Research Journal of Environmental Sciences

ISSN 1819-3412

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Environmental Sciences 4 (1): 50-56, 2010
ISSN 1819-3412

Genetic Diversity in Ecotypes of Two *Agropyron* Species using RAPD Markers

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Abstract: This study conducted to analyze genetic diversity in two *Agropyron* species, *A. pectiniforme* and *A. elongatum*, by RAPD analysis. Random Amplified Polymorphic DNA (RAPD) analysis using 12 primers produced 142 polymorphic bands with lengths ranging 564 to 2,000 bp. On the basis of Nei's gene index, the genetic diversity within ecotypes varied from 0.1014 to 0.178. The highest and lowest of this index were obtained in ecotype of 6951 (from *A. pectiniforme*; 0.178) and ecotype of 225 (from *A. elongatum*; 0.1014), respectively. The results showed high variation within ecotypes. The average gene diversity within (H_S), total (H_T) and coefficient of gene differentiation (G_{ST}) were 0.13, 0.27 and 0.47, respectively. Analysis of molecular variance (AMOVA) displayed significant variance within and among *Agropyron* ecotypes. The variance within ecotypes (61.08) was about two times higher than between ecotypes (31.24). Cluster analysis based on RAPD data using Nei's genetic distance categorizes the entries into four clusters. Using principle coordinate analysis, the first three coordinates accounted for the 52.84% of the total variation. Classifying the ecotypes by the two coordinates verified the results of cluster analysis.

Key words: AMOVA, gene differentiation, gene diversity, *Agropyron elongatum*, *Agropyron pectiniforme*

INTRODUCTION

Agropyron, as a range plant grows at the most of the rangeland of Iran. In world, *Agropyron* genus composed of 150 species, a 100 species existing over Asia. About 19 species of herbaceous and perennial plants have been found in North, Northwest and Central regions of Iran (Bor, 1970). The nearest relatives to *Agropyron* are *Aegilops* and *Triticum* (Refoufi *et al*., 2001) belonging to Triticeae family and can be crossed with each other. Today, plant geneticists are making many attempts to identify *Agropyron* species and transfer their useful genes for improving and developing new varieties for increasing disease resistance potential (Baden, 1991). Yellow dwarf disease resistance genes in *Agropyron* species have been introduced to wheat and barley (Bor, 1970). Three wheat lines has been crossed with *A. elongatum* and observed chromosome pairing and banding to produce a substitution disomic line, CI5321. The chromosome D1 of wheat is changed with chromosome 1 of *Agropyron* and become resistant to mosaic virus (Jiang *et al*., 1993). Also, there are many attempts to transfer the target genes from *Agropyron* particularly *elongatum* species to bread wheat (Liu *et al*., 2008, 2009; Cui *et al*., 2009).

Agropyron species have wide adaptation and grow in different climates. Therefore, gene pool conservation and its accurate application can be used in plant breeding programs in order to improvement rangeland and increasing forage production. Since, there is high variation within and among different species of *Agropyron*, so selection response for improving important traits is high and

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the gene pool can be more successfully used in breeding projects. In a breeding program, knowing of genome and ploidy level is very important. Therefore, such studies have been made on some *Agropyron* species (Assadi, 1995). Karyology of four *Agropyron* species has been investigated and tetraploid state with 7 chromosomes in genome base (2n = 4x = 28) has been demonstrated and differed from other attributes (Asghari *et al*., 2007).

Genetic distance based on genetic structure of biological population, may be stated by genotypic frequencies (genotypic distance) and or by different allele frequencies in loci (gene distance). Gene distance has positive association with heterosis (Falconer, 1996). Many researches have been made on measuring of genetic variation in *Agropyron* using protein and DNA markers (Che and Li, 2007; Refoufi and Esnault, 2008). Generally, determination of genetic variation is made by morphological, biochemical and molecular traits. Morphological traits are measured at field or greenhouse so they are cost consuming and are affected by environmental factors. Isozyme and storage proteins may be affected by environments, tissues and plant developmental stages (Kato and Yokoyama, 1992). Markers based on PCR e.g., RAPD is a useful way to overcome above difficulties (Virk *et al*., 1996). The RAPD markers are random and unlimited for number. There is no need to knowing of genome sequence to design RAPD primers. On the other hand, application of this markers are cost saving and their efficiency have been verified in genetic variation studies (Callow *et al*., 1997). Welsh *et al*. (1991) showed RAPD can be applied to study all organisms and is more valuable to identify various strains. RAPD analysis has been used widely to assess genetic variation in other plants (Huff, 1997; Garcia *et al*., 2002; Rajasekar *et al*., 2005; Narasimhan *et al*., 2006; Rout, 2006). In this study, RAPD analysis was applied to study genetic diversity among and within ecotypes of two *Agropyron* species.

MATERIALS AND METHODS

In this study, 10 various ecotypes from two species of *Agropyron* (*A. pectiniforme* and *A. elongatum*) were assessed during 2009 in molecular plant breeding laboratory of Agriculture Faculty, University of Mohaghegh Ardabili, Ardabil, Iran. Names and accession numbers of these ecotypes were shown in Table 1. These ecotypes were grown in greenhouse and leaf samples were harvested at rosette stage from seven individual plants of each ecotype for DNA extraction. The DNA of individual plants was extracted using the CTAB procedure according to Maroof *et al*. (1984). The quality and quantity of DNA samples were assessed using spectrophotometer (Techne, England) and 0.8% agarose gel electrophoresis. All of the DNA samples were diluted to 25 ng μL^{-1} and used in PCR reactions. Fifty RAPD primers (Metabione, Germany) were used to analyze polymorphism in the plants and polymorphic primers were used to genotyping. PCR reaction for RAPD analysis was performed in a volume of 15 μL contained 50 ng of DNA template, 2 mM $MgCl_2$, 0.05 mM each dNTP, 0.132 μM primer, 1U Taq DNA polymerase and 1×PCR buffer. For RAPD primers the amplification profile consisted of a 5 min initial denaturation step at 94°C followed by 40 cycles of denaturing at 94°C for 30 sec, annealing at 37°C for 30 sec, extension step at 72°C for 2 min and a final

Table 1: Species, accession No. and origin of studied ecotypes of *Agropyron*

Species	Accession No.	Origin	Gene diversity
A. pectiniforme	62	External	0.1025
	6951	Tehran	0.1800
	7819	Kerman	0.1600
	7852	Kerman	0.1221
	7854	Yasuj	0.1547
A. elongatum	223	External	0.1322
	224	Qazvin	0.1457
	225	Qazvin	0.1094
	3202	Mazandaran	0.1014
	6990	Yasuj	0.1439

extension step at 72°C for 10 min. The RAPD amplified products were analyzed using 1.5% agarose gels and ethidium bromide staining. Each polymorphic marker bands were scored as 1 (presence) and 0 (absence) and obtained data were analyzed using NTSYS pc 2.0, POPGENE 1.31 and Arlequin 3.11 software.

RESULTS AND DISCUSSION

Results showed that from 50 studied primers, 12 primers were highly polymorphic and banding pattern resulted from these 12 primers were located in the intervals between 564-2000 bps and totally produced 142 polymorphic bands. The average band production for each polymorphic primer was 11.83. The name and sequence of the studied primers was outlined in Table 2. According to Table 2, the primers, Oligo-17 and Oligo-23, produced highest and lowest number of polymorphic bands, respectively. Also, ecotypes of 224 (*A. elongatum*) and ecotype of 225 (*A. elongatum*) had most and least amplified bands, respectively (not shown data). On the basis of Nei's gene index (Nei and Chakravarti, 1977), the genetic diversity within ecotypes varied from 0.1014 to 0.178. The highest and lowest of this index were obtained in ecotypes of 6951 (from *A. pectiniforme*; 0.178) and 3202 (from *A. elongatum*; 0.1014), respectively. Gene diversity in other studied ecotypes was in average level. The results showed high diversity within ecotypes. The amount of gene diversity of ecotypes was shown in Table 1. The estimated average gene diversity within (H_S), total (H_T) and coefficient of gene differentiation (G_{ST}) were 0.13, 0.27 and 0.47, respectively.

Cluster analysis based on RAPD data using Nei (1972) genetic distance (Table 3) categorized the entries into 4 clusters (Fig. 1). First and second groups composed of 6 and 2 ecotypes, respectively and the remained entries located on two different clusters. The first cluster encompassed the ecotypes belonged to *A. pectiniforme* together with one ecotype of *A. elongatum* and second cluster included two

Table 2: Name, sequence and number of polymorphic bands produced by studied primers

Name	Sequence	No. of bands
Oligo-1	CCTGGGCTT C	11
Oligo-2	CCTGGGCTT G	11
Oligo-3	CCTGGGCTT A	15
Oligo-5	CCTGGGTTC C	9
Oligo-6	CCTGGGCCT A	15
Oligo-12	CCTGGGTCC A	13
Oligo-17	CCTGGGCCT C	17
Oligo-19	GCCGGGTTT A	12
Oligo-23	CCCGCCTTC C	7
Oligo-24	ACAGGGGTG A	12
Oligo-25	ACAGGGCTC A	8
Oligo-29	CCGGCCTTA C	12

Table 3: Coefficient of similarity (above diagonal) and distance (below diagonal) between studied ecotypes based on Nei (1972) genetic distance method

		A. pectiniform					*A. elongatum*				
Species	Ecotype No.	62	7852	7819	6951	8754	6990	224	223	225	3202
A. pectiniforme	62	***	0.873	0.871	0.840	0.826	0.743	0.697	0.807	0.666	0.745
	7852	0.137	***	0.940	0.916	0.858	0.806	0.748	0.869	0.749	0.879
	7819	0.129	0.060	***	0.942	0.889	0.835	0.759	0.908	0.757	0.818
	6951	0.160	0.084	0.058	***	0.908	0.809	0.761	0.900	0.759	0.831
	8754	0.174	0.152	0.111	0.092	***	0.823	0.783	0.900	0.715	0.825
A. elongatum	6990	0.257	0.194	0.165	0.191	0.177	***	0.788	0.867	0.793	0.826
	224	0.303	0.252	0.241	0.239	0.217	0.212	***	0.792	0.734	0.747
	223	0.193	0.141	0.092	0.100	0.100	0.133	0.208	***	0.796	0.864
	225	0.334	0.261	0.243	0.241	0.285	0.207	0.266	0.204	***	0.817
	3202	0.255	0.231	0.182	0.169	0.175	0.174	0.253	0.156	0.183	***

***p = 0.01

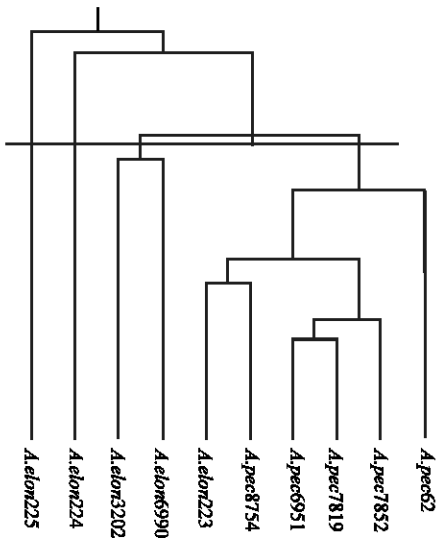

Fig. 1: Dendrogram obtained from cluster analysis using UPGMA method based on Nei´s (1972) genetic distance

Table 4: Analysis of molecular variance (AMOVA) based of RAPD data for studied species and ecotypes

Source of variation	df	SS	Variance components	Variance (%)	Gene differentiation
Between species	1	13.60	0.19**	7.68	0.76
Ecotypes/species	8	55.48	0.77**	31.74	0.33
Within ecotypes	60	90.85	1/51**	61.08	0.38

**p = 0.01

ecotypes of *A. elongatum*. Other ecotypes of *A. elongatum* located in two different clusters. In order to study of primer efficiency that used in grouping, each primer was separately analyzed. Primers of Oligo-1 and Oligo-29 were more efficient than others to distinguish ecotypes (Fig. 3, 4). These may be applied to distinguish the two species and ecotypes.

Analysis of molecular variance (Table 4) displayed significant variance within and among *Agropyron* ecotypes. The variance within ecotypes (61.08%) was about two times of between ecotypes (31.24) and only 7.68% of totally estimated variance belonged to between species. According to these results it was concluded that some of studied ecotypes may be presented as potential gene resources for using in further breeding aims. Separately analysis of variance for each of the two species showed significant differences between and within ecotypes. Estimated variance between and within ecotypes of *A. elongatum* were 47.07 and 52.93% with coefficient of gene differentiation of 0.47. Also, it was resulted that within ecotype variance of *A. pectiniforme* was higher than *A. elongatum* and the estimated average gene diversity between and within ecotypes and coefficient of gene differentiation were 30.45, 69.55 and 0.30%, respectively.

The estimated genetic distance of 10 ecotypes based on Nei (1972) genetic distance were ranged between 0.060 to 0.334% and the highest estimated values belonged to *A. pectiniforme* (ecotype No. 62) and A. *elongatum* (ecotype No. 225) (Table 3). Because of higher genetic difference between *A. elongatum* 225 and *A. pectiniforme* 62, it was recommended to use these ecotypes in order to make crossing for design further breeding efforts and benefiting heterosis in hybridization.

Using Principle Coordinate Analysis (PCoA), the first three coordinates accounted for the 52.84% of the total variation. First, second and third components determined in turn 29.28, 19.28 and 14.28% of total variance and it was concluded that the adequate coverage of selected primers and suitable sampling were existed (Fig. 2). Therefore, the grouping based on the first two components confirmed cluster analysis results.

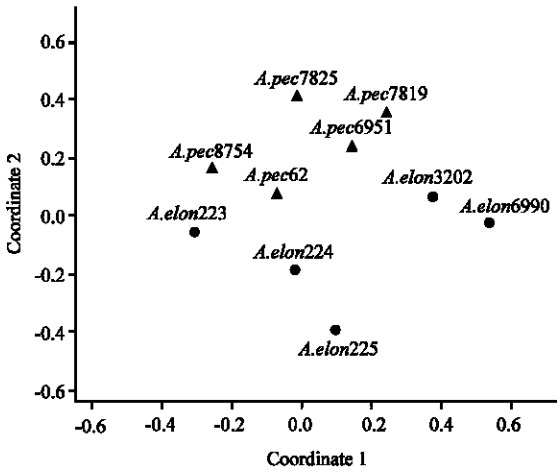

Fig. 2: Grouping of studied ecotypes using the first and second coordinates

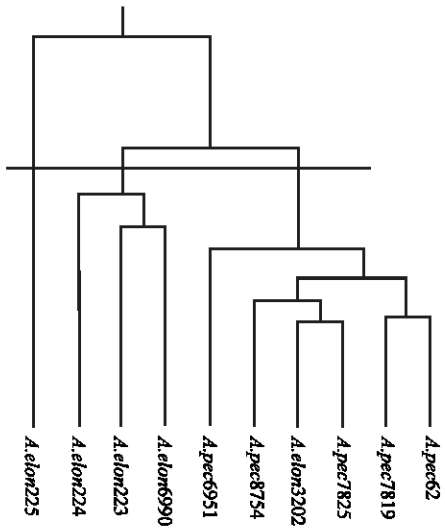

Fig. 3: Dendrogram obtained from cluster analysis using UPGMA method based on Oligo-1 markers data

This study suggested RAPDs is a good technique to analyze *Agropyron* diversity in respect to its time and cost saving. Nevertheless, the most important disadvantage of RAPD is dominant nature of the amplified markers. The results displayed high genetic variability in *Agropyron*. Analysis of molecular variance (AMOVA) indicated that high levels of variation existed in within ecotypes. This result with low gene differentiation is expected in cross-pollinated species (Hamrick and Godt, 1996). Several reports about genetic analysis in grasses using molecular markers showed variation within was greater than between population (Che and Li, 2007; Refoufi and Esnault, 2008). Since, the half of the ecotypes in this study was open pollinated, the obtained results were expected. Separately analyses of molecular variance for the two species showed *elongatum* species, a self and some cross pollinated, had same variation of within and among ecotypes. While in *pectiniforme* species, a cross pollinated, variance of within was about twice of between. These are corresponded to expected genetic structure of self and cross pollinated species, respectively.

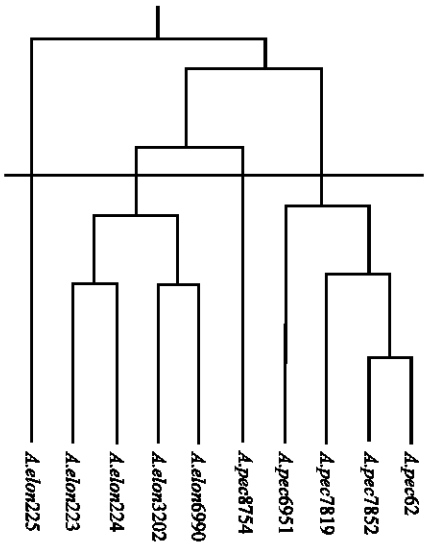

Fig. 4: Dendrogram obtained from cluster analysis using UPGMA method based on Oligo-29 (b) markers data

The UPGMA clustering of *Agropyron* ecotypes and PCoA established by RAPDs, had not direct relationships with geographical pattern. Moreover, some genotypes from the two species were clustered together. Since, the two species have close relativeness, $RAPD_S$ could unravel genetic similarity at noncoding regions of genome. That is two species may be different morphologically but have too similarities at genome level. $RAPD_S$ proved to be a suitable technique to analyze genetic variation between and within species in *Agropyron*.

In conclusion, high genetic variability found within *Agropyron* ecotypes, specialy in *Pectiniforme* species, which is a cross pollinated species. This variability can be used in plant breeding programs, in order to improvement of range plants and increasing of forage production. Also, the ecotypes of each species with high genetic distance can be used in crossing programs in order to producing mapping populations and utilization of heterosis.

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