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Analysis of Genetic Diversity in Improved Varieties and Iranian Landraces of Alfalfa Using EST, POX, GOT and MDH Allozyme Markers

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Abstract
Genetic diversity of five improved varieties (Kaysari, Kadi, Ranger, Mesmir, Sea-River) and seven Iranian landraces (Gharayonje, Amozeynadin, Rahnani, Tazekand, Shazand, Hamedani, Yazdi) of alfalfa were assessed using Esterase (EST), Peroxidase (POX), Glutamate Oxaloacetate Transaminase (GOT) and Malate Dehydrogerase (MDH). GOT and MDH were monomorphic, whereas EST and POX showed polymorphic banding patterns. In total, four allozyme loci were detected for EST and POX. There were no significant differences between improved varieties and landraces for allelic frequency in the polymorphic markers. However, average heterozygosity in landraces (0.623) was higher than that of improved varieties (0.594). Genetic distance among populations was less than 0.02. F_{ST} of landraces versus improved varieties was 0.003 showing no differentiation based on the studied marker systems. The value of F_{IS} was about 0.089, which indicated that inbreeding effect was larger than the genetic drift in alfalfa. Applying x^2 test showed that Hamedani was significantly different from nine varieties and Shazand from seven varieties. Based on cluster analysis, alfalfa landraces and improved varieties were not arranged in separate groups. This means that there were not appreciable differences between these groups for allozyme markers.

Keywords: Alfalfa; Allozyme markers; Genetic distance; Genetic diversity

Introduction

Alfalfa (*Medicago sativa* L.) is widely grown as a perennial forage crop in the world. It is used in a number of different forms such as green mass, hay, silage, etc. (Labombarda *et al*. 2000). It is cultivated in about 32 million hectares over the world (Michaud *et al*., 1988). Alfalfa is autotetraploid (2n=4x=32), allogamous and a seed-propagated species. These factors contribute to genetic complexity of alfalfa at both individual and population levels (Labombarda *et al*. 2000).

Electrophoresis is a versatile biochemical technique for detecting genetic variation. Protein molecules migrate in an electric field because they are charged (Hamrick and Godt 1990). The allozyme was defined as the variant proteins produced by allelic forms of the same locus (Buth 1984). Polymorphism can be defined as the multiple phenotypic forms of a character attributable to the alleles of a single gene or the homologs of a single chromosome (Acquaah 1992). If in an analysis a gel pattern is interpretable, the banding pattern is regarded as an electrophoretic phenotype (Wendel 1989), which usually consists of one or more colored bands for each individual analyzed. In some cases, it may be simple and consist of a single invariant band in the whole sample. In contrast, some enzymes may show complex phenotypes with 15 or more bands per individual. Thus a correct interpretation of banding patterns in genetic terms requires the proper determination of the pertinent factors that

influence the electrophoretic phenotype (Weeden 1983).

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 (Tucak *et al*. 2008). Genetic variation and differentiation within and among wild and cultivated populations of the *M. sativa* complex has been studied with the use of allozymes (Quiros 1982; Birouk and Datter 1989; Jenczewski *et al*. 1998, 1999a,b; Morales Corts and Crespo Martinez 2000) and various DNA fragment markers, such as RFLP (Brummer *et al.* 1991), RAPD (Ghérardi *et al*. 1998; Jenczewski *et al.* 1998,1999b; Mengoni *et al*. 2000), SSR (Mengoni *et al*. 2000; Flajoulot *et al*. 2005; Greene *et al.* 2008), ISSR (Touil *et al*. 2008) and AFLP (Greene *et al*. 2008).

One of the most useful measures of population structure is F-statistics (Wright 1951), which describes the amount of inbreeding-like effects within subpopulations (F_{IS}), among subpopulations (F_{ST}) and within the entire population (F_{IT}) (Flajulot *et al*. 2005). A theoretical framework for the analysis of population structure in autotetraploid species is available (Ronfort *et al.* 1998). There is other possible explanation only by the band variability – mixed phenotypes (Etoh and Ogura 1981; Karkouri *et al*. 1996; Lehman, 1997). The objective of this work was to measure genetic diversity of 12 populations of alfalfa by allozymic loci.

Materials and Methods

Plant materials

Seven Iranian landraces and five improved varieties of alfalfa were studied (Table 1). From each improved variety and landrace, 35 individuals were planted in separate pots and leaf samples were collected for enzyme extraction and allozyme analyses.

Enzyme extraction and electrophoresis

The fresh and healthy leaves of adult plants were crashed with separate mortar and pestle in a tris-HCl extraction buffer, pH 7.5 (tris 50 mM, sucrose 5%, ascorbic acid 50 mM, sodium metabisulfite 20 mM, PEG 2% and 2ME 0.1% before use) with a ratio of 0.5 mg/µl and centrifuged at 4ºC and 10,000 rpm for 10 minutes using small Eppendof tubes. Enzyme extracts (supernatant) were immediately absorbed onto 3×5 mm wicks cut from Whatman 3 mm filter paper and loaded onto 7% horizontal slab acrylamide gel (16×10×0.6 cm). Electrophoresis was carried out at 4 ^{o}C for 3 h (constant current of 30 mA, and voltage of 180 V). After electrophoresis, the gels were stained for isozymes by applying standard histochemical methods described by Soltis and Soltis (1990). Electrophoretic allozyme phenotypes (hereafter zymograms) were genetically interpreted as one-banded for homozygotes or multiple-banded for heterozygotes, with balanced and unbalanced heterozygotes recorded by the differential staining intensity of bands. Taking into account the known monomeric versus dimeric structures of enzymes, multilocus genotypes of each population was determined for each individual in the population and allozymic variations were scored for seven gene loci. Loci were numbered consecutively and

alleles at each locus were labeled alphabetically, beginning from the most anodal form in all cases.

Table 1. List of alfalfa populations under study and their origin

Ecotypes	Locality
Ghara-Yonje	Maraghe-Iran
Amo-Zeynetdin	Tabriz-Iran
Rahnani	Esfahan-Iran
Taze-Kand	Naghade-Iran
Shazand	Arak-Iran
Hamedani	Hamedan-Iran
Yazdi	Yazd-Iran
Kaysari	Turkish
Ranger	USA
Kadi	unknown
Mesmir	Russia
Sea-River	Australia

populations. A cluster analysis for allozyme data was performed using the average linkage algorithm. Allozymic genetic diversity among the populations was estimated by the observed (H_o) and expected heterozygosities (H_e)(Nei 1972). H_e values were calculated assuming chromosomal segregation. Allozyme frequencies at each locus were also calculated for each population. Data were analyzed by using the POPGENE 32 (Yeh and Yang 1999) software.

Results and Discussion

Enzyme electrophoresis showed consistent staining for four enzymes (EST, POX, GOT and MDH) encoded by seven putative loci. All the enzymes migrated anodally. Three loci were monomorphic (POX-a, GOT, MDH) and four of them were polymorphic (Figure 1). The proportion of polymorphic loci (P) was estimated to be 57.14%. This proportion, as a measure of genetic variation, was higher than the value (26%) reported by Nevo and Clever (1978) for 15 plant species. Results showed highest allelic frequencies in a_2 at EST-A, b_2 at EST-B, b_1 at

Data analysis

For polymorphic markers, percentage of polymorphic loci (P), allele frequency, observed heterozygosity (H_o), genetic diversity or expected heterozygosity ($H_e = 1- \sum p_i^4$) were calculated. A x^2 test was performed to verify the existence of equilibrium in the populations. To determine whether the allele frequencies were different in the populations, another x^2 test was performed according to Workman and Niswander (1970) as follows:

$$\chi^2 = \frac{2NV_P}{\bar{p}\bar{q}}$$

where N is the total sample size, $\bar{p}$ and $\bar{q}$ are the weighted averages of two allele frequencies and V_p is the weighted variance (Hederick 2005). Wright's F-statistics (Wright 1951), which is based on the decrease in the proportion of heterozygosity, was used to measure the amount of inbreeding-like effects within each population (F_{IS}) and among populations (F_{ST}). Nei's unbiased genetic distance and genetic identity value were calculated for pairwise comparisons of

Ghara-Yonje to 0.736 in Shazand. There was no considerable difference between landraces and improved varieties with respect to H_e. Observed heterozygosity values were lower than expected heterozygosity in all of the populations except in Ghara-Yonje, Yazdi and Kaysari populations (Table 3).

POX-B and c_3 at POX-C in all populations, except b_1 at EST-B for the Hamedani population (Table 2). The observed mean heterozygosities (H_o) were ranged from 0.501 in Sea-River to 0.683 in Kaysari indicating no noticeable differentiation between landraces and improved varieties. Expected mean heterozygosity (H_e), as a measure of genetic diversity, was ranged from 0.566 in

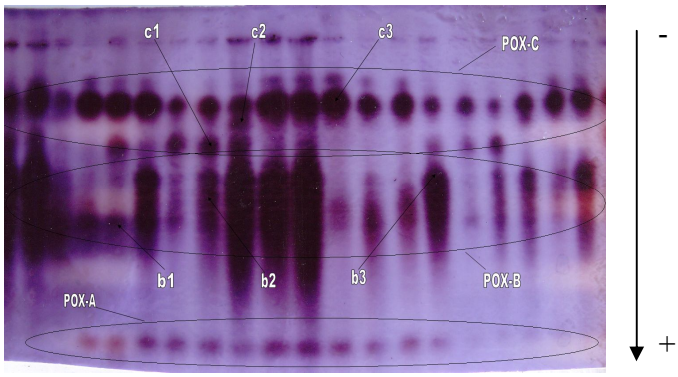

Figure 1. Examples of monomorphic and polymorphic bands of POX

Table 2. Allele frequencies of polymorphic loci in 12 alfalfa populations

		EST-A		EST-B			POX- B			POX- C		
Germplasm	Population	a_1	a_2	b_1	b_2	b_3	b_1	b_2	b_3	c_1	c_2	c_3
	Ghara-Yonje	0.080	0.920	0.260	0.700	0.040	0.83	0.12	0.05	0.2	0.06	0.74
	Amo-Zeynetdin	0.120	0.880	0.210	0.670	0.120	0.63	0.3	0.07	0.29	0.05	0.67
	Rahnani	0.140	0.860	0.120	0.730	0.150	0.69	0.3	0.01	0.15	0.16	0.69
Landrace	Taze-Kand	0.120	0.880	0.120	0.840	0.040	0.74	0.25	0.01	0.2	0.06	0.74
	Shazand	0.140	0.860	0.420	0.550	0.030	0.54	0.45	0.01	0.28	0.01	0.71
	Hamedani	0.170	0.830	0.520	0.390	0.090	0.66	0.25	0.09	0.12	0.12	0.76
	Yazdi	0.090	0.910	0.100	0.680	0.220	0.63	0.29	0.08	0.21	0.02	0.77
	Kaysari	0.100	0.900	0.260	0.710	0.030	0.5	0.38	0.12	0.21	0.07	0.73
	Ranger	0.150	0.850	0.110	0.790	0.100	0.71	0.24	0.05	0.24	0.11	0.65
Improved	Kadi	0.080	0.920	0.300	0.620	0.080	0.6	0.37	0.03	0.08	0.1	0.82
	Mesmir	0.100	0.900	0.100	0.840	0.060	0.65	0.24	0.11	0.24	0.09	0.67
	Sea-River	0.080	0.920	0.160	0.760	0.080	0.74	0.18	0.08	0.29	0.15	0.56
	Landrace	0.120	0.880	0.250	0.650	0.100	0.67	0.28	0.05	0.21	0.07	0.73
	Improved	0.100	0.900	0.190	0.740	0.070	0.64	0.28	0.08	0.21	0.10	0.69
	Mean	0.110	0.890	0.220	0.690	0.090	0.66	0.28	0.06	0.21	0.08	0.71

Table 3. Observed mean and expected mean heterozygosity for all loci in all populations of alfalfa under study

Germplasm	Population	H_o	H_e
Landrace	Ghara-Yonje	0.588	0.566
	Amo-Zeynetdin	0.632	0.705
	Rahnani	0.621	0.676
	Taze-Kand	0.564	0.574
	Shazand	0.654	0.736
	Hamedani	0.662	0.725
	Yazdi	0.646	0.645
Improved	Kaysari	0.683	0679
	Ranger	0.619	0.662
	Kadi	0.598	0.632
	Mesmir	0.570	0.615
	Sea-River	0.501	0.636
	Landrace	0.623	0.661
	Improved	0.594	0.645

Estimated parameters of F_{IS}, F_{IT} and F_{ST} in all populations by four polymorphic loci are shown in Table 4. Genetic divergence of landraces from improved varieties, estimated by the F_{ST} parameter, was low (F_{ST} = 0.029) which indicates that the studied enzyme systems were not able to differentiate the landrace populations from the improved varieties. Furthermore, F_{ST} and F_{IT} values were 0.003 and 0.092, respectively in two groups of alfalfa populations (Table 5).

Nei's (1972) genetic distances between pairs of alfalfa populations are shown in Table 6. The genetic distances of populations were ranged from 0.013 to 0.02, with a value of 0.016 between landraces and improved varieties (Table 6).

In order to examine Hardy-Weinberg equilibrium, chi-square values for all populations are presented in Table 7. The values were ranged from 0.013 for POX-B in Kaysari to 9.886 in Amo-Zeynetdin for POX-C. In EST-A and EST-B, most of the populations were in equilibrium but in POX-B and POX-C most of them were not in equilibrium.

A chi-square test comparing pairs of alfalfa populations indicated that Hamedani was significantly different from nine varieties, Shazand from seven varieties, Ghara-Yonje from four varieties, etc. This means that Hamedani population had maximum differentiation from other populations. Alfalfa landraces and improved varieties didn't show significant difference and were close together (Table 8).

Clustering of the populations based on polymorphic loci produced two clusters. The largest cluster included Ghara-Yonje, Taze-Kand, Amo-Zeynetdin, Yazdi, Rahnani, Mesmir, Sea-River and Kaysari (Figure 2).

The cultivated alfalfa (*Medicago sativa* L.) is characterized by a great genetic variability, which enables it to adapt to different environments.

Table 4. Summary of F-statistics for studied loci in 12 populations of alfalfa

Locus	$\overline{H_S}$	F_{IS}	F_{ST}	F_{IT}
EST-A	0.380	0.076	0.011	0.086
EST-B	0.725	0.063	0.060	0.119
POX-A	-	-	-	-
POX-B	0.780	0.052	0.029	0.080
POX-C	0.732	0.081	0.018	0.097
GOT	-	-	-	-
MDH	-	-	-	-
Mean	0.654	0.068	0.029	0.096

Table 5. Summary of F-statistics for studied loci using landraces and improved groups of alfalfa

Locus	$\overline{H_S}$	F_{IS}	F_{ST}	F_{IT}
EST-A	0.372	0.062	0.001	0.063
EST-B	0.758	0.105	0.008	0.112
POX-A	-	-	-	-
POX-B	0.809	0.089	0.001	0.090
POX-C	0.743	0.100	0.002	0.101
GOT	-	-	-	-
MDH	-	-	-	-
Mean	0.670	0.089	0.003	0.092

Table 6. Nei's genetic distance among alfalfa populations under study

Population	Ghara-Yonje	Amo-Zeynedin	Rahnani	Taze-Kand	Shazand	Hamedani	Yazdi	Kaysari	Ranger	Kadi	Mesmir
Amo-Zeynetdin	0.017										
Rahnani	0.02	0.015									
Taze-kand	0.02	0.017	0.018								
Shazand	0.015	0.013	0.013	0.014							
Hamedani	0.015	0.013	0.013	0.014	0.013						
Yazdi	0.019	0.016	0.017	0.019	0.014	0.014					
Kaysari	0.017	0.015	0.015	0.017	0.014	0.013	0.017				
Ranger	0.019	0.015	0.016	0.019	0.013	0.013	0.016	0.015			
Kadi	0.019	0.016	0.016	0.018	0.015	0.015	0.018	0.017	0.016		
Mesmir	0.019	0.016	0.017	0.02	0.014	0.013	0.018	0.017	0.018	0.017	
Sea-river	0.02	0.016	0.017	0.019	0.013	0.013	0.017	0.017	0.017	0.016	0.019
Landraces vs improved varieties	0.016										

Table 7. Chi-square values for all populations of alfalfa under study

		Locus			
Germplasm	Population	EST-A	EST-B	POX-B	POX-C
	Ghara-Yonje	0.422ns	0.262ns	6.549*	4.727*
	Amo-Zeynetdin	0.914ns	1.378ns	0.13ns	9.886**
	Rahnani	0.879ns	0.116ns	0.722ns	5.431*
Landrace	Taze-Kand	0.206ns	2.354ns	1.964ns	4.968*
	Shazand	0.761ns	2.805ns	0.09ns	4.762*
	Hamedani	1.966ns	1.858ns	4.786*	3.25ns
	Yazdi	0.283ns	1.3ns	4.129*	7.15**
	Kaysari	0.692ns	0.417ns	0.013ns	8.137**
	Ranger	1.259ns	8.5**	0.722ns	5.110*
Improved	Kadi	1.506ns	3.805ns	1.852ns	0.104ns
	Mesmir	0.662ns	0.162ns	1.832ns	7.369**
	Sea-River	2.886ns	0.476ns	1.75ns	0.508ns
	Landrace	2.754ns	4.534*	12.520**	41.941**
	Improved	0.171ns	4.031*	0.106ns	17.575**

*,** Significant at 5% and 1% levels of probability, respectively

Table 8. Comparison of allele frequencies of alfalfa populations based on chi-square test

Population	Ghara-Yonje	Amo-Zeynetdin	Rahnani	Taze-Kand	Shazand	Hamedani	Yazdi	Kaysari	Ranger	Kadi	Mesmir	Mean
Amo-Zeynetdin	6.161											6.161
Rahnani	6.088	1.539										3.814
Taze-Kand	3.815	2.953	0.624									2.464
Shazand	13.019**	4.603	10.363*	11.932**								9.979
Hamedani	11.482**	9.057*	14.476**	13.756**	2.635							10.281
Yazdi	6.966	2.580	1.283	1.314	9.602*	14.490**						6.039
Kaysari	11.769**	2.175	5.839	7.792	2.287	8.270*	4.063					6.028
Ranger	6.734	2.091	0.233	1.131	12.049**	16.129**	2.233	7.515				6.014
Kadi	7.217	4.146	7.285	6.686	3.871	6.601	4.529	2.361	9.664*			5.818
Mesmir	8.620*	1.969	0.590	1.607	12.485**	19.413**	0.893	5.739	0.893	8.500*		6.071
Sea-River	5.988	2.904	2.914	3.986	13.805**	18.328**	4.954	9.376*	2.243	11.595**	2.890	7.180
Landrace vs Improved	0.833											

*,** Significant at 5% and 1% levels of probability, respectively

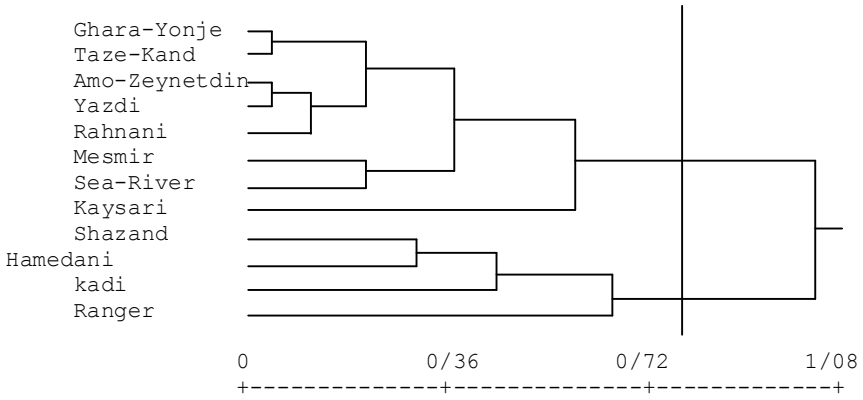

Figure 2. Dendrogram showing the genetic similarity among 12 populations of *Medicago sativa* based on allozyme data

the allozyme marker was 0.014, which is also in accordance with our findings. Results of this study showed that the populations had low genetic distance from each other and most of the diversity was within populations. Flajoulot *et al*. (2005) also reported similar results. This study provides evidence that allozyme markers are not suitable enough to differentiate alfalfa landraces as well as improved varieties. Therefore, other tools for varietal (populations) identification should be investigated.

Kaljund and Jaaska (2010) reported that all populations and subpopulations have high values of the expected heterozygosity (H_e) and genetic diversity measure ranged between 0.795 and 0.893 that is nearly consistent with our findings. Mengoni *et al*. (2000), Brummer *et al*. (1991) and Puppilli *et al*. (1996) also confirmed the high heterozygosity of alfalfa populations by using RAPD, SSR and RFLP markers. Jenczewski *et al*. (1999b) used allozyme and RAPD markers in alfalfa populations and reported that F_{ST} value for

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