy shoved all bands, on the other hand Mahmudiye had only one band, Dilburnu, Mollakasim and L-1312 had two bands, the others ecotypes had more then three bands, respectively (Table-3).

Glutamate (GDH) enzyme: GDH enzyme on gels formed a total of 4 bands. The R_f values of these bands were calculated as 0.19, 0.32, 0.61, 0.78, respectively. While the Arrow ecotypes was observed in only the first band, Alaköy and Arrow ecotypes was observed in only the third band. All bands existed in Gulsinberk ecotypes. Dilburnu and Burcu ecotypes were only first and second bands (Table-3).

Superoxide dismutase (SOD): Result of electrophoresis analysis showed that a total of 7 bands were formed by SOD enzyme. Their R_f values were 0.07, 0.18, 0.37, 0.47, 0.53, 0.58 and 0.66, respectively. While band 1 existed in all individuals of Mahmudiye, Dilburnu, Gulsinberk, Dönemeç, Gülgören, Alaköy ecotypes, it did not exist in other ecotypes. Second band revealed the most of the ecotypes except Mahmudiye, Dilburnu, and Burcu. The seventh band was observed in only Arrow and Otluca ecotypes. Accordingly, Burcu ecotype had only two bands (Table-4).

Malate dehydrogenase (MDH) enzyme: There were 5 bands formed by MDH enzyme. Their R_f values were 0.17, 0.2, 0.40, 0.42 and 0.44, respectively. All of the ecotypes Gülgören ecotypes did not exist any bund

TABLE-3
BAND PATTERNS FOR PEROXIDASE (PRX), POLYPHENYL OXIDASE (PPO) AND GLUTAMATE (GDH) ENZYMES FORMED RELATIVE MOBILITY VALUES (R_f) (– BAND SEEN ON THE GEL)

Ecotypes/ Cultivar	PRX (R_f)				PPO (R_f)							GDH (R_f)			
	.047	.067	.10	.11	.13	.15	.22	.38	.49	.65	.73	.19	.32	.61	.78
Mahmudiye	–		–	–	–	–						–		–	
Arrow					–	–	–	–	–	–	–	–			
Dilburnu					–	–	–					–	–		
Cayirbasi					–	–	–			–	–		–	–	
Gulsinberk		–		–	–	–	–	–	–			–	–	–	–
Otluca				–	–	–	–	–					–	–	–
Hidirkoy			–	–	–	–			–	–	–	–	–	–	
Adiguzel			–	–	–	–	–	–	–	–		–		–	–
Donemec	–	–	–	–			–	–	–	–			–	–	–
Kayseri	–		–		–	–	–			–	–	–	–		
Mollakasim	–		–	–					–	–			–		–
Ahlat	–	–	–	–	–	–		–	–		–			–	–
Kopruler		–	–	–	–	–	–	–		–		–	–	–	
Diableverde	–		–	–	–	–			–	–	–		–	–	–
Gulgoren	–		–	–	–	–	–			–	–	–			–
Alakoy	–		–	–		–	–	–	–	–	–			–	
L-1312	–		–	–	–			–			–			–	
Burcu	–		–	–	–	–	–		–	–		–	–		

on this enzyme. While, Cayirbasi ecotypes existed in only third band. On the other hand Kayseri cultivar had four bands. A second band existed in Arrow, Otluca, Kayseri and L-1312 ecotypes (Table-4).

A dendogram formed from data based on band pattern to determine the degree of relationship of ecotypes is shown in Fig. 1, using UPGMA as the clustering method. The enzyme results showed the presence of 3 clusters among the *Medicago sativa* ecotypes. Cluster 1 consists of Dilburnu, Çayirbasi, Arrow, Mahmudiye ecotypes. Cluster 2 was divided into 2 sub clusters and Kayseri, Mollakasim, Dönemeç ecotypes formed a sub clusters. Otluca, Hidirköy, Gülsinberk ecotypes formed a sub clusters. Cluster 3 was divided into 2 sub clusters and L1312 and Burcu ecotypes formed a sub cluster. Ahlat, Köprüler, Gülgören, Alaköy, Diableverde ecotypes formed a sub clusters. The highest genetic distance was between Dilburnu, Diableverde and the least was between Dilburnu and Çayirbasi.

18 Ecotypes and cultivars used as material, differences were detected based on the number of band PRX, PPO, GDH, SOD and MDH enzymes formed and their R_f value. There was also variation within ecotypes based on these five enzymes.

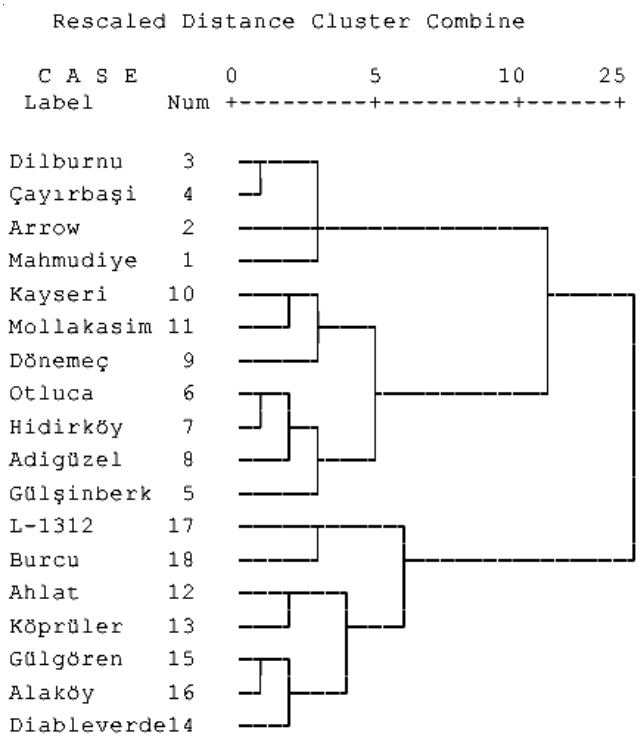

Fig. 1. Dendogram made according to band pattern

The range of this variation was pronounced in some ecotypes based on these enzyme studied in this work. For example, less variation was observed between Arrow, Dilburnu, and Çayirbasi ecotype based on PRX enzyme, Mahmudiye did not revealed any variation based on PPO enzyme, and the list variation based on GDH, SOD and MDH enzymes. The reason for this variation detected within ecotypes may be genetic composition, which is probably due to heterozygosis because of out cross-pollination of alfalfa. Morphological differentiation studied by Small[21] on *Medicago sativa* sis, in relation to ploidy level, population structure studied Maureira *et al.*[22]. Genetic control of alfalfa seed yield and its components examined[7,8,23]. Also genetic variation in alfalfa pointed out by Julier *et al.*[24], forage quality, morphology and seed yield[2]. Rumbaug *et al.*[3] stated that geographic, edaphically and vegetation regions are extremely diverse effect on alfalfa.

Similar and different izoenzyme bands between ecotypes existed based on the five enzymes with R_f value 0.13 band containing PRX enzymes and two bands (R_f value 0.11, 0.13) containing PPO enzyme, and one band (R_f 0.61) containing GDH enzyme, two bands (0.47, 0.61) containing SOD enzyme and one band (0.40) pertaining to MDH enzyme were detected as common bands in the most ecotypes and cultivars.

TABLE-4
BAND PATTERNS FOR SUPEROXIDE DISMUTASE (SOD) AND MALATE (MDH) ENZYMES FORMED RELATIVE MOBILITY VALUES (R_f) (– BAND SEEN ON THE GEL)

Ecotypes/ Cultivar	SOD (R_f)							MDH (R_f)				
	.07	.18	.37	.47	.53	.58	.66	.17	.2	.4	.42	.44
Mahmudiye	–			–			–	–		–		–
Arrow		–		–	–	–			–		–	
Dilburnu	–		–	–	–			–		–		
Cayirbasi		–	–	–			–			–		
Gulsinberk	–	–	–		–					–		–
Otluca		–		–		–			–	–	–	
Hidirkoy		–	–	–			–	–		–		
Adiguzel		–	–	–			–	–			–	
Donemec	–	–		–	–		–	–	–	–	–	
Kayseri		–	–		–		–		–	–	–	–
Mollakasim		–	–	–			–	–	–			
Ahlat		–	–	–				–	–	–	–	
Kopruler		–	–	–	–			–	–		–	
Diableverde		–	–		–		–	–	–	–		–
Gulgoren	–	–			–		–					
Alakoy	–	–	–		–		–	–	–		–	–
L-1312		–		–					–	–	–	
Burcu				–	–		–	–		–	–	

There were major differences between populations with respect to the five enzymes. The studies which used the same population[2,7,8,25] and these studies revealed similar findings. There were significant differences between ecotypes with respect to morphological, phonological and biological and agronomical properties. Forage yields, stem and leaf properties based on close relationships of ecotypes were investigated and the dendogram analysis was similar to that of the studies mentioned above.

In conclusion, variations exist both within ecotypes and between ecotypes based on isoenzyme polymorphism. Therefore isoenzyme profiling could be an efficient way to explore variation of *Medicago sativa*. Differentiation of hybrid and inbred plants can be easily performed in perennial plants such as alfalfa. The results presented here are a contribution to characterization of *Medicago sativa* ecotypes. The isoenzymes technique was shown to be adequate for discrimination of *Medicago sativa* ecotypes (accessions), although the accuracy of these results should also be confirmed with studies using markers such as RAPD, micro satellite, and AFLP. All those application might contribute considerably to reduce the length of time taken in breeding programs.

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