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R-RAP: A new marker for genetic characterization and evaluation of relationships among different *Aegilops* species

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ABSTRACT

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Genetic drift of germplasm in cultivated wheat is good motivation for studying genetic diversity in its wild relatives. In this study, R-RAP, a new retrotransposon-based marker, was applied to assess the structure and genetic diversity of 30 accessions of *Aegilops* sp. originating from different geographical regions of Iran. The 15 R-RAP primer combinations revealed a total of 338 bands ranging from 16 (LTR1061 – OPA02) to 29 (LTR2116 – OPG06), with an average of 22.5 bands per primer. The size of the amplified bands varied from 100 to 1500bp. The total average of polymorphism information content (PIC) was 0.30. The highest PIC value (0.46) was for primer LTR1061-OPG06. Of the three studied *Aegilops* species, *Ae. tauschii* possesses the highest level of genetic diversity. UPGMA cluster separated the three *Aegilops* species into three discrete groups. Results show that R-RAP markers could be a reliable marker system for organizing genetic variability and detecting useful diversity for wheat breeding purposes. The different diversity parameters calculated in this study demonstrated the high diversity present in *Aegilops* accessions.

Keywords: *Aegilops*, genetic variability, polymorphism information content (PIC), R-RAP markers

INTRODUCTION

By 2020, world demand for wheat (*Triticum aestivum* L.) will be 40% greater than it is today (Monneveux *et al*., 2000). To satisfy the demand for increased global food production, plant breeding is adopting new approaches to develop improved cultivars and increase crop yields (Landjeva *et al*., 2007). In recent decades, the narrow genetic basis of modern wheat cultivars has been amply demonstrated (Alnaddaf *et al*., 2011). Hence, it is necessary to broaden the genetic base of wheat through the introgression of novel genes from wild relatives that contain numerous unique alleles that are absent in modern wheat cultivars.

The wild relatives of bread wheat are considered potential sources of useful alleles for bread wheat improvement (Khalighi *et al*., 2008), because the high polymorphism found in wild relatives of cultivated crops can be important for crop improvement/breeding purposes (Cenkci *et al*., 2008). Several *Aegilops* species participated in wheat evolution and played a major role in wheat domestication; the genus *Aegilops* thus represents the largest portion of wheat's secondary gene pool that could be used in wheat improvement programs (Kilian *et al*., 2011).

Earlier the theories of evolution were based on morphological and geographical variations among organisms (Sharma *et al*., 2008). But recently molecular markers have been a powerful tool for assessing genetic relationships, taxonomy, physiology, embryology, plant breeding, ecology, genetic engineering, etc. So far, several DNA-based markers have been introduced. These markers differ from each other in the degree of polymorphism, dominance or co-dominance, distribution in genome, repeatability, dependence or independence from DNA sequencing information, costs, etc.

RAPD was one of the first developed methods that were widely used in all genetic programs. RAPD used a short primer (almost 10 nucleotides), and no additional information was needed to learn about the genome sequence; its speed, low cost and low technical requirements made RAPD a desirable marker for many years (Sawalha *et al*., 2008). On the other hand, given the many potential priming sites for these sequences, the low annealing temperature that causes low reproducibility, and the

development of new methods such as AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat) which solved RAPD problems, this system was eliminated from molecular markers today (Kalendar *et al*., 2011).

Several molecular marker methods based on retrotransposons (RTNs) were also developed (Waugh *et al*., 1997; Flavell *et al*., 1998; Kalendar *et al*., 1999). The dispersion, ubiquity, and prevalence of RTNs in plant genomes provide an excellent basis for developing marker systems. Inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP), two RTN-based markers, require no DNA digestion and have been applied as molecular markers in genetic diversity studies as well as for studying RTN integration events in plant genomes (Kalendar *et al*., 1999; Abdollahi Mandoulakani *et al*., 2008, 2009).

R-RAP (RAPD-retrotransposon amplified polymorphism), a new method based on LTR retrotransposons and RAPD primers developed by Aalami *et al*. (2012) has made it possible to produce new bands from a combination of RAPD and LTR primers. Their proposal represents a departure from IRAP and RAPD restrictions, because it overcomes not only low annealing temperatures and, therefore, low RAPD reproducibility, but also the problem with IRAP, i.e., that for LTRs it would either produce no bands at all or bands with low resolution (Aalami *et al*., 2012). This new combination may actually produce amplicons from new genomic regions that previously could not be amplified by other primers, thus providing new information about DNA sequences and plant evolution. Compared to other retrotransposon-based methods, R-RAP needs no restriction enzymes or silver staining such as S-SAP, nor prior information about SSR motifs such as REMAP. It also solves IRAP problems but is still as easy to perform as IRAP; at the same time, it can be as efficient as all of these methods (Aalami *et al*., 2012).

Diversity in different *Aegilops* species has been evaluated using isozymes (Nakai, 1989), RAPD and AFLP (Pester *et al*., 2003), SSR (Naghavi *et al*., 2009, Moradkhani *et al*., 2012) and ISSR markers (Han-Yu *et al*., 2006). The objective of the present study was to use new R-RAP markers for assessing the genetic diversity and relationships of different *Aegilops* species in order to use them in diverse wheat breeding programs.

MATERIALS AND METHODS

Plant materials and DNA isolation

Plant materials consisted of 30 accessions: 20 *Ae. tauschii* accessions, five *Ae. crassa* accessions and five *Ae. cylindrica* accessions (Table 1 and Fig. 1) that were kindly provided by the Seed and Plant Improvement Institute, Karaj, Iran. These accessions were selected as representative of the main geographic distribution of each species. After seed germination and growth, fresh leaves were used for DNA extraction according to Saghai-Maroof's method (Saghai-Maroof *et al*., 1984). DNA was quantified using a spectrophotometer and the concentration of all samples was set at 10 ng/µl.

Table 1. List of the *Aegilops* accessions used in this study.

Taxon	Ploidy level	Genome (Badaeva *et al*., 1998)	Accession numbers	Origin
Ae. tauschii	**2x**	**D**	**1772, 2115, 2043, 2033, 2031, 1746, 1229, 1461, 2020, 843, 2120, 2207, 312, 699, 667, 1745, 50084, 1770, Aladizgeh, Guilan**	**Iran**
Ae. crassa	**4x**	**XD**	**598, 723, 730, 675, 721**	**Iran**
Ae. cylindrica	**4x**	**CD**	**591, 588, 622, 592, 575**	**Iran**

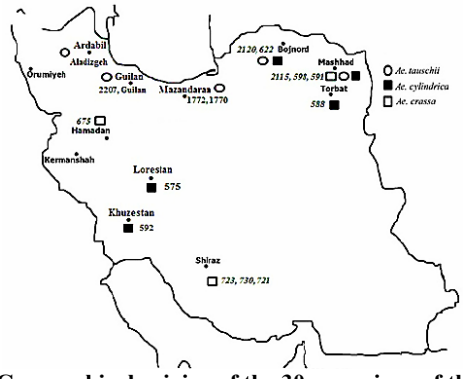

Fig. 1. Geographical origins of the 30 accessions of the three *Aegilops* species used in this study.

R-RAP reactions

Fifteen R-RAP primer combinations (Table 2) were used to analyze genetic diversity and relationships in 30 accessions of *Aegilops*. Because of the great difference between the annealing temperature (Ta) of RAPD and LTR primers, Ta for R-RAP reactions was optimized using gradient PCR. R-RAP PCR reactions were carried out in a volume of 10 µl containing 50 ng template DNA, 2 mM $MgCl_2$, 0.4 mM dNTPs and 0.4 µM of each primer in 1x PCR reaction buffer. The amplification reaction was performed in the Eppendorf Master cycler (Eppendorf North America, New York,

United States) with an initial 5-min denaturation at 94°C, then 35 amplification cycles: 40 seconds denaturation at 94°C; 40 seconds annealing at 54-61°C and 2 minutes extension at 72°C. Final extension was carried out at 72°C for 5 minutes. Amplified products were electrophoresed on 1.8% agarose gel containing 1X TBE (Tris Borate EDTA) buffer at a constant voltage of 100V for two to three hours. Gels were then stained using 0.5 µg/ml ethidium bromide and a photograph was taken under UV light using the Bio-Rad gel documentation system.

Table 2. Name and sequences of the RAPD and retrotransposon primers used in this study.

RAPD		Retrotransposon primers	
Primer name	**Sequence (5'-3')**	**Primer name**	**Sequence (5'-3')**
OPG06	GTCTACGGCA	LTR1061	AGAGGGGAATGTGGGGGTTTCC
OPG10	AGGGCCGTCT	LTR2116	CGAACCTGGGTAAAACTTCGTGTC
OPA02	TGCCGAGCTG	Sukkula	GATAGGGTCGCATCTTGGGCGTGAC
OPA10	GTGATCGCAG	-	
OPJ13	CCACACTACC	-	

Data analysis

Amplified fragments were scored independently as 1 or 0 for their presence or absence at each position, and the obtained binary data were used for analysis. A genetic similarity (GS) matrix was computed based on simple matching coefficient of similarity, and subsequently used to carry out cluster analysis using the UPGMA algorithm. To verify the adjustment between genetic similarity matrices and respective dendrogram-derived matrices (cophenetic matrix), the cophenetic correlation coefficient was estimated. All of these computations were implemented in NTSYS 2.02 software (Rohlf, 2000).

To compare the effectiveness of the 15 primer combinations, assayed number of loci, percentage of polymorphic loci, number of loci with a frequency higher than or equal to 5%, number of private loci, number of less common loci with frequency lower than or equal to 25% and 50%, mean of expected heterozygosity (He) and standard error (SE) of He, number of effective alleles (Ne), number of observed alleles (Na), Shannon's information index (I) and marker index (MI) (Powell *et al*. 1996) were calculated in GenAlEx 6.4 (Peakall and Smouse, 2006). Also the polymorphic information content (PIC) was calculated to measure the discriminating power of each primer combination using the formula of Roldan-Ruiz *et al.* (2000), $PIC = 2P_i(1 - P_i)$, where P_i is the frequency of the amplified fragments and $1 - P_i$ is the frequency of nonamplified fragments.

RESULTS AND DISCUSSION

Fifteen R-RAP primers (Table 2) were used to analyze genetic diversity and relationships in 30 accessions belong to three different *Aegilops* species including *tauschii*, *crassa* and *cylindrica* (Table 1). Analyses of the amplification patterns of accessions belonging to the studied *Aegilops* species showed a difference in position and number of generated bands. The selected 15 primer combinations generated a total of 338 bands ranging from 16 (LTR1061 – OPA02) to 29 (LTR2116 – OPG06), with an average of 22.5 bands per primer ranging in size from 100 to 1500bp. Of the 338 bands, over 99% (337 in total) were polymorphic (Table 3).

The high level of polymorphism observed in this study corroborated the results reported by Naghavi *et al*. (2007) in *Ae. tauschii* accessions.

Table 3. R-RAP primer combinations used in this study, plus annealing temperature, total loci, polymorphic loci, polymorphic information content (PIC) and marker index (MI).

Primer combinations	Annealing temperature	Total loci	Polymorphic loci	PIC	MI
LTR1061 – OPG06	54	20	20	0.46	9.2
LTR1061 – OPA02	54	16	15	0.26	3.9
LTR1061 – OPG10	54	23	23	0.31	7.1
LTR1061 – OPA10	54	21	21	0.41	8.6
LTR1061 – PJ13	54	23	23	0.27	6.2
LTR2116 – OPG06	61	29	29	0.38	11.0
LTR2116 – OPA02	61	23	23	0.24	5.5
LTR2116 – OPG10	61	25	25	0.25	6.2
LTR2116 – OPA10	61	27	27	0.33	8.9
LTR2116 – PJ13	61	20	20	0.29	5.8
Sukkula – OPG06	61	21	21	0.27	5.7
Sukkula – OPA02	61	24	24	0.32	7.7
Sukkula – OPG10	61	26	26	0.27	7.0
Sukkula – OPA10	61	20	20	0.21	4.2
Sukkula – PJ13	61	20	20	0.23	4.6
Total/means	-	338	337	0.30	6.80

High polymorphism revealed that R-RAP could resolve genetic variation among plant germplasm, identify cultivars and estimate genetic relationships. The highest and lowest PIC values were 0.46

(LTR1061 – OPG06) and 0.21 (Sukula – OPA10), respectively, with an average value of 0.30. PIC estimates the discriminatory power of a marker by taking into account not only the number of alleles at a locus, but also their relative frequencies. Primers LTR2116 – OPG06 and LTR1061 – OPA02 represented the lowest and the highest marker index (MI) values, respectively (Table 3). The high marker index or diversity index is a reflection of marker efficiency in simultaneously analyzing a large number of bands rather than a reflection of the level of polymorphism detected (Powell *et al*., 1996). The patterns of R-RAP bands produced by the LTR1061-OPA02 primer is shown in Fig. 2.

The characteristics of amplified R-RAP loci using 15 R-RAP primer combinations for each species were reported in Table 4. The percentage of R-RAP polymorphic loci in the studied species varied from 47.63 (*Ae. cylindrica*) to 95.86 (*Ae. tauschii*), averaging 68.24. The frequency of all the R-RAP-amplified loci was more than 5%. Forty-one private loci were detected, suggesting that fewer amplified loci are prevalent in the studied species. Mean heterozygosity varied from 0.18 (*Ae. cylindrica*) to 0.31 (*Ae. tauschii*), averaging 0.24 (Table 4).

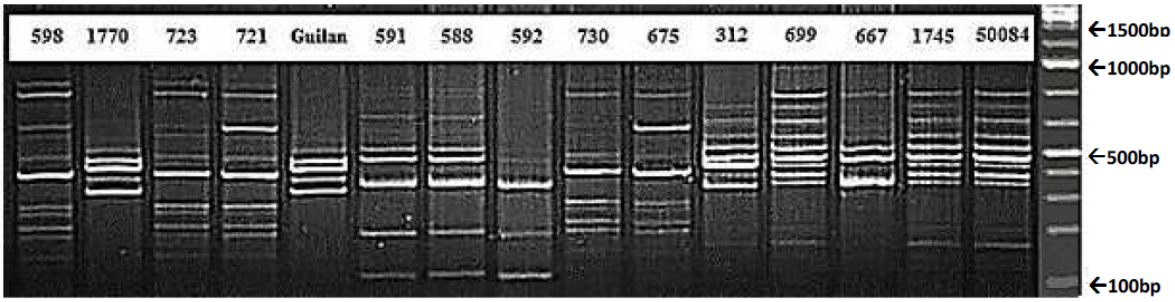

Fig. 2. Polymorphism detected by R-RAP primer combination. Polymorphism detected by OPG10-Sukkula primer and 100bp plus DNA ladder (Fermentas) in base pairs (*Ae. tauschii*: 1770, Guilan, 312, 699, 667, 1745, 50084; *Ae. crassa*: 598, 723, 721, 730, 675; *Ae. cylindrica*: 591, 588, 592).

Table 4. Characteristics of amplified R-RAP loci in the studied *Aegilops* species.

Characteristic	*Ae. tauschii*	*Ae. crassa*	*Ae. cylindrica*
Number of loci	333	255	213
Percentage of polymorphic loci (%)	95.86	61.24	47.63
Number of loci with frequency of ≥5%	333	255	213
Number of private loci	40	0	1
Number of less common loci (≤25%)	0	0	0
Number of less common loci (≤50%)	0	0	0
Mean of heterozygosity	0.31	0.23	0.18
Standard error of mean heterozygosity	0.008	0.011	0.011

Information on genetic variability within each species is summarized in Table 5. The highest genetic diversity was found in *Ae. tauschii* (0.58) and the lowest was found in *Ae. crassa* (0.52). In addition, among tetraploid species, genetic diversity was higher in *Ae. cylindrica* (0.54) compared to *Ae. crassa* (0.52). The mean of Nei's gene diversity index (Nei, 1973) was highest in *Ae. tauschii* (0.42), and lowest in *Ae. cylindrica* (0.35). Also, the mean of I index was highest in *Ae. tauschii* (0.62), and lowest in *Ae. crassa* (0.48). Among the studied species, *Ae. tauschii* had the highest values in all estimated parameters, indicating that in this species there is great potential for discovering useful genes that wheat breeding programs could utilize. The high level of variation in *Ae. tauschii* may be attributed to its wide geographical distribution in Iran, as reported by Naghavi *et al*. (2007).

Genetic similarity between accessions based on R-RAP primers ranged from 0.481 (between accessions 730 and Aladizgeh) to 0.952 (between accessions 1772 and 2115) with a mean value of 0.68. Cluster analysis was performed using the UPGMA algorithm based on simple matching similarity coefficient. The three studied species were divided into three discrete groups (Fig. 3), which

Table 5. Genetic diversity estimates for three *Aegilops* species based on R-RAP data.

Species	Sample size	I (s.d.)	He (s.d.)	Ne	Na
Ae. tauschii	20	0.62 (0.27)	0.42 (0.18)	2.71	3.56
Ae. crassa	5	0.48 (0.19)	0.36 (0.21)	2.38	3.42
Ae. cylindrica	5	0.53 (0.23)	0.35 (0.24)	2.24	3.08

I: Shannon's information index, He: Nei's gene diversity,
Ne: Effective number of alleles, Na: Observed number of alleles.

indicates that these primers can be used efficiently for discriminative diversity and evolutionary programs in the mentioned species. Also, *Ae. cylindrica* (with CD genome) was more closely related to polyploid *Ae. crassa* than to D genome (*Ae. tauschii*) species (Hedge *et al*., 2000). Thus, the R-RAP results obtained in this study indicate good resolution for distinguishing among the three *Aegilops* species based on their genome composition.

To assess and partition total genetic variation among and within species, AMOVA (Analysis of Molecular Variance) was performed based on the three species using R-RAP data. The level of genetic variation was higher within species (82%) than among species (18%). This finding can be attributed

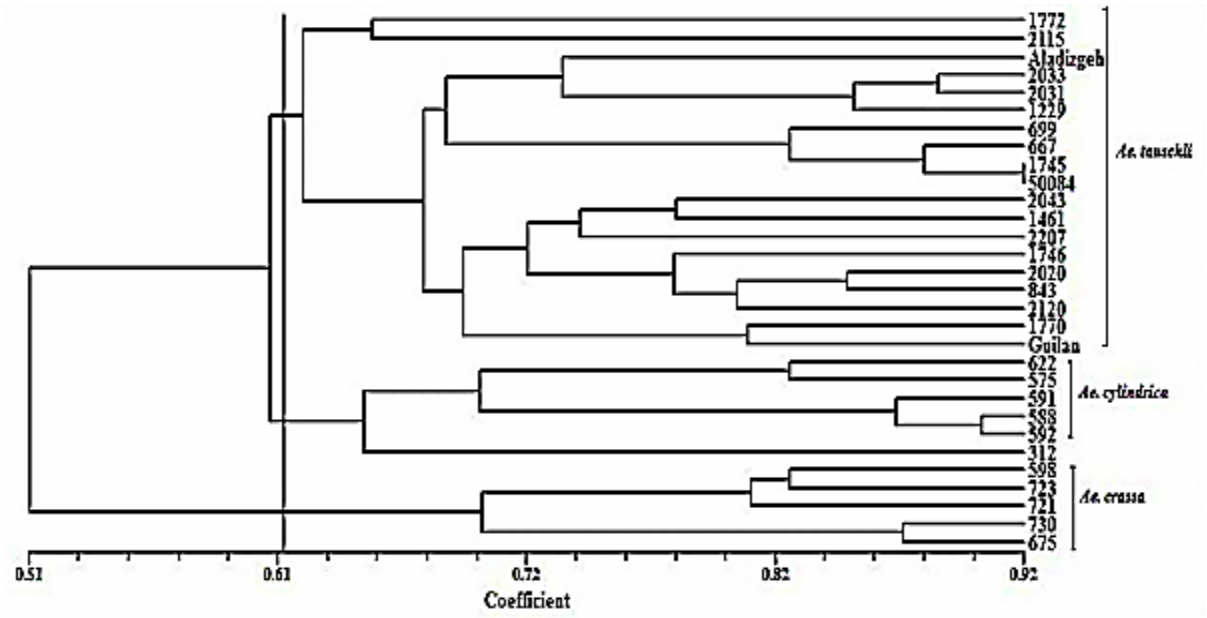

Fig. 3. Dendrogram of 30 studied accessions belonging to three different species of *Aegilops* using simple matching similarity coefficients and the UPGMA clustering algorithm based on R-RAP data.

to the extensive genotype collection site (Fig. 1). Also, the ΦPT (PhiPT) value, estimated with AMOVA, amounted to 0.178 ($p < 0.01$) based on permutations across the full data set. This AMOVA showed significant genetic differences among groups of species.

In summary, in the present study, R-RAP analysis of accessions of *Aegilops* species possessing different genomes revealed extensive allelic variation. Interestingly, we also observed a high degree of variation as well as the highest number of unique alleles within *Ae. tauschii* accessions, indicating the *Ae. tauschii* gene pool is a great potential source of novel genes for bread wheat improvement. High levels of genetic diversity in *Ae. tauschii* in Iran were found by Saeidi *et al*. (2006) and Naghavi *et al*. (2009) using SSR and AFLP markers. However, we found a greater genetic distance between *Ae. tauschii* and *Ae. crassa*, indicating more differences between the D-genome of these two species. According to Badaeva *et al*. (1998), the D^{cr1}-genome of 4x *Ae. crassa* derived from the D-genome of *Ae. tauschii* but was modified substantially during speciation.

The present study also shows that *Ae. cylindrica* accessions are not highly variable and it seems that only a few accessions of the D-genome of *Ae. tauschii* entered into this tetraploid genome. Some results suggested that the D-genome of common wheat and the CD-genome of *Ae. cylindrica* were inherited from different biotypes of *Ae. tauschii* (Badaeva *et al*., 1998; Caldwell *et al*., 2004). However, *Ae. cylindrica* is known to hybridize spontaneously with wheat (Gandilyan and Jaaska, 1980; Snyder *et al*., 2000) and, therefore, could be used as a potential source of desirable gene transfers in breeding programs. Plants with cytoplasmic male sterility are commonly observed when the nuclear genome of common wheat (*Triticum aestivum*) interacts with *Aegilops crassa* cytoplasm (Murai and Tsunewaki, 1993; Ogihara *et al*., 1997); this posits a new system of hybrid seed production. Compared to the other two species, hybridization between *Ae. crassa* and hexaploid wheat is very difficult; for this reason, embryo rescue methods are recommended to facilitate the

transferral of genes from *Ae*. *crassa* to bread wheat.

Finally, our results suggest that these primers can be used efficiently for discriminative, diversity and evolutionary programs in the mentioned species and that there is a high or moderate level of genetic variation in the D-genome of *Aegilops* species as revealed by R-RAP analysis. Therefore, in the context of germplasm management and utilization of wheat genetic resources, *Aegilops* species provide a readily available source of potentially useful variation for wheat improvement.

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