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Genetic Diversity of Synthetic Alfalfa Generations and Cultivars Using Tetrasomic Inherited Allozyme Markers

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ABSTRACT

Enzyme electrophoresis was used to measure genetic variation within, and divergence among, three generations of recently bred synthetic alfalfa generations (Syn_1, Syn_2, and Syn_3) originating from a polycross of 12 selected parents and several cultivars. Three isozyme loci, exhibiting tetrasomic inheritance in 10-day seedlings, were detected from five enzymatic systems analyzed by polyacrylamide slab gel electrophoresis for about 100 individuals of each alfalfa population. Very high levels of heterozygosity (ranging from 0.521 to 0.699) were observed within alfalfa populations, using polymorphic loci. The reduction in heterozygosity was about 5% from Syn_1 to Syn_2 and from Syn_2 to Syn_3. The last open pollinated generation was found to be in W-H equilibrium as well as Ghara-yonja, a native ecotype under examination, using χ^2-test. Application of Wright's *F*-statistics revealed that the estimated overall inbreeding coefficient, (F_{IT}), of 9.4% was mainly related to inbreeding or double reduction in alfalfa (F_{IS}= 8.61%) rather than random genetic drift or population differentiation (F_{ST}= 1.6%). Therefore, due to very large intra-population diversity, the breeding program of the synthetic alfalfa did not generate a large variety differentiation. However, the use of seedling allozymic loci can be applied successfully for estimation of the population genetic parameters.

Keywords: Alfalfa, Allozymes, Genetic diversity, Tetrasomic inheritance.

INTRODUCTION

Alfalfa (*Medicago sativa* L.), is the most important forage crop grown in the temperate regions. It is cultivated over 32 million hectares worldwide (Michaud *et al*., 1988) and over 680 thousands hectares in Iran (Anonymous, 2007), especially in the Northwest region, which is considered as one of the two alfalfa origins (Sinskaya, 1950). In Iran, cultivated alfalfa mainly consists of several adaptive landraces including Ghara-yonja and Hamadani as well as occasionally introduced cultivars such as Ranger or Maopa.

Tetrasomic inheritance (2n= 4x= 32) in autotetraploid alfalfa (Stanford, 1951; Demarly, 1954) and pronounced inbreeding depression (McCoy and Bingham, 1988), have caused the genetic improvement of alfalfa to be less than other major crops. Improved varieties are synthetic cultivars, usually obtained through three or four generations of open pollinated reproduction of polycross seeds of selected parents (Tysdal and Crandal, 1948; Flajulot *et al*., 2005).

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). In contrast to diploids, the correlation of genes between individuals within population with respect to genes between populations (F_{ST}) may vary among loci due to double reduction (Wricke and Weber, 1986) and appropriate number of loci may be required for estimating demographic

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parameters of autotetraploid populations. The allozymic markers showing tetrasomic inheritance patterns can be used to perform genetic diversity analysis.

The objective of this work was to measure population structure of three generations belonging to a newly produced synthetic alfalfa (Syn_1, Syn_2, and Syn_3), some parental populations, and improved varieties by allozymic loci.

MATERIALS AND METHODS

Plant Material

A total of 600 alfalfa seedlings were analyzed from six populations of alfalfa including three generations of a synthetic alfalfa (Syn_1, Syn_2 and Syn_3), one parental landrace, Ghara-yonja, and two cultivars, namely, Ranger and Sea-river, released in the USA and Australia, respectively. Syn_1 was obtained by polycrossing of 12 selected ecotypes in 2002 arranged in an isolated latin-square design after a period of four years of screening 30 parental ecotypes from Northwest of Iran by progeny test, at the Research Station of University of Tabriz (Valizadeh *et al.*, 2002). Syn_2 and Syn_3 were produced by isolated open pollination in 2004 and 2005, respectively. Gara-yonja, which was collected from Maragheh region in East-Azarbaijan Province, is the most common landrace grown in Northwest of Iran.

Enzyme Extraction and Electrophoresis

Hypocotyls and cotyledons of l0-day old seedlings 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^{-1} and centrifuged at 4ºC and 8,000g for 20 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. Electrophoresis was performed using Poulik gel buffer and tris 32 mM, boric acid 0.6 mM, and EDTA-Na_2 0.1 mM electrode buffer at pH 8.8. Five enzymatic systems were stained on sliced gels: Esterase (Est), glutamate oxaloacetate transaminase (Got), catalase (Cat) and malate dehydrogerase (Mdh), according to Soltis and Soltis (1990) and peroxidase (Pox) according to Olson and Varner (1993). Putative loci were designated sequentially, with the most anodally migrating isozyme designated *A*, the next one *B*, etc. Likewise, alleles were designated sequentially with the most anodally migrating allele designated 1.

Data Analysis

Some standard genetic parameters including proportion of polymorphic loci (P) and genetic diversity, or expected heterozygosity (He=$1-\Sigma p_i^4$), were calculated for about 100 individuals in each alfalfa population. A χ^2 test was performed to verify the existence of equilibrium in populations with one locus and two allele models, by keeping the most common allele and pooling the other alleles, when necessary. To determine whether the allele frequencies were significantly different, a χ^2 test was performed according to Workman and Niswander (1970):

$$\chi^2 = \frac{2NV_{(p)}}{\bar{p}\bar{q}}$$

Where, *N* is the total sample size, $\bar{p}$ and $\bar{q}$ are the weighted averages of the two allele frequencies, and $V_{(p)}$ is the weighted variance (See Hederick (2005) for more details). Due to the limited number of loci, no statistical analysis was used for the parameters estimations.

Nei's (1972) genetic distance measures were calculated for all pairs of populations to estimate genetic divergence among the 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-like effects

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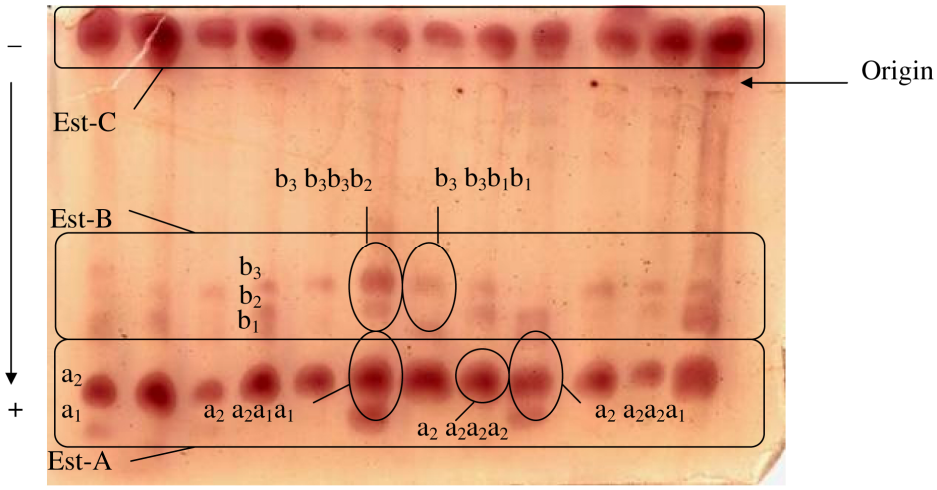

Figure 1. Banding pattern of esterase for 12 alfalfa seedlings, showing two polymorphic loci (Est-A and Est-B) and one monomorphic locus (Est-C). The allele's nomenclature and the genotypes of some individuals are indicated.

within each population (F_{IS}), among populations (F_{ST}), and within the entire germplasm under study, using Autotet software.

RESULTS AND DISCUSSION

Eight putative loci were resolved from five enzymatic systems. These were *Got-A*, *Got-B*, *Mdh-A*, *Cat-A*, *Est-A*, *Est-B*, *Pox-B*, and *Pox-C*. The four former loci were monomorphic and the four latter were polymorphic. Therefore, the proportion of polymorphic loci (P) was estimated to be 50%. This proportion, as a measure of genetic variation, was higher than the 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 in alfalfa.

The *Est-A*, *Est-B*, and *Pox-C* loci exhibited unambiguous tetrasomic inheritance (Figures 1 and 2) for two, three and three allozymes, respectively, in all populations and were used to study the populations genetic structure. The polymorphic *Pox-B* locus was not included in this study due to faint or inconsistent staining. A summary of the allele frequencies for three polymorphic loci are given in Table 1. All populations shared the same highest allelic

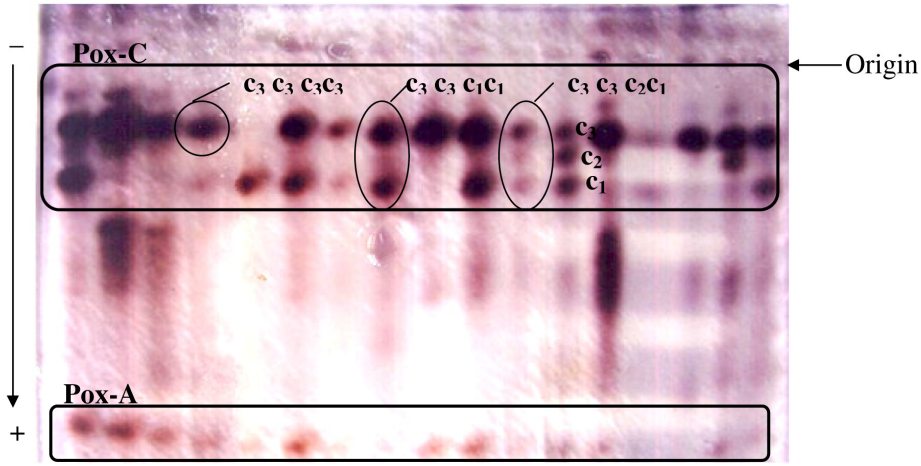

Figure 2. Banding pattern of peroxidase for 17 alfalfa seedlings, showing one unambiguous polymorphic locus (Pox-C).The allele's nomenclature and the genotypes of some individuals are indicated.

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Table 1. Allele frequencies for three polymorphic loci in the studied populations of alfalfa.

Alfalfa population	N[a]	*Est-B*			*Est-A*		*Pox-C*		
		b_1	b_2	b_3	a_1	a_2	c_1	c_2	c_3
Ranger	69	0.149	0.366	0.486	0.062	0.938	-	-	-
Sea-river	70	0.143	0.500	0.357	0.104	0.896	-	-	-
Ghara-yonja	94	0.181	0.352	0.468	0.188	0.812	0.283	0.042	0.675
Syn_1	100	0.214	0.339	0.446	0.120	0.880	0.303	0.037	0.660
Syn_2	102	0.172	0.360	0.468	0.127	0.873	0.281	0.071	0.648
Syn_3	111	0.243	0.279	0.478	0.113	0.887	0.291	0.051	0.658
Average		0.183	0.366	0.450	0.119	0.881	0.288	0.052	0.660

[a] Number of individuals (seedlings) analyzed for each population and each enzyme system.
- : Data not available

Table 2. Observed (expected) levels of heterozygosity in alfalfa populations based on polymorphic loci.

Alfalfa population	Allozymic loci			Mean heterozygosity
	Est-B (3 alleles)	*Est-A* (2 alleles)	*Pox-C* (3 alleles)	
Ranger	0.843 (0.926)**	0.198 (0.225)*	-	0.521 (0.575)
Sea-river	0.846 (0.921)^{ns}	0.320 (0.354)*	-	0.583 (0.637)
Ghara-yonja	0.872 (0.936)^{ns}	0.526 (0.564)*	0.690 (0.790)^{ns}	0.696 (0.753)
Syn_1	0.906 (0.945)^{ns}	0.366 (0.400)**	0.780 (0.820)*	0.684 (0.722)
Syn_2	0.853 (0.938)*	0.368 (0.420)^{ns}	0.760 (0.810)**	0.660 (0.721)
Syn_3	0.833 (0.938)*	0.333 (0.380)^{ns}	0.730 (0.810)^{ns}	0.632 (0.709)

Data not available
ns, *, ** Non-significant difference from equilibrium and significant at 5 and 1% levels of probability, based on two alleles model of χ^2- test.

frequencies at three loci: b_3 at *Est-B*, a_2 at *Est-A* and c_3 at *Pox-C*, with the exception of b_2 at *Est-B* for Sea-river, a cultivar that originated from Australia. The observed and expected values of heterozygosity based on these loci are shown in Table 2. Very high levels of heterozygosity were found within alfalfa population, especially for *Est-B* and *Pox-C* loci. The observed mean heterozygosities (Ho) ranged from 0.521 in the improved variety Ranger to 0.696 in Ghara-yonja landrace. The Syn_1 generation exhibited the highest *Ho* of 0.684. But, the two successive open pollinated generations showed reduction in heterozygosity. The reduction was about 5% from Syn_1 to Syn_2 and also from Syn_2 to Syn_3. The expected mean heterozygosity (He), as a measure of genetic diversity, ranged from 0.575 to 0.753, indicating high within-population variability. This result was in agreement with the *He* level of 0.665 to 0.717 in some cultivars evaluated by using seven SSR loci (Flajoulot *et al*., 2005). 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, or RFLP markers.

Results obtained on genetic distances (Nei, 1972) between pairs of alfalfa populations are shown in Table 3. Apparently, cultivar Sea-river, originating from Australia, was a more distant population, but, Ranger did not show any appreciable divergence from other alfalfa populations.

Estimation of F_{ST}, F_{IS}, and F_{IT} for three generations of Syn_1, Syn_2 and Syn_3 and one of their 12 polycrossed parents (Ghara-yonja) by three polymorphic loci (Table 4) indicated that, in spite of small differences in the effect of the three allozymic loci on the above mentioned parameters, similar results were obtained from all loci. Therefore, the overall

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Table 3. Nei's genetic distances between alfalfa populations based on analyzed polymorphic loci.

	Ranger	Sea-river	Ghara-yonja	Syn_1	Syn_2	Syn_3
Ranger	-	0.0224*	0.0075	0.0051	0.0021	0.0115
Sea-river		-	0.0260*	0.0250*	0.0211*	0.0486**
Ghara-yonja			-	0.0042	0.0019	0.0071
Syn_1				-	0.0012	0.0026
Syn_2					-	0.0058
Syn_3						-
Mean distance	0.0097	0.0281	0.0093	0.0076	0.0058	0.0151

* and **: Significant at 5 and 1% levels of probability, according to Workman and Niswander (1970) χ^2-values.

Table 4. Average F-statistics in alfalfa populations (three synthetic generations and Ghara-yonja landrace) obtained from three allozymic loci.

Loci	*F* components		
	F_{IS}	F_{ST}	F_{IT}
Pox-C	0.0796	0.0022	0.0817
Est-B	0.0801	0.0080	0.0874
Est-A	0.0986	0.0245	0.1156
Average	0.0861	0.0116	0.0948

inbreeding coefficient (F_{IT}) was mainly due to inbreeding effect (F_{IS}) or double reduction in alfalfa rather than random genetic drift or population differentiation (F_{ST}). The mean values of F_{IT}, F_{IS}, and F_{ST} were estimated as 0.0948, 0.0861 and 0.0116, respectively.

Accordingly, due to very large intra-population diversity in alfalfa, the three generations of synthetic alfalfa did not generate a large amount of differentiation compared to Ghara-yonja landrace. Flajoulot *et al.* (2005) also reported low amount of F_{ST} parameter (between 0.001 and 0.009), but significant for some pairs of alfalfa populations.

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بررسی تنوع ژنتیکی نسل‌های سنتتیک و ارقام یونجه با استفاده از وراثت تتراسومیک نشانگرهای آلوزیمی

م. ولیزاده، م. مهیجی، ن. یاسین‌زاده ، ص. نصراله‌زاده و م. مقدم

چکیده

برای اندازه‌گیری تنوع ژنتیکی درون و بین ارقام یونجه و نیز سه نسل از یک جمعیت سنتتیک (Syn_1، Syn_2 و Syn_3) به دست آمده از تلاقی پلی کراس بین ۱۲ والد، از الکتروفورز آنزیم‌ها استفاده شد. از بین پنج سیستم آنزیمی مورد مطالعه در حدود ۱۰۰ گیاهچه ۱۰ روزه از هر جمعیت با الکتروفورز در ژل افقی پلی اکریلامید، سه مکان ژنی ایزوزیمی تشخیص داده شد که وراثت تتراسومیک نشان دادند. با بررسی این مکان‌های پلی مورف، سطح بسیار بالایی از هتروزیگوسی (بین ۰/۵۲۱ تا ۰/۶۹۹) در داخل جمعیت‌های یونجه مشاهده شد. کاهش میزان هتروزیگوسی از Syn_1 به Syn_2 و از Syn_2 به Syn_3 حدود ۵٪ بود. با بکار بردن آزمون χ^2 ثابت گردید که نسل اخیر (Syn_3) همانند اکوتیپ بومی قره یونجه در تعادل هاردی ـ وینبرگ قرار دارد. کاربرد آماره F رایت نشان داد که میزان ضریب خویشامیزی کل F_{IT}= ۹/۴٪ برآورده شده عمدتاً (F_{IS} = ۸/۶۱٪) به خویشامیزی یا کاهش دوگانه قابل انتساب است تا رانش تصادفی ژنتیکی یا تمایز جمعیتی (F_{ST}= ۱/۶٪). بنابراین، به دلیل وجود تنوع بسیار بالای درون ـ جمعیتی، یونجه سنتتیک نتوانست تمایز واریته‌ای چشمگیری تولید کند. با وجود این، استفاده از مکان‌های آلوزیمی می‌تواند به شکل موفقیت آمیزی برای برآورد پارامترهای ژنتیکی جمعیت‌ها و ارقام یونجه بکار رود.

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