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Molecular genetic diversity of *Aegilops triuncialis* L. revealed by IRAP markers

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Abstract

Iran is considered as one of center of diversity for *Aegilops triunsialis* species is a genetic source for wheat (*Triticum eastivum*) improvement. Therefore, study of genetic variation of this plant in its center of diversity extremely important regarding study of evolution and breeding purposes. The genetic variation among 40 accessions of *Ae. triunsialis* collected from northwest, west and southwest of Iran were analysed using 5 IRAP (Inter-retrotransposon Amplified Polymorphism) markers. Five plants from each accession were chosen, DNA (Desoxyribonucleic acid) extracted, concentrations adjusted and bulked. A high polymorphism was observed for IRAP markers with an average polymorphism of 94.51. The PIC (polymorphism information content) value varied from 0.32 to 0.44 which the high value of PIC for RTR-10 and RTR-2 primers confirmed a high efficiency of these primers. AMOVA (Analysis of Molecular Variance) indicated that the major proportion (90%) of the total variation was within accessions while, 10% was among accessions. Three dendrograms based on Sokal & Sneath coeffecient of dissimilarity and NJ (Neighbor Joining) placed the 40 accessions in three clusters. The average genetic distance was 57 percent that means this species have a high diversity in this area. The result of classification shows low relation between genetic divergence and geographical origins.

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Introduction

Measurement of the diversity of a group of related taxa allows geneticists to understand evolution and plant breeders to exploit wider pools of diversity. Studying the geographical distribution of plant lineages can help define evolutionary history and genetic exchange, providing insights into the factors that shape the genetic diversity of a taxon and crop origins (Saeidi *et al.*, 2008). Barb goatgrass (*Aegilops triuncialis* L.) whit UUCC genome (2n = 4x = 28) is a winter annual that is native to Mediterranean Europe and western Asia. This goatgrass species able hybridize whith winter wheat (*Triticum aestivum* L.) (Davy, 2008; Nakai, 1981). Wild *Aegilops* species related to cultivated wheat (*Triticum* spp.) possess numerous genes of agronomic interest and can be valuable sources of resistance to diseases, pests and extreme environmental factors. *Aegilops* species played an important role in the evolution of cultivated wheat (Monneveux *et al.*, 2000). Molecular markers (DNA markers) reveal neutral sites of variation at the DNA sequence level. By neutral is meant that, unlike morphological markers, these variations do not show themselves in the phenotype, and each might be nothing more than a single nucleotide difference in a gene or a piece of repetitive DNA. They have the big advantage that they are much more numerous than morphological markers, and they do not disturb the physiology of the organism (Jones *et al.*, 1993).Molecular markers have been widely used in genetic analysis and breeding of plant species, with a multitude of applications (Ritschel 2004). DNA-based techniques (RFLP, QTL, RAPD, AFLP, ISSR, IRAP) are used to evaluate variation at the DNA sequence level (Cenkci, 2008; Zietkiewicz *et al.*,1994; Kalendar *et al.*, 1999). Transposable elements, particularly the retrotransposons, comprise much or most of plant genomes; their replication generates genomic diversity and makes them an excellent source of molecular markers. The retrotransposon-based marker methods rely on PCR amplification between a conserved retrotransposon feature, most often the long terminal repeat (LTR), and another dispersed and conserved feature in the genome. The inter-retrotransposon amplified polymorphism (IRAP) method displays insertional polymorphisms by amplifying the segments of DNA between two retrotransposons. It has been used in numerous studies of genetic diversity (smykal, 2011).

In the present study, IRAP marker was employed to investigate the genetic diversity and genetic differentiation of *Ae. triuncialis*. The main objectives of this study are to (a) characterizing the level of genetic diversity in this Wild species related to cultivated wheat (*Triticum* spp.), (b) reveal the pattern of genetic differentiation among populations and (c) assess feasible approaches for *Ae. triuncialis* conservation.

Materials and methods

Plant materials

Forty accessions of *Ae. triuncialis* collected from Thirteen provinces of northwest, west and southwest of Iran in 2005 (Table 1). Accessions were divided into six groups according to their geographical origin [Azarbaijan sharghi (1) 5 accessions; Ardebil, Zanjan and Tehran (2) 6 accessions; Kurdestan and Hamedan (3) 6 accessions; Kermanshah, Lorestan and Ilam (4) 12 accessions; Khozestan, Chaharmahal Bakhtiari and Kohkeloye va Boyerahmad (5) 6 accession; Fars (6) 5 accession. The seeds from each accession (mainly from one individual) were mixed and a sample of five seeds used for DNA extraction.

DNA extraction and PCR (Polymerase chain reaction) amplification

Total genomic DNA was isolated from young leaves of greenhouse-grown plants according to CTAB (N, N, N, Cethyl ammonium bromide) Protocol (Doyle & Doyle, 1990) with minor modifications. To reveal the level of genetic variation for each accession, DNA from five plants were bulked and analysed. five IRAP primers were tested (Yang *et al.*, 2007) (Table 2). IRAP PCR reactions were performed in GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA, USA) in a 20μL reaction mixture containing 30 ng DNA, 1X PCR buffer, 2 mM $MgCl_2$, 0.8 pM of each primer, 0.4 mM dNTP (10mM) and 1 unit of Taq polymerase. The PCR reaction condition consisted of:

94 ℃, 4 min; 35 cycles of 94 ℃, 40 s, annealing at the Tm specified in Table 2 for 40 s and 72 ℃ for 2 min ramping 3 s per cycle, with a final extension at 72 ℃ for 5 min. PCR products were analysed by electrophoresis on 1.5 % (w/v) agarose gels and detected by ethidium bromide staining.

Table 1. Geographical origins from where accession of *A. triuncialis* collected.

No.	Origin site	Province	No.	Origin site	Province
1	Ivandare	Kurdestan	21	Shiraz	Fars
2	Ivandare	Kurdestan	22	Ardal	Chaharmahal Bakhtiari
3	Eslam abad	Kermanshah	23	Bonab	Azarbaijan Sharghi
4	Harsin	Kermanshah	24	Ahar	Azarbaijan Sharghi
5	Marivan	Kurdestan	25	Norabad	Lorestan
6	Kamyaran	Kurdestan	26	Sepid Dasht	Lorestan
7	Shorab	Lorestan	27	Maharlo	Fars
8	Bardaj	Fars	28	Harsin	Kermanshah
9	Ahar	Azarbaijan Sharghi	29	Kah Firoz	Fars
10	Borojen	Chaharmahal Bakhtiari	30	Divandare	Kurdestan
11	Meshkinshahr	Azarbaijan Sharghi	31	Sepid Dasht	Lorestan
12	Sepidan	Fars	32	Dorod	Lorestan
13	Yasoj	Kohkeloye va Boyerahmad	33	Saadat Abad	Tehran
14	Sareein	Ardebil	34	Zanjan	Zanjan
15	Dhdoz	Khozestan	35	Heiran	Ardebil
16	Yasoj	Kohkeloye va Boyerahmad	36	Khohkeloye	Kohkeloye va Boyerahmad
17	Sareein	Ardebil	37	Sarabele	Ilam
18	Miandoab	Azarbaijan Sharghi	38	Asadabad	Hamedan
19	Zanjan	Zanjan	39	Sarabele	Ilam
20	Aleshtar	Lorestan	40	Sarabele	Ilam

Table 2. The selected primers with their sequences and the temperature melting (Tm).

Marker	Primer	Sequence (5´-3´)	Tm(℃)
	RTR-1	TATGCTGACCAAGGTGGTAC	61.19
	RTR-2	AAGTTGTCTGAGGCTTATGTGACTT	66.09
IRAP	RTR-7	TCCGCTGTGCAGTAGTGTTTAGTG	68.39
	RTR-8	CTTACCTCTCCCATACATCACCA	64.22
	RTR-10	AACGTGTTAATGTCGCTTTGTC	62.81

Analysis of diversity

The presence (1) or absence (0) of clear and distinguishable fragments of particular mobility was scored from the gels for each accession. Based on the results obtained, the index the polymorphism information content (PIC) (Nei, 1973)were calculated for each primer. Nei's genetic diversity (h), Shannon's information index (I) and percentage of polymorphic loci (PPL) across all the six groups were also analyzed by GenAlEx software version 6.5 (Peakall & Smouse, 2012). Genetic distance (Nei, 1972) among the groups were calculated using GenAlEx software (version 6.5). Genetic distance of among accessions were calculated using DARwin software (version 5) (Perrier & Jacquemoud-Collet, 2006).Dendrograms showing distance between different accessions of *Ae. triuncialis* were then constructed by a distance-based method. The cophenetic (COPH) value matrix was

computed for each tree matrix generated based on a particular distance coefficient, and matrix correlation (r) and Mantel test were computed using XLSTAT 2008 (Addinsoft USA, New York, NY)to measure the degree of relationship between the cophenetic matrix and distance matrix. The dendrograms of Sokal & Sneath coefficient showed the highest value of cophenetic coefficient (83 % for ISSR, 89 % for IRAP and 87 % for integrated data). The dendrograms were constructed using the Neighbor joining method implemented in DARwin software, version 5. principle component analysis using a standardized data matrix (Darroch and Mosimann, 1985)were conducted using DARwin software (version 5). In order to describe variability among and within groups, the nonparametric Analysis of Molecular Variance (AMOVA) procedure was used as delineated in Excoffier *et al.* (1992), where the variation was partitioned within groups and among groups. This analysis was performed with the GenAlEx software version 6.5.

Table 3. The numbers of bands, numbers of polymorphic bands, Polymorphism (%) and polymorphic information content IRAP primers in 40 accessions of *Ae. trunciallis*.

Marker	Primer	No. of Bands	No. of Polymorphic Bands	Polymorphism (%)	PIC1
	RTR-1	18	18	100	0.35
	RTR-2	23	22	95.65	0.41
IRAP	RTR-7	12	12	100	0.38
	RTR-8	13	10	76.92	0.32
	RTR-10	21	21	100	0.44
Total	-	87	83	-	-
Mean	-	17.4	16.6	94.51	0.38

Table 4. Nei's genetic distances and genetic identities among 6 groups of *Ae. triuncialis*.

Group	1	2	3	4	5	6
1	***	0.071	0.074	0.067	0.109	0.163
2	0.932	***	0.053	0.052	0.093	0.123
3	0.929	0.948	***	0.052	0.074	0.125
4	0.935	0.949	0.949	***	0.042	0.079
5	0.897	0.911	0.929	0.959	***	0.080
6	0.850	0.885	0.882	0.924	0.923	***

Above diagonal, genetic distance; below diagonal, genetic identities.

IRAP primers variation

Five IRAP primers have produced severale amplifications (Fig 2, Table 3).

According to IRAP analysis 87 bands were detected whit the number ranging from 12 to 23, with an average of 17. 4. Polymorphic bands comprised 94.51% (83 bands) of the bands and the mean was 16.6 per primer. The PIC value varied from 0.32 to 0.44 with an average of 0.38. The lowest and the highest number of polymorphic bands per assay unit were 10 (RTR-8 primer) and 22, 21, (RTR-2, RTR-10 primers, respectively). The lowest and the highest PIC value per assay unit were 0.32 (RTR-8 primer) and 0.44, 0.41 (RTR-10, RTR-2 primers, respectively) (Table 3).

Genetic relationships

The average genetic distance among 40 accessions of *Ae. triuncialis* with IRAP markers ranged from 0.32 to 0.93 with an average of 0.57. Dendrogram based on Sokal & Sneath coeffecient of dissimilarity and Neighbor Joining placed the 40 accessions in three clusters (Fig 3). Principal Coordinates Analysis

showed the first two could explain the total variation of 27.70 percent.

On the basis of allele frequencies for ISSR and IRAP markers, the genetic distance among all group ranged from 0.042 to 0.163 with an average of 0.0838. Highest genetic distance values were found between group 1 and 6 while the lowest genetic distance values were detected between group 4 and 5 (Table 4). Genetic diversity among groups.

Table 5. Genetic variation parameters of *Ae. triuncialis* groups based IRAP primers.

groups	No. of accession	h[1]	I[2]	P[3] (%)	Ne
1	5	0.14 ± 0.01	0.21 ± 0.01	41.56	1.28 ± 0.02
2	6	0.27 ± 0.01	0.40 ± 0.01	70.13	1.48 ± 0.02
3	6	0.21 ± 0.01	0.31 ± 0.01	60.61	1.36 ± 0.02
4	12	0.28 ± 0.01	0.42 ± 0.01	77.49	1.49 ± 0.02
5	6	0.22 ± 0.01	0.33 ± 0.01	61.47	1.39 ± 0.02
6	5	0.22 ± 0.01	0.32 ± 0.01	58.01	1.39 ± 0.02
	Average	0.22 ± 0.005	0.33 ± 0.008	61.54 ± 4.97	1.39 ± 0.01

[1]Nei´s gene diversity; [2]Shannon's information index; [3]Percentage of polymorphic loci, [4]Effective

Table 6. analysis of molecular variance (AMOVA) for *Ae. triuncialis* groups.

Source of variation	df	Mean sum of squares	Variance components	Percentage of variation	Φ_{st}	P-value
Among lines	5	130.05	26.01	10	0.09	<0.01
Within lines	34	525.76	15.46	90		
Total	39	655.82		100		

In individual groups, the percentage of polymorphic loci (P) ranged from 41.56 % to 77.49 %, with an average of 61.54 %. Nei's gene diversities (h) varied from 0.14 to 0.28, with an average of 0.22, Shannon's indices (I) ranged from 0.21 to 0.42 with an average of 0.33 and Effective number of alleles (Ne) ranged from 1.28 to 1.49 with an average of 1.39. Also, the values of the parameters h, Ne and I showed a similar trend. Among 6 *Ae. triuncialis* groups, groups 2 and 3 had the highest genetic variation and group 1 had the lowest genetic variation (Table 5).

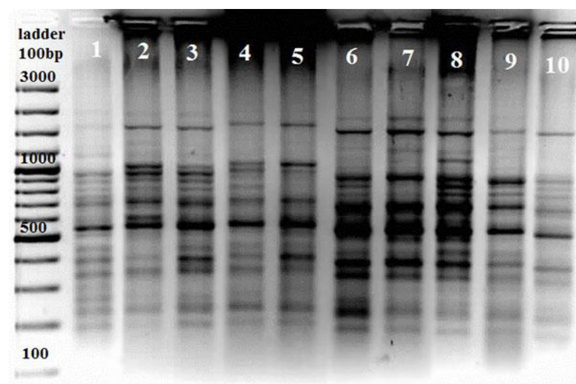

Fig. 1. The agarose gel electrophoresis profile of inter retrotransposon amplified polymorphism with the primer RTR-10. The numbers 1 to 10 designate the accessions number in Table 1.

Analysis of Molecular Variance

To assess the diversity among and within *Ae. triuncialis* groups, an AMOVA was performed from the distance matrix. AMOVA showed highly significant (P≤0.01) genetic differences among groups. From the total genetic diversity, 90 % is attributed to variation of 6 *Ae. triuncialis* groups and 10 % related to differences among groups (Table6).

Disscution

The high value of PIC and number of polymorphic bands for RTR-2, RTR-10 primers confirmed a high efficiency of these primers. IRAP marker with average PIC value of 0.38 and average polymorphic percentage of 94.51 showed highe variation. This outcome suggests that IRAP primers are more useful for evaluating genetic variation among *Ae. triuncialis* species. Principal Coordinates Analysis showed the first two could explain 27.70 percent of the total variation that indicate primers suitable dispersion in

the genome level.

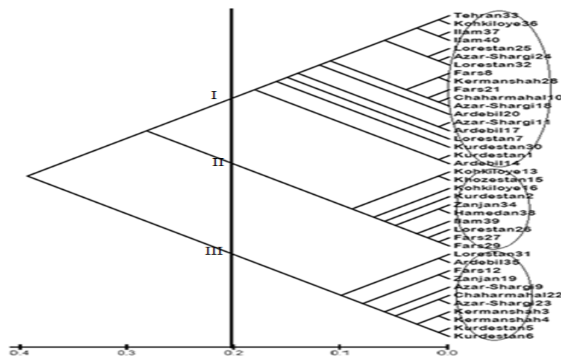

Fig. 2. NJ dendrogram generated using IRAP data and Sokal & Sneath dissimilarity coefficient. The relationships among 40 accessions of *Ae. triuncialis* are divided into 3 groups (Clusters I , II and III).

The average genetic distance useing Sokal & Sneath distance matrix was 0.57 that means this species have a high diversity in northwest, west and southwest of Iran and could be used in breeding and crossing programs to breed a new *Ae. triuncialis* genotype. The result of classification show that there was low relationship between genetic divergence and geographical origins so that accessions from similar geographical regions (e.g. Lorestan 31 versus Lorestan 25 or Lorestan 26) belonges to separate clusters. Conversely, accessions from different geographical conditions (such as Azarbaijan Sharghi 23 and Fars 12) were clustered in one part of the dendrogram means these species have a monotonous diversity in northwest, west and southwest of Iran. Since wild wheats are habitates in the West of Iran or east of Fertile Crescent (van Slageren, 1994) this result could be expected. Usually when a species is native to a country occurs low genetic divergence could be observed among accessions and for this reason geographic divisions does not matches with genetic diversity. Due to the weedy characteristics of *A. cylindrica* and its presence as a common weed in wheat fields. The seeds of *A. cylindrica* are often harvested with wheat grains and transported across a wide geographical area, which provides more opportunity for hybridization among accessions (Farkhari, *et al.*, 2007). Our results revealed a mean of Nei's genetic distances among *Ae. triuncialis* groups of 0.0838, which indicates low and non significant variation. Also analysis of molecular variance (AMOVA), Nei's genetic distances and grouping of populations based on NJ and Sokal & Sneath distance matrix, suggested that the genetic variation within *Ae. triuncialis* groups is higher than genetic variation among *Ae. triuncialis* groups.

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