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Discrimination of alfalfa half-sib families by allozyme banding pattern and its relationship with forage yield attributes

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Abstract

Electrophoretic variation of three allozyme systems coding by seven gene loci were studied on 12 alfalfa half-sib families. Obtained from alfalfa polycross nursery performed in Faculty of Agriculture, University of Tabriz. Polyacrylamide gel electrophoresis was used to evaluate the levels of genetic variations and population structure. The level of polymorphic loci (P) in families was 0.571 and observed mean heterozigosities (H_o) were ranged from 0.59 in Selvana to 0.77 in Galebani half-sibs. Expected mean heterozygosities (H_e) were ranged from 0.332 in Selvana to 0.439 in Chaleshte. The families were found to be in Hardy-Weinberg equilibrium by using x^2 test. Wright's *F* statistics revealed that the estimated overall inbreeding coefficient, (F_{IT}), of 0.091 was mainly related to inbreeding or double reduction in alfalfa (F_{IS}= 0.078) rather than random genetic drift or population differentiation (F_{ST}= 0.013). The mean of distance coefficient in families were ranged from 0.94 to 0.99 and the lowest distance obtained for Chaleshte and Galebani. The presence of esterase allozyme of Est-b1 was recognized to be related to the fresh weight and leaf weight and peroxidase allozyme Pox-b2 band was correlated significantly to the fresh weight in the studied half-sib families.

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Introduction

Alfalfa (*Medicago sativa* L.), is the most important forage crop grown in the temperate regions. It is cultivated over 32 million hectares worldwide (Flajoulot *et al.*, 2005) and over 680 thousand hectares in Iran (Anonymous, 2007), specially in the northwest region, where is considered as one of the two alfalfa origins (Sinskaya, 1950). The cultivated varieties and ecotypes, having the genetic constitution of 2n = 4x = 32, are autotetraploid with allogamous fertilization. This describes the great variability of this species (Muslera and Ratera, 1984). The existence of large variability, even at an intravarietal level, together with the environmental effect and life cycle of alfalfa makes it difficult to use morphological or agronomic characters as descriptors of the varieties (Cordero and Crespo, 1995).

Electrophoresis of allozymes was initiated in 1957, when Hunter and Markert (1957) exploited the idea of using the catalytic properties of enzymes to reveal their presence with histochemical methods. Markert and Moller (1959) proposed the term isozyme to describe "the different molecular forms in which proteins may exist with the same enzymatic specificity". This functional definition was intended to be broad, to cover all molecular forms of enzymes. The codominant nature of expression of allozymes allowed the unambiguous identification of heterozygous and homozygous individuals. In the last decade, a wide range of molecular techniques has been introduced to study the variation in alfalfa. Same of these techniques are and isozyme markers (Morales Corts and Crespo 2000; Valizadeh *et al.*, 2011). random amplified polymorphic DNA (RAPD) (Noparvar *et al.*, 2008), simple sequence repeat (SSR) (Zhi Peng *et al.*, 2007; Li *et al.*, 2009) and expressed sequence tags (ESTs) (Bahar *et al.*, 2006). Different enzymes can easily be visualized after completion of electrophoresis by using published staining recipes such as Harris and Hopkinson (1976) and Pasteur *et al.*, (1988). Isozyme markers are advantageous over conventional morphological markers because they allow seedling screening and save considerable greenhouse and field space for the breeder. Allelic frequency differences in isozymatic loci have been used for characterizing allogamous varieties (Nielsen *et al.*, 1985; Charmet and Balfourier, 1994). Within the genus *Medicago,* Quirós (1980, 1981, 1982, 1983) carried out analyses using the isozymatic systems of esterases, acid phosphatases, leucine aminopeptidases, and peroxidases.

The aims of this study were to (a) evaluate the levels of genetic variations in half-sib populations of alfalfa and (b) examine the genetic differentiations based on allele frequencies.

Material and methods

Plant materials used in this study consisted of 12 half-sib families (Table 1) that were obtained from 12 parental ecotypes or cultivars arranged in a latin square polycross nursery, at the Research Station of University of Tabriz (Valizadeh *et al.*, 2002). Thirtyfive individuals of each population were grown in separate pots in field condition and analyzed in the from of completely randomized design.

Table 1. List of the parental ecotypes and cultivars, and their used to produce polycross progeny alfalfa.

Ecotypes and Cultivar	Locality	Country
Leylan-Hamid	Arak	Iran
Galebani	Marand	Iran
Ghara-Yonje	Maraghe	Iran
Maman-Famenin	Hamedan	Iran
Amo-Zeynetdin	Tabriz	Iran
Taze-kand	Nagadeh	Iran
Zoghal-Aghaj	Tabriz	Iran
Selvana	Urmia	Iran
Shazand	Arak	Iran
Maopa	-	USA
Ranger	-	USA
Chaleshte	Chahar mahale Bakhtiyary	Iran

Plant height, fresh weight, dry weight, leaf weight, stem weight and leaf/stem ratio were measured for each individual plant at harvest time in 12 alfalfa half sib families.

Electrophoresis

The crude extract of fresh and healthy leaves from adult plants were prepared 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 (0.6*15*12 cm) using TBE (Tris-Borate-EDTA) electrode buffer (pH= 8.8). Electrophoresis was carried out at 4ºC for 3 h (constant current of 30 mA, and voltage of 180 V). Allozymic variations were scored for seven gene loci (Fig. 1). Three enzymatic systems were studied on sliced gels: Esterase (Est) and catalase (Cat) according to Soltis and Soltis (1990) and peroxidase (Pox) according to Olson and Varner (1993). Loci were numbered consecutively and alleles at each locus were labeled alphabetically, beginning from the most anodal form in both cases.

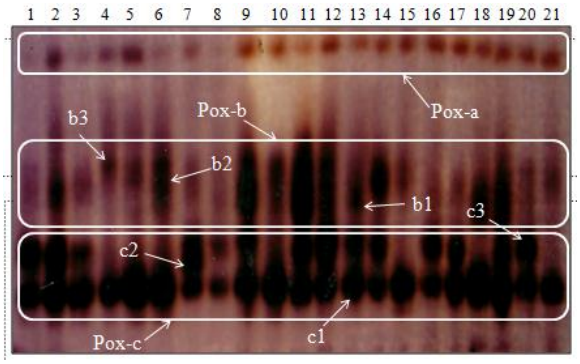

Fig. 1. various zymograms of peroxidase isozymes are exemplified by 21 selected alfalfa plants, showing two polymorphic loci (Pox-B and Pox-C) and one monomorphic locus (Pox-A).

Data analysis

Allozyme frequencies at each locus were calculated for each half sib family on the basis of tetrasomic inheritance in alfalfa. Data were analysed using the POPGENE 32 (Yeh and Yang, 1999) software. Some standard genetic parameters, including percentage of polymorphic loci (P), effective number of alleles (n_e), observed heterozygosity (H_o), and genetic diversity ($H_e = 1- \sum p_i^4$) were colculated. A x^2 test was performed to verify the existence of Hardy-Weinberg equilibrium in populations. Wright's F-statistics (Wright, 1951), which is based on the decrease in the proportion of heterozygosity, was used to evaluate the amount of inbreeding effects within each half sib families (F_{is}). Furthermore, among half sib families (F_{st}) and genetic identity value were calculated for pairwise comparisons of populations. A cluster analysis was performed using the unweighted pair-group method using arithmetic averages (UPGMA). A dendrograms was constructed using the computer POPGENE software package.

Results and discussion

Enzyme electrophoresis resulted in clear and consistent bands for three enzymes encoded by seven putative loci at Est, Pox and Cat system. All enzymes migrated anodally. Three loci were monomorphic (Pox-a, Cat-a and Cat-b) and four of them were polymorphic (Pox-b, Pox-c, Est-a and Est-b) (Table 2). Therefore, the proportion of polymorphic loci (P) was estimated to be 57.14%. This proportion as a measure of genetic variation was higher than the amount of 26% reported by Nevo (1978) for 15 plant species. This high (P) value could be attributed to the tetrasomic inheritance as well as self-incompatibility nature in alfalfa. The Nei's distance (1972) coefficient of families were ranged from 0.0035 to 0.0594 (Table 3), with the lowest mean distance value obtained for Chaleshte (0.0133) and the highest mean value for Maopa (0.0378) fallowed by Ghara-Yonje (0.0377).

The observed mean heterozygosities were ranged from 0.59 in Selvana to 0.773 in Galebani (Table 4). The expected mean heterozygosity (H_e), as a measure of genetic diversity, ranged from 0.66 in Leylan-Hamid to 0.81 in Ranger indicating very high within population variability. Brummer *et al.* (1991), Puppilli *et al.* (1996), Mengoni *et al.* (2000) and Valizadeh *et al.* (2011) also reported very high heterozigosity of alfalfa populations using RAPD, SSR, RFLP and allozymes markers. Furthermore, values of observed heterozygosity were lower than those of expected heterozygosity in all families (Table

4). Chi-square values were measured for all half sib families in each locus and overall loci. It was shown that all of half-sib families were in Hardy-Weinberg equilibrium (Table 4). This could be altributed to their random mating in polycross nursery.

Table 2. Allele frequencies of polymorphic loci in 12 alfalfa half-sib families.

Loci	Allele	Half-sib families											
		Leylan-Hamid	Gale-bani	Ghara-Yonje	Maman-Famenin	Amo-Zeyne-ddin	Taze-Kand	Zogha-lAghaj	Selv-ana	Shaz-and	Maopa	Ran-ger	Chale-shte
Est-a	a1	0.17	0.23	0.27	0.26	0.38	0.23	0.26	0.13	0.26	0.22	0.36	0.23
	a2	0.83	0.77	0.73	0.74	0.62	0.77	0.74	0.87	0.74	0.78	0.64	0.77
Est-b	b1	0.24	0.26	0.22	0.25	0.25	0.26	0.29	0.21	0.28	0.13	0.28	0.25
	b2	0.58	0.58	0.53	0.52	0.59	0.55	0.53	0.56	0.55	0.55	0.64	0.65
	b3	0.18	0.16	0.25	0.23	0.16	0.19	0.18	0.23	0.17	0.32	0.08	0.10
Pox-b	b1	0.39	0.33	0.36	0.32	0.34	0.42	0.41	0.24	0.32	0.46	0.29	0.34
	b2	0.36	0.44	0.39	0.49	0.54	0.51	0.41	0.45	0.50	0.30	0.50	0.44
	b3	0.25	0.23	0.25	0.19	0.12	0.07	0.18	0.31	0.18	0.24	0.21	0.22
Pox-c	c1	0.19	0.20	0.24	0.15	0.17	0.30	0.11	0.10	0.12	0.18	0.25	0.20
	c2	0.04	0.06	0.04	0.02	0.06	0.02	0.05	0.02	0.07	0.03	0.00	0.02
	c3	0.77	0.74	0.72	0.83	0.77	0.68	0.84	0.88	0.81	0.79	0.75	0.78

Table 3. Nei's genetic distance among 12 alfalfa half sib families.

	Leylan-Hamid	Gale-bani	Ghara-Yonje	Maman-Famenin	Amo-Zeyne-ddin	Taze-Kand	Zoghal-Aghaj	Selv-ana	Shaz-and	Maopa	Ranger	Chale-shte
Leylan-Hamid												
Galebani	0.0045											
Ghara-Yonje	0.0064	0.0056										
Maman-Famenin	0.0120	0.0047	0.0035									
Amo-Zeyneddin	0.0311	0.0156	0.0202	0.0156								
Taze-Kand	0.0213	0.0140	0.0247	0.0236	0.0170							
Zoghal-Aghaj	0.0077	0.0067	0.0261	0.0157	0.0154	0.0169						
Selvana	0.0148	0.0126	0.0140	0.0093	0.0422	0.0444	0.0245					
Shazand	0.0136	0.0041	0.0115	0.0044	0.0085	0.0156	0.0057	0.0164				
Maopa	0.0145	0.0276	0.0466	0.0430	0.0521	0.0394	0.0238	0.0376	0.0394			
Ranger	0.0270	0.0118	0.0171	0.0163	0.0075	0.0214	0.0207	0.0360	0.0119	0.0593		
Chaleshte	0.0065	0.0022	0.0170	0.0129	0.0159	0.0161	0.0091	0.0173	0.0070	0.0332	0.0094	
Mean	0.0145	0.0144	0.0377	0.0146	0.0219	0.0237	0.0156	0.0244	0.0125	0.0378	0.0216	0.0133

Table 4. Summery of genetics parameters and chi-square values for all half sib families under study.

Population	P	n_e	H_o	H_e	χ^2
Leylan-Hamid	0.571	1.7190	0.7187	0.6636	0.2509^{ns}
Galebani	0.571	1.7457	0.7731	0.7336	0.0698^{ns}
Ghara-Yonje	0.571	1.6561	0.7428	0.7765	0.0231^{ns}
Maman-Famenin	0.571	1.5746	0.7070	0.7083	0.0611^{ns}
Amo-Zeyneddin	0.571	1.7263	0.7723	0.8027	0.0180^{ns}
Taze-Kand	0.571	1.7701	0.7711	0.7438	0.0677^{ns}
Zoghal-Aghaj	0.571	1.6598	0.7182	0.7088	0.0871^{ns}
Selvana	0.571	1.5606	0.5994	0.5579	0.2896^{ns}
Shazand	0.571	1.7280	0.6773	0.7265	0.1820^{ns}
Maopa	0.571	1.5605	0.7011	0.6953	0.0745^{ns}
Ranger	0.571	1.9931	0.6882	0.8112	0.0781^{ns}
Chaleshte	0.571	1.8054	0.7046	0.7188	0.0321^{ns}

P = Percentage of polymorphic loci; ne = Mean effective number of alleles; Ho = Mean observed heterozygosity; He = Mean expected heterozygosity; ns = Non-significant difference from equilibrium.

F_{is}, F_{it} and F_{st} Parameters estimated for all half sib families using four polymorphic loci are included in Table 5. The mean value of F_{st} which measures differentiation among populations was considerably low (F_{st} = 0.0137) with respect to the mean value of F_{it} (F_{it} = 0.0913) which measures gene fixation due to inbreeding.

Table 5. Summary of F-statistics and gene flow for all loci.

Locus	$\overline{H_S}$	Fis	Fit	Fst
Est-a	0.66	0.0760	0.0984	0.0243
Est-b	0.88	0.0780	0.0821	0.0049
Pox-a	-	-	-	-
Pox-b	0.93	0.0891	0.0973	0.0089
Pox-c	0.61	0.0717	0.0874	0.0168
Cat-a	-	-	-	-
Cat-b	-	-	-	-
Mean		0.0786	0.0913	0.0137

Correlations between different allozyme frequencies and mean values of agronomic traits in alfalfa half-sib families are presented Table 6. Three of four loci showed significant correlations with some studied traits. Est-a1, Est-b1 and Pox-b2 showed medium to high significant correlations with alfalfa fresh weight respectively and Est-b1showed a significant correlation with leaf weight. Many authors have found that the changes in allozyme frequencies are associated with yield or other agronomic traits. Stuber and Moll (1972) reported a high correlation between Acp-1 and yield. Pollak *et al.* (1984) also found associations of Acp-1 with yield, maturity and leaf variables, as well as Got-1, Pox-1 and Adh-1. Kahler (1983) observed changes in Adh-1, Mdh-2 and Acp-1 were related to yield selection. Stuber *et al.* (1980) found associations between Acp-1, Glu-1, Pgm-1 and Mdh-1 and yield. Van and Jin (2011) reported association of Est with yield components on rice. Pham (1988) reported that Pgi-2 was a marker for spikelet fertility and plant height in a Japonica-Indica rice cross.

In conclusion, it was shown that there is a large genetic variability in alfalfa populations under study. However, due the nature of alfalfa breeding system, within population variability was much higher than interpopulation variation indicating that further selection could be carried out on each of the half-sib families under study. Furthermore, significant correlation of several isozyme loci whit fresh weight and leaf weight in alfalfa suggest that there markers may be used to select indirecty for forage yield after the stability of here markers were verified in other experiments.

Table 7. Correlation coefficient between agronomic characteristics allozyme zymograms in 12 alfalfa half-sib families.

Loci		Dry weight	Fresh weight	Leaf weight	Stem weight	Leaf/stem ratio	Plant height
Est-a	1	0.4865	0.5545^{+}	0.4946	0.3598	-0.1762	0.2333
	2	-0.4865	-0.5545	-0.4946	-0.3598	0.1762	-0.2333
Est-b	1	0.3027	0.5914*	0.5785*	-0.1569	-0.0283	0.3680
	2	-0.0958	-0.1058	0.1350	-0.2139	0.4182	-0.2313
	3	-0.1428	-0.3302	-0.4730	0.2397	-0.2438	-0.1015
Pox-b	1	-0.3186	-0.3616	-0.3067	-0.0461	0.3845	-0.2106
	2	0.2930	0.7169**	0.3357	-0.1186	-0.1018	0.0711
	3	-0.0184	-0.4454	-0.0769	0.1751	-0.2557	0.1232
Pox-c	1	-0.0603	-0.2908	0.0375	-0.0927	0.1669	-0.4121
	2	-0.1144	0.3657	-0.1425	-0.1548	-0.0949	0.4980
	3	0.1087	0.1729	0.0140	0.1589	-0.1432	0.2528

$^{+}$,*,** Significant at 7, 5 and 1% probability levels, Respectively.

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