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Araştırma Makalesi/*Research Article (Original Paper)*

Assessment of genetic diversity in Aegilops species in North-West of Iran using ISSR marker

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Abstract: Inter-Simple Sequence Repeat (ISSR) and ACID-PAGE analyses were used to evaluate genetic diversity and relationships of *Aegilops* species from North-West of Iran. Thirty-three accessions of six *Aegilops* species including [*Ae. taucshii* (D), *Ae. cylindrica* (CD), *Ae. umbellulata* (U), *Ae. triuncialis* (UC), *Ae. biuncialis* (UM) and *Ae. crassa* (DM)] were studied in ISSR study. Twelve of the accessions were employed in the ACID-PAGE study. Eleven ISSR primers produced 171 polymorphic bands, which were used to construct the dendrograms. In ISSR method, the studied *Aegilops* species were subgrouped and it is identified that the species with common genome standed in the same group. ACID-PAGE analysis revealed high rate of polymorphism among *Aegilops* accessions. Therefore, results showed that there was potencial genetic diversity in the *Aegilops* species from North-West of Iran, and it could be useful genetic stock in any breeding perspectives. Specially the intra specific genetic diversity could be the feasible genetic base for any selection activities.

Keywords*:* ACID-PAGE, *Aegilops*, ISSR, Genetic diversity

İran'ın Kuzey-Batı *Aegilops* Türlerinin Arasındaki Genetik Çeşitliliğininin ASİT-PAGE Analizleri ve ISSR Markırları ile Değerlendirilmesi

Özet: Basit tekrarlı diziler arası polimorfizm (ISSR) ve ASİT-PAGE analizleri İran'ın Kuzey-Batı *Aegilops* türlerinin akrabalık ilişkileri ve genetik çeşitliliğini değerlendirmek için kullanılmıştır. Altı adet *Aegilops* türüne [*Ae. taucshii* (D), *Ae. cylindrica* (CD), *Ae. umbellulata* (U), *Ae. triuncialis* (UC), *Ae. biuncialis* (UM) ve *Ae. crassa* (DM)] ait otuz üç aksesyon ISSR çalışmasında kullanılmıştır. Aksesyonlardan on iki tanesi ASİT-PAGE çalışmalarına dahil edilmiştir. On bir adet ISSR primerinden elde edilen 171 adet polimorfik ISSR bandı dendrogramlar oluşturmak için kullanılmıştır. ISSR yönteminde, üzerinde çalışılan *Aegilops* türleri alt gruplara ayrılmıştır ve ortak genoma sahip türlerin aynı gruplar içerisinde yer aldığı belirlenmiştir. ASİT-PAGE analizi *Aegilops* aksesyonları içerisinde yüksek oranda polimorfizmi ortaya çıkarmıştır. Bu nedenle, sonuçlar İran'ın Kuzey-Batı *Aegilops* türleri içerisinde potansiyel bir genetik çeşitlilik olduğunu ve herhangi bir ıslah yaklaşımında bunların yararlı genetik stok olabileceklerini göstermiştir Özellikle, tür içi özel genetik çeşitlilik herhangi bir seleksiyon faaliyetleri için uygun genetik tabanı olabilecektir.

Anahtar kelimeler: ACID-PAGE, *Aegilops*, ISSR, Genetik çeşitlilik

Introduction

Genetic diversity has decreased in many important crops like wheat and this reduction is partly related to modern agriculture methods. The reduction of diversity not only reduces the output of breeding programs, but also causes genetic hemogeneity and sensitivity of agricultural products against biotic and abiotic stresses (Ciaffi et al. 1993). Wild relatives of crops are uniqe and plant breeders are using them as valuable genetic resources because they contain benefitial genes having resistance to both biotic or abiotic stresses (Nevo 1998). The *Aegilops* genus from *poaceae* family consists of 22 species, i.e, *Ae. taucshii*,

Ae. crassa, Ae. cylindrica, Ae. umbellulata, Ae. biuncialis, Ae. triuncialis and etc, that 10 of them are diploid, 10 are tetraploid and two species are hexaploid (Van Slageren 1994). The purpose of agriculture revolution in the 21st century, is to increase food production by genetic base as the most hopeful cases are about progenitores of cultivated crops that are genetic resources of resistance to stresses (Nevo 1998; Konstantinos et al. 2010).

Wild relatives of wheat can indicate valuable resources of genetic diversity for improving resistance to stresses, yield and the quality of cultivated wheat (Ki-hyun et al. 2010). According to researches, using the genes of wild relatives, obtained a uniqe position among plant breeders in the 1970s and 1980s (Hajjar et al. 2007). The *Aegilops* genus can play an important role in improving of cultivated wheat, because of including wild relatives of this noticeable crop (Shneider et al. 2008).

Many of the *Aegilops* species contain benefitial genes for resistance to biotic stresses: *Ae. comosa* containing the genes Yr8 and Sr24 (McIntosh 1982), *Ae. triuncialis* containing Lr tr, Lr58 (Kuraparthy et al. 2007), Cre7 and H30 genes (Montes et al. 2008), *Ae. umbellulata* containing Lr9 (Sears et al. 1956), Lr and Sr genes (Chhuneja et al. 2007). *Aegilops* species can also help to increase biomass production of cultivated wheat by increasing the size of flag-leaf as *Ae. tauschii* (D genome) (Monneveux et el. 2000). Inter-Simple Sequence Repeat (ISSR) markers detect polymorphism well by targeting multiple microsatellite loci that are distributed in any genome (Reddy et el. 2002). In *Aegilops*, ISSRs have been used by researches for diversity assessments (Gong et al. 2006) and mapping (Boyko et al. 2002).

Endosperm proteins of wheat generally consist of two classes of storage proteins, gliadin and glutenin. Classification of these proteins is on the basis of their solubility in different solvents. Genetic and biochemical aspects of these proteins have attracted attention of researchers in recent years due to their importance in determining the rate of diversity, technological and nutritional properties of cultivated wheats (Ciaffi(1) et al. 1993). ACID-PAGE method has been used for analysing the rate of protein diversity. In a study Sofalian et al. (2009) proved that ACID-PAGE is a suitable method to discriminate wheat variety and species. Also results of this study confirmed that the genetic variation among seed storage proteins of wild relatives were considerable. The importance of genetic erosion in cultivated wheat and breeding purposes are the reasons to assess the genetic diversity of these accessions to reach the wheat resistance and yield improvement through introgression of their genomes. Hence this study carried out to evaluate the genetic diversity of Northwest Iranian *Aegilops* species using ISSR markers and ACID-PAGE method.

Materials and method

Plant materials

The plant material consisted of 33 samples (Table 1) collected from 3 provinces from Northwest of Iran: West Azerbaijan, East Azerbaijan and Ardebil (Figure 1). These samples including six *Aegilops* species [*Ae. taucshii* (D), *Ae. cylindrica* (CD), *Ae. umbellulata* (U), *Ae. triuncialis* (UC), *Ae. biuncialis* (UM), *Ae. crassa* (DM)] that collected from Northwest of Iran (Figure 1). Twelve of those 33 accessions were used for ACID-PAGE method (Table 1).

Figure 1. Geographical regions of the studding *Aegilops* species in this study.

Genomic DNA extraction

Four to five healthy young leaves were collected from four to five plants of each sample and all DNA samples were extracted using CTAB method (Saghaei-Maroof et al. 1984).

PCR amplification

PCR reactions were carried out in a 20 µl volume containing 2µl PCR buffer, 0.2 µl dNTPs, 0.8 µl $MgCl_2$, 1.6 µl ISSR primer, 0.26 U Taq DNA polymerase (Hy Test Ltd.) and 4 µl genomic DNA. ISSR amplifications were performed in a TECHNE thermocycler (Model TC 512, UK). The temperature profile for ISSR amplification included one cycle of 7 min at 94 °C, followed by 44 cycles of amplification. Each cycle of amplification had a denaturation step at 94 °C for 30 seconds, an annealing step at 45 °C – 54 °C (Table 2.), for 45 seconds and an extension step at 72 °C for 2 min. After the final cycle, the samples were held for 7 min at 72 C°. Amplification products were analysed by electrophoresis in 1.2% agarose gels containing ethidium bromide in 1XTAE buffer. The ISSR bands were visualized and photographed using DOC-CF08.XD (UVITEC). ISSR amplified bands were recorded as 1 when present and 0 when absent. Only reproducible amplification bands that were clearly visible on the photographs were scored for the construction of the data matrix.

Table1.List of samples used in the study

Number	Species	Genome	Collection Site
1	*Ae. triuncialis*	UC	Khalkhal
2	*Ae. triuncialis**	UC	Marand
3	*Ae. triuncialis*	UC	Jolfa
4	*Ae. triuncialis**	UC	Ahar
5	*Ae. triuncialis*	UC	Sarab
6	*Ae. triuncialis*	UC	Horand
7	*Ae. triuncialis*	UC	Maraghe
8	*Ae. triuncialis*	UC	Kaleibar
9	*Ae. triuncialis*	UC	Neor
10	*Ae. triuncialis*	UC	Maku
11	*Ae. triuncialis*	UC	Tabriz
12	*Ae. triuncialis*	UC	Malekan
13	*Ae. umbellulata*	U	Shabestar
14	*Ae. umbellulata*	U	Sufian
15	*Ae. taucshii**	D	Ahar
16	*Ae. taucshii**	D	Ardebil
17	*Ae. cylindrica*	CD	Khalkhal
18	*Ae. cylindrica**	CD	Sarab-Bostanabad
19	*Ae. cylindrica*	CD	Maraghe
20	*Ae. cylindrica*	CD	Namin
21	*Ae. cylindrica**	CD	Sarab-Duzduzan
22	*Ae. biuncialis**	UM	Marand
23	*Ae. biuncialis*	UM	Kaleibar
24	*Ae. biuncialis**	UM	Shabestar
25	*Ae. biuncialis*	UM	Noghduz
26	*Ae. biuncialis*	UM	Moghan
27	*Ae. biuncialis*	UM	Meshkin
28	*Ae. biuncialis*	UM	Marand countryside
29	*Ae. crassa**	DM	TN-01-911
30	*Ae. crassa**	DM	TN-01-944
31	*Ae. crassa**	DM	TN-01-947
32	*Ae. crassa*	DM	TN-01-1107
33	*Ae. crassa**	DM	TN-01-1166

*Selected accessions for ACID-PAGE method

ISSR primers

A total of 34 ISSR primers were evaluated in a preliminary experiment using six populations. Eleven of the primers were selected for the main experiments based on the quality and reliability of their amplification and the polymorphism levels they revealed. The primers actually used for data analysis in this study are listed in Table 2. The random ISSR primers tested were synthesized by BIONEER (made in Korea Republic).

ACID-PAGE

ACID-PAGE method was performed based on procedure described by Metakovsky and Novoselskaya (1984).

Data analysis

Shanon information index calculated for estimation of inter-specie genetic diversity. Total genetic diversity (H_T), within diversity (H_S) and G_{ST} were calculated on the basis of Nei (1973) and genetic distance matrix calculated on the basis of Nei (1978). Grouping the accessions carried out using complete linkage clustering method and simple matching coefficient, all using the appropriate procedures of the computer program NTSYS-pc v2, Genelex 6.4, POPGENE 32. We used principal coordinate analysis (PCoA) using the simple matching coefficient to test the goodness of fit of the complete linkage cluster analysis (Mohammadi and Prasanna 2003).

Table 2. Primer number, nucleotide sequences, total bands and polymorphic bands of 11 ISSR primers used for analysing the 33 accessions of the present study.

Primer number	Sequence 5′-3′	Total bands	Polymorphic bands	Ann. Temp. °C
1	5′ AGAC AGACGC 3′	207	22	47
3	5′ AGAGAGAGAGAGAGAGC 3′	101	16	52
8	5′ GACGACGACGACG 3′	131	17	53
9	5′ TCTCTCTCTCTCTCTCC 3′	114	16	53
12	5′ TTGTTGTTGTTGTTGC 3′	126	16	47
13	5′ ACACACACACACACACYG 3′	102	15	54
16	5′ CACACACACACAAG 3′	125	15	50
17	5′ AGAGAGAGAGAGAGAGG 3′	99	13	45
19	5′ AGAGAGAGAGAGAGAGT 3′	94	16	53
23	5′ GTGTGTGTGTGTGTGTYG 3′	103	12	54
31	5′ CACCACCACGC 3′	174	21	46
	Sum	1376	179	
	Mean	125.09	16.27	

Results

In a preliminary experiment, 34 ISSR primers were used for DNA amplification of three accessions of six species. Eleven primers were selected (Table 2), which yielded in total 1376 bands of which 179 were polymorphic (Figure 2). Amplified bands per primer ranged from 12 (primer number 23) to 22 (Primer number 1) with an average of 15.8 bands. On the basis of Nei's genetic index, the genetic diversity within species varied from 0.01 to 0.07 (Table 3). The highest were obtained in *Ae. triuncialis* and *Ae. biuncialis* populations (0.07), and the lowest was obtained in *Ae. umbellulata* population (0.01). The results showed low variation within different populations of *Aegilops*. Also, 179 loci totally. Average genetic diversity within (H_S) was 0.04, total (H_T) was 0.31 and G_{ST} was 0.84. Between genetic diversity figured out 0.27 that indicates high variation between the species.

Table 3. Genetic diversity on the basis of Nei's genetic index, Shannon index, number and percentage of polymorphic loci, according to within specie data of *Aegilops* species.

Population	Number of polymorphic loci	Nei index	Shannon index	Percentage of polymorphic loci
Ae. triuncialis	39	0.07	0.11	20.53%
Ae. umbellulata	7	0.01	0.02	3.68%
Ae. tauschii	14	0.03	0.05	7.37%
Ae. cylindrica	17	0.03	0.05	8.95%
Ae. biuncialis	39	0.07	0.11	20.53%
Ae. crassa	27	0.05	0.08	14.21%

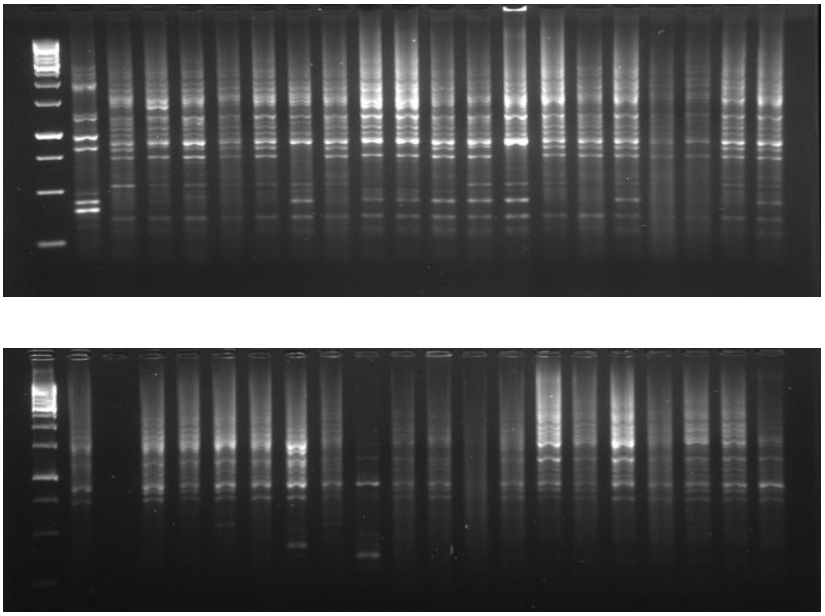


Figure 2. ISSR amplification pattern of *Aegilops* genotypes using number 13 ISSR. The accessions of each specie is given by number referring , and number 13, 14, 15 and 16 have replications.

According to the A-Page results 23 bands were counted (Figure 3). Ahar population of *Ae. triuncialis* produced the most bands with 20 bands and TN-01-1166 population of *Ae. crassa* produced the least bands with 9 bands. Nei index (mean genetic diversity) for all loci was 0.431 and Shanon index was 0.621. Mean polymorphism percentage for all populations was 89.17. These results show high rate of diversity of populations. Also Nei index for ω, γ and β areas were 391, 492 and 0.460. In addition, there were bands in α area in all accessions except Sarab-Bostanabad accession of *Ae. cylindrica* specie.

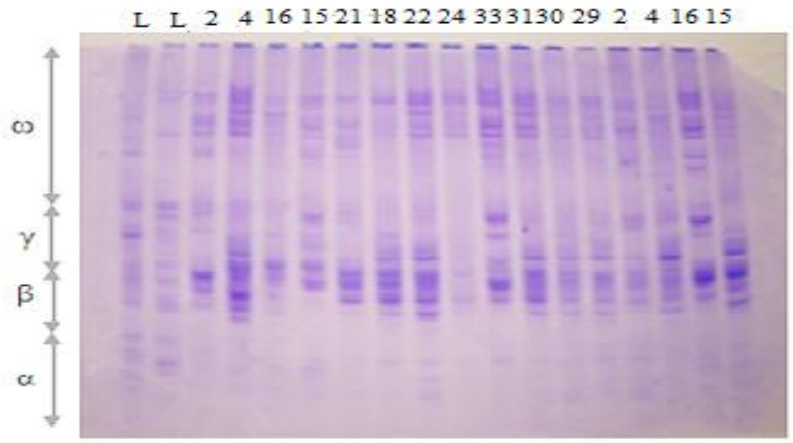


Figure 3. A-PAGE pattern of *Aegilops* accessions. The accessions of each specie is given by number referring, and number 15, 16, 4 and 2 have replications.

Genetic relationships among accessions and the rate of genetic diversity

Genetic similarities among accessions were calculated based on ISSR and A-PAGE data. Based on ISSR data the genetic similarity between all pairs of accessions using Nei index ranged from 0.50 to 0.98. Nei index indicated that Marand and Noghduz accessions of *Ae. biuncialis* specie had the most genetic similarity (0.98) and the lowest similarity (0.50) was between TN-01-1107 accession of *Ae. crassa* specie and 2 accessions of *Ae. triuncialis* specie collected from Neor and Tabriz. On the other hand A-PAGE data based on Nei index showed that Sarab-Duzuza and Sarab-Bostanabad accessions of *Ae. biuncialis* had the most similarity and we had the lowest similarity between Shabestar accession of *Ae. biuncialis* and Ahar accession of *Ae. tauchii*.

Using ISSR and A-PAGE data, cluster analysis was carried out by the Complete Linkage method based on Nei genetic similarity coefficients. To test the goodness of fit of the Complete Linkage cluster analysis with ISSR and A-PAGE data sets, the cophenetic correlation coefficients were calculated. For the ISSR data the correlation value r = 0.891, and for the A-PAGE data the correlation value r = 0.813 showed a goodness of clustering. Based on ISSR revealed three main groups. A representative dendrogram constructed with the Complete Linkage based on Nei coefficient using ISSR data is shown in Figure 4, and a dendrogram constructed with the Complete Linkage based on Jaccard coefficient using A-PAGE data is shown in Figure 5. Based on ISSR data first group included accessions of *Ae. triuncialis*, second group included accessions of *Ae. umbellulata* and *Ae. biuncialis* and the third group included accessions of *Ae. taucshii*, *Ae. cylindrica* and *Ae. crassa*. Standings of accessions based on A-PAGE method have been shown in Figure 5.

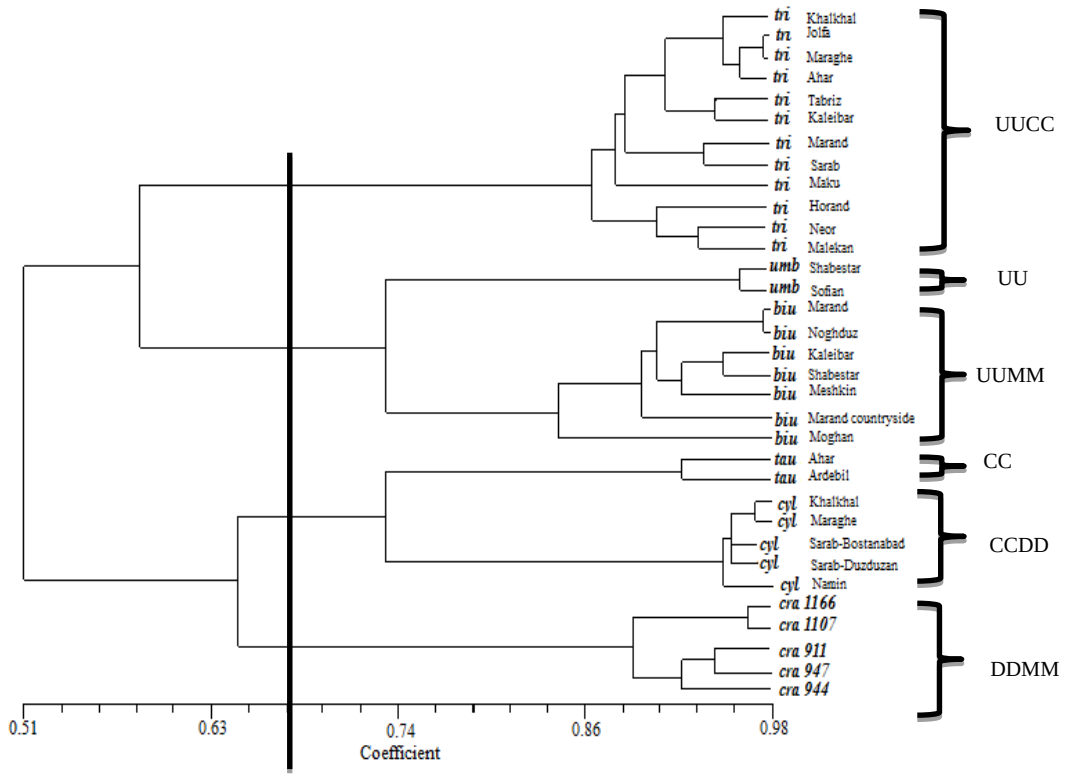


Figure 4. Dendrogram of the *Aegilops* accessions used in the study based on the ISSR data. The Nei coefficient was used to estimate genetic similarities.

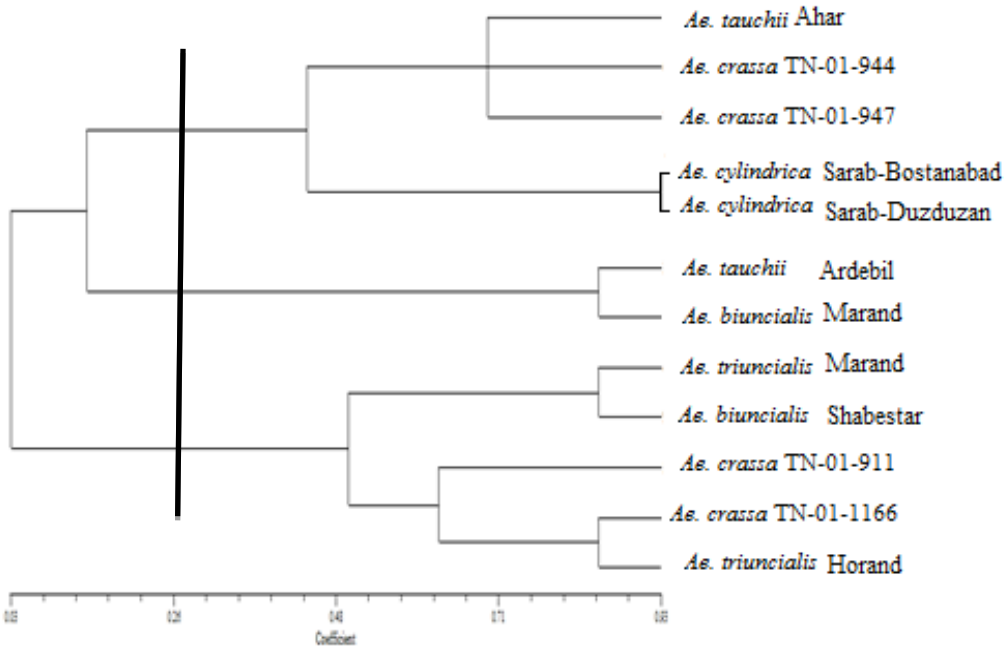
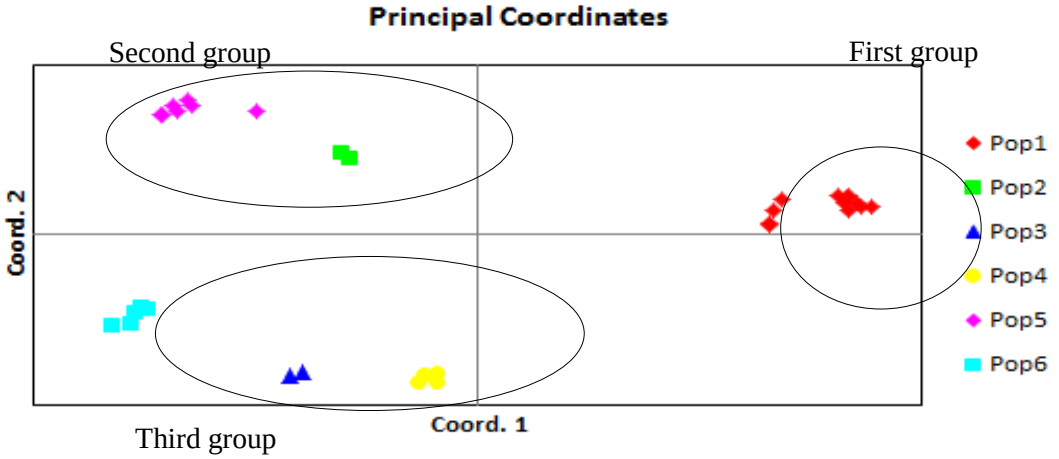


Figure 5. Dendrogram of the *Aegilops* accessions used in the study, based on the A-PAGE data. The Jaccard coefficient was used to estimate genetic similarities

Principal Coordinate Analysis (PCoA) was carried out based on Nei similarity coefficients for ISSR data and on Jaccard similarity coefficients for A-PAGE data. For ISSR, the first two principal coordinate axes of PCoA based on Nei coefficient explained nearly 72% of the total variation in all cases and emphsized the clustering method (Figure 6). The total variation explained by the first two axes of PCoA with Jaccard coefficient was more than 66%, for A-PAGE data and emphsized the clustering method (Figure 7).



Pop1= *Ae. triuncialis* Pop2= *Ae. umbellulata* Pop3= *Ae. tauschii* Pop4= *Ae. cylindrica*
Pop5= *Ae. biuncialis* Pop6= *Ae. Crassa*

Figure 6. PCoA diagram based on the ISSR data and the Nei coefficient.

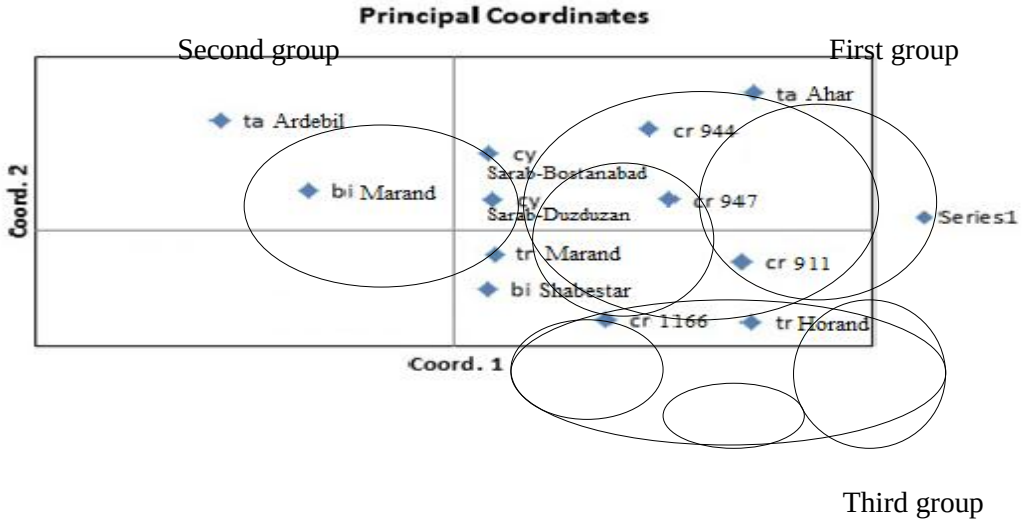


Figure 7. PCoA diagram based on the A-PAGE data and the Jaccard coefficient.

Discussion

A-PAGE and ISSR marker technology was used in *Aegilops* genus diversity studies revealing various levels of polymorphism. In ACID-PAGE method Known as a good biochemical marker (Sofalian et al. 2009), we had high rate of genetic diversity among studied accessions (Mean polymorphism percentage for all populations was 89.17). These results are consonant with Sofalian et al.'s (2009) results about high rate of genetic diversity in Iranian wild wheat progenitors. In breeding programs, these accessions can be used for enhancing the rate of genetic diversity of wheat germplasm. In ISSR method estimated 0.27 diversity among species that is a high rate of diversity between species and is consonant with the reports of Konstantinos et al. (2010), Okuno et al. (1998), Monte et al. (1999) and Mighdadi et al. (2006) about

between species polymorphism of *Agilops* and output of ISSR markers. Also results showed that the accessions with similar genome stand near each other in the dendrogram (Figure 4) that shows a relation among them maybe have the same parents. We should notice that comparing with ISSR analysis, ACID-PAGE analysis showed higher rate of genetic diversity and we know that ISSR analyses DNA but ACID-PAGE analyses proteins, and the reason of showing high rate of genetic diversity by ACID-PAGE method might be the U-chromatin segments in the genome that not express, so, do not produce products as proteins and frequency of these segments is over 95%.

Conclusion

Aegilops accessions were grouped according to the species and their genomes. The ISSR data and the Nei coefficient grouped the accessions in clusters according to Van Slageren's (1994) grouping in sections. The high level of polymorphism was detected in the gliadin subunits of the wild wheat relatives by ACID-PAGE method. The data derived from ISSR marker types helped in a comprehensive realization of the genetic relationship between the species and the accessions.

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