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Genetic diversity among and within alfalfa populations native to Azerbaijan based on RAPD analysis

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Genetic variation in ten indigenous populations of *Medicago sativa* from Azerbaijan was investigated by randomly amplified polymorphic DNA (RAPD) analysis. A total of 80 and 78 fragments were scored using ten arbitrary primers for individual and bulk analysis, respectively. The percentage of polymorphic loci was 67.95% for bulk analysis whereas for individual-based analysis it was 100%. In spite of the low gene diversity, the bulk analysis was highly efficient in discriminating among populations based on genetic distances. Analysis of molecular variance (AMOVA) showed that approximately 80% of the total genetic variation was attributable to intra-population variance. However, measurements of Φ_{st} (F statistics analog) were significant for all pairwise population comparisons. The within-populations considerable variation was expected due to the allogamy and autotetraploidy of alfalfa. Based on the differentiation among groups, the coancestry coefficient was the most effective in clustering populations. Among the four surveyed eco-geological traits, elevation of the region has affected populations' genetic structure more severely than any other. However, no significant differentiation between groups was observed.

Key words: alfalfa, diversity, molecular variance, RAPD, synthetic variety.

INTRODUCTION

Alfalfa (*Medicago sativa*), known also as lucerne, is native to western Asia and eastern Mediterranean. It is the world's oldest forage crop having the highest feeding value of all commonly grown hay crops (Duke, 1981; Frame *et al*., 1998; Anon, 2002). The chromosome number is 4n = 32 for the tetraploid and 2n = l6 for the diploid, sativa *vs* falcata, respectively.

The importance of genetic diversity in crops can be examined on the basis of two different perspectives. The first perspective is that genetic diversity may be a necessary condition to achieve high productivity and yield stability whereas the second perspective refers to the unique aspect of genetic resources to be used in breeding programs (Gepts & Papa, 2003). Therefore, the ability to identify genetic variation is *sine qua non* to the effort to select and conserve the genetic resources and use them efficiently.

Past limitations such as partly represented genetic diversity and the effect of natural selection on distribution of diversity associated with pedigree and morphological data, along with physiological and cytological markers, have been largely circumvented by the development of DNA markers (Soltis *et al*., 1992; Kidwell *et al*., 1994; Cruzan, 1998; Ghérardi *et al*., 1998) such as RFLPs (Botstein *et al*., 1980; Tavoletti *et al*., 1996; Pupilli *et al*., 2000), RAPDs (Williams *et al*., 1990), AFLPs (Zabeau & Vos, 1993; Julier *et al*., 2003) and SSRs (Tautz, 1989; Morgante & Olivieri, 1993; Provan *et al*., 1999). Their applicability could be extended in determining heterotic groups and identifying parents (Brummer, 1999). However, molecular markers are not successful in predicting heterosis (Riday *et al*., 2003; Riday & Brummer, 2004). As a result, the use of molecular markers to select parents

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has the dynamics to allow simultaneous maintenance of genetic diversity and performance.

RAPD markers, which use one or sometimes two short arbitrary primers (usually 8-10 bases) to amplify anonymous stretches of DNA (Yu & Pauls, 1994), have been applied in gene mapping, population genetics, molecular evolutionary genetics, and plant/animal breeding. This is mainly due to the speed and cost-effectiveness of the RAPD technique in generating many numbers of markers in a short time period (Welsh & McClelland, 1990; Williams *et al*, 1990; McClelland & Welsh, 1994; Skroch & Nienhuis, 1995; Jones *et al*., 1997; Bardakci, 2001). RAPDs have been used particularly in the investigation of the genetic relationships among accessions of several single species including tetraploid alfalfa (Barcaccia *et al*., 1994; Ghérardi *et al*., 1998; Mengoni *et al*., 2000; Thoquet *et al*., 2002; Arzani & Samei, 2004), and the phylogenetic relationships among species, as well (Excoffier *et al*., 1992; Huff *et al*., 1993; Dulloo *et al*., 1997; Bai *et al*., 1998; Gustine & Huff, 1999; Gustine *et al*., 2002; Bolaric *et al*., 2005).

Most commercial alfalfa cultivars are synthetics developed by randomly crossing selected parents (Busbice, 1969; Hill *et al*., 1988). Therefore, any selected genotype would be considered as a candidate parent to be incorporated into breeding programs and this is an important objective for almost all forage breeding programs (Breese & Tyler, 1986; Mumm & Dudley, 1994; Abo-elwafa *et al*., 1995; Maughan *et al*., 1995; Dubreuil *et al*., 1996; Yang *et al*., 1996; Menkir *et al*., 1997; Rumbaugh *et al*., 1998). Difficulties associated with distinguishing genetically diverse individuals have forced many breeders to include more parents in their synthetic cultivars in order to minimize inbreeding and conserve genetic variability both for yield and other traits. In spite of this the high amount of genetic variability for forage yield observed within many alfalfa cultivars suggests that a subset of the original parents could be crossed to produce higher yielding synthetic cultivar (Hill & Elgin, 1981). This could be easily accomplished using molecular markers for the identification of genetically diverse populations, and use at least four of them as parents to avoid inbreeding (Busbice, 1970; Smith *et al*., 1990; Bingham *et al*., 1994). Therefore, it would be interesting to utilize molecular marker analysis to study local alfalfa landraces for their genetic diversity.

A survey was conducted to assess the genetic variation among and within ten alfalfa populations from different regions of Azerbaijan-Iran based on RAPD polymorphism analysis. The objective of this research was to study their variation and the possibility for their use in future breeding programs.

MATERIALS AND METHODS

Plant material

Ten different *Medicago sativa* populations, grown in different locations of East Azerbaijan, Iran (Table 1) were used to evaluate variability via RAPD analysis.

Genomic DNA preparation

Collected seeds from each population were stored at 4°C. For the RAPD assay, a random seed sample from each population was used. Seeds were germinated in a 16/8 hrs day-night photoperiod and room temperature. A total of 300 *in vitro* germinated single seeds (30 seeds per population) were randomly selected for individual DNA extraction. Genomic DNA extraction from excised cotyledons was processed following the Kang & Yang (2004) method. Single seed-derived cotyledons were homogenized in a 50 μl DNA extraction buffer (500 mM NaCl, 100 mM Tris-HCl pH 7.5, 50 mM EDTA pH 7.5 and 10% SDS). In a next step, another 50 μl of extraction buffer were added and centrifuged at 10000 g for 5 min at 4°C. The supernatant was transferred in a clean tube for isopropanol extraction using centrifugation at 5000 g for 5 min at 4°C. The resulted pellets were washed with 70% ethanol, dried, and resuspended in 50 μl sterile fresh TE buffer. The quantity and quality of genomic DNA extracts were determined using spectrophotometrical and electrophoretical (1% w/v of agarose gels) procedures. For the analysis of bulk samples, genomic DNA extraction was performed using a mixture of 30 excised plantlets per population.

In vitro template amplification

RAPD reactions were conducted on a Programmable Thermal Cycler (Touchgene Gradient, model: FTGRAD2D, Techne Ltd) using the following profile: 4 min at 94°C for initial denaturation, 40 cycles of 1 min at 93°C for denaturation, 1 min at 40°C for annealing, and 100 sec at 72°C for extension. Final extension time was 6 min at 72°C. The total volume for reactions was 25 μl. Each reaction contained 1 μl of DNA template (25 ng), 1 μl of arbitrary primers (at a final concentration of 4 pmol μl^{-1}), 12.5 μl of CinnaGen PCR Master Mix (CinnaGen PCR Master Kit, Cat. No. PR8250C) and 10.5 μl sterile deionized

TABLE 1. Geological, ecological and agricultural traits of the populations studied

Pop	City or Township	Village	Elevation	Topography	Climate	Planting type
1	Bostan Abad	Bashkand	2400	Mountainous	Very cold	Dry farming
2	Heris	Khoja	1650	Mountainous	Temperate	Semi-dry farming
3	Varzghan	Khervang	1450	Mountainous	Cold	Dry farming
4	Sarab	Baftan	1700	Mountainous	Cold	Irrigated farming
5	Bostan Abad	Garachai	2000	Mountainous	Cold	Dry farming
6	Varzgan	Almord	1100	Mountainous	Cold	Dry farming
7	Tabriz	Satello	1200	Plateau	Warm	Irrigated farming
8	Varzghan	Dizajsafarali	1700	Mountainous	Cold	Irrigated farming
9	Maraghae	Kordedeh	2000	Mountainous	Cold	Dry farming
10	Maraghae	Goltapae	1800	Mountainous	Cold	Dry farming

water. Amplified fragments were separated on 1.5% agarose gel. Following electrophoresis, gels were stained in 0.1 g ml^{-1} ethidium bromide (EtBr) and then documented with a Bio-Doc Analyze System (Biometra) under UV illumination.

Analysis of locus profiles

Marker patterns were scored as presence (1) or absence (0) for each locus separately. Genetic analysis was carried out using the POPGEN software (ver. 1.32) to compute the number of polymorphic loci per population, Nei's gene diversity (Nei, 1973) and the genetic distances among populations (Nei, 1972, 1978).

The hierarchical variance components and the F statistic analogs (Φ statistics) for the RAPD phenotypes were measured via the AMOVA procedure (Excoffier *et al*., 1992; Huff *et al*., 1993; Huff, 1997) using Arlequin 3.11 (Excoffier *et al*., 2005). The permutational procedure was set at 2000 on the original inter-individual squared distance matrix to provide significance tests for each of the hierarchical variance components and related F statistic analogs.

To cluster the populations, the matrix of pairwise Nei's measures of genetic distances (Nei, 1978) and the Φ statistics as inter-population distances were used for the sequential agglomerative hierarchical nested clustering method (SAHN) as available in NTSYS (ver. 2.02) based on the UPGMA algorithm (Sneath & Sokal, 1973). Cophenetic correlations were also determined by the MXCOMP program of NTSYS to test the association between input and output of the distance matrix (Mantel, 1967).

Analyses were performed in two ways: 30 single samples per population (individual analysis) and a mixture of 30 samples (bulk analysis).

RESULTS

An informative and reproducible amplification of RAPD fragments was established using the arbitrary primers (Fig. 1, Table 2).

Individual analysis

A total of 80 polymorphic loci (8 loci per arbitrary primer) ranging from 200 to 3000 bp (Table 2) were identified and used to analyze genetic diversity within and among populations. The maximum and minimum numbers of bands observed by primers B7 and OPJ4 were 11 and 5, respectively (Table 2). Population numbers 9 and 10 were the most polymorphic baring 68 and 67 bands, respectively whereas population numbers 2 and 1 were the least polymorphic (Table 3). In terms of Nei's gene diversity, population numbers 9 and 5 showed the maximum and minimum genetic diversity, respectively (Table 3).

Population clustering (Sneath & Sokal, 1973) based on Nei's genetic distance (Lamboy, 1994) is shown in Figure 2. Populations were divided into three groups with a matrix correlation of 0.607 (Mantel, 1967; Rohlf, 1972; Smouse *et al*., 1986). The

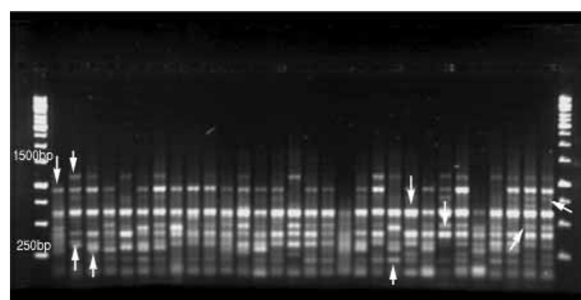

FIG. 1. RAPD fragments for Khervang population using the primer OPJ13. Arrows indicate the scored bands.

TABLE 2. Description of arbitrary oligonucleotide primers used for RAPD analysis

Primer	Sequence 5′ - 3′	Individual analysis: Number of bands	Individual analysis: Band size range (approx.)	Bulk analysis: Number of bands	Bulk analysis: Band size range (approx.)
OPJ4	CCGAACACGG	5	550-1250	5	550-1250
B1	GGTTCGCTCC	7	350-900	12	150-800
B6	TGCTCTGCCC	10	230-1400	7	230-1400
B7	GGTGACGCAG	11	250-1500	10	250-1500
B8	GTCCACACGG	8	270-1100	7	270-1100
OPJ13	CCACACTACC	9	220-1200	4	350-900
B10	CTGCTGGGAC	10	370-3000	10	370-2700
OPA1	CAGGCCCTTC	6	300-1600	6	250-700
OPJ19	GGACACCACT	6	300-1100	7	200-800
OPJ20	AAGCGGCCTC	8	200-1250	10	200-1250

TABLE 3. Population polymorphism based on individual and bulk analysis

Population	Polymorphic loci (no.)	Polymorphic loci (%)	Loci: Low frequency[1]		Loci: High frequency[2]		Nei's gene diversity
1	54	67.50	33	61.1%	2	3.7%	0.2008
2	50	62.50	24	48.0%	7	14.0%	0.1992
3	63	78.75	35	55.5%	7	11.0%	0.2554
4	54	67.50	32	59.2%	5	9.2%	0.2019
5	55	68.75	45	81.8%	3	5.4%	0.1674
6	53	66.25	26	49.0%	6	11.0%	0.2222
7	57	71.25	31	54.3%	4	7.0%	0.2269
8	61	76.25	32	52.4%	4	6.5%	0.2386
9	68	85.00	37	54.4%	0	0.0%	0.2728
10	67	83.75	31	49.2%	2	2.9%	0.2868
Total	80	100.00	47	58.7%	9	11.2%	0.2800
Bulk samples for all populations	53	67.95	–	–	–	–	0.2241

[1] allele 0 > 0.7, [2] allele 1 > 0.7

AMOVA (Excoffier *et al*., 1992; Huff *et al*., 1993; Huff, 1997) based on the population grouping did not show significant differences among any groups based on either of the three models (Table 4A) rendering the dendrogram data (Fig. 3) inefficient. On the contrary, AMOVA data revealed effective differentiation among populations within groups (Fig. 3) and furthermore among individuals within populations.

The hierarchical variance components were computed (Excoffier *et al*., 1992; Huff *et al*., 1993; Huff, 1997) in order to define the RAPD ability to differentiate genetically among alfalfa populations (Table 4B). Among the four grouping models, variation among groups was significant only when based on elevation indicating that elevation was the force that differentiated the populations at the specific areas. Data indicated that the ecological criterion was more effective than the genetic models. On the contrary, in all groupings models, variation among populations within groups and among individuals within populations was significant in accordance to data previously discussed (Table 4A).

Clustering based on F_{ST} values (data not shown) was effective since all population pairwise differences, as proportion of the total variance, were significant ($p < 10^{-5}$) based on 2000 random permutations.

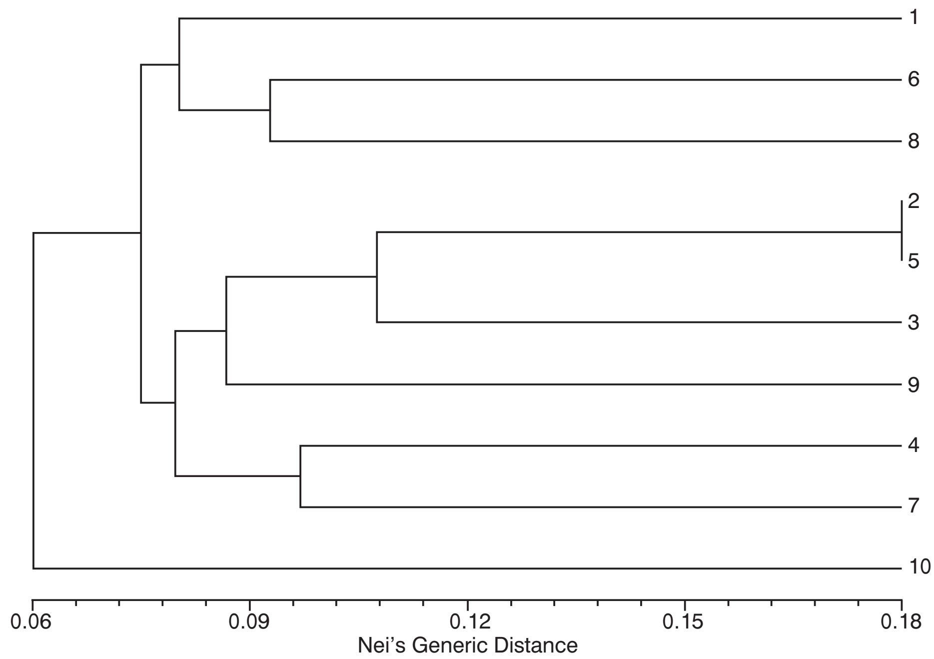

FIG. 2. Sequential agglomerative hierarchical nested cluster analysis (SAHN) based on Nei's measures of genetic distances in individual analysis (see Table 1 for population identification).

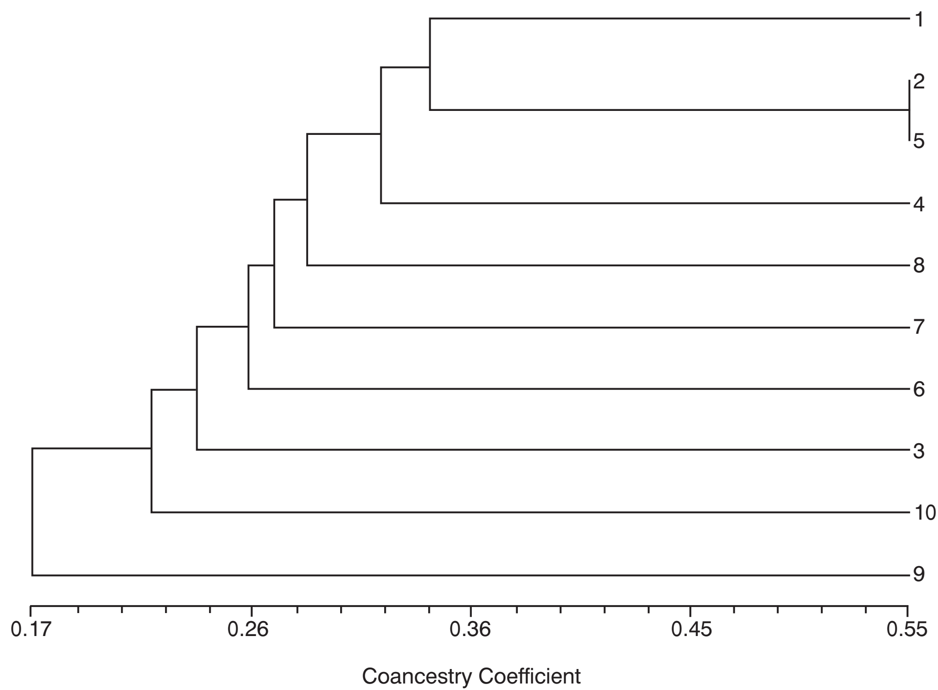

FIG. 3. Sequential agglomerative hierarchical nested cluster analysis (SAHN) based on coancestry coefficient in individual analysis (see Table 1 for population identification).

TABLE 4. The hierarchical variance components of various grouping models

A

	Grouping models					
	Based on coancestry coefficient[1]		Based on F_{ST}[2]		Based on Nei's genetic distance[3]	
Source of variation	Variation (%)	*p*	Variation (%)	*p*	Variation (%)	*p*
Among groups	0.41	0.355	–0.86	0.600	–2.05	0.870
Among populations within groups	20.59	$< 10^{-5}$	21.41	$< 10^{-5}$	22.14	$< 10^{-5}$
Among individuals within populations	79.00	$< 10^{-5}$	79.45	$< 10^{-5}$	79.90	$< 10^{-5}$

[1] First group includes populations 3, 6, 7, 9 and 10 and the second group includes the others

[2] First group includes populations 1, 2, 5, 6 and 8 and the second includes the others (clustering based on F_{ST} not shown)

[3] First group includes populations of 1, 6, 8 and 10 and the second includes the others

B

	Grouping models based on eco-geological characters							
	Elevation[1]		Topography[2]		Weather[3]		Farming type[4]	
Source of variation	Variation (%)	*p*	Variation (%)	*p*	Variation (%)	*p*	Variation (%)	*p*
Among groups	3.88	0.047	–3.80	0.804	–1.06	0.513	–3.30	0.970
Among populations within groups	18.41	$< 10^{-5}$	22.25	$< 10^{-5}$	21.37	$< 10^{-5}$	22.76	$< 10^{-5}$
Among individuals within populations	77.72	$< 10^{-5}$	81.55	$< 10^{-5}$	79.69	$< 10^{-5}$	80.54	$< 10^{-5}$

[1] Based on elevation (≥ 1699 m and < 1699 m)

[2] Based on topography (plateau and mountainous)

[3] Based on weather (between very cold-cold and temperate-warm)

[4] Based on irrigation pattern (between dry farming and irrigated or semi-dry farming)

Bulk analysis

For all populations and primers the number of scored loci was 78 ranging from 150 to 2700 bp. The percentage of polymorphic bands was 67.95% of the total bands (Table 3) as compared to data of the individual-based strategy discussed previously. B1 and OPJ13 primers showed the maximum and minimum band numbers, respectively (Table 2). Nei's bulk gene diversity index was 0.2241 as compared to the range 0.1674-0.2868 for the individual data (Table 3).

The cophenetic correlation for the clustering based on Nei's genetic distances between bulks (Fig. 4) was 0.60479. The resulted groups were not in agreement with our eco-geological knowledge of the populations (Table 1).

DISCUSSION

Results indicated fewer polymorphic bands using the bulk analysis as compared with the individual analysis (Table 3), probably because of higher genetic variation within population relative to that among populations (Table 4A, 4B).

Separation of the population number 10 from all other populations (Fig. 2) indicates its geological distance from the other populations studied. The efficiency of clustering based on the coancestry coefficient was sufficient to observe the apparent genetic separation of the geologically distant populations 9 and 10 as well as to assign population numbers 1, 2 and 5 on a branch with small geological distances (Figs 3, 5, Table 1). Mount Sahand stands as a physical barrier between these two populations from all

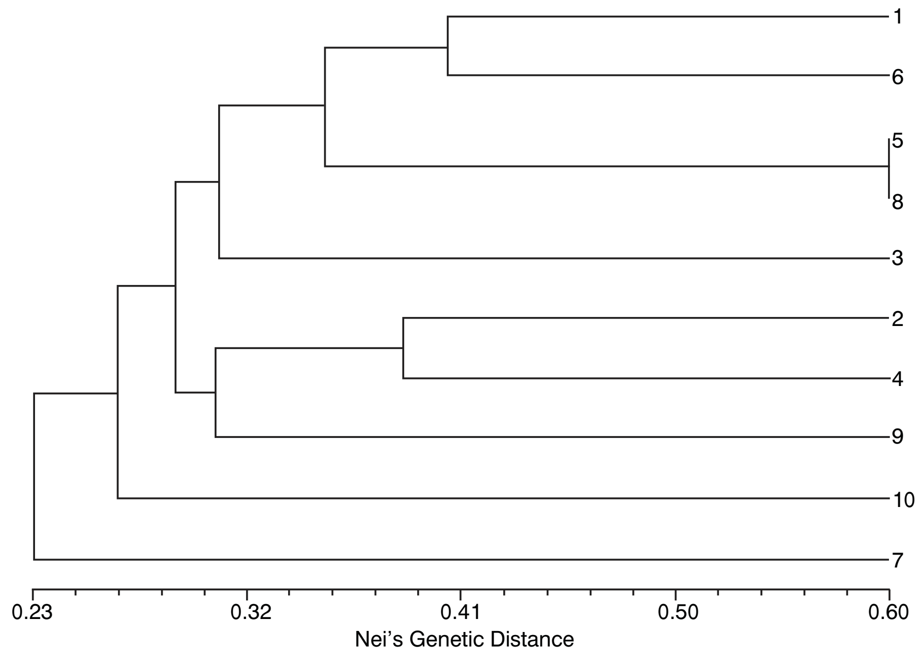

FIG. 4. Sequential agglomerative hierarchical nested cluster analysis (SAHN) based on Nei's measures of genetic distance pertinent to bulk analysis (see Table 1 for population identification).

others (Fig. 5). These data were further corroborated from the coancestry coefficient based clustering (Table 4A).

Bulk-based grouping (Fig. 4) did not indicate any differentiation based on AMOVA (data not shown) using the information obtained from the marker patterns of individuals within populations. The diversity in the bulk analysis was underestimated because of the elimination of the within population variation which was the largest source of variation. This has resulted to reduce the mean of Nei's gene diversity from 0.28 in the individual analysis to 0.22 in the corresponding bulk analysis (Table 3). High or low frequency loci could negatively affect the actual amount of the heterogeneity as it has been reported by Kidwell *et al*. (1994). In our study, such conditions were common for most of the loci, ranging from 52% to 87% of the polymorphic bands and depending on the population (Table 3). Furthermore, low abundance amplicons were probably eliminated in the bulk PCR reactions because of the high competition among templates especially when they were faint. These two factors have probably caused the apparent reduction in the variance observed among groups in the bulk analysis (data not shown) as compared with the individual analysis (Table 4A, 4B). This further resulted in increasing the inter-bulk dissimilarity (Table 5) by eliminating the intra-population diversity. In this sense, the greater the number of samples in the bulk analysis, the lower will be their apparent diversity (Yu & Pauls, 1993; Kidwell *et al*., 1994; Segovia-Lerma *et al*., 2003). Therefore, the presence of 30 individuals per bulk sample in our study was an additional reason

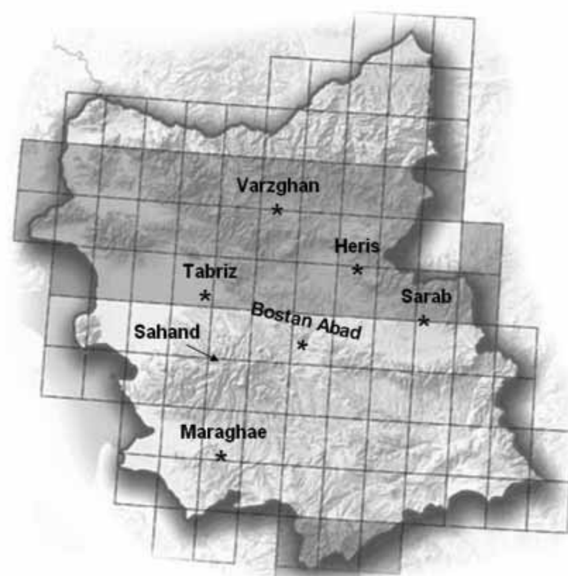

FIG. 5. Map of East Azerbaijan-Iran (photo from National Cartographic Center of Iran; http://www.ncc.org.ir).

TABLE 5. Nei's genetic distances for individual and bulk analysis shown in the upper and lower diagonal, respectively

Population (no.)	1	2	3	4	5	6	7	8	9	10
1	–	0.0849	0.0472	0.0915	0.1108	0.0836	0.0349	0.0752	0.0568	0.0546
2	0.3677	–	0.0758	0.0552	0.1794	0.0274	0.0663	0.1125	0.0855	0.0696
3	0.1978	0.2296	–	0.0729	0.1378	0.0679	0.0487	0.0856	0.0799	0.054
4	0.3864	0.3864	0.3137	–	0.1241	0.0603	0.0963	0.0794	0.0893	0.0602
5	0.4855	0.3677	0.4855	0.2792	–	0.1537	0.1295	0.0602	0.0918	0.0973
6	0.4055	0.3677	0.3314	0.1520	0.2963	–	0.051	0.0920	0.0701	0.0552
7	0.1226	0.2792	0.0662	0.2963	0.3864	0.3137	–	0.0831	0.0446	0.0563
8	0.3137	0.2136	0.2136	0.2963	0.5955	0.3137	0.2624	–	0.057	0.0406
9	0.2136	0.3494	0.2136	0.2624	0.3494	0.3494	0.1372	0.2296	–	0.0451
10	0.2624	0.2624	0.1978	0.2792	0.4447	0.2963	0.1823	0.2136	0.1520	–

Mean for lower diagonal: 0.2916
Mean for upper diagonal: 0.0745

for the declined apparent diversity. In spite of these limitations bulk analysis could be effective in differentiating among populations and/or cultivars. However, the wide range of high-low frequency bands observed, resulting in an expected bias, did not allow us to differentiate among groups.

Higher variation within than among populations was observed, consistent with reported results (Crochemore *et al*., 1996; Dehghan-Shoar *et al*., 1997; Ghérardi *et al*., 1998; Flajoulot *et al*., 2005). The allogamy of alfalfa, its autotetraploidy and sexual propagation result in large genetic variation at the intra-population or variety levels (Kidwell *et al*., 1994) and did not allow effective genetic discrimination among populations. Even though, promising individuals and/or alleles are usually found in any population. The high within-population variation can hinder the rate of improvement for polygenic traits such as forage yield, especially in synthetic varieties (Hill *et al*., 1988).

Summarizing our data, the coancestry coefficient based clustering seemed to be the most efficient method for among group differentiation whereas bulk analysis was efficient for a large scale assay and inter-population or cultivar identification, although not effective for understanding the evolution of biological diversity. Molecular markers may did not seem to be useful for selecting genotypes to be used directly as parents for synthetic cultivar development, but they could be used to identify new promising germplasm complementary to the existing. Finally, the heterotic group identification could be efficient by using both classical and molecular methods based on either of the approaches studied.

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