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Full Length Research Paper

Assessment of genetic diversity in *Triticum* spp. and *Aegilops* spp. using AFLP markers

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Genetic diversity among some wild relatives of wheat was estimated using amplified fragment length polymorphism (AFLP) and morphological markers. Thirty one *Triticum* and *Aegilops* genotypes including twenty-four *Triticum* and *Aegilops* accessions belonging to five diploid (*Triticum baeoticum*, *Triticum monococcum*, *Aegilops umbellulata*, *Aegilops caudata* and *Aegilops tauschii*), five tetraploid (*Triticum dicoccoides, Triticum dicoccum, Aegilops crassa* (4x)*, Aegilops cylindrica, Aegilops triuncialis*) and two hexaploid (*Triticum compactum*, *A. crassa* (6x)) species sampled from different eco-geographical regions of Iran; a durum wheat cultivar 'Langdon', a local wheat cultivar 'Roshan', a wheat cultivar 'Chinese spring' and four synthetic hexaploid wheats were evaluated. Genetic diversity among wheat accessions was estimated using 14 *Pst*I:*Mse*I primer pair combinations. Of the approximately 414 detected AFLP markers, 387 (93.5%) were polymorphic with 28 bands per used primer pair. Cluster analysis of 31 accessions belonging to the 15 species by UPGMA cluster analysis based on Jaccard's similarity estimates for AFLP data divided all accessions into two major clusters reflecting almost their genome composition. The first one included wheat species having A and AB genomes, while second cluster included wheat species having C, D, AB, CD, UC, DM, DDM and ABD genomes. The genetic similarity coefficients ranged from 0.12 between *Ae. glabra* and accession number 3 of *T. monococcum* and 0.57 between *A. crassa* (6x) and *Ae. crassa* (4x-6x). Two *Aegilops* species of *A. umbellulata* and *A. caudata* were ranked as the second most related species.

Key words: Amplified fragment length polymorphism (AFLP), genetic diversity, wheat, *Triticum* spp., wild wheat, *Aegilops* spp.

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is the most widely grown plant in the world due to its physiological characteristics that adapt wheat cultivars for production in a wide range of eco-geographical conditions and the chemical and physical properties of the wheat gluten that contribute to the wide use of wheat grain for many different food products. It is the staple food for 35% of the world's population, and is becoming increasingly important in the developing world (CIMMYT 2003). To meet the demand for developing high yielding and stress-resistant wheat cultivars, it is desirable to increase the genetic base of this crop. There has been a growing concern about the remaining variability in the bread wheat gene pool which is grossly insufficient to address current and future breeding objectives (Rejesus et al., 1996). In last decades, the narrow genetic basis of modern wheat cultivars is well evident, as breeders prefer using either improved cultivars as parents or advanced breeding materials to accelerate the development of new cultivars. While in the beginning, selection was utilized to isolate pure lines from heterogeneous landraces or natural populations, today improved cultivars were used as parents in wheat breeding programs. It is therefore necessary to broaden the genetic base of wheat. The wild relatives of bread wheat, *T. aestivum* L., are considered as potential sources of useful alleles for bread- wheat improvement.

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The genus *Aegilops* L. and *Triticum* L. have contributed two of the three (B and D) and one of the three (A) bread wheat genomes, respectively. The genus *Aegilops* contains 22 species comprising both diploids and polyploids, that originated from center of origin (van Slageren, 1994). Consequently, study of the genetic diversity of the genetic resources of such species may provide significant information regarding their potential for breeding purposes.

Genetic erosion caused by modern cultivation procedure has narrowed the genetic base of many crops, including bread wheat. Wild relatives and related species can be successfully crossed with bread wheat (Jiang et al., 1993; Sharma, 1995; Arzani et al., 2000). Consequently, genes from the wild relatives can be introgressed in the cultivated wheats through recombination of the homologous chromosomes, and undesirable gene linkages can be mostly broken by repeated backcrossing to cultivated wheat (Friebe et al., 1996). Amphiploids from interspecific crosses between tetraploid wheats and *Aegilops* species, as their close relatives, are useful bridging germplasm for introduction of desirable alien characters to bread wheats (Friebe et al., 1996).

Iran is very rich in habitat diversity due to the diversity in its geomorphology, topography and climate. This has helped the survival of a diverse plant species in the wild. Amongst the Iranian flora, there are some of the most important food crops such as wheat and its wild relatives. These species represent a large reservoir of useful characteristics that can be exploited for wheat improvement. Many agronomically interesting characterisics, comprising resistance to biotic and abiotic stresses have been transferred from these species to wheat (Jiang et al., 1993; Friebe et al., 1996). Accessions of *Aegilops tauschii,* particularly those from Iran have already proved a diverse genetic storehouse of potentially useful characteristics for incorporation into modern wheat cultivars (Lagudah et al., 1993; Thompson and Haak, 1997). Nishikawa et al. (1980) and Dvorak et al. (1998) suggested that the region of SE and SW Caspian sea including Iran and Armenia, were the geographical place of origin of *T. aestivum* L. Comparative study of microsatellite diversity in wheat germplasm among a wide range of world areas indicated that the greatest genetic diversity originated from Iran (Huang et al., 2002).

Traditionally, germplasm has been characterized based on agronomic and morphological studies, but recently use of molecular markers to study diversity of crop species has become common. DNA markers have the advantage of directly detecting sequence variation among cultivars. The use of micro-satellites (simple sequence repeats, SSRs) and amplified fragment length polymorphisms (AFLPs) are routine methods for quickly and efficiently estimating relationships between lines and populations of many plant species. AFLP is an efficient, reproducible technique which combines the reliability of RFLP and the power of PCR technique (Vos et al., 1995). AFLP has been widely used to discriminate between different accessions of a number of plants species including *T. aestivum* L. (Barrett and Kidwell 1998; Barrett et al., 1998; Bohn and Melchinger, 1999; Ridout and Donini, 1999; Soleimani et al., 2002; Almanza-Pinzon et al., 2003).

Quantification of genetic diversity in cultivated and wild crops has important implications for breeding programs and for the conservation of genetic resources. The primary objective of this study was to understand the extent and pattern of genetic diversity among diploid and polyploid wild species of wheat using AFLP marker and to compare these genetic diversity estimates with those of using morphological traits.

MATERIALS AND METHODS

Plant materials

A collection of twenty-four *Triticum* and *Aegilops* accessions belonging to five diploid (*T. baeoticum* (Boiss. em. Schiemann.) [syn.= *T. monococcum* (L.) ssp. aegilopoides Link em. Thell.], *T. monococcum* (L.), *Ae. umbellulata* (Zhuk.), *Ae. caudata* (L.) and *A. tauschii* (Coss.)), five tetraploid (*T. dicoccoides* (Koern. ex Asch. & Graebn) [=*T. turgidum* L. ssp. *dicoccoides* (Koern. ex Asch. and Graebn) Thell.], *T. dicoccum* (Schrank ex Schübler) [=*T. turgidum* (L.) ssp. *dicoccum* (Schrank ex Schübler) Thell.], *Ae. crassa* Boiss (4x), *Ae. cylindrica* Host., *A. triuncialis* L.) and two hexaploid (*T. compactum* [=*T. aestivum* L. ssp. *compactum* (Host) MacKey], *Ae. crassa* Boiss (6x)) species and sampled from different eco-geographical regions of Iran was used (Table 1). Four synthetic hexaploid wheat lines provided by CIMMYT, a durum wheat and two bread wheat cultivars were also used in this study.

AFLP analysis

Single plant from each accession was selected and then selfed seeds of the selected plant of each accession were used. Leaves from ten plants derived from individual selfed plant of each accession were combined and their DNA was extracted. DNA extraction was performed according to Dellaporta et al. (1983) method for young leaf tissue with modifications. One hundred milligrams of frozen leaf samples were ground with liquid nitrogen followed by addition of 400 µl of extraction buffer. The supernatant removed after the first centrifugation was transferred to a clean tube and centrifuged for the second time at 12,000 g for 10 m, followed by ethanol precipitation. DNA concentration was determined by comparing its intensity with those of DNA standard (λDNA with known concentrations) on the ethidium bromide stained 1% agarose gel.

AFLP analysis was based on established protocol (Vos et al., 1995). Fourteen *Pst*I:*Mse*I primer combinations were used. Five hundred nanograms of DNA from each genotypes were digested with *Tru*91 (an isoschizomer of *Mse*I) and *Pst*I restriction enzymes. A total of 250 ng genomic DNA was restricted with *Mse*I and *Pst*I (5U of each) for 2 - 3 h at 37°C in 1 x restriction buffer OPA (One-Phor-all; Pharamacia) in a final volume of 50 µl. After controlling for complete digestion, 10 µl of a ligation mix [50 pmol *Mse*I adapter, 5 pmol *Pst*I adapter, 10 mM ATP, 1 x OPA buffer and 1 U T4 DNA ligase (Promega)] was added and the samples incubated for 3 h at 37°C. The digestion-ligation reaction was terminated by incubating at 65°C for 10 m, and products were then diluted 1:5 in TE buffer [10 mM Tris, 1 mM EDTA].

Table 1. Wild wheat relatives (*Triticum* and *Aegilops* accessions), synthetic wheat lines, durum and bread wheat cultivars used for diversity study.

S/N	Species	Genome	Growing status	Location	Origin
1	*T. baeoticum* (1)	A	Wild	Semirom-Isfahan	Central Iran
2	*T. baeoticum* (2)	A	Wild	Kohkiloye-Boyerahmad	SW Iran
3	*T. baeoticum* (3)	A	Wild	Lorestan	West Iran
4	*T. baeoticum* (4)	A	Wild	Shirvancherdavel Ilam	West Iran
5	*T. baeoticum* (5)	A	Wild	Khoramabad Lorestan	West Iran
6	*T. baeoticum* (6)	A	Wild	KarajTehran	Iran
7	*T. baeoticum* (7)	A	Wild	PiranshahrKordestan	West Iran
8	*T. monococcum* (1)	A	Wild	Shirvancherd-Ilam	West Iran
9	*T. monococcum* (2)	A	Wild	Shirvancherd-Ilam	West Iran
10	*T. monococcum* (3)	A	Wild	Lorestan	West Iran
11	*T. monococcum* (4)	A	Wild	Kermanshah	West Iran
12	*T. turgidum* ssp. *dicoccoides*	AB	Wild	Semirom-Isfahan	Central Iran
13	*T. turgidum* ssp. *dicoccum*	AB	Wild	West Azerbaijan	NW Iran
14	*T. compactum*	ABD	Wild	Maco-Azerbaijan	NW Iran
15	*Ae. tauschii*	D	Wild	Ilam-Ilam	West Iran
16	*Ae. crassa* (6x)	DDM	Wild	Ilam-Ilam	West Iran
17	*Ae. crassa* (mixoploid 4x & 6x)	DM-DDM	Wild	Ilam-Ilam	West Iran
18	*Ae. crassa* (4x)	DM	Wild	Ilam-Ilam	West Iran
19	*Ae. glabra*		Wild	Ilam-Ilam	West Iran
20	*Ae. umbellulata*	U	Wild	Ilam-Ilam	West Iran
21	*Ae. caudata*	C	Wild	Ilam-Ilam	West Iran
22	*Ae. cyclindrica*	CD	Wild	Ilam-Ilam	West Iran
23	*Ae. triuncialis* (1)	UC	Wild	Ivan- Ilam	West Iran
24	*Ae. triuncialis* (2)	UC	Wild	Ilam-Ilam	West Iran
25	Altar84/*Ae. tauschii*	ABD	Synt. wheat	Genetic Resources	CIMMYT
26	Croc 1/ *Ae. tauschii*	ABD	Synt. wheat	Genetic Resources	CIMMYT
27	Yar/ *Ae. tauschii*	ABD	Synt. wheat	Genetic Resources	CIMMYT
28	Srn/ *Ae. tauschii*	ABD	Synt. wheat	Genetic Resources	CIMMYT
29	*T. turgidum* ssp. *durum* cv. Langdon	AB	Durumwheat	Genetic Resources	CIMMYT
30	*T. aestivum* cv. Chinese spring	ABD	Bread wheat	Genetic Resources	CIMMYT
31	*T. aestivum* cv. Roshan	ABD	Bread wheat	Isfahan	Iran

A pre-amplification reaction was performed in a 50 µl reaction containing 5 µl of template DNA, 150 ng of *Mse*I and *Pst*I primer, 0.25 mM dNTPs, 10 x PCR buffer, 1.2 mM MgCl2 and 1 U *Taq* Polymerase (Accutherm, GeneCraft, Germany). Samples were subjected to pre-amplification thermocycle profile [initial denaturetion step 94°C (2 min), 40 cycles of 94°C (30 s), 56°C (30 s), 72°C (1 min) and a final extension 72°C (5 min)]. The pre-amplified DNA was diluted (1:5) and used for selective amplification. Selective amplifications were performed in a 20 µl reaction containing: 5 µl of the diluted template DNA, 60 ng *Mse*I, *Pst*I primer each having three selective nucleotides (with exception of primer combinations 7 and 10 each of which having two selective nucleotides for *Pst*I), 10 x PCR buffer, 1.2 mM MgCl2 and 1 U *Taq* Polymerase (Accutherm, GeneCraft, Germany). Samples were subjected to the selective amplification thermocycle profile of initial denaturation step 94°C (2 min); 12 cycles of 94°C (30 s), 65°C (30 s) (step -0.7°C/cycle for cycles 2 - 12), 72°C (1 min); 23 cycles of 94°C (30 s), 56°C (30 s), 72°C (1 min) and a final extension 72°C (5 min). All amplifications were conducted in Techgene thermocycler (Biometra, Germany).

The selective amplification product were resolved in a 6% polyacrylamide gel prepared with 1x TBE as the running buffer and electrophoresed at 80 W for 2 h using Biometra Model S2 gel electrophoresis equipment with Biometra Model PS9009TC power supply. After electrophoresis, the amplification products were visualized by silver staining according to the protocol of Bassam et al. (1991).

Data analysis

Polymorphic bands from AFLPs were individually identified by their specific migration rates in the electrophoretic analyses. Once bands were properly and distinctively identified, binary (0/1) matrices were constructed to compare the patterns. Jaccard's similarity coefficients were generated by the SIMQUAL subroutine from the NTSYS-pc 2.02e (Exeter Software, Setanket, NY) statistical package. This similarity algorithm was chosen because it ignores 0/0 matches, and is appropriate for molecular marker data producing only dominant bands (such as AFLPs). Cluster analyses along with their corresponding dendrograms were generated by the UPGMA, with the SAHN and TREE subroutines from the NTSYS-pc statistical package. The goodness-of-fit of the clustering was tested

Table 2. *Pst*I- and *Mse*I-selective nucleotide combinations used for AFLP analysis. Sequences of the adapters and primers used in the pre-amplification indicated at the bottom rows.

S/N	Primer combination		Polymorphic bands		
	***Pst*I-**	***Mse*I-**	**Total bands**	**Number**	**%**
1	AAG	CAA	16	14	87.5
2	ACT	CTA	41	38	92.7
3	AGG	CTA	35	34	97.1
4	AGG	GCG	40	37	92.5
5	AGT	CAT	38	37	97.4
6	AGG	CTT	17	16	94.1
7	AG	GCG	47	45	95.8
8	ACG	CAA	17	16	94.1
9	ACT	CAT	20	19	95
10	AG	CTT	22	20	90.9
11	ACT	CTT	20	18	90
12	AAG	CGT	51	47	92.2
13	ACG	CGT	35	33	94.3
14	ACG	CTG	15	13	86.7
Total			414	387	93.5
PstI adapter		Ligation	5'-CCTACGCAGTCTACGAG-3'		
			3'-ACGTGGATGCGTCAG-5'		
MseI adapter			5'-GACGATGAGTCCTGAG-3'		
			3'-GACTCAGGACTCAT-5'		
Primers		Pre-amplification			
PstI			5'-GACTGCGTACATGCAG-3'		
MseI			5'-GATGAGTCCTGAGTAA-3'		

using the MXCOMP program, which directly compares the original similarity matrix and the cophenetic value matrix, as suggested by Rohlf (1997).

RESULTS AND DISCUSSION

Analysis of the 31 *Triticum* and *Aegilops* genotypes with fourteen AFLP primer pairs identified a total of 414 reliably detectable fragments, of which 387 (93.5%) were polymorphic between two or more accessions (Table 2). Primer combinations 7 (P-AG/M-GCG) and 12 (P-AAG/M-CGT) produced the greatest polymorphic bands (45 and 47, respectively) and primer combinations 14 (P-ACG/M-CTG) and 1 (P-AAG/M-CAA) produced the lowest polymorphic bands (13 and 14, respectively) (see Table 2). The average number of bands per primer combination was 30 with a level of polymorphism ranging from 86.7% to 97.4%. An example of an AFLP pattern produced using the *Pst*I-ACG/*Mse*I-CGT primer combination is presented in Figure 1.

The genetic similarity coefficients ranged from 0.12 between *A. glabra* and accession number 3 of *T. monococcum* and 0.57 between *A. crassa* (6x) and *A. crassa* (4x-6x). Two *Aegilops* species of *A. umbellulata* and *A. caudata* were ranked as the second most related species. The mean of similarity index for 31 studied genotypes was 25% with the lowest mean of similarity index (32%) belonging to the accessions of *T. monococcum*. Mean of similarity index for hexaploid synthetic wheats was estimated as 0.34%.

Cluster analysis of 31 accessions belonging to the 14 species was performed using NTSYS-pc statistical package. A dendrogram was generated by UPGMA cluster analysis based on Jaccard's similarity estimates for AFLP data revealed two main groups (Figure 2). The measure of goodness-of-fit of a cluster analysis is given by the ''cophenetic correlation'' (r). The dendrogram had a matrix correlation r = 0.8 which is interpreted as a good fit. The two main distinct groups (I and II) had closely corresponded with their genome composition. Group I contained 13 accessions belonging to *T. dicoccoides*, *T. dicoccum* and *T. monococcum* species. Studies on the sequencing of the internal transcribed spacer (ITS)-2 region of nuclear ribosomal DNA showed that the A genome of *T. dicoccoides* originated from *T. monococcum* (Zhang et al., 2002). One of the two enzyme used for DNA digestion in AFLP, *Pst1*, is a methylation sensitive enzyme and will only cut hypomethylated regions (Almanza-Pinzon et al., 2003). Hence, the biased distribution of AFLP markers toward A genome and high genetic similarity between A genome of the einkorn and emmer wheats could be attributed to their similar cluster-

Figure 1. AFLP polyacrylamide gel profile of 31 wheat genotypes (14 species including wild, synthetic and cultivated species) using primer combination *Pst*I-ACG-*Mse*I-CGT. M is a lane of 50 bp molecular ladder and numbers correspond to the genotypic numbers listed in Table 1.

ing pattern.

Group II contained 18 genotypes (the remaining 59%), of which 11 were the accessions of *Aegilops* spp, 4 were synthetic hexaploid wheats and one durum and two were bread wheat cultivars. Group II contained broader species diversity, whilst the group I contained accessions which were collected from the diverse geographical areas of Iran. It is also interesting to note that the first cluster only included wheat species containing A and AB genomes, while second cluster included wheat species containing C, D, AB, CD, UC, DM, DDM and ABD genomes.

Group I and II can further be divided into two (IA and IB) and four (IIA, IIB, IIC and IID) sub-groups, respectively. Group IA had the only two tetraploid (AB genome) accessions belonging to each of *T. dicoccoides* and *T. dicoccum* species. *T. dicoccoides* is a wild tetraploid species whereas *T. dicoccum* is its domesticated form. However, these two species were separated when a transect line was placed at approximately 0.35 on the distance scale within the dendrogram (Figure 2). Group IIB contained all 11 *T. monococcum* accessions that used in this study. In spite of the genetic similarity of accessions of this sub-group, they were collected from six geographical regions of Iran with more or less same climatic conditions. This may in turn indicate the similar evolutionary pattern of *T. monococcum* accessions. Group IIA contained five *Aegilops* speciess including *A. glabra*, *A. umbellulata*, *A. caudata*, *A. cylindrica* and *A. tauschii*. This sub-group mainly contains the C, D and CD genome compositions. Group IIB contained only one accession belonging to *T. compactum*. Group IIC contained eight genotypes comprising two accessions of *T. triuncialis*, three synthetic hexaploid wheats, one durum wheat and two bread cultivars. Group IID contained three accessions of *A. crassa* and a line of synthetic hexaploid wheat. In this sub-group D genome is common in both *A. crassa* and the synthetic hexaploid wheat and differed for having M and AB genomes, respectively.

In the present study, a higher AFLP polymorphism was observed among 14 wheat species than the previous reports which only used one or a few wheat species (Barrett et al., 1998; Soleimani et al., 2002; Almanza-Pinzon et al., 2003). The availability of large numbers of

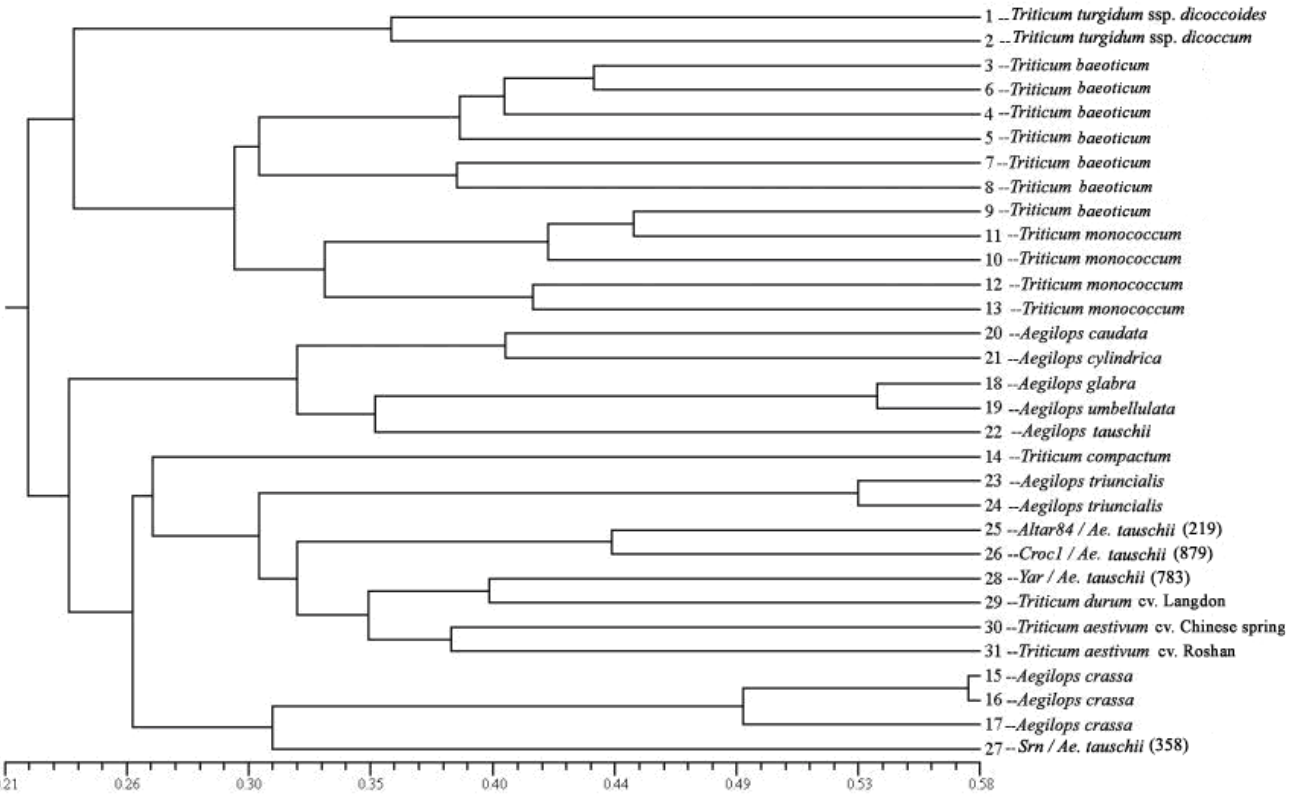

Figure 2. Dendrogram generated for 31 wheat genotypes belonging to 14 species using UPGMA cluster analysis based on Jaccard's similarity estimates for AFLP data.

fragments defining independent genetic loci with highly reproducible polymorphism detection enables the efficient evaluation of genetic diversity. A low level of polymorphism observed in wheat especially among cultivated lines and/or cultivars (Reif et al., 2005). A narrow genetic diversity among durum wheat genotypes was also found (Soleimani et al., 2002; Shoaib and Arabi, 2006). It has been suggested that genetic diversity in wheat is narrow due to a modern breeding (Huang et al., 2002; Reif et al., 2005). Nevertheless, there has been growing public concern that the tremendous enhancements of yield by modern breeding would go hand in hand with a large decrease in diversity, which could threaten future selection progress. Therefore, increasing the genetic diversity through the introgression of novel materials including wild relatives which contain numerous unique alleles that are absent in modern wheat cultivars should be emphasized.

Expectedly, *T. monococcum* to *T. monococcum* subsp *baeoticum* and likewise, *T. dicoccom* to *T. dicoccoides* were very closely related considering their genetic similarity coefficients. This finding is in complete agreement with that of Yildirim and Akkaya (2006). This result also supports those classifications that recognize *baeoticum* as a subspecies of *T. monococcum* not as a distinct species of *T. baeoticum* (http://www.k-state.edu/wgrc/Taxonomy/taxintro.html for details).

Cluster analysis of 31 *Triticum* and *Aegilops* genotypes based on morphological characters, with Dice coefficient followed by UPGMA algorithm, revealed eight major clusters (Arzani et al., 2005). The degree of relationship between the distance estimates based on AFLPs and morphological markers was studied as correlation among all similarity matrices. The correlation between the two measurements correlated was highly significant (r = 0.63**). Despite the differences between similarity matrices computed based on the two markers (range= 0.16 – 1.0 and mean = 0.54 for morphological markers and range= 0.12 – 0.57 and mean= 0.25 for AFLP markers), similarities in the two measurements were identified according to both the correlations of the similarity indices and discriminating of genotypes in the cluster analyses. Lage et al. (2003) used 5 morphological markers with 13 agronomic (quantitative) traits and AFLPs to assess the genetic diversity of synthetic hexaploid wheats and their comparison of genetic distances showed no correlation between the two diversity measurements. Our study of AFLP and morphological diversity in 31 wheat genotypes belonging to 14 species shows that the wild relatives are

valuable sources for increasing variation to incorporate novel germplasm in the cultigens.

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