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Genetic variability and phylogenetic relationships studies of *Aegilops* L. using some molecular markers

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Abstract

Studying of genetic relationships among *Aegilops* L. species is very important for broadening the cultivated wheat genepool, and monitoring genetic erosion, because the genus *Aegilops* includes the wild relatives of cultivated wheat which contain numerous unique alleles that are absent in modern wheat cultivars and it can contribute to broaden the genetic base of wheat and improve yield, quality and resistance to biotic and abiotic stresses of wheat. The use of molecular markers, revealing polymorphism at the DNA level, has been playing an increasing part in plant biotechnology and their genetics studies. There are different types of markers, morphological, biochemical and DNA based molecular markers. These DNA-based markers based on PCR (RAPD, AFLP, SSR, ISSR, IRAP), amongst others, the microsatellite DNA marker has been the most widely used, due to its easy use by simple PCR, followed by a denaturing gel electrophoresis for allele size determination, and to the high degree of information provided by its large number of alleles per locus. Day by day development of such new and specific types of markers makes their importance in understanding the genomic variability and the diversity between the same as well as different species of the plants. In this review, we will discuss about genetic variability and phylogenetic relationships studies of *Aegilops* L. using some molecular markers, with theirs Advantages, and disadvantages.

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Introduction

The eroding genetic base of cultivated wheat, Rapid changes in climatic and environmental stress conditions have led researchers to investigate the possibility of using the genetic diversity present in wild relatives of cultivated crops (Arzani *et al.,* 2005). Its have been sustaining under intensive stress conditions, by modifying themselves to adapt to newly emerging conditions (Nevo *et al.,* 2002). Great interest has been focused to the genus *Aegilops* L., which is closely related to *Triticum* constitutes a precious source of economically important traits for wheat improvement (Holubec *et al.,* 1993; Nevo *et al.,* 2002) particularly those associated with disease resistance (Bouktila, 2001; Martin-Sanchez *et al.,* 2003) and other economically desirable attributes. The genus *Aegilops* contains 22 species comprising both diploids and polyploids that originated from centre of origin (Van Slageren, 1994). Use of morphological traits may be helpful but often inadequate in differentiation of closely related cultivars. Certain morphologically different variants may be phylogeneticly closely related. In addition morphological traits are highly influenced by the environment (Fang *et al.,* 1998). However; molecular tools provide abundant information, are highly efficient and are insensitive to environmental factors. These techniques allow the analysis of variation at the genomic level and permit detection of genetic variation at the genomic level. Therefore, information obtained from the molecular level could be used to assess genetic relationships among the major germplasm groups. A better understanding of the effectiveness of the different molecular markers is considered a priority step towards germplasm classification and characterization, and a prerequisite for more effective breeding programs (Belaj *et al.,* 2003). In *Aegilops* L., a wide species of DNA based markers has been used in order to study their genetic variation as well as phylogenic relationship among different species, and some of the important examples are: RADP (Konstantinos *et al.,* 2010; Mahjoub *et al.,* 2010; Schoenenberger *et al.,* 2005; Mahjoub *et al.*, 2009), SSR (Bandopadhyay *et al.,* 2004; Naghavi *et al.*, 2005), ISSR (Jam Baranduzi *et al.,* 2013; Laya *et al.,* 2014), AFLP (Kaya *et al.,* 2011; Khalighi *et al.,* 2008), IRAP (Hojjatollah *et al.,* 2008; Fathi *et al.*, 2014). To this end, the application of molecular markers based on DNA sequences are largely depends on the type of markers employed, distribution of markers in the genome, type of loci they amplify, level of polymorphism and reproducibility of products (Virk *et al.,* 2001; Fernandez *et al.,* 2002). Despite this, a new marker type, named SNP, for Single Nucleotide Polymorphism, is now on the scene and has gained high popularity, even though it is only a bi-allelic type of marker. The development of such new and specific types of markers makes their importance in understanding the genomic variability and the diversity between the same as well as different species of the plants. It is necessary to update DNA marker based techniques from this review, to conclude DNA markers and their application and provide base platform information to the researchers working in the area to be more efficiently expertise.

In this review, we summarize genetic variability and phylogenetic relationships studies of *Aegilops* L. with some applications of molecular markers while showing their advantages and disadvantages

Molecular markers in genetic variability studies in Aegilops L.

Knowledge of genetic variation and genetic relationship among genotypes is an important consideration for classification, utilization of germplasm resources and breeding. The genetic diversity and structure of plant populations reflect the interaction of many factors, including the long-term evolutionary history of the species (e.g. shifts in distribution patterns, habitat fragmentation, and population isolation), mutation, genetic drift, mating system, gene flow and selection (Schaal *et al.,* 1998). All of these factors can lead to complex genetic structuring within populations, and losses of genetic diversity, with severe potential consequences since genetic variation at the intra specific level is a prerequisite for future adaptive change or evolution (Schaal *et al.,* 1991). Molecular markers allow the

analysis and detection of variation at the genomic level. Therefore, information obtained from the molecular level could be used to assess genetic relationships among the major germplasm groups. A better understanding of the effectiveness of the different molecular markers is considered a priority step towards germplasm characterization, and a prerequisite for more effective breeding programs (Belaj *et al.,* 2003). They represent one of the most powerful tools for the analysis of genomes and enable the association of heritable traits with underlying genomic variation (Duran *et al.,* 2009).

RAPD markers

The introduction of DNA markers based on the PCR technology has led to the development of several novel genetic assays that can be used for many purposes in plant genetic analysis such as cultivar identification and gene mapping. RAPD is a simple, sensitive and fast DNA molecular marker technique to randomly amplify DNA fragments under low-stringency conditions by short oligonucleotides (Williams *et al.,* 1990). They require no prior knowledge of the DNA sequence and can amplify a large number of DNA fragments for reaction. RAPD markers resulting from PCR amplification of genomic DNA sequences recognized by ten-mer primers of arbitrary nucleotide sequence (Williams *et al.,* 1990), they have proved to be valuable in *Aegilops geniculata* Roth populations similarities and estimation of their relationships (Mahjoub *et al.,* 2010).They provide a fast and easy approach for genetic variability and phylogenetic relationships studies of *Aegilops* L. This assay has the advantage of being readily employed, requiring very small amounts of genomic DNA, and eliminating the need for blotting and radioactive detection (Cipriani *et al.,* 1996). This technique, regardless of its sensitivity to reaction conditions, problems with repeatability, and amplifying of nonhomologous sequences has been successfully used for the assessment of genetic diversity in plants (Maria *et al.,* 2008). Factors such as speed, efficiency and amenability to automation make it one of the most suitable methods for germplasm management with respect to estimating diversity, monitoring genetic erosion and removing duplicates from germplasm collections (Khadari *et al.,* 2003). Another advantage of using RAPD markers for introgression studies is that these markers occur in randomly amplified genome regions, and if sequenced and compared to nucleotide databases they may provide some information on eventual genes or traits introgressed from one species to another (Schoenenberger *et al.,* 2005).

RAPD analyses generally require purified, high molecular weight DNA, and precautions are needed to avoid contamination of DNA samples because short random primers are used that are able to amplify DNA fragments in a variety of organisms. Altogether, the inherent problems of reproducibility make RAPDs unsuitable markers for transference or comparison of results among research teams working in a similar species and subject. As for most other multilocus techniques, RAPD markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous. RAPD markers were found to be easy to perform by different laboratories, but reproducibility was not achieved to a satisfactory level (Jones *et al.,* 1997) and, therefore, the method was utilized less for routine identifications.

Konstantinos *et al.* (2010) evaluated the genetic variability and relationships of thirty-eight accessions of seven Greek *Aegilops* species using nineteen RAPD and ten ISSR markers. Cenkci *et al.* (2008) suggested that RAPD analysis can be used to distinguish wild *Triticum* and *Aegilops* species and wheat cultivars. In addition, RAPD technique can be used to develop genome-specific markers.

Aloui Mahjoub *et al.* (2012) used nineteen RAPD markers to study the genetic diversity in natural populations of *Aegilops geniculata* Roth and *Triticum durum* Desf from Tunisia, the result show that genetic diversity within populations was relatively high. Nei's genetic diversity (H) and Shannon's index (I) were 0.324, 0.484 respectively.

Global AMOVA showed that genetic variation within populations accounted 80% occurring (ΦPT =0.205 p<0.05). The total genetic diversity (Ht) and the within population genetic diversity (Hs) were 0.3195 and 0.1516 respectively, Total gene diversity was attributable mostly to diversity within population, indicating that the groups of populations were likely to differ genetically. Genetic differentiation was low in the two closely related species. The amount of gene flow (Nm) among groups of populations was also low. Despite the relatively restricted geographical range covered by the investigation, studied groups of populations exhibited a pronounced genetic divergence at different hierarchical levels. Therefore, dendrogram based on Nei's genetic distance indicated segregation of *Aegilops geniculata* groups of populations and *Triticum durum* into two main clear pattern clusters.

SSR markers

Simple sequence repeats (SSRs) or microsatellites are short sequence elements composed of tandem repeat units one to seven base pairs (bp) in length (Tautz, 1989). SSRs are becoming increasingly widespread because it is co-dominant, multi allelic, highly polymorphic genetic markers and appropriate for genetic diversity studies, evenly distributed throughout the genome and regarded to be the most reliable marker (Goldstein *et al.,* 1999; Jannati *et al.,* 2009).

Because the technique is PCR-based, only low quantities of template DNA (10–100 ng per reaction) are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high and analyses do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap (Ghislain *et al.,* 2004). This decreases significantly the analytical costs. Furthermore, the screening of microsatellite variation can be automated, if the use of automatic sequencers is an option EST-SSR markers are one class of marker that can contribute to 'direct allele selection', if they are shown to be completely associated or even responsible for a targeted trait (Sorrells and Wilson, 1997).Yu *et al.* (2004) identified two EST-SSR markers linked to the photoperiod response gene (ppd) in wheat. The EST-SSR loci have been integrated, or genome-wide genetic maps have been prepared, in several plant (mainly cereal) species. A large number of genic SSRs have been placed on the genetic maps of wheat (Nicot *et al.,* 2004, Holton *et al.,* 2002, Gao *et al.,* 2004). Microsatellites can also be implemented as monolocus, codominant markers by converting individual microsatellite loci into PCR-based markers by designing primers from unique sequences flanking the microsatellite. Microsatellite containing genomic fragment have to be cloned and sequenced in order to design primers for specific PCR amplification. This approach was called sequence-tagged microsatellite site (STMS) (Beckmann and Soller, 1990**).** In the longer term, development of allele-specific markers for the genes controlling agronomic traits will be important for advancing the science of plant breeding.

In this context, genic microsatellites are but one class of marker that can be deployed, along with single nucleotide polymorphisms and other types of markers that target functional polymorphisms within genes. The choice of the most appropriate marker system needs to be decided upon on a case by case basis and will depend on many issues, including the availability of technology platforms, costs for marker development, species transferability, information content and ease of documentation.

One of the main drawbacks of microsatellites is that high development costs are involved if adequate primer sequences for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although microsatellites are in principle codominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. The potential presence of null alleles

increases with the use of microsatellite primers generated from germplasm unrelated to the species used to generate the microsatellite primers (poor “cross species amplification”). Null alleles may result in a biased estimate of the allelic and genotypic frequencies and an underestimation of heterozygosity.

Furthermore, the underlying mutation model of microsatellites (infinite allele model or stepwise mutation model) is still under debate. Homoplasy may occur at microsatellite loci due to different forward and backward mutations, which may cause underestimation of genetic divergence. A very common observation in microsatellite analysis is the appearance of stutter bands that are artifacts in the technique that occur by DNA slippage during PCR amplification. These can complicate the interpretation of the band profiles because size determination of the fragments is more difficult and heterozygotes may be confused with homozygotes. However, the interpretation may be clarified by including appropriate reference genotypes of known band sizes in the experiment.

Bandopadhyay *et al.* (2004) were used 64 EST-SSRs in 18 species of *Triticum-Aegilops* complex to identify genus specific and genome specific EST-SSRs and to estimate the level of DNA polymorphism detected by them in these 18 species of the complex, any polymorphism detected using EST-SSRs, may reflect better the relationships among *Triticeae*. And they indicated that the SSRs derived from the functional portion of the genome of bread wheat may be successfully used in cultivated and wild relatives of wheat belonging to *Triticum-Aegilops* complex for comparative genomics studies such as genome analysis, localization of expressed genes, discrimination among different species, etc. Therefore, EST-SSRs may be used in studies on polymorphism, genetic diversity, gene mapping and synteny conservation across different species of *Triticeae*. Naghavi *et al.* (2005) were used 21 simple microsatellite primers to determine the genetic relationship of the D genome among hexaploid wheat *Triticum aestivum* and 3 *Aegilops* species *Aegilopsd tauschii*, *Aegilops cylindrica* and *Aegilops crassa*. They reported that different genotypes of *Aegilops tauschii* could be involved in the evolution of polyploid species. A high level of variation and also the highest number of unique alleles observed within *Aegilops crassa* accessions, indicating that *Aegilops crassa* is a good potential source of novel genes for bread wheat improvement. The conclusion of their study confirms the usefulness of SSR markers to study wheat genetic diversity. Additionally, the results obtained from their study could be useful for improving the understanding of diversity in and management of germplasm collections.

ISSR markers

Inter-simple sequence repeat (ISSR) marker is a PCR based method that can rapidly differentiate closely related individuals. They involve the amplification of DNA segments between two identical microsatellite repeat regions. ISSR markers use degenerate primers to reveal a large number of fragments per PCR reaction, and thus are able to efficiently distinguish between closely related individuals (Zietkiewicz *et al.,* 1994). They have high reproducibility possibly due to the use of longer primers (16–25-mers) as compared to RAPD primers (10-mers), which permits the subsequent use of high annealing temperature (45°–60°C) leading to higher stringency. ISSR uses a single primer of 16–25 bp long microsatellites. This primer consists of a di, tri, tetra or penta nucleotide which can be anchored at the 3° or 5° end with 2–4 arbitrary degenerate nucleotides. This technique combines most of the benefits of AFLP and SSR markers with the universality of RAPD (Pradeep Redy *et al.,* 2002). Being polymorphic (Bornet and Branchard, 2001) and ubiquitous in the genome ISSR markers, have the advantages of SSR markers, while by passing the major obstacle to the development of SSR markers, that is, the need to know the flanking sequences. Hence, ISSR markers are suitable for use in species where extensive information on DNA sequences are not yet available (Meloni *et al.,* 2006), they are scored as dominant markers and inherited in Mendelian fashion (Ratnaparkhe *et al.,* 1998). It is widely used

by the research community in various fields of plant science such as breeding, germplasm conservation and genetic mapping (Pradeep Redy *et al.,* 2002).

This technique overcomes most limitations such as low reproducibility and high cost (Zietkiewicz *et al.,* 1994; Pradeep Redy *et al.,* 2002). ISSR provide a useful and relatively simple method for cultivar fingerprinting (Luro *et al.,* 1995). Jam Baranduzi *et al.* (2013) investigated the genetic diversity in thirty-three accessions of six *Aegilops* species by using eleven ISSR markers and their results revealed that 171 polymorphic bands were produced; low variation within different accessions of *Aegilops* and genetic similarity separated three major cluster groups.

Laya *et al.* (2014) used 10 ISSR primers to estimate genetic diversity among 14 durum breeding lines. The result showed that the primers produced 85 polymorphic fragments. The average of PIC index was 0.33, that showed a good efficiency of primers to separate the genotypes. Cluster analysis using UPGMA method and Dice similarity coefficient categorized the genotypes into five main groups in which the check genotypes were classified in the separated groups.

AFLP markers

Molecular markers can provide information needed to select genetically diverse parents for developing breeding and mapping accessions, among which the AFLP markers have been successfully used to determine genetic diversity in many plant species (Pillay and Myers, 1999). AFLP markers are generated by selective amplification of a subset of restriction fragments from total genomic DNA (Mueller and Wolfenbarger, 1999). The reproducibility, heritability, effectiveness and reliability of these amplified DNA products have substantial advantages when compared with other marker systems (Russell *et al.,* 1997). The PCR-based AFLP markers are amenable to automation for high-throughput genotyping and, being anonymous, do not require any sequence information (Rouf Mian *et al.,* 2002). AFLP fingerprinting is considerably informative, allowing the survey of variation in more than 50 co-amplified restriction fragments in each AFLP reaction (Yildirim and Akkaya, 2006). The use of AFLP in genetic marker technologies has become the main tool due to its capability to disclose a high number of polymorphic markers by single reaction (Vos *et al.,* 1995). Li-COR IR2 automated DNA sequencers and associated software have been demonstrated to efficiently generate and analyze complex AFLP patterns of various genomes (Qui *et al.,* 1999). We applied AFLP markers to characterize the genetic diversity and relationships among different populations of *Aegilops* in Turkey using Li-COR instrument.

The disadvantages include the need for purified, high molecular weight DNA, the dominance of alleles, and the possible non-homology of comigrating fragments belonging to different loci. In addition, due to the high number and different intensity of bands per primer combination, there is the need to adopt certain strict but subjectively determined criteria for acceptance of bands in the analysis. Special attention should be paid to the fact that AFLP bands are not always independent. For example, in case of an insertion between two restrictions sites the amplified DNA fragment results in increased band size. This will be interpreted as the loss of a small band and at the same time as the gain of a larger band. This is important for the analysis of genetic relatedness, because it would enhance the weight of non-independent bands compared to the other bands. However, the major disadvantage of AFLP markers is that these are dominant markers.

Kaya *et al.* (2011) studied the genetic diversity and relationship of 55 accessions of genus *Aegilops*, including the species *Aegilops triuncialis* L. (UUCC), *Aegilops geniculata* Roth (MMUU), *Aegilops cylindrica* Host (CCDD) and *Aegilops umbellulata* Zhuk (UU) using the combinations of 16 AFLP selective primers. Similarly, Khalighi *et al.* (2008) assessed the genetic diversity and morphological traits of thirty one *Triticum* and *Aegilops* genotypes using 414 AFLP markers.

Soleimani *et al.* (2002) studied the genetic variation in 13 modern Canadian durum wheat cultivars based upon amplified restriction fragment polymorphism (AFLP). The result, showed of the approximately 950 detected AFLP markers, only 89 were polymorphic. The ancestry of Canadian durum wheat cultivars was traced back to 125 cultivars, selections, and breeding lines including 17 landraces. Mean pairwise genetic distance based on the kinship coefficient was 0.76. On the other hand, AFLP-based mean pairwise genetic distance was 0.40. Even though there was a large difference between the means of the two diversity measures, a moderate positive correlation (r=0.457, p<0.002) was detected between the two distance matrices. Cluster analysis with the entire AFLP data divided all cultivars into three major groups reflecting their breeding origins.

IRAP markers

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, 2006). Retrotransposon insertional polymorphism is sufficiently great to support not only analyses on the whole genome level within species, but also gene mapping projects within the generally narrower germplasm of cultivated varieties (Queen *et al.,* 2004).

A virtue of IRAP is its experimental simplicity. All that is needed is simple PCR followed by electrophoresis to resolve the PCR products. IRAP can be carried out with a single primer matching either the 5’ or 3’ end of the LTR but oriented away from the LTR itself, or with two primers. Nearby TEs may be found in different orientations in the genome (head-to-head, tail-to-tail or head-totail) increasing the range of tools available to detect polymorphism depending on the method and primer combinations. If two primers are used, they may be from the same retrotransposon family or from different families. The PCR products, and therefore the fingerprint patterns, result from amplification of hundreds to thousands of target sites in the genome. The pattern obtained will be related to the TE copy number, insertion pattern and size of the TE family. IRAP fingerprints with single primers often generate bands from 500 to 3000 bases, lengths that are not convenient for capillary electrophoresis. To reduce the size of the DNA products to be separated and visualized, fluorescent primers may be used in the PCR reaction and the amplicon DNA digested with a four-base-specific restriction enzyme such as *TaiI* or *TaqI* after the PCR reaction. In this way, IRAP can be adapted to analyses on capillary sequencing platforms.

A major disadvantage of this method is the need for retrotransposon sequence information to design family-specific primers. LTR primers can be readily used across species lines, among closely related genera and even sometimes between plant families (Lou and Chen, 2007; Sanz *et al.,* 2007). In this case, primers designed to conserved TE sequences are advantageous. Moreover, TEs are dispersed throughout the genome and often interspersed with other elements and repeats. By combining PCR primers from different classes of repeats and families of LTRs, PCR fingerprints can be improved. Deployment of a retrotransposon marker system into a species, in which the methods have not been previously used, requires PCR primers that recognize a retrotransposon and, in the case of RBIP, the flanking sequences. The retrotransposon targets that can be amplified by heterologous primers developed in a different species tend to be members of old families of elements present before the divergence of the plant clades in question. Jing *et al.* (2005) estimated the average age of segregating retrotransposon insertion sites in Pisum as being

approximately 2 Myr. This result is roughly similar to estimates made by SanMiguel *et al.* (1998) and Vitte *et al.* (2004) in maize and rice, respectively, but may be biased toward younger elements because structural disruptions make old insertions harder to characterize. Nevertheless, these ages are comparable to divergence times between some closely related species (or even the genera Homo and Pan), suggesting that some retroelements may be useful in recently diverged clades. In this context, Fathi *et al.* (2014) studied molecular genetic diversity of *Aegilops triuncialis* L. revealed by IRAP markers. The result of classification showed low relation between genetic divergence and geographical origins. Hojjatollah *et al.* (2008) tested the IRAP method and applied to characterize the diversity of *Aegilops tauschii* collected across Iran. He also evaluated the method for analysing the relationships at infraspecific (subspecies and varieties) level to develop phylogeographic models for the distribution of these taxa.

Conclusion

Knowledge of the levels and distribution of genetic diversity are important for designing conservation strategies for threatened and endangered species. Preservation of the genetic diversity represented in all the plant ecosystems throughout the world has become a major issue of international concern. The loss of increasingly large numbers of plant species through habitat destruction threatens the availability of a diverse plant germplasm base which will be needed to feed future generations. Advances in biotechnology, especially in the area of molecular biology techniques provide some important tools for improved conservation and management of plant genetic resources. The present review highlights the usage of different marker system for studying genetic diversity across DNA level in the genus *Aegilops* L. All the marker techniques provided useful information on the level of polymorphism and genetic diversity in *Aegilops* L. and have potential use in studies of diversity, linkage mapping, accession identification, and germplasm organization. Selection of the best marker system depends on aim of research and ploid level of studied being. In *Aegilops* L., a wide variety of DNA based markers has been used in order to study their genetic variation as well as phylogenic relationship among different genera. RAPD and SSR are the markers of choice in *Aegilops* breeding research, because of their variability, ease of use, accessibility of detection and reproducibility. IRAP, ISSR, AFLP are also used to study the genetic diversity of *Aegilops* throughout the world.

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