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Molecular Phylogeny of *Aegilops* L. and *Triticum* L. Species Revealed by Internal Transcribed Spacers of Ribosomal Genes

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ABSTRACT

Phylogenetic analysis of *Triticum* L. and *Aegilops* L. species was performed using the nuclear ribosomal Internal Transcribed Spacer (ITS) sequences. The full length of PCR products for ITS1 and ITS2 ranged from 650 bp to 700 bp, respectively. Sequence divergences between species were estimated following aligning. The average G+C contents of the ITS regions was 60.8% for ITS2 and 61.5% for ITS1. The phylogenetic analyses were constructed using the Neighbor-Joining (NJ) method based on pairwise genetic distances. The resulting NJ tree successfully separated *Triticum* and *Aegilops* species and displayed three clusters, einkorn wheats, polyploid wheats, and *Aegilops*. Our results confirmed that the A genome of bread wheat is more related to *T. urartu* than *T. boeticum*. In the case of the D genome, the affinity between *Ae. tauschii* and bread wheat was greater than other D genome-bearing species of *Aegilops* (*Ae. crassa* and *Ae. cylindrica*). Obtained results also revealed that *Ae. speltoides* was separated from *Aegilops* cluster and grouped with polyploid wheats. The close relationship between *Ae. speltoides* and polyploid wheats indicates that the former is the most likely donor of the B genomes to wheats. The present study verified the potential of ITS regions in phylogenetic studies and strongly supported the evolution of cultivated wheats, which occurred through hybridization and polyploidization between *Triticum* and *Aegilops* species.

Keywords: PCR products, Phylogenetics, Polyploid wheats, Rdna.

INTRODUCTION

Triticum L. and *Aegilops* L. (family Poaceae), two important genetic and economic resources genera of the tribe Triticeae, are closely related. Genomic constitution of the different *Triticum* and *Aegilops* species were established by Kihara (1954) using the numerous cytogenetic analyses. These finding subsequently reviewed by molecular, cytoplasmic and cytological studies (e.g., Wang *et al*., 2000; Huang *et al*., 2002; Sallares and Brown, 2004; Baum *et al*., 2009, Vakhitov *et al*., 2003). These studies have described 22 *Aegilops* and 5 *Triticum* species, including diploid (2n= 2x= 14), tetraploid (2n= 4x= 28) and hexaploid (2n= 6x= 42) cytotypes. Common wheat or bread wheat (*Triticum aestivum* L.), has been described as an allohexaploid species (BA^uD) derived through hybridization and allopolyploidization. Two or three diploid ancestors have shared their homeologus genomes in derivate allopolyploid species (Petersen *et al*., 2006). The origin of the polyploid wheat genomes (A, B, D, and G) has been frequently the subject of numerous studies (Vakhitov *et al*. 2003; Petersen *et al*., 2006; Baum *et al*., 2009; Golovnina *et al*., 2007, 2009).

The genus *Aegilops* played a significant role in the evolution and domestication of wheat through natural hybridization (Sliai and Amer, 2011). *Aegilops* is classified into six sections or subgenera (Eig, 1929; Kihara, 1954; Vakhitov *et al*., 2003). The seven

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basic genomes in the genus are C, D, M, N, S, T and U (Sasanuma *et al*., 2004).

The A genome of the bread wheat is the only wheat-specific genome and was proposed to be originated from einkorn wheat species; *T. boeoticum, T. monococcum*, and *T. urartu.* The genome of *T. urartu* is described as A^u while *T. boeoticum* and *T. monococcum* have the A^b genome (Vakhitov *et al*., 2003; Golovnina *et al*., 2009).

Some *Aegilops* species are considered as the donors of the two other genomes of wheat (Baum *et al*., 2009; Sliai and Amer, 2011). It has been found that B and G genomes of polyploid wheat species were closely similar to the S genome in the *Sitopsis* section of *Aegilops* (Zhang *et al*., 2002; Sallares and Brown, 2004; Kilian *et al*., 2011; Haider, 2013). While *Ae. tauschii* L., from section *Vertebrata*, served as a donor of the D genome (Vakhitov *et al*. 2003; Sasanuma *et al*. 2004; Golovnina *et al*. 2009; Bordbar *et al*., 2011) .The close genetic relationship between *Aegilops* species and polyploid wheats attracted a lot of attention to reveal the phylogenetic relationships between them.

Phylogenetic relationships between *Aegilops* and *Triticum* have been investigated based on morphological, cytogenetical, and molecular studies, for which it is necessary to find an appropriate molecular marker (Alnaddaf *et al*., 2012, 2013; Kilian *et al*., 2011). DNA markers have been used widely in botanical studies and provided new information on phylogeny and genetic relationships of different species of *Triticum–Aegilops* (e.g., Huang *et al*., 2002; Sallares and Brown, 2004; Yamane and Kawahara, 2005; Petersen *et al*., 2006; Poczai and Hyvonen, 2010).

Of the available molecular techniques, analysis of nuclear ribosomal DNA (nrDNA) regions has been proved useful for identifying genetic variation and inferring evolutionary relationships in all eukaryote groups. The rDNA spacer regions could have potential to phylogenetic studies because of their universality, simplicity, and small size (Nepolo *et al*., 2010; Poczai and Hyvonen, 2010). The rDNA regions are parts of repeat units of tandem copies, which are located at the chromosomal sites known as Nucleolus Organizing Regions (NORs) (Alvarez and Wendel, 2003; Haque *et al*., 2009).

Each rDNA gene comprises the 18S, 5.8S and 28S rRNA sub-units which called Internal Transcribed Sequenes (ITS), and an InterGenic Spacer (IGS), that found universally in plants. These gene families contain conserved (transcribed) and non-conserved (partly transcribed) regions, each part of which evolves with different rates (Alvarez and Wendel, 2003; Haque *et al*., 2009; Poczai and Hyvonen, 2010).

The conserved regions (18S and 28S genes) of rDNA display relatively little variation and are analyzed to infer phylogenetic relationships at higher taxonomic levels (e.g., family) (Nepolo *et al*., 2010; Poczai and Hyvonen, 2010). Other parts, i.e. the spacer regions (ITS and IGS), which evolve more quickly and are highly polymorphic, can be easily used in plant systematic at the low-level (generic or population) (Singh *et al*., 2008; Haque *et al*., 2009).

For over a decade, to infer plants phylogenetic and evolutionary relationships, and detect their genetic diversity, the most widely used marker in plants was the ITS. The sequence length of ITS, which has three components, namely, the ITS1, ITS2 and 5.8S, varies between 500 and 750 bp in angiosperms to 1,500–3,500 bp in other seed plants (Alvarez and Wendel, 2003; Calonje *et al*. 2009).

The nature of the ITS region, its biparental inheritance, its high number of copies, and existence in virtually all organisms make it useful in studies concerning parentage of polyploids, hybrid formation and phylogenetics (Barkman and Simpson, 2002; Albach and Chase, 2004; Fehrer *et al*., 2007). Since the first application of ITS by Porter and Collins (1991), it has been used widely for phylogeny reconstruction in Triticeae (Goryunova *et al*., 2005; Calonje *et*

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al., 2009; Haque *et al*., 2009; Logacheva *et al*., 2010; Nepolo *et al*., 2010; Poczai and Hyvonen, 2010; Alnaddaf *et al*., 2013; Bordbar *et al*., 2011).

The present study aimed to compare the sequence variations of the ITS regions in accessions of 12 *Triticum* and *Aegilops* species, representing different genomes, to infer their phylogenetic relationships.

MATERIALS AND METHODS

Plant Material

A set of 71 accessions from 12 species belonging to *Aegilops* and *Triticum*, representing different genomes (A, B, C, D, M, S and U) and different ploidy levels (diploid, tetraploid and hexaploid), were included in this study. Seeds of accessions, which were selected to display various geographical distributions of *Aegilops* and *Triticum* species in Iran, were provided by the Gene Bank of the University of Ilam, Ilam province.

One accession of wild barley, *Hordeum spontaneum* (K. Koch) Thell, was chosen as an out-group due to its close relationship with *Aegilops* and *Triticum*. The ploidy level, genome type, and source of the plant materials used in this study are shown in Table 1.

DNA Extraction and PCR Amplification

Total genomic DNA was extracted from fresh leaves of a single plant of each accession, using the CTAB (CetylTrimethyl Ammonium Bromide) method (Doyle and Doyle, 1987). The quality of isolated DNA was checked by electrophoresis on 0.8% (90V at 45 minutes) agarose gel.

The ITS region was amplified by two primer pairs (Table 2). PCR Reactions were carried out in 50 µL reaction mixture, consisted of 25 µL of mastermix (containing Taq polymerase, dNTPs, $MgCl_2$ and a reaction buffer), 1 µL of each primer (forward and reverse), 2 µL of genomic DNA and 21 µL sterile water. Cycling conductions were adjusted according to the following PCR program: an initial denaturation at 94°C for 4 minutes and 38 thermal cycles each of which included denaturation at 92°C for 30 seconds, annealing at TA (Table 2) for 1 minute and extension at 72°C for 45 seconds). Another denaturation at 94°C for 30 seconds and annealing for 30 seconds, was conducted. The final extension was performed at 72°C for 5 minutes.

Purification and Sequencing

Amplified fragments were separated by electrophoresis on 1.5% agarose gel prepared with 1X TAE (Tris-Acetate EDTA). A 100 bp DNA ladder was used to estimate the molecular weights of the amplified products. In order to purify and sequence PCR products, 25-30 µL of each of the latter were delivered to Bioneer Company, Korea. Sequencing reactions were carried out in the forward direction.

Data Analysis

Sequences were aligned by MEGA5.0 based on Clustul W. Polymorphism, nucleotide composition, and nucleotide pair

Table 2. The sequences of primers used for PCR amplification of ITS regions.

Primer	Strand	Sequence	TA(°C)
ITS1	Forward	*5'-GAGGAAGGAGAAGTGGTAAC-3'*	48
	Reverse	*5'-CTGGGGTCGCGGTCGA-3'*	
ITS2	Forward	*5'-TATCTTTAGAGGAAGGA-3'*	30
	Reverse	*5'-GATATGCTTAAACTCAGC-3'*	

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Table 1. Details of plant materials and their collection places.

Code	Species	Genome		Longitude	latitude	Elevation (meter)	Source
1	*T. aestivum*	BA^uD	6x	45° 23'16.21" E	36° 57' 22.80" N	1319.08	West Azerbaijan, Naghadeh, Iran
2	*T. aestivum*	BA^uD	6x	55° 9' 34.77" E	37° 14' 26.40" N	37.68	Golestan, Gonbad, Iran
3	*T. aestivum*	BA^uD	6x	49° 59' 53.65" E	36° 16' 25.17" N	1305.22	Qazvin, Iran
4	*T. aestivum*	BA^uD	6x	51° 51' 46.22" E	32° 0' 29.19" N	1825.67	Isfahan, Shahreza, Iran
5	*T. aestivum*	BA^uD	6x	49° 52' 12.31" E	31° 49' 26.26" N	851.42	Khuzestan, Izeh, Iran
6	*T. aestivum*	BA^uD	6x	46° 10' 23.85" E	33° 7' 3.59" N	153.97	Ilam, Mehran, Iran
7	*T. boeticum*	A^b	2x	48° 53' 3.85" E	33° 13' 2.22" N	1030.13	Lorestan, Sepiddasht, Iran
8	*T. boeticum*	A^b	2x	50° 38' 16.37" E	36° 12' 53.95" N	1856.64	Alborz, Taleghan, Iran
9	*T. boeticum*	A^b	2x	46° 29' 31.92" E	34° 48' 22.59" N	1313.41	Kermanshah, Javanrood, Iran
10	*T. boeticum*	A^b	2x	45° 5' 49.69" E	37° 2' 11.17" N	1438.34	West Azerbaijan, Oshnavieh, Iran
11	*T. boeticum*	A^b	2x	46° 34' 9.26" E	33° 45' 57.47" N	1014.23	Ilam, Sarableh, Iran
12	*T. turgidum*	BA^u	4x	48° 40' 14.23" E	31° 19' 5.98" N	20.81	Khuzestan, Ahvaza, Iran
13	*T. turgidum*	BA^u	4x	48° 17' 59.96" E	38° 15' 13.45" N	1347.95	West Azerbaijan, Ardabil, Iran
14	*T. turgidum*	BA^u	4x	51° 35' 15.08" E	30° 40' 6.18" N	1839.00	Kohgiluye and Boyer-Ahmad, Yasooj, Iran
15	*T. turgidum*	BA^u	4x	47° 8' 10.37" E	35° 57' 19.29" N	1927.23	Kordestan, Iran
16	*T. turgidum*	BA^u	4x	59° 37' 0.32" E	36° 15' 37.66" N	981.90	Razavi Khorasan, Mashhad, Iran
17	*T. turgidum*	BA^u	4x	46° 10' 23.85" E	33° 7' 3.59" N	153.97	Ilam, Mehran, Iran
18	*T. urartu*	A^u	2x	46° 14' 7.70" E	34° 16' 50.52" N	1549.51	Kermanshah, Kerand, Iran
19	*T. urartu*	A^u	2x	46° 16' 40.70" E	36° 14' 20.20" N	1454.44	Kordestan, Saqqez, Iran
20	*T. urartu*	A^u	2x	46° 10' 32.45" E	35° 31' 15.96" N	1309.00	Kordestan, Marivan1, Iran
21	*T. urartu*	A^u	2x	51° 27' 16.51" E	30° 51' 36.07" N	2240.39	Kohgiluye and Boyer-Ahmad, Sisakht, Iran
22	*T. urartu*	A^u	2x	50° 58' 51.74" E	32° 16' 13.66" N	2112.48	Chaharmahal and Bakhtiari, FarrokhShahr, Iran
23	*T. urartu*	A^u	2x	47° 35' 46.78" E	34° 46' 40.42" N	1690.33	Kermanshah, Sonqor, Iran
24	*T. urartu*	A^u	2x	47° 26' 9.46" E	34° 23' 25.72" N	1363.96	Kermanshah, Behistun, Iran
25	*T. urartu*	A^u	2x	47° 26' 9.46" E	34° 23' 25.72" N	1363.96	Kermanshah, Iran
26	*T. urartu*	A^u	2x	46° 10' 32.45" E	35° 31' 15.96" N	1309.00	Kordestan, Marivan2, Iran
27	*Ae. triaristata*	UM	4x	48° 20' 56.17" E	32° 27' 13.73" N	148.07	Khuzestan, Andimeshk, Iran
28	*Ae. triaristata*	UM	4x	48° 1' 45.74" E	33° 29' 17.22" N	1008.11	Lorestan, Veysian, Iran
29	*Ae. triaristata*	UM	4x	47° 22' 58.42" E	33° 8' 38.04" N	661.51	Ilam, Darreh Shahr, Iran
30	*Ae. triaristata*	UM	4x	47° 53' 10.60" E	33° 18' 53.48" N	813.45	Lorestan, Afrineh, Iran
31	*Ae. triaristata*	UM	4x	46° 24' 55.01" E	33° 38' 5.91" N	1382.58	Ilam, Banganjab, Iran
32	*Ae. crassa*	DM	4x	52° 16' 34.44" E	30° 2' 42.21" N	1885.61	Fars, Sepidan, Iran
33	*Ae. crassa*	DM	4x	45° 59' 13.95" E	37° 44' 39.54" N	1409.40	East Azarbaijan, Azarshahr, Iran
34	*Ae. crassa*	DM	4x	47° 35' 46.78" E	34° 46' 40.42" N	1690.33	Kermanshah, Sonqor, Iran
35	*Ae. crassa*	DM	4x	51° 17' 40.59" E	31° 58' 47.25" N	2241.89	Chaharmahal and Bakhtiari, Borujn, Iran
36	*Ae. crassa*	DM	4x	46° 24' 55.01" E	33° 38' 5.91" N	1382.58	Ilam, Iran
37	*Ae. crassa*	DM	4x	47° 36' 16.62" E	34° 16' 18.89" N	1567.97	Kermanshah, Harsin, Iran

Table 1 continued…

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Continued of Table 1.

Code	Species	Genome		Longitude	latitude	Elevation (meter)	Source
38	*Ae. cylindrica*	CD	4x	47° 8' 10.37" E	35° 57' 19.29" N	1927.23	Kordestan, Iran
39	*Ae. cylindrica*	CD	4x	47° 36' 16.62" E	34° 16' 18.89" N	1567.97	Kermanshah, Harsin, Iran
40	*Ae. cylindrica*	CD	4x	47° 39' 53.46" E	38° 23' 41.38" N	1419.37	Ardabil, Meshgin Shahr, Iran
41	*Ae. cylindrica*	CD	4x	48° 30' 31.40" E	36° 40' 58.82" N	1699.49	Zanjan, Iran
42	*Ae. cylindrica*	CD	4x	48° 52' 7.37" E	38° 25' 20.84" N	-23.59	Gilan, Astara, Iran
43	*Ae. ovata*	UM	4x	47° 39' 53.46" E	38° 23' 41.38" N	1419.37	Ardabil, Meshgin Shahr, Iran
44	*Ae. ovata*	UM	4x	46° 18' 26.34" E	33° 49' 47.28" N	1177.85	Ilam, Eyvan, Iran
45	*Ae. ovata*	UM	4x	46° 31' 40.34" E	34° 6' 47.47" N	1335.05	Kermanshah,Eslamabad-e Gharb, Iran
46	*Ae. ovata*	UM	4x	47° 4' 6.09" E	38° 29' 21.95" N	1376.54	East Azarbaijan, Ahar, Iran
47	*Ae. ovata*	UM	4x	46° 34' 9.26" E	33° 45' 57.47" N	1014.23	Ilam, Sarableh, Iran
48	*Ae. ovata*	UM	4x	47° 39' 53.46" E	38° 23' 41.38" N	1419.37	Ardabil, Meshgin Shahr, Iran
49	*Ae. speltoides*	S	2x	45° 34' 39.67" E	34° 30' 57.25" N	354.25	Kermanshah, Qasr-e Shirin, Iran
50	*Ae. speltoides*	S	2x	53° 41' 16.97" E	32° 25' 40.47" N	1139.54	Iran
51	*Ae. speltoides*	S	2x	53° 41' 16.97" E	32° 25' 40.47" N	1139.54	Iran
52	*Ae. speltoides*	S	2x	47° 22' 58.42" E	33° 8' 38.04" N	661.51	Ilam, Darreh Shahr, Iran
53	*Ae. speltoides*	S	2x	47° 22' 58.42" E	33° 8' 38.04" N	661.51	Ilam, Darreh Shahr, Iran
54	*Ae. speltoides*	S	2x	47° 25' 28.32" E	32° 59' 19.03" N	878.85	Ilam, Abdanan, Iran
55	*Ae. tauschii*	D	2x	49° 35' 32.69" E	37° 16' 51.41" N	2.04	Gilan, Iran
56	*Ae. tauschii*	D	2x	48° 17' 59.96" E	38° 15' 13.45" N	1347.95	Ardabil, Iran
57	*Ae. tauschii*	D	2x	47° 4' 6.09" E	38° 29' 21.95" N	1376.54	East Azarbaijan, Ahar, Iran
58	*Ae. tauschii*	D	2x	50° 38' 35.04" E	36° 55' 36.58" N	8.99	Mazandaran, Ramsar, Iran
59	*Ae. tauschii*	D	2x	48° 30' 31.40" E	36° 40' 58.82" N	1699.49	Zanjan, Iran
60	*Ae. tauschii*	D	2x	54° 51' 23.84" E	36° 54' 26.59" N	124.82	Golestan, Aliabad-e Katul, Iran
61	*Ae. triuncialis*	UC	4x	53° 41' 16.97" E	32° 25' 40.47" N	1139.54	Iran
62	*Ae. triuncialis*	UC	4x	47° 1' 36.07" E	35° 54' 49.15" N	1844.94	Kordestan, Divandarreh, Iran
63	*Ae. triuncialis*	UC	4x	48° 23' 55.75" E	33° 34' 54.62" N	1496.27	Lorestan, Iran
64	*Ae. triuncialis*	UC	4x	48° 34' 44.95" E	38° 24' 46.70" N	1242.12	Gilan, Astara, Heyran, Iran
65	*Ae. triuncialis*	UC	4x	49° 52' 12.31" E	31° 49' 26.26" N	851.42	Khoozestan, Izah, Iran
66	*Ae. triuncialis*	UC	4x	51° 17' 40.59" E	31° 58' 47.25" N	2241.89	Chaharmahal and Bakhtiari, Borujn, Iran
67	*Ae. umbellulata*	U	2x	46° 34' 9.26" E	33° 45' 57.47" N	1014.23	Ilam, Sarableh, Iran
68	*Ae. umbellulata*	U	2x	46° 31' 40.34" E	34° 6' 47.47" N	1335.05	Kermanshah, Eslamabad-e Gharb, Iran
69	*Ae. umbellulata*	U	2x	52° 16' 34.44" E	30° 2' 42.21" N	1885.61	Fars, Sepidan, Iran
70	*Ae. umbellulata*	U	2x	46° 55' 6.98" E	33° 13' 40.04" N	1212.94	Ilam, Dehloran, Meymeh, Iran
71	*Ae. umbellulata*	U	2x	47° 36' 39.89" E	33° 31' 38.84" N	1186.03	Lorestan, Kuhdasht, Iran
72	*H. spontaneum*	H	2x	46° 17' 56.14" E	33° 41' 45.48" N	1040.31	Ilam, Chavar, Iran

frequencies, and number of variable sites was estimated by MEGA 5.0. Pairwise genetic distance of accessions and species were performed by DARwin 6.0. The phylogenetic trees were constructed based on Neighbor Joining, using MEGA 5.0. Bioplot of species was conducted using DARwin 6.0 based on Principal Component Analysis (PCA) and dissimilarity matrix of species.

RESULTS AND DISCUSSION

The nucleotide sequences of the ITS fragment by two spacers were determined in *Aegilops* and *Triticum*. A single band of 650bp was observed in PCR products generated for ITS1 in all species analyzed. As for ITS2, the size of the single band generated in all species was 700 bp (Figure 1). Some accessions generated no PCR products. This may be due to mis-matching of primers to the target sites (probably, point mutation in these regions). Alnaddaf *et al*. (2013) and Bordbar *et al*. (2011) used the spacer regions to determine the genetic relationships among *Aegilops* and *Triticum* species. The entire length of ITS amplified in these two studies ranged from 600-602 bp. Wang *et al*. (2000) studied diploid species of *Aegilops* using ITS region. The ITS region varied from 601 to 607 bp. Similar results were obtained by Sharma *et al*. (2002).

ITS fragments showed polymorphism among accessions. A low variability of nucleotide sequences (16%) was revealed for ITS1 *Aegilops* species. However, the higher level of ITS1sequence variation (36%) was found in *Triticum*. Divergence of ITS2 fragments ranged from 13% for *Triticum* species to 30% for *Aegilops* species. However, 40 and 34% of nucleotides were variable among all accessions (*Triticum* and *Aegilops* species) in ITS1 and ITS2 fragments, respectively. Conservation rate for ITS nucleotide sequences was relatively high for *Triticum* (60%) and *Aegilops* (66%) genera.

In the present study, the level of polymorphism in targeted loci was higher than those reported earlier in the ITS sequences for different species of Triticeae (Wang *et al*., 2000; Sharma *et al*., 2002; Goryunova *et al*., 2005; Alnaddaf *et al*., 2013). This is not surprising because there were many accessions, which were sampled from 12 species having seven different genomes and were collected from various geographical regions. Additionally, in all previous works, divergences were reported within species, whereas our results are based on interspecific polymorphism, which is a possible reason for the relatively higher variation observed here.

In both spacer regions (ITS1 and ITS2), the highest and lowest nucleotide numbers belonged to C and T, respectively. A similar result was obtained by Goryunova *et al*. (2005).

The average G+C contents of the sequences varied from 60.8% in ITS2 to 61.5% for ITS1. Analysis of nucleotide pair frequencies for ITS1indicates high rate of identity among aligned sequences. Therefore, 94.84% of nucleotide pairs were found as identical pairs and only 2.50% and 2.66% of sequences emerged as transitional pairs and transversional pairs, respectively.

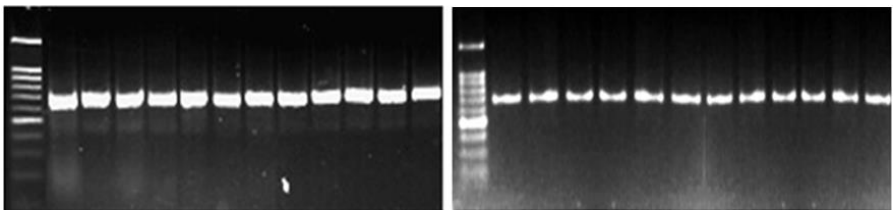

Figure 1. Amplification of ITS region in 4 *Triticum* and 8 *Aegilops* species, Left: ITS1, Right: ITS2. The first well on the left of each gel: 100 bp DNA ladder.

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As for ITS2, there were 95.12% identical pairs, 2.37% transitional pairs and 2.51% transversional pairs (Table 3).

Our observations strongly coincides with previous works that studied the ITS region of *Triticum-Aegilops*. Wang *et al*. (2000) reported that the G+C content of the *Aegilops* species ranged from 61.1-62.9%. The G+C% of *Triticum* and *Aegilops* species in the study of Goryunova *et al*. (2005) ranged from 60.3 to 63.9%. Moreover, Zhang *et al*. (2002) mentioned that the G+C content of the ITS regions varied from 59 to 65%. On the other hand, Nalini *et al*. (2007) and Sharma *et al*. (2002) reported that the G+C% was 59-61.75 and 58-61% both in common wheat, respectively.

There is a relationship between the GC content and complex secondary structures. The high GC content displays durability of the DNA and RNA secondary (Alvarez and Wendel, 2003).The high GC content of ITS sequences is also associated with evolution and adaptation to the changing environment (Alnaddaf *et al*., 2013).

The NJ dendrograms of accessions were conducted based on pairwise genetic distances (data not shown) for each spacer (Figures 2 and 3). The trees were relatively identical and divided the accessions in three main clusters. The first included einkorn wheats (*T. urartu* and *T. boeticum*), the second cluster comprises of two subgroups of polyploid wheats (*T. aestivum, T. turgidum*) and *Ae. speltoides*. The *Aegilops* cluster contained several subgroups. *Ae. tauschii*, *Ae. crassa* and *Ae. cylindrica* (only in ITS1 tree) clearly formed separated subgroups, while the differentiation between other *Aegilops* species was comparatively ambiguous. *Ae. ovata* and *Ae. umbellulata* and only one accession of *Ae. triaristata* were grouped together. Other species also formed a mixed subgroup. There was a high similarity between the results of the two spacers. Mantel's test also showed an average correlation (r= 0.18) between distance matrix of ITS1 and ITS2 that was statistically significant (P= 0.0001). Therefore, the rest analyses were conducted based on combined data.

The pairwise genetic distances of species ranged between 0.021 and 0.115. The lowest distance was between *Ae. ovata* and *Ae. umbellulata*, which may be due to the similarity of their genome (including U genome). The highest similarity value was between *T. boeticum* and *Ae. crassa*. These species showed wide intraspecific divergences (0.078, 0.068) (Table 4).

As Table 4 shows, *Ae. umbellulata* and *Ae. tauschii* have the lowest intraspecific variation. The low level of intraspecific variation of *Ae. tauschii* was supported by similar previous works (Sasanuma *et al*., 2004).

The general, topology of the dendrogram of species was similar to the dendrogram of accessions. Three major groups were recognized. The *Aegilops* group included all the *Aegilops* species except *Ae. speltoides*, which was clustered with polyploidy wheats. This finding was also supported by evidence based on genetic distance between species (see Figure 4 and Table 4).This divergence is probably due to its breeding system since

Table 3. Characteristics of nucleotide sequences of ITS1 and ITS2.

Primer	%Nucleotide Composition		%Nucleotide pair frequencies							
ITS1	T	16.90	TT	15.86	CT	0.79	AT	0.16	GT	0.31
	C	33.00	TC	0.47	CC	31.41	AC	0.47	GC	0.31
	A	21.60	TA	0.16	CA	0.47	AA	20.26	GA	0.63
	G	28.50	TG	0.31	CG	0.31	AG	0.47	GG	27.33
ITS2	T	17.50	TT	16.24	CT	0.70	AT	0.30	GT	0.40
	C	32.10	TC	0.60	CC	30.86	AC	0.30	GC	0.30
	A	21.70	TA	0.30	CA	0.30	AA	20.48	GA	0.60
	G	28.70	TG	0.30	CG	0.30	AG	0.40	GG	27.50

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Table 4. Pairwise genetic distance within and among species.

Groups		Amonggroups											
	Within groups	*T. aestivum*	*T. boeticum*	*T. turgidum*	*T. urartu*	*Ae. triaristata*	*Ae. crassa*	*Ae. cylindrica*	*Ae. ovata*	*Ae. speltoides*	*Ae. tauschii*	*Ae. triuncialis*	*Ae. umbellulata*
T. aestivum	0.019												
T. boeticum	0.087	0.100											
T. turgidum	0.024	0.026	0.096										
T. urartu	0.040	0.077	0.065	0.073									
Ae. triaristata	0.029	0.059	0.084	0.056	0.062								
Ae. crassa	0.068	0.092	0.115	0.087	0.093	0.072							
Ae. cylindrica	0.027	0.057	0.082	0.054	0.059	0.029	0.068						
Ae. ovata	0.029	0.056	0.087	0.054	0.062	0.035	0.069	0.034					
Ae. speltoides	0.023	0.048	0.087	0.044	0.063	0.047	0.079	0.044	0.045				
Ae. tauschii	0.017	0.058	0.081	0.055	0.057	0.039	0.069	0.033	0.035	0.040			
Ae. triuncialis	0.020	0.057	0.082	0.054	0.059	0.023	0.069	0.025	0.033	0.044	0.036		
Ae. umbellulata	0.013	0.055	0.084	0.051	0.058	0.030	0.067	0.028	0.021	0.038	0.031	0.028	

Ae. speltoides is the only cross-pollinated species and the most divergent among *Aegilops* and *Triticum* species (Giorgi *et al*., 2002). Such species contains higher levels of variation at the DNA level than self-pollinating species (Sasanuma *et al*., 2004). Differences between *Ae. speltoides* and the other *Aegilops* species have been detected in previous studies (Giorgi *et al*., 2002; Wang *et al*., 2000; Sallares and Brown, 2004; Yamane and Kawahara, 2005; Sliai and Amer, 2011).

Here, *Ae. speltoides* formed a cluster with *T. aestivum* and *T. turgidum*. This indicates that *Ae. speltoides* genome is closely related to the B genome of polyploied wheats. The vast majority of previous studies proposed *Ae. speltoides* as the donor of the B genome to polyploidy wheats (e.g., Khlestkina and Salina, 2001; Zhang *et al*., 2002; Sallares and Brown, 2004; Petersen *et al*., 2006; Kilian *et al*., 2007b; Eilam *et al*., 2007; Golovnin *et al*., 2007; Chalupska *et al*., 2008; Haider, 2013).

Other morphological and cytoplasmic evidence revealed the similarity of *T. aestivum* to the *Sitopsis* species of *Aegilops* to which *Ae. speltoides* belongs (Sasanuma *et al*., 2004; Baum *et al*., 2009).

In Golovnina *et al*. (2007) study, *Ae. speltoides* branched out before the separation of wild diploid *Triticum* and *Aegilops* species. This made Yamane and Kawahara, (2005) and Golovnina *et al*. (2007) suggest the inclusion of *Ae. speltoides* into a new genus. In 1929, Eig separated and placed this species in another subsection *Truncata* Eig.

According to the constructed trees, two einkorn species *T. boeticum* and *T. urartu* were grouped in one cluster, which supported the similarity of their genomes (A with corresponding indices). There was a debate regarding which species was the donor of the A genome of polyploid wheats. The diploid species *T. monococcum* and *T. boeoticum* were suggested as the A genome donor of polyploidy wheats (e.g., Sallares and Brown, 1999, Khlestkina *et al*., 2001). Some other data, however, revealed that only *T. boeoticum* contributed to the formation of *T. zhukovskyi* and several artificial polyploids (Goncharov *et al*., 2007).

In this study, in order to clarify the origin of the A genome of polyploid wheat species, we focused on pairwise distance of species

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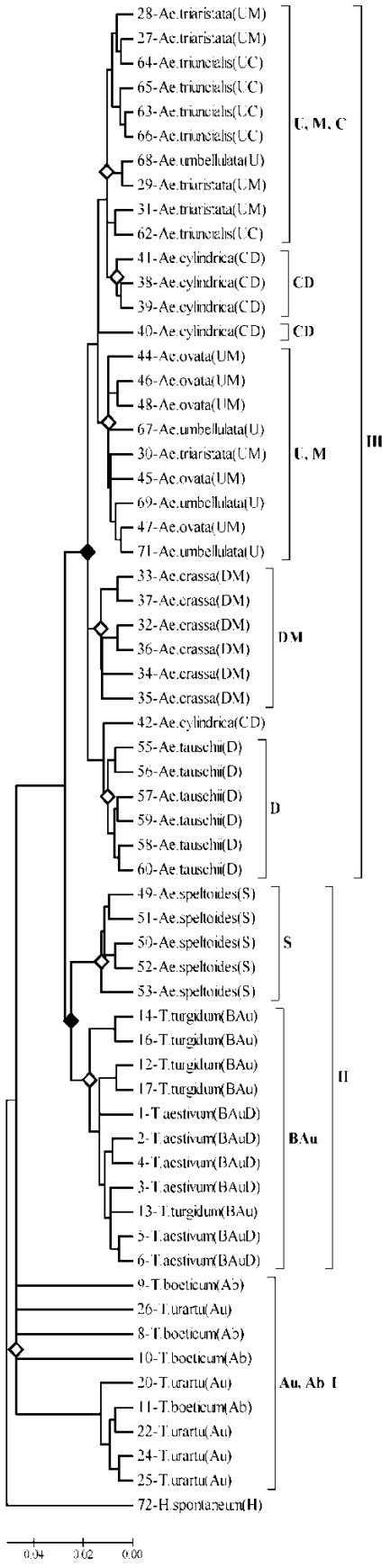

Figure 2. Phylogenetic tree of *Triticum* L. and *Aegilops* L., accessions from 12 different species based on ITS1 sequences.

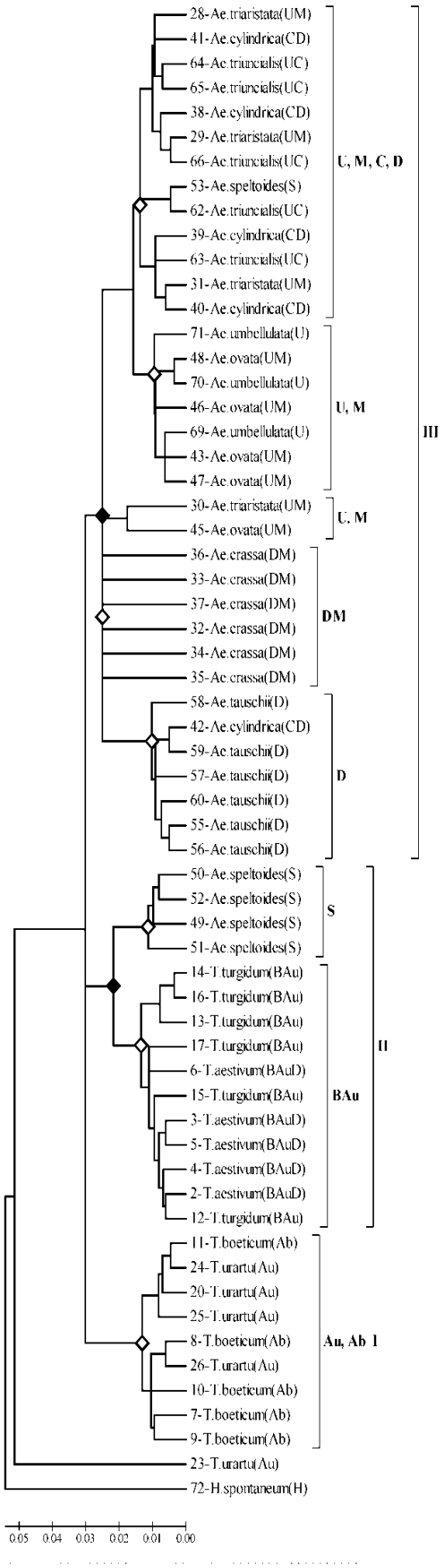

Figure 3. Phylogenetic tree of *Triticum* L. and *Aegilops* L., accessions from 12 different species based on ITS2 sequences.

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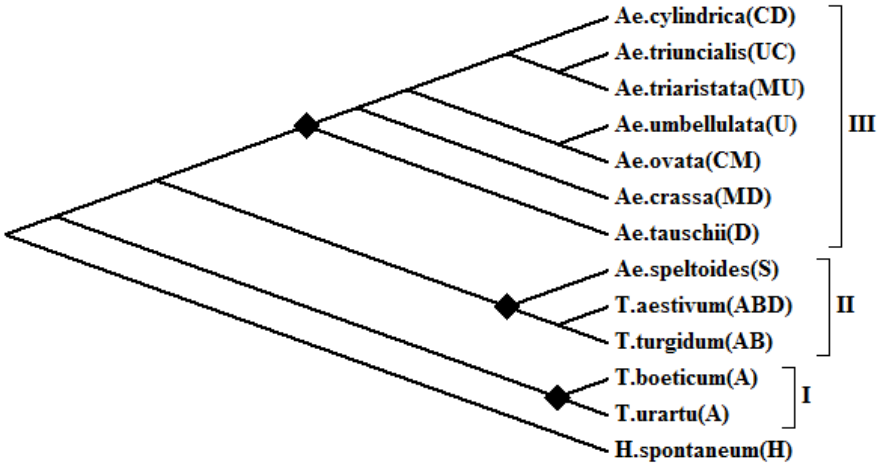

Figure 4. Phylogenetic tree of *Triticum* and *Aegilops* species constructed from ITS1+ITS2 sequences.

(Table 4). The higher distance between *T. boeticum* and common wheat compared to the observed distance between the latter and *T. urartu*, increasingly confirmed *T. urartu* as donor of the A genome to polyploid wheat. The same results were revealed by other studies such as Galili *et al*. (2000), who suggested *T. urartu* as the donor of the A genome of *T. turgidum* and *T. timopheevii*.

Konarev *et al*. (1979) proposed that the A genome of *T. turgidum* was derived from *T. urartu* and that of *T. timopheevii* from *T. monococcum* based on the immunological character of seed storage proteins. Nishikawa *et al*. (1992) suggested that the A genome of *T. turgidum* was possibly derived from both *T. boeoticum* and *T. urartu*. Similar results generated by other studies based on nucleotide sequences of rDNA (Zhang *et al*., 2002; Baum, *et al*. 2009) and other molecular data (Galili *et al*., 2000; Huang *et al*., 2002; Kilian *et al*., 2007a) clearly showed that *T. urartu* was the ancestor of the A genome of *T. turgidum* and *T. timopheevii*.

Regarding the *Aegilops* cluster, *Ae. tauschii* (D genome) and the closely related species *Ae. crassa* (DM genome) were divided in two separate groups, although the D genome of *Ae. crassa* was, most probably, inherited from *Ae. tauschii* (Badaeva *et al*., 2002). The position of *Ae. tauschii* in NJ trees (Figures 2, 3, and 4) confirms its close relationship to bread wheat.

The closer genetic distance between *Ae. tauschii* and polyploid wheats compared to that observed between the latter and the other D genome-bearing species (*Ae. crassa* and *Ae. cylindrica*) may indicate that the source of the D genome in polyploidy wheats is *Ae. tauschii* (Table 4).

The close relationship between *Ae. tauschii* and common wheat was inferred by Huang *et al*. (2002) and Dizkirici *et al*. (2016). The formation of hexaploid wheat by hybridization among diploid *Ae. tauschii* and *T. turgidum* made Bordbar *et al*. (2011), Kilian *et al*. (2011), and Haider (2013) conclude that *Ae. tauschii* could serve as the donor of the D genome. The same results were also clearly revealed based on morphological comparison of various *Aegilops* and wheat species and their chromosomes (Pathak, 1940; Riley and Chapman, 1960). Zhang *et al*. (2002) crossed *T. turgidum* with *Ae. tauschii* and generated a synthetic hexaploid wheat. Interestingly, the latter conclusion may explain the main role of *Ae. tauschii* in bread making properties of common wheat and also the existence of *Ae. tauschii* as a weed among tetraploid wheat species (Vakhitov *et al*., 2003).

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A large body of molecular studies reviewed the phylogenetic relationships of the three wheat genomes. Dizkirici *et al*. (2016) showed a close relationship between each of *Ae. speltoides* and *Ae. tauschii* and polyploid wheats using sequences of the *ITS rDNA* and *matK* genes. Buchner *et al*. (2004) analyzed sequences of transporter genes, which confirmed the close relationship between *T. aestivum* and *Ae. tauschii*, and *T. urartu* and *Ae. speltoides*. Based on *18S rRNA* gene sequence (Sallares and Brown, 2004), *Ae. tauschii* was nominated as the D genome donor of *T. aestivum* and *Ae. speltoides* as the B genome donor. These evidences pointed to the outcrossing of *Ae.speltoides* as the female parent of tetraploid wheats with *T. urartu* as the male parent.

In conclusion, all of the species tended to be clustered according to their genomes since the similar genomes were clustered together. *T. boeticum* and *T. urartu* (A genome), were found in one cluster. *T. aestivum* and *T. turgidum*, which have A and B genomes, were also grouped in one clade, *Aegilops* species were also grouped based on their genomes, and the species that carried the same genome showed the close position. Among *Aegilops* species, only *Ae. speltoides* was distinct and was grouped with *Triticum* (Figures 4 and 5).

Bioplot of species displayed distribution, distance and evolutionary relationships of species (Figure 6). According to the results generated, two einkorn species (*T. urartu* and *T. boeticum*) were distinct from other species, and two *Triticum* species (*T. turgidum* and *T. aestivum*) were positioned close to *Aegilops* species. This proves the close evolutionary relationships between *Triticum* and *Aegilops*. The results also confirmed that *Aegilops* species are progenitors of polyploid wheats.

The rDNA spacers have been proved to be useful as phylogenetic markers at both higher and lower level evolutionary studies. Here, the ITS region allowed revealing genetic relationships between *Triticum* and *Aegilops* species. Data obtained here also allowed to separate *Aegilops* and *Triticum*. The two genera were incorporated into a single genus, *Triticum* Bowden by Vakhitov *et al*. (2003). This taxonomic revision was disapproved using several cytogenetic and molecular studies, which argue that *Aegilops* and *Triticum* are two distinct genera. In addition, Van-Slageren (1994) separated *Ae. mutica* from *Aegilops* and placed it into a mono-specific genus called *Amblyopyrum* Eig. Therefore, there are three genera: *Triticum*, *Aegilops* and *Amblyopyrum* (Van-Slageren, 1994; Vakhitov *et al*., 2003; Yamane and Kawahara, 2005; Baum *et al*., 2009; Poczai and Hyvonen, 2010; Sliai and Amer, 2011).

The phylogenetic relationships among *Aegilops* groups (C, D, M, and U genomes) were unclear, and our findings were not able to identify the relationships among these species in more detail. Some *Aegilops* species such as *Ae. ovata*, *Ae. umbellulata*, *Ae. triaristata*, *Ae. triuncialis* and *Ae. cylindrica* were revealed (based on ITS2 sequences) to be closely related as they appeared as mix groups. Such ambiguity clarifies the necessity of searching for a

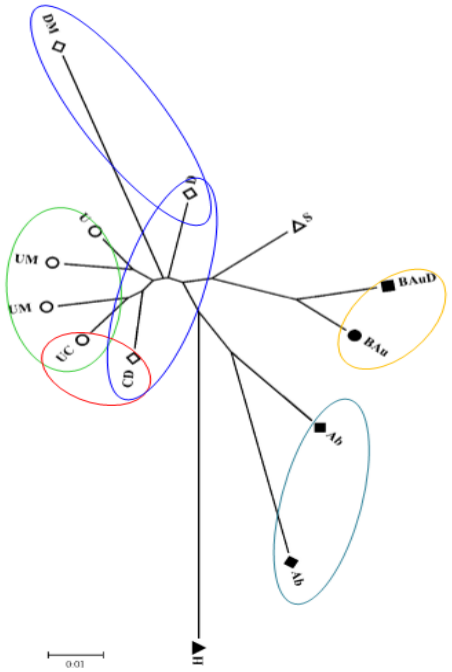

Figure 5. Genome relationship of Triticum and Aegilops species constructed from ITS1+ITS2 sequences.

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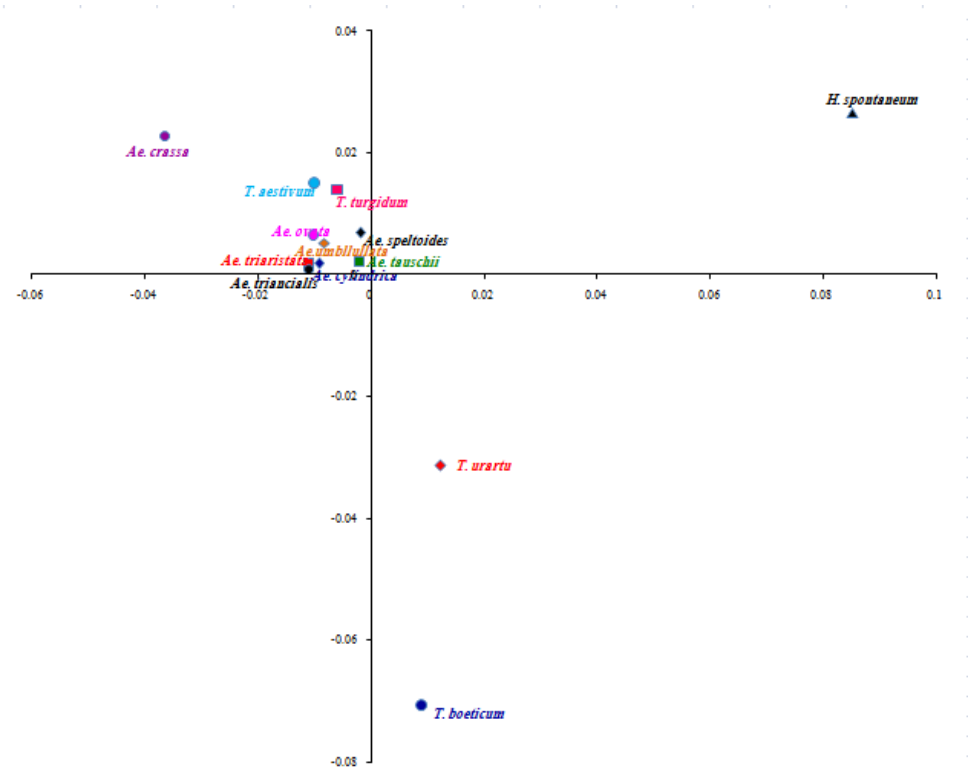

Figure 6. The chart of bioplot obtained from principal component analysis in *Aegilops* and *Triticum* species.

method that is more capable for revealing phylogenetic relationships and speciation in depth.

It is apparent that cytogenetic analysis is one of the most effective methods for studying phylogeny and evolution of related species. Therefore, to take the potential results, the sampling must be extended to include more representative accessions of all genera and genomes within the Triticeae. Due to this relatively weak phylogenetic signal, constructing analyses combining ITS and cytogenetic data can be performed. Such data would provide a robust phylogenetic signal.

Hybridization and introgression are natural processes occurring among closely related species. All wild relatives of cultivated wheat have the potential to hybridize with wheat. This causes gene flow from diploid to polyploidy Triticeae (Bordbar *et al*. 2011; Kilian *et al*. 2011). It seems possible to recognize the relatives of wheat and extend gene flow from diploid species into common wheat, in order to improve disease resistance and other useful traits in the latter.

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فیلوژنی مولکولی گونه‌های .L *Aegilops* و .L *Triticum* با استفاده از فاصله-اندازهای بین رونوشت‌های ژن‌های ریبوزومی

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چکیده

تحلیل فیلوژنی گونه‌های .L *Triticum* و .L *Aegilops* با استفاده از توالی‌های بین رونوشت‌های ژن‌های ریبوزومی هسته‌ای (ITS) انجام شد. طول کل محصولات PCR در ITS1 و ITS2 به ترتیب از ۶۵۰ جفت باز تا ۷۰۰ جفت باز متغیر بود. پس از هم ترازی، تنوع توالی‌ها بین گونه‌ها محاسبه شد.

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محتوای G+C نواحی ITS بین ۶۰/۸٪ در ITS2 تا ۶۱/۵٪ در ITS1 متغیر بود. تحلیل فیلوژنی با استفاده از روش نزدیک‌ترین همسایه (NJ) و بر مبنای فواصل ژنتیکی جفتی، انجام گرفت. نتایج درخت NJ به خوبی توانست گونه‌های دو جنس *Triticum* L. و *Aegilops* L. را از هم جدا کند و سه کلاستر ایجاد کرد، گندم‌های اینکورن، گندم‌های پلی‌پلوئید و آژیلوپس‌ها. نتایج ما نشان داد که ارتباط بین ژنوم A گندم نان و *T. urartu* نسبت به *T. boeticum* بیشتر است. در ارتباط با ژنوم D، نزدیکی بیشتری بین گندم نان و *Ae. Tauschii* نسبت به سایر گونه‌های آژیلوپس حامل ژنوم D (*Ae. crassa* و *Ae. cylindrica*) مشاهده شد. نتایج به دست آمده همچنین نشان دادند که *Ae. speltoides* از سایر گونه‌های جنس آژیلوپس جدا بود و در کلاستر گندم‌های پلی‌پلوئید قرار گرفت. رابطه نزدیک بین *Ae. speltoides* و گندم‌های پلی‌پلوئید نشان داد که این گونه محتمل‌ترین دهنده‌ی ژنوم B به گونه‌های گندم می‌باشد. مطالعه حاضر، پتانسیل نواحی ITS را در مطالعات فیلوژنی تأیید کرد و قویاً تکامل گندم‌های زراعی را که از طریق هیبریداسیون و پلی‌پلوئیداسیون بین گونه‌های *Triticum* L. و *Aegilops* L. اتفاق می‌افتد را تصدیق کرد.

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