Genetic similarities among Iranian populations of *Festuca*, *Lolium*, *Bromus* and *Agropyron* using amplified fragments length polymorphism (AFLP) markers

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Abstract

The study of genetic variation and phylogenetic relationships is essential for the efficient selection of superior plant material and conducting introgression breeding programs. In Iran, despite the wide geographical distribution of grasses no report is available on the genetic diversity and relationships of cool season grass populations. In this study amplified fragment length polymorphism (AFLP) was used to study 42 populations from eight species of *Festuca arundinacea* Schereb., *Festuca. pratensis* Huds., *Festuca. rubra* L., *Festuca. ovina* L., *Lolium perenne* L., *L. rigidum Gaud., Bromus tomentellus* Boiss. and *Agropyron cristatum* (L) Gaertn. The number of amplified products ranged from 11 to 78 per primer combination and a total of 497 markers were scored. Jaccard's genetic similarity coefficients among populations ranged from 0.15 to 0.88 showing high levels of inter and intra-specific genetic diversity. The cluster analysis and principle coordinate analysis (PCOA) reflected the phylogenetic relationships among species and clearly demonstrated differences in the degree of similarity among accessions. Results indicated that AFLP is a useful technique to reveal genetic diversity at different taxonomic levels of grasses and might facilitate the selective introgression of useful genes in plant breeding programs.

Key words: Genetic similarity; Grasses; AFLP marker

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INTRODUCTION

Forage and turf grasses have an important role in sustainable agriculture and contribute extensively to the world economy. They play a major role in meat and dairy production and are important in soil conservation, environmental protection, and outdoor recreation (Wang *et al*., 2001).

The genus *Festuca* contains approximately 100 species, some of which are commonly used as forage and turf grasses. They belong to the grass family Poaceae, subfamily Pooideae, and tribe *Poeae* (Soreng and Davis, 1998). Based on leaf texture these are divided in two subgeneric types, including the coarse fescues (e.g. *Festuca arundinacea* and *Festuca pratensis*) and fine fescues (e.g. *Festuca rubra* and *Festuca ovina*) (Turgen, 1985). Tall fescue (*Festuca arundinacea*) is the most important perennial forage and turf grass species of this genus and is widely grown throughout the temperate regions of the world (Saha *et al*., 2005; Sleper, 1985). Tall fescue is a hexaploid (2n=6x=42) consisting of three genomes ($PPG_1G_1G_2G_2$) with the P genome derived from diploid *Festuca pratensis* (2n=2x=14) and the G_1G_2 genome from the tetraploid *Festuca arundinacea* var *glaucescens* (2n=4x=28) (Sleper, 1985; Seal, 1983). The genus *Lolium*, which is closely related to the *Festuca*, contains several diploid species including the outcrossing perennial *Lolium perenne*, the annual *Lolium multiflorum*, and the self-pollinating annual *Lolium temulentum*, all of which are cross-compatible (Saha *et al*., 2004). Rigid ryegrass (*Lolium rigidum*) is

Table 1. The names, Codes, and Origins of accessions surveyed using AFLP.

Accession Number	Accession Name	Species	Accession Code*	Origin
1	FAL2	*Festuca arundinacea* Schreb.	6000.39	Yazdabad, Iran
2	FAL5	*Festuca arundinacea* Schreb.	6000.13	Semirom, Padena, Iran
3	FAL6	*Festuca arundinacea* Schreb.	6000.78	Yasooj, Iran
4	FAL7	*Festuca arundinacea* Schreb.	-	Semirom, Iran
5	FAL9	*Festuca arundinacea* Schreb.	RCAT064772	Tyukod, Hungary
6	FAM3	*Festuca arundinacea* Schreb.	6000.11	Fozveh, Iran
7	FAM5	*Festuca arundinacea* Schreb.	6000.30-1	Fozveh, Iran
8	FAM6	*Festuca arundinacea* Schreb.	RCAT042281-1	Pakozd, Hungary
9	FAM9	*Festuca arundinacea* Schreb.	6000.112	Daran, Iran
10	FAM11	*Festuca arundinacea* Schreb.	10D2	Mobarakeh, Iran
11	FAN1	*Festuca arundinacea* Schreb.	14D (Rebel)	NewJersey, USA
12	FAN2	*Festuca arundinacea* Schreb.	6000.79	Semirom, Iran
13	FAN3	*Festuca arundinacea* Schreb.	RCAT064767-1	Pacin, Hungary
14	FAN6	*Festuca arundinacea* Schreb.	3D	Gelogerd, Iran
15	FAN8	*Festuca arundinacea* Schreb.	-	Charmahal, Iran
16	FAN9	*Festuca arundinacea* Schreb.	RCAT042279-1	Kecskemet-Solt, Hungary
17	FAN10	*Festuca arundinacea* Schreb.	06477	Anon, Hungary
18	FAO4	*Festuca arundinacea* Schreb.	RCAT042279-2	Kecskemet-Solt, Hungary
19	FAO5	*Festuca arundinacea* Schreb.	RCAT064767-2	Pacin, Hungary
20	FAO6	*Festuca arundinacea* Schreb.	6000.38	Yazdabad, Iran
21	FAO10	*Festuca arundinacea* Schreb.	RCAT041815-1	Sarkad, Hungary
22	FAA4	*Festuca arundinacea* Schreb.	1000.52	Fozveh, Iran
23	FAG9	*Festuca arundinacea* Schreb.	1000.247	Fozveh, Iran
24	FAJ6	*Festuca arundinacea* Schreb.	12000.26	Anonymous, Poland
25	FAV3	*Festuca arundinacea* Schreb.	4000.44	Semnan, Shahrood, Iran
26	FPL8	*Festuca pratensis* Huds.	6000.70	Fozveh, Iran
27	FPM4	*Festuca pratensis* Huds.	6000.67	Borojen(Nasirabad), Iran
28	FPN11	*Festuca pratensis* Huds.	2D	Koohrang, Iran
29	FPO7	*Festuca pratensis* Huds.	6000.81	Gorgan, Iran
30	FRP1	*Festuca rubra* L.	RCAT042391	Tarhos, Hungary
31	FRP2	*Festuca rubra* L.	RCAT042387	Derekegyhaz, Hungary
32	FRP3	*Festuca rubra* L.	6000.21	Fozveh, Iran
33	FRP4	*Festuca rubra* L.	RCAT042393	Veszto, Hungary
34	FOP7	*Festuca ovina* L.	6000.107	Semirom, Iran
35	LPI1	Lolium perenne L.	12000.30	Yazdabad, Iran
36	LPI5	*Lolium perenne* L.	12000.46	Yazdabad, Iran
37	LRJ11	*Lolium rigidum* Gaud.	12000.18	Fozveh, Iran
38	LRJ12	*Lolium rigidum* Gaud.	12000.55	Fozveh, Iran
39	ACC4	*Agropyron cristatum* (L) Gaertn.	1000.33	Tehran, Iran
40	ACC8	*Agropyron cristatum* (L) Gaertn.	1000.102	Fozveh, Iran
41	BTR3	*Bromus tomentellus* Boiss.	2000.51	Yasooj-margoon, Iran
42	BTQ8	*Bromus tomentellus* Boiss.	2000.9	Tehran, Iran

*The accession codes are given by Iranian or Hungarian gene banks.

another annual diploid in this group.

Russian bromegrass (*Bromus tomentellus*) is a perennial grass with a wide geographical distribution in the most arid and semiarid regions of Iran, as well as neighboring countries (Rechinger, 1973). Ecotypes of *B. tomentellus* are well adapted to both cool-moist and cool-dry environments and are grown at different densities on approximately 15 million hectares of native rangelands in Iran, mixed with other grasses and legumes (Mirlohi *et al*., 2006). Crested wheatgrass

(*Agropyron cristatum*) is a cross-pollinating diploid, containing the basic P-genome (Dewey, 1984; Love, 1984), and is widely distributed in the natural habitats of Iran.

Among molecular techniques for genetic assessment, amplified fragment length polymorphisms (AFLP) is a DNA marker system based on a combination of polymerase chain reaction (PCR) and restriction enzyme analyses and reveals high levels of polymorphism. AFLP is highly reproducible, less sensitive to reaction conditions and does not require DNA sequence information (Krauss and Peakall, 1998; Vos *et al*., 1995). This technique have been effectively used to study genetic variation, characterize accessions and asses genetic similarities between and within genera and species of grasses (Vergara and Bughrara, 2004; Wu *et al*., 2004; Vergara and Bughrara, 2003; Guthridge *et al*., 2001). Mian *et al*. (2002) used AFLP markers to determine genetic diversity and to distinguish 18 populations of tall fescue from USA, using the DNA bulk strategy. Fjellheim and Rognli (2005) assessed 12 Nordic cultivars and one Icelandic natural population of meadow fescue (*Festuca pratensis* Huds.) by the AFLP marker technology and found high levels of genetic diversity. Guthridge *et al*. (2001) assessed genetic diversity within and between perennial ryegrass (*Lolium perenne* L.) populations using AFLP markers and interpreted their findings in terms of breeding history of genotypes.

Reliable characterization of native plant germplasm is an essential step towards better use of wild genetic resources in plant improvement programs. The aim of the present study was to analyze genetic diversity and inter-population variability in different species of *Festuca*, *Lolium*, *Bromus*, and *Agropyron* native to Iran using AFLP markers.

MATERIALS AND METHODS

Plant materials: Forty two populations belonging to eight species of the genera *Festuca*, *Lolium*, *Bromus*, and *Agropyron* were used in this work (Table 1). Iranian accessions were collected from different geo-

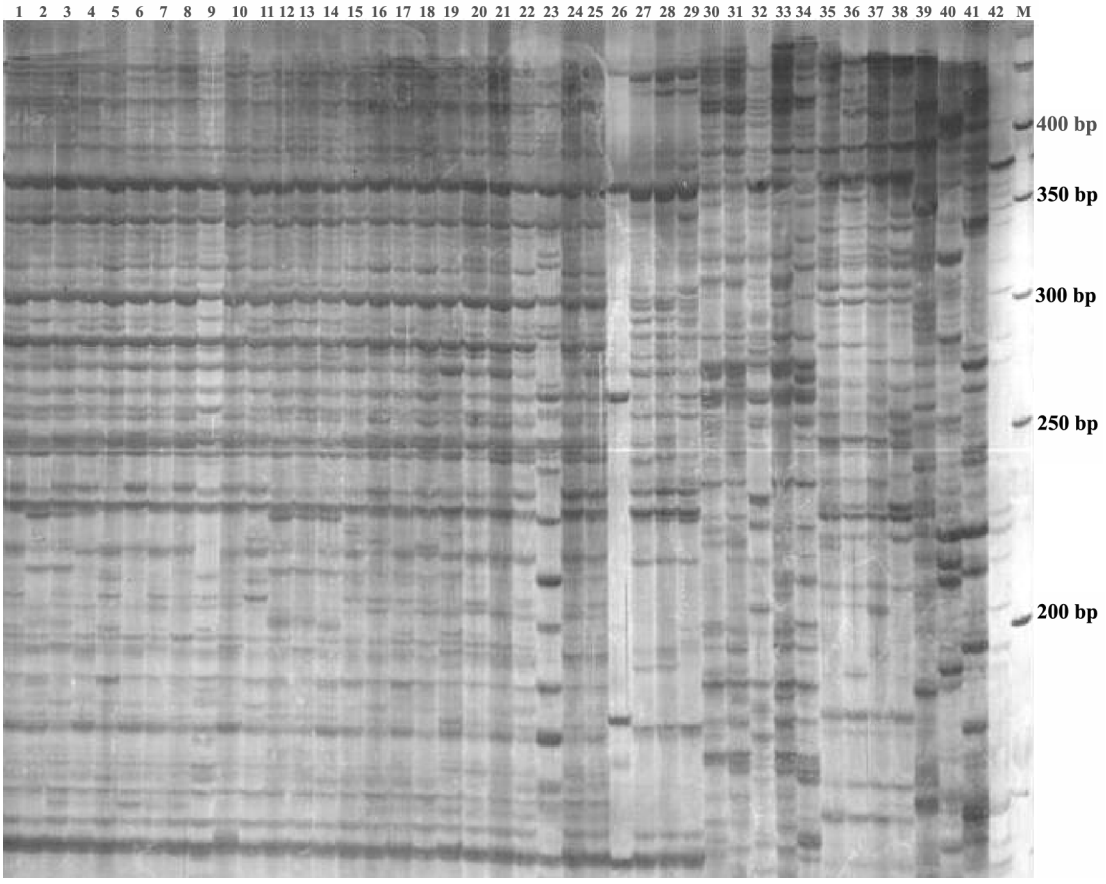

Figure 1. AFLP profile of 42 grass accessions using *Eco*RI-ATC and *Mse*I-CGC primer combinations (Information for each lane is presented in table 1, accessions number 1 to 42).

graphical regions nation wide. Out of the 42 accessions, 13 originated from Hungary, Poland and USA, kindly provided by the gene bank of the Hungarian Institute of Agrobotany (HIFA), Tapioszele, Hungary. All accessions were germinated and grown in a greenhouse before use in DNA extraction.

DNA extraction and amplified fragments length polymorphism (AFLP) profiling: For DNA extraction, young leaf tissue was equally sampled from 30 plants of each accession and bulked together. Genomic DNA was isolated according to the procedure described by Dellaporta *et al*., (1983). DNA was quantified by spectrophotometer (Beckman-DU 530, Germany) and its quality was checked by agarose gel electrophoresis (Biorad, Germany).

Isolated genomic DNA (approximately 300ng) was digested with *Eco*RI and *Mse*I restriction enzymes at 37ºC for 3 h. The restricted DNA fragments were ligated to *Eco*RI and *Mse*I adaptors overnight at 37ºC and the product was then diluted (1:5). Pre-amplification reactions were performed with *Eco*RI+*C* and *Mse*I+*C* AFLP primers. The amplification products were diluted (1:5) and stored at -20ºC until use for selective amplification. Selective amplification was carried out with 12 combinations of *Eco*RI+3 and *Mse*I+3 primers (Table 2) in a final volume of 20 µl containing 4 µl of the diluted pre-amplification product, 15 ng of the *Eco*RI and *Mse*I primers, 1X PCR buffer, 20 mM $MgCl_2$, 1.0 U of *Taq* DNA polymerase (Roche company, Germany) and 0.2 mM dNTPs (deoxynucleotide triphosphates).

The selective amplification product was mixed with 10 µl of the loading buffer, and the mixture was denatured at 95ºC for 4 min and immediately placed on ice. Five µl of each of the denatured samples was loaded onto a 6% (w/v) polyacrylamide gel containing 7 M urea and electrophoresis was conducted with constant power (100 W) at a constant temperature of 50ºC for 2.5 h in a Biometra S_2 sequencing gel. After electrophoresis, gels were fixed for 30 min in 10% (V/V) acetic acid solution and immediately afterwards, stained with silver nitrate (Pillay and Myers, 1999). A typical sample of stained gel is shown in Figure 1.

Data analysis: For data analysis, AFLP bands throughout the gel profile were scored as present (1), absent (0) or ambiguous (9), at least twice. The NTSYSpc v.2.02 software was used to generate genetic similarity matrices, create a dendrogram and corresponding cophenetic matrices, and calculate the cophenetic correlation (Rohlf, 1997). Cophenetic matrix correlation values were calculated to measure goodness of fit of the tree matrices and were interpreted according to Rohlf (1997) as follows: less than 0.7, very poor fit; 0.7-0.8, poor fit; 0.8-0.9, good fit; and 0.9-1.0, very good fit. Genetic similarities were calculated based on the Jaccard coefficients (Rohlf, 1997). Dendrograms were generated with the unweighted pair group method using arithmetic average (UPGMA)

Table 2. Primer combinations and the number of polymorphic AFLP bands scored in the studied grass species.

No	Primer combinations*	Total number of bands	Number of polymorphic bands	Number of monomorphic bands	Percentage of Polymorphic bands
1	E-AAA, M-CGA	47	45	2	95.74
2	E-ATG, M-CCT	78	74	4	94.87
3	E-AAC, M-CGG	20	19	1	95.00
4	E-AAT, M-CGT	66	63	3	95.45
5	E-ATC, M-CGC	71	65	6	91.55
6	E-ATA, M-CTA	48	44	4	91.66
7	E-AGG, M-CCC	11	10	1	90.90
8	E-AAG, M-CTC	31	28	3	90.32
9	E-AAT, M-CTG	35	30	5	85.71
10	E-AAT, M-CTT	30	24	6	80.00
11	E-AGG, M-CAA	40	37	3	92.50
12	E-ACT, M-CAC	20	18	2	90.00
Total		497	457	40	91.95
Mean		41.4	38	3.33	7.66

*E: pre-amplification primer of *EcoRI* (5'-GACTGCGTACCAATTC-3'); M=pre-amplification primer of *MseI* (5-'GATGAGTCCTGAGTAA-3').

clustering method. Principal coordinate analysis (PCOA) was also conducted to identify the number of groups based on the Eigen vectors.

RESULTS

A total of 497 fragments were scored from 12-primer combinations, ranging in size from 50 to 500-bp (Table 2). Out of the 497 scored bands, 457 (92%) were polymorphic. The number of polymorphic bands for each primer combination varied from 10 to 74. The E-ATG/M-CCT primer combination produced the greatest number of polymorphic fragments (74 bands), while the E-AGG/M-CCC primer combination produced the lowest number (10 bands) (Table 2).

Genetic similarity coefficients (SC) based on AFLP markers ranged from 0.15 to 0.88 in these accessions. The highest SC (0.88) for pair wise comparisons among the genotypes was obtained between two tall fescue accessions (FAM6 and FAO10) from Hungary. The lowest SC value (0.15) was for the pair wise comparisons of *A. cristatum* and *L. rigidum*.

Cluster analysis provided a better illustration of genetic similarities among accessions. The UPGMA cluster tree generated by similarity coefficient matrix is shown in Figure 2. To test the dendrogram goodness of fit, the cophenetic correlation was calculated and interpreted according to Rohlf (1997). The cophenetic correlation was 0.96 indicating the high goodness of fit

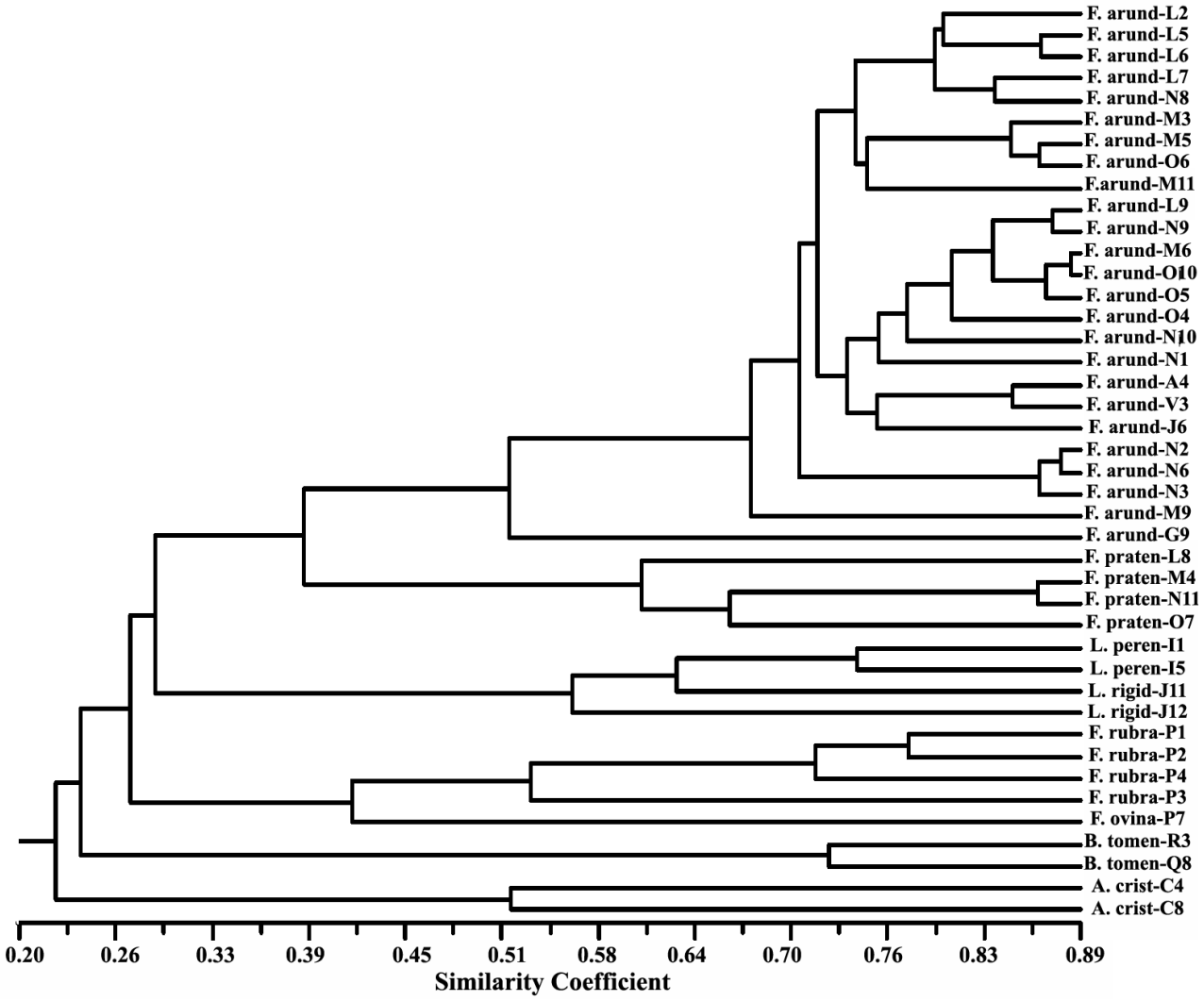

Figure 2. UPGMA dendrogram of the 42 accessions of different grass species based on AFLP markers. For name and origin of the accessions see Table 1.

of the similarity indices. According to this interpretation the evident patterns, were considered significant because the correlation between the SC matrix and the cophenetic matrix was as r = 0.96 (data not shown).

The AFLP analyses provide measure of genetic variation within and among studied grass genera. All accessions within a species were clearly clustered in a single group. At the similarity coefficient of 0.26, cluster analysis grouped *Agropyron* and *Bromus* populations in separate clusters and distinguished them from other genera. *Agropyron* and *Bromus* belong to tribes Triticeae and Bromeae respectively while other genera fall in the Poeae tribe. At the similarity coefficient of 0.30, fine leaf fescues (*F. ovina* and *F. rubra*) were separated from coarse leaf fescues (*F. arundinacea* and *F. pratensis*) and *Lolium* species. The AFLP profile showed that coarse fescues have more genomic similarity with *Lolium* species than with fine fescues.

At the similarity coefficient of 0.42, the accessions were grouped into seven major clusters, each corresponding to a separate species except for the two *Lolium* species in that were placed in one cluster (Fig. 2). *Festuca ovina* was the only accession in this study that was separated from the other accessions, showing greater inter-specific than intra-spesific variation at the genomic level.

In the cluster containing *F. pratensis* accessions, the two accessions, FPM4 and FPN11, had the highest similarities. These two accessions were also related in terms of geographical locations (Table 1). The last and largest cluster in this grouping included all 25 accessions of the tall fescue. The accessions of this cluster were subdivided into six subclusters, most of which fell into subclusters congruent with their geographical origins. Two accessions, FAM9 and FAG9 (both from Iran), did not group with any other entry and consisted of separate clusters.

DISCUSSION

Most of the primer combinations tested in this study revealed workable patterns and high DNA polymorphism among grass populations. For this set of primers none of the accessions shared identical DNA marker profiles indicating that the collection did not contain duplications. The high level of polymorphism has facilitated analysis of the genetic diversity among accessions and in a few situations specific AFLP markers were also found for some species. Affirmation of these markers in other collections may assist in developing specific probes to effectively discriminate fescue species.

The coarse fescues had more genomic similarity with *Lolium* species than fine fescues. Xu and Sleper (1994) have indicated that the genetic distance between *F. Pratensis* and *L. perenne* was the lowest when compared to these of other species of *Festuca* based on RFLP markers. Darbyshire and Warwick (1992) have evaluated the phylogeny of *Festuca* and related genera using chloroplast DNA restriction site variation and have reported high similarity between *F. arundinacea* and *L. perenne*. The closely related *Festuca* and *Lolium* genera have also been reported by Stammers *et al*. (1995).

In this study, greater inter-specific than intra-spesific variation at the genomic level was observed. For example the only *F. ovina* accession clearly separated from the other accessions. Although the four *F. rubra* accessions were grouped in one cluster, AFLP could separate the Iranian *F. rubra* (P3) accession from the other three Hungarian populations. This reflects the possible role of geographical regions in intra-specific genetic variability.

Two Iranian accessions of *F. arundinacea* (M9 and G9) did not group with the entries of similar geographic origin and each was placed in a separate cluster. In many molecular systems, the lack of genetic differentiation between accessions of definite identity and distinct geographic origin is usually attributed to the random nature of genomic DNA amplification, which is the case in AFLP (Roldan-Ruiz *et al*., 2000). This may also be due to the uncultivated nature (lack of artificial selection) and high intr-aspecific genetic variation in the Iranian tall fescue populations.

Principle coordinate analysis (Fig. 3), in which PC1 accounted for 50.5% of the total variation and PC2 accounted for 28.1%, was generally consistent with results from the cluster analysis in groupings of the species and accessions. The PC2 values for *F. rubra* and *F. ovina* accessions were high. These values were medium for accessions of *F. pratensis* and low for accessions of *F. arundinacea*. The values of PC1 for accessions of *F. ovina* were low, medium for accessions of *F. rubra* and *F. pratensis* and high for accessions of *F. arundinacea*. The FAG9 and to a lesser extent FAM9 were located far apart from the other accessions (Fig. 3). This was very much in accordance with grouping of these two accessions in the clustering (Fig. 2), indicating their greater genetic divergence

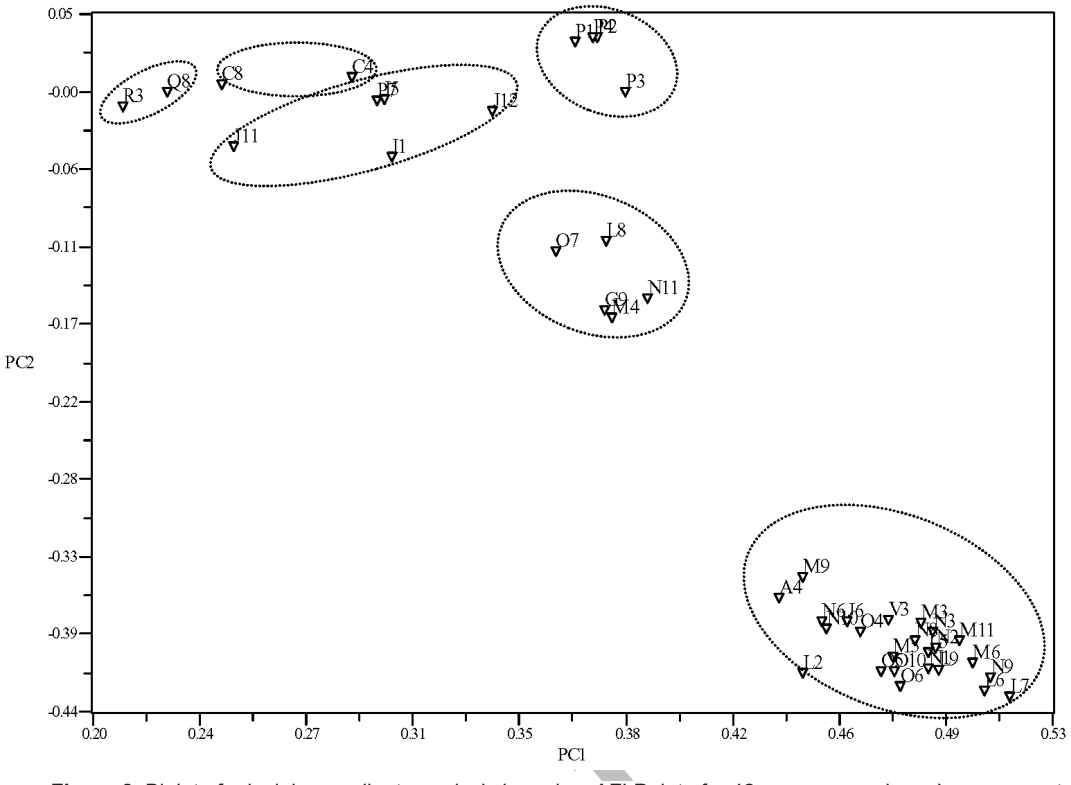

Figure 3. Biplot of principle coordinate analysis based on AFLP data for 42 grass accessions. In agreement with cluster analysis, accessions of similar species are closer together in each group. The number of entries are the same as accession numbers in table 1 which are abbreviated in this Figure (e.g. FAG9 = G9).

from other accessions. These accessions may be good candidates for breeding programs in constructing mapping populations or as parents of synthetic varieties.

In conclusion, genetic similarity coefficients between populations showed high levels of inter and intra-specific genetic diversity in the studied germplasm. Cluster analysis reflected the phylogenetic relationships among species and clearly demonstrated differences in the degree of similarity between genotypes. The close molecular homology between some grasses specially *Lolium* and coarse fescues, may reflect the possibility of producing new valuable germplasm through introgression breeding. In order to improve forage quality and digestibility, tall fescues and meadow fescues are sometimes intercrossed with other related species such as *Lolium perenne*. (Yamada *et al*., 2005). The untapped Iranian genetic resources of these grass species may be utilized for similar objectives. There was considerable congruity between AFLP groupings and geographical distribution of accessions. The high level of polymorphism indicated the capacity of AFLP markers in assessment of phylogenetic relationships and genetic variation studies. Genome specific amplified products will be useful molecular markers to discriminate species, verify hybrids and monitor gene introgression for inter-specific and intergeneric hybridization. Iranian tall fescue accessions contain a high degree of genetic variability, are very much diverged from accessions of other geographical regions, and thus can be exploited in breeding programs.

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