ASSESSMENT OF GENETIC VARIATION IN ALFALFA POPULATIONS USING RAPD MARKERS

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Abstract. Alfalfa (*Medicago sativa*) is a major perennial forage legume. Random Amplified Polymorphic DNA markers were used to assess genetic diversity within and between populations collected from different area of East Azarbaijan province, Iran. A total of 135 and 109 fragments were amplified using 10 arbitrary primers in individual and bulk samples showing 97.04 and 100% polymorphism, respectively. Analysis of molecular variance (Amova) revealed higher genetic variation within population than between population compared with that of between populations. The result indicates that RAPD markers could provide useful information for a breeding program and the method could be useful for estimating genetic relatedness among the heterogeneous crop like alfalfa.

Keywords: alfalfa, genetic variation, RAPD, analysis of molecular variance

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most cultivated forage legumes in the world due to its ability to fix atmospheric nitrogen molecule and its high protein content. Analysis of genetic variation within and between populations is essential for assessment of future risk of genetic erosion in alfalfa and for development of sustainable conservation and genetic improvement strategies. Alfalfa is autotetraploid (2n=4x=32), allogamous and seed-propagated, therefore successful analysis of genetic diversity in alfalfa has been hampered by the statistical methods available [13, 39].Various marker types have been used to assess genetic diversity in alfalfa, including isozymes [33], $RFLP_s$ [2, 4, 20, 32, 41] SSR_s [12, 26, 31, 40], and AFLP [17, 36, 46], RAPDs [14, 42, 43, 45]. RAPD technology is a simple,fast and cheap fast method for characterization of genetic diversity in species and population [16], although use of $RAPD_s$ is not appropriate for any application, such as parentage analysis [19, 35]. Brummer [3] proposed that identification and maintenance of distinctly divergent populations may be necessary in order to develop heterotic groups in alfalfa. Molecular markers have been proven as useful tools for assigning population into heterotic groups in crop plants [6, 15, 24, 27].The analysis of the genetic differentiation among the Lucerne populations showed the importance of the diversity available for breeding. Although molecular analysis of individual genotypes provides useful information about population genetic structure, this approach is not practical for analysis of large numbers of individuals and populations. Hence, the bulking of DNA over multiple alfalfa genotypes was proposed [45]. They evaluated four populations using RAPD markers and preliminarily demonstrated the usefulness of the DNA-bulking approach. High variation within population is reported in alfalfa populations and landraces [9]. Crochemore *et al.* [8] used RAPD for analysis of genetic diversity in west Europe alfalfa populations and found high within population variation compared with that of between-populations. Considering that an important part of the genetic variation is a within-population variation, this pattern of diversity underlines the large possibilities of genetic improvement which are possible through an exploitation of the genetic variability available in each group. The position of the wild material, both for the morphological characteristics the molecular markers and the wide variation within the wild population show that the wild population of *Medicago sativa* represents massive reservoirs of variation for future breeding of this species [7].

MATERIAL AND METHODS

Plant material: Ten local cultivated alfalfa populations (*Medicago sativa*) from East Azarbaijan province were used in the study (Table 1). This study was carried out in experiment field of institute ABRII.

Genomic DNA isolation: For DNA extraction, 300 seeds (30 seeds per population) were randomly selected. Genomic DNA from seed was extracted based on Kang & Yang [18] protocol. Single seed was homogenized in a 200ul DNA extraction buffer consisting 500mM NaCl, 100mM Tris-Hcl pH7.5, 50mM EDTA pH 7.5 and 10% SDS in sterile eppendorf tube using pestle. The homogenates were centrifuged at 10000 rpm at 4^0C for 10 min. Supernatant was taken into fresh tube and DNA was precipitated using isopropanol. The DNA was pelleted by centrifugation at 5000 rpm for 5 min. The resulted pellets were washed with 70% ethanol. After air drying, the pellet was dissolved in 50 ul TE pH 8.0. The quantity and quality of DNA extracts were determined using spectrophotometer and 1% agarose gel electrophoresis. For bulk analysis, genomic DNA extraction was performed using a mixture of 30 excised plants per population in 4 repeats.

Table 1. Geological and ecological characteristics of the studied alfalfa populations

Pop number	Pop name	Altitude (meter)	Topography	Climate
1	Siahrood	750	Plateau	Temperate
2	Zolbin	1650	Mountainous	Cold
3	Zavieh	1850	Mountainous	Cold
4	Ahar-horand	630	Plateau	Warm
5	Osku	1300	Plateau	Warm
6	Hashtrood	1400	Mountainous	Cold
7	Heris	1130	Mountainous	Cold
8	Malekan	1800	Plateau	Temperate
9	Sefidekhan	2000	Mountainous	Cold
10	Chaltab	1950	Mountainous	Cold

RAPD analysis: Ten random primers namely OPJ4, B1, B6, B7, B8, OPJ13, B10, OPA1, OPJ19, OPJ20 were selected based on their polymorphic banding pattern among the lucerne cultivars.(Table 2, Fig 5). PCR amplification was performed in a final volume of 25µl containing 1ul of DNA (30ng), 1µl primer (4pmol ul^{-1}), 13µl of PCR master kit (cinnagene PCR masterkit, cat, NO.RR8250c) and 10µl sterile deionized water. The amplification was carried out in a thermal cycler (Touchgene gradient, model: FTGRAD2D, Techne Ltd). Amplification program consisted of denaturation at 94°c for 5 min followed by 40 cycles of 93^0C for 60 sec, primer annealing at 40^0C for 60 sec and primer extension at 72^0C for 100 sec and one cycle of final extention 72^0C for 5 min, PCR products were separated on a 1.5% agarose gel containing ethidium bromide solution (0.5 ug/ml of gel solution) in 1x TAE buffer (pH 8.0). The size of the amplicons was determined using size standard marker (1Kb bp ladder plus or DNA ladder mix, MBI Fermentas, Lithuania). DNA fragments were visualized under UV light, documented in Gel Doc (Bio-Rad USA Biometra) and photographed.

Data analysis: Each RAPD band was scored as "1" and "0" for presence and absence, respectively. Genetic analysis was carried out using the POPGEN software ver 1.32 [44] to compute number of polymorphic loci per population, Nei's gene diversity [29], Shannon's Information index [22] and the genetic distances among population [28, 30]. Pair wise F_{ST} estimation and hierarchical analysis of molecular variance (AMOVA) [11] were carried out using ARLEQUIN 3.11 software [10].The studied populations were grouped based on Nei's pairwise measures of genetic [30] and F_{ST} using UPGMA sequential agglomerative hierarchical nested clustering method (SAHN) of NTSYS software ver. 2.02 [34].

RESULTS

A total 135 band were amplified using 10 primers and 131 bands (97.04 percent) were polymorphic. The amplified fragments size ranged from 200 to 2500 bp (Table2). The maximum and minimum numbers of bands were 19 and 8, which were amplified by primers B1 and B8, respectively. Populations No. 2 and 4 showed the highest (66) and lowest (6) number of fragment (Table 3). Nei's genetic diversity index, the maximum and minimum genetic diversity observed in population numbers 6 and 2 were 0.1999 and 0.1478, respectively (Table 4). Based on Nei's gene diversity within and total genetic diversity average (Hs) , (HT) were 0.1666 and 0.2224 respectively and (Gst) of among population for total bands was 0.2511. The highest genetically distance between different alfalfa populations, based on the Nei's genetic distance (1972), was between populations number 9 and number 3 which is about 0.1442 and the lowest genetically distance was between populations number 5 and 6 which is 0.0371 (Table 5).The variety between the populations was estimated as 29.98 percent and within the populations was estimated as 70.02 (Table 6). To analyze individuals inside populations with the aim of classification of the populations based on the matrix of the Nei's genetically distance [28] and FST correlation, UPGMA and CLINK algorithms were used and the UPGMA algorithm was used because of its higher cophenetic correlation coefficient of 0.6189 [23, 37] (Fig 1 and 2). The populations were divided into two separate groups.

Principal Coordinate Analysis (PCA): The principal component analysis was used as a complementary method for cluster analysis. The gained results demonstrated that the two first axes justified 48.8 percent of the primary changes of the indicators. (The first axis 29.2 percent and the second axis 19.2 percent).The three dimensional representation of populations based on the two first axes is shown in the figure 4.

Bulk analysis: For all populations and primers the number of scored loci was 109 ranging from 200 to 2500bp. B1 and OPJ19 primers showed the maximum and minimum band numbers, respectively (Table 2). The genetic distance calculated from RAPD patterns obtained with bulk DNA samples ranged from 0.0833 to 0.2341 (Table 5). Population number 1 was the most polymorphic, baring 71 bands, whereas population number 8 was the least polymorphic baring 40 bands (Table 3). In terms of Nei's gene diversity, population numbers 1 and 8 showed the maximum and minimum genetic diversity, respectively (Table 4). Population clustering [38] based on Nei's genetic distance [21] is shown in Figure 3.

Table 2. Details of RAPD analysis of 10 alfalfa population

Primer	Sequence (*5'-3'*)	Individual analysis	Range of amplicons	Bulk analysis	bp
		Number of bands		Number of bands	
B1	GGTTCGCTCC	19	2000-250	13	1400-200
OPA1	CAGGCCCTTC	16	1400-250	11	1500-250
OPJ4	CCGAACACGG	15	1300-250	11	1300-250
OPJ19	GGACACCACT	15	2100-250	9	1400-200
B6	TGCTCTGCCC	14	1850-350	10	1900-300
B7	GGTGACGCAG	14	1700-250	10	1300-250
B10	CTGCTGGGAC	12	1400-300	11	2100-300
OPJ13	CCACACTACC	12	1850-250	12	1800-250
OPJ20	AAGCGGCCTC	10	1250-300	12	1500-200
B8	GTCCACACGG	8	1100-300	10	1100-250

Table 3. Population polymorphism based on individual and bulk analysis

Population	Individual analysis		Bulk analysis	
	Polymorphic loci (no.)	Polymorphic loci (%)	Polymorphic loci (no.)	Polymorphic loci (%)
1	69	51.11	71	65.14
2	66	48.89	62	56.88
3	68	50.37	70	64.22
4	66	48.89	45	41.28
5	79	58.52	62	56.88
6	81	60.00	70	64.22
7	73	54.07	65	59.63
8	75	55.56	40	36.7
9	68	50.37	70	64.22
10	70	51.85	67	61.47
Mean	71.4	52.96	62.2	57.06

Table 4. Nei's gene diversity and shannon's information index for Individual and bulk analysis

Population	Individual analysis		Bulk analysis	
	Nei's gene diversity	Shannon's Information index	Nei's gene diversity	Shannon's Information index
1	0.1730	0.2617	0.2564	0.3765
2	0.1478	0.2253	0.1961	0.2981
3	0.1569	0.2377	0.2376	0.3547
4	0.1629	0.2449	0.1385	0.2113
5	0.1757	0.2696	0.2052	0.3087
6	0.1999	0.3024	0.2263	0.3422
7	0.1666	0.2548	0.2065	0.3125
8	0.1784	0.2697	0.1261	0.1919
9	0.1485	0.2273	0.2425	0.3598
10	0.1559	0.2382	0.2364	0.3492
Mean	0.1666	0.2532	0.2071	0.3105

Table 5. Nei's genetic distance for individual and bulk analysis in the lower and upper diagonal, respectively

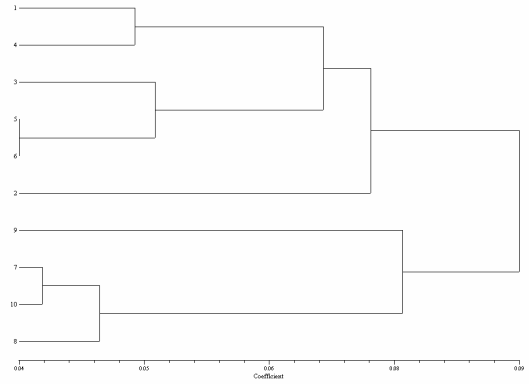
Population number	1	2	3	4	5	6	7	8	9	10
1	0	0.1180	0.1150	0.1226	0.1324	0.1359	0.1098	0.2000	0.0833	0.0852
2	0.0779	0	0.1431	0.1463	0.1209	0.1474	0.0956	0.1647	0.1115	0.1256
3	0.0869	0.0634	0	0.1787	0.1726	0.1087	0.1377	0.1757	0.1197	0.1150
4	0.0492	0.0779	0.0760	0	0.1342	0.2341	0.1204	0.1577	0.1646	0.1998
5	0.0511	0.0648	0.0469	0.0565	0	0.1723	0.1161	0.1320	0.1529	0.1489
6	0.0694	0.0858	0.0558	0.0742	0.0371	0	0.1443	0.2314	0.1491	0.1306
7	0.0736	0.1031	0.1005	0.1055	0.0633	0.0599	0	0.1714	0.0924	0.1128
8	0.0843	0.1027	0.1058	0.0954	0.0578	0.0510	0.0490	0	0.2170	0.2262
9	0.0554	0.1427	0.1442	0.1112	0.1091	0.0907	0.0847	0.0769	0	0.1101
10	0.0619	0.0993	0.1178	0.0957	0.0570	0.0614	0.0395	0.0420	0.0704	0

Table 6. Amova design for individual population

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among Population	9	1445.127	4.9556	29.98
Within Population	290	3363.867	11.59954	70.02
Total	299	4808.993	16.56521	-
Fixation Index	FST : 0.29976			

Table 7. Amova design for bulk population

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among Population	9	12.650	0.0000	0.000
Within Population	30	55.500	1.85000	100.00
Total	39	68.150	1.73889	-
Fixation Index	FST: 0.000			



Matrix correlation: r = 0.61892

Figure 1. Dendrogram of RAPD molecular data based on Nei's measures of genetic distances in individual analysis using unweighted pair group method of arithmetic means (UPGMA)

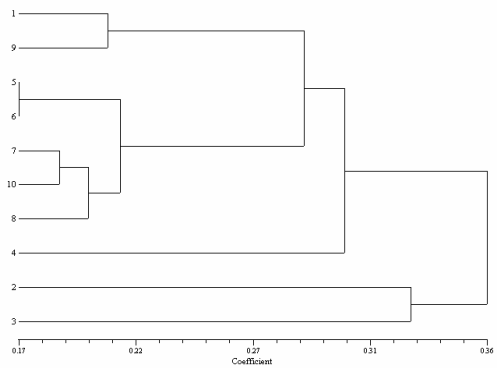


Figure 2. Dendrogram of RAPD molecular data based on FST in individual analysis using unweighted pair group method of arithmetic means (UPGMA)

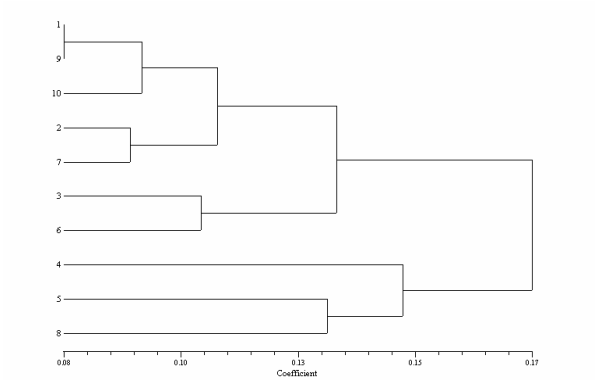


Figure 3. Dendrogram of RAPD molecular data based on Nei's measures of genetic distances in bulk analysis using unweighted pair group method of arithmetic means (UPGMA)

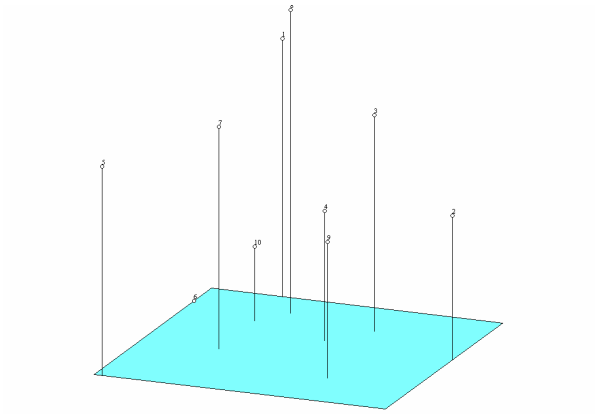


Figure.4. Principal co-ordinate analysis of individual population

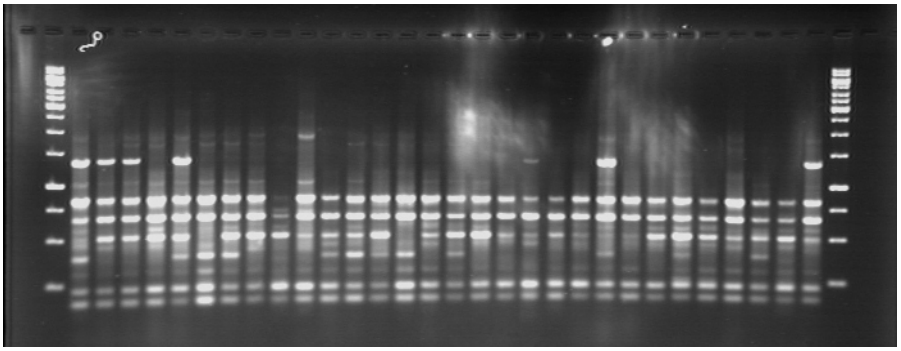


Figure 5. RAPD fragments for siahroad population using the primer B_6

DISCUSSION

The results showed that the used primers contain a high percent of multi-figure indicators. Having a high number of bands for each primer shows a high variety between the studied populations [1]. The average number of polymorphic bands, the individual samples was higher than bulk samples.(Table3).The presence of this band in individual plants was either due to non-specific nature or mis-priming of DNA templates with primers at a low annealing temperature usually kept for RAPD with random primers which was otherwise observed absent in bulk DNA samples [5]. Michelmore [25] reported that are RAPD markers could not be detected in bulk samples, they were represented less than 10% of the total DNA.Considering the fact that in a bulk sample, in repeated appearances of the same population, homogeneous bands were not observed and variety inside the populations of bulk samples were more than the variety between the populations of different groups (Table7),Chandra [5] in this case does not conform with reality and according to the gained results and Michelmore results it can be said that since variety inside populations by considering cross pollination of alfalfa is high, in bulk samples in each repetition the bands which were stronger and monomorphic,appeared in most of the populations and the weak bands with less populations were deleted .The classification of populations, the efficiency of clustering and dendrogram drawing show the separation of populations with far geographical distance. In this classification populations number 1 and 4 which are geographically closer and have similar geographical altitudes were categorized under the same subclass. The populations number 2, 3, 5, and 6 were also categorized under one subclass. The populations number 2 and 3 are closer to each other and have similar altitudes and the populations number 5 and 6 have similar altitudes and Sahand mountain is located between two populations.Dehghan- Shoar [9] have also reported the compatibility of genetic variety pattern with the geographical distribution pattern in Iran's alfalfa populations. In the classification of populations using bulk samples, populations 5 and 8 which are close to each other were put into one group and the population number 4 which has less altitude in comparison with other groups was separated from other groups. Populations number 1, 9, 10, 2 and 7 were put into one group and populations 3 and 6 which are close to each other were also put together in one group in the classification.The variation within population was large in alfalfa cultivars on the same set of population, Crochemore [8] Dehghan-shoar , Flajoulot and Gherardi [9, 13, 14] showed that, for the RAPD markers, it was larger than the variation between population.The allogamy of alfalfa, its autotetraploidy and sexual propagation caused large genetic variation within [20].In molecular data if the 2-3 first variants are able to justify only a small section of the changes of molecular data, it shows that the used molecular indices were from different parts of the genome and sample gathering was made from different parts of the genome. Thus, according to the gained results for the former components and latter components, it can be concluded that the chosen indices have had good genomic distribution. The results show that the individual samples are more suitable to show variation between the populations and thus are more suitable for the classification purpose.The result indicates RAPD markers could provide useful information for breeding program and the method could be useful for estimating genetic relatedness among the heterogeneous crop like alfalfa.

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