DETERMINATION OF GENETIC DIVERSITY OF DIFFERENT BARLEY GENOTYPES GROWN IN KHYBER PAKHTUN KHWA PROVINCE USING RAPD MARKERS

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Abstract

Plant breeders in Pakistan mostly relied upon phenotypic traits as indices of genetic variation among different varieties which may genotypically be closely related or vice versa. The main objective of the current study was to determine genetic diversity among 12 different barley genotypes grown in Khyber Pakhtun Khwa province of Pakistan, The results indicated that different barely genotypes showed various levels of polymorphism for the loci detected by seven RAPD primers. A total of 205 alleles were observed giving an average of 17.08 bands genotype^{-1}. Among the seven primers used in the current study, primers C06 yielded on an average maximum number of bands (3.3) genotype^{-1}. While primer G05 produced on an average minimum number of bands (1.75) genotype^{-1}. Range of genetic distance observed was 0 to 60%. Maximum genetic distance was observed for genotype serial number 4 and 11 while 11 and 12 showed no genetic distance. Total average genetic distance calculated for all seven RAPD primers was 41.26%. Three major groups with at least two sub-groups were identified among these barely genotypes. Genotype serial number 3, 4 and 5 were found to be genetically related with a genetic distance of 13.0% and genotype serial number 4 and 11 were genetically very distant with 60% genetic distance. On the basis of dendrogram, these genotypes were grouped into three groups. (1) Group A comprises of genotype serial number 7 and 8 (2) Group B comprises of genotype serial number 2, 3, 4, 5, 9, 10, 10, 11 and 12 (3) Group C genotype serial number 1 and 6.

Introduction

Barley is an economically important crop, ranking fourth in the world cereal production. It is used as a fodder crop, brewing malt and for human consumption. Barley can be grown in wide range of environments ranging from high mountains to deserts, and is more salt tolerant than any other cereal crop. Wild barley possess high genetic variation in several useful characters including earliness, biomass yield, protein content, resistance against powdery mildew and leaf rust (Nevo, 1992). The cultivated barley, *Hordeum vulgare*, including its wild relatives *H. vulgare ssp spontaneum*, belongs to the primary gene pool of *Hordeum*. The total number of barley accessions in the gene banks, including redundant material, has been estimated around 280,000 (Fernandez *et al.,* 2002).

Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity among species and population. It has been reported that different markers revealed various levels of variation (Graham *et al.,* 2004; Fu *et al.,* 2006; Sargent *et al.,* 2007; Lewer *et al.,* 2008; Brennan *et al.,* 2008; Mattia *et al.,* 2008; Cerbert *et al.,* 2008; Ahmed *et al.,* 2009; Bakht *et al.,* 2011). RAPD (Randomly Amplified Polymorphic DNA), a PCR based marker, has the advantages of being readily used and require minute amount of genomic DNA. It does not require blotting and radioactive detection. DNA finger prints can be generated with Random Amplified Polymorphic DNA (RAPD) having short nucleotide sequences of arbitrary nature as primers (Fernandez *et al.,* 2002; Nazari & Pakniyat, 2008) and does not need any prior knowledge of DNA sequence; still reveals a high level of polymorphism. RAPD markers are based on the amplification of unknown DNA sequences using short and random oligonucleotide primers. RAPD PCR is used as a tool in breeding programs of various crops for the assessment of genetic variability between genotypes. Keeping in view the importance of RAPD markers in different breeding programs, the present study was initiated to determine the genetic diversity among different barley genotypes grown in Khyber Pakhtun Khwa province of Pakistan for future breeding programs.

Materials and Methods

This research project was initiated at the Institute of Biotechnology and Genetic Engineering (IBGE,) KPK Agricultural University Peshawar, Pakistan to determine the genetic variations among 12 barley genotypes (Table 1) using seven different RAPD primers (Table 2).

Preparation of DNA samples: Genomic DNA was extracted from young leaves of barley genotypes according to the method of Weining & Langridge (1991) with slight modifications. Leaf material was collected and subsequently frozen in liquid nitrogen. Samples were then crushed into fine powder. Five hundred microliter extraction buffer (1% SDS, 100 mM NaCl, 100 mM Tris, 100 mM EDTA, pH 8.5 by HCl) was added to the crushed leaf material and mixed thoroughly with equal volume (500 μl) of phenol:chloroform:isoamyl alcohol (ratio 25:24:1). Samples were centrifuged at 5000 rpm for 5 minutes. The aqueous phase was re-extracted with equal volume of chloroform, centrifuged at 5000 rpm for 5 minutes. One tenth volume of sodium acetated (pH 4.8) and equal volume of isopropanol was then added and mixed gently to precipitate the DNA. The samples were kept at -20ºC for 30 minutes and centrifuged at 5000 rpm for 5 min to pellet the DNA. The pellet was washed with 100, 80 and 75 percent ethanol, dried and re-suspended in 50 μl of TE buffer. For complete dissolution, DNA samples were kept in water bath at 65 degree centigrade for 20 minutes. Quantity and quality of DNA sample was determined by spectrophotometer at 260 and 280 nm wave lengths.

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Table 1. Barley genotypes under study grown in different areas of Khyber Pakhtun Khwa Province.

S. No.	Genotype No.	Area of cultivation	S. No.	Genotype No	Area of cultivation
1.	004259	D.I Khan	7	004470	Mingora
2.	004260	D.I Khan	8	004471	Unknown
3.	004347	KPK	9	004613	Mingora
4.	004375	Dir	10	004620	Peshawar
5.	004377	Hazara	11	004644	Chitral
6.	004469	D.I Khan	12	005103	Haripur

Table 2. Sequence information and amplification conditions of the RAPD primers.

S.No.	Primer	Sequence	Size (bp)	Mol. weight	Melting temp	% GC
1.	GL Decamer A-20	GTTGCGATCC	10	3.29	29.5^{0}C	60
2.	GL Decamer C-06	GAACGGACTC	10	3037	29.5^{0}C	60
3.	GL Decamer G-08	TCACGTCCAC	10	2942	29.5	70
4.	GL Decamer G05	CTGAGACGGA	10	3077	29.5^{0}C	60
5.	GL Decamer G-13	CTCTCCGCCA	10	2924	33.6^{0}C	70
6.	GL Decamer D-13	GGGGTGACGA	10	3133	33.6^{0}C	60
7.	GL Decamer G-04	AGCGTGTCTG	10	3059	29.5^{0}C	60

GL= Genelink Technology USA.

Polymerase chain reaction (PCR): PCR was conducted according to the protocols of Devos & Gale (1992) with certain modifications. PCR thermal profile is given in Table 3. For PCR, RAPD primers obtained from the Gene Link Technology, USA was used for the genetic diversity analysis (Table 2). PCR reaction was carried out in 25 µl reaction mixture, having 1 µl template DNA, 1 µl RAPD primer, 15 µl of ddH$_2$O and 7 µl of PCR mix (composed of 460 µl H$_2$O, 500 µl buffer, 10 µl of each dNTPs and 300 µl MgCl$_2$). PCR reaction mixture except DNA in the above mentioned volume was pooled to sterilized PCR tubes and mixed thoroughly by gentle pipetting. DNA was then added individually to each PCR tube and centrifuged briefly to homogenize the constituents and subjected to the thermal profile in the Thermocycler (GeneAmp 2700). Amplification products were electrophoresed on 2% agarose/TBE gel and visualized by staining with ethidium bromide under the ultra violet light and photographed.

Table 3. PCR thermal profile of the reaction for all primers.

Step	Temperature	Duration
Hot start	94ºC	4 min
Denaturation	94ºC	1 min
Primer annealing	34ºC	1 min
Extension	72ºC	2 min
Total cycles (35)		
Final extension	72ºC	10 min

Data analysis: Data on all unambiguous polymorphic RAPD fragments were identified and scored on the basis of presence (1) or absence (0). Similarity matrix, generated according to the coefficient of Dice (1945) was used for the un-weighted pair-group method with arithmetic averaging (UPGMA) (Sokal & Michener, 1958). Bi-variate analysis was performed to generate dissimilarity matrix. A dendogram indicating the estimated similarity among the barley genotypes was constructed with computer program "Popgene 32" version 1.31.

Result and Discussion

In the present study all the barley genotypes showed various levels of genetic polymorphism for the loci detected by all seven primers. In total 205 alleles were observed in 12 genotypes for the seven primers giving an average of 17.08 bands genotype^{-1} with a size range of 150 to 2000 base pairs (bp). Qian *et al.,* (2001) reported a total of 199 bands ranging from 220 to 2000 base pairs (bp) while investigating genetic variation within and among population of wild rice (*Oryza granulata*) from China by RAPD and ISSR markers. Among the seven primers used in the current study, primer C06 yielded on an average maximum number of bands (3.3) genotype^{-1}, while primer G05 and D13 yielded lowest number of bands genotype^{-1} (Figs. 1, 2,3 and 4). Fragment sizes ranging from 400-1350 bp were determined by Baum *et al.,* (1997) when RAPD primers were used for the determination of genetic diversity in wild barley. Presence of a particular band in all varieties is known as monomorphic while absence was regarded as polymorphic. Absence of bands may be due to the inability of primer to anneal because of no complementary binding site (Clark & Lanigan, 1993). Genotype serial number 4 and 11 showed maximum genetic distance (60%) followed by genotype serial number 1 and 7 with genetic distance of 58% while no genetic distance (0%) was observed for genotypes serial number 11 and 12 (Tables 4 and 5). The total average genetic distance calculated for all the seven primers was 41.26%. In the current study genetic similarity was in the range of 40 to 100%. These findings are in contrast to the earlier data reported by Elena *et al.,* (2003) where they revealed genetic similarity of 22.7% to 84.0% while Russell *et al.,* (1997) reported 87.9% to 91.7% genetic similarity in two rows spring barley using RAPD markers.
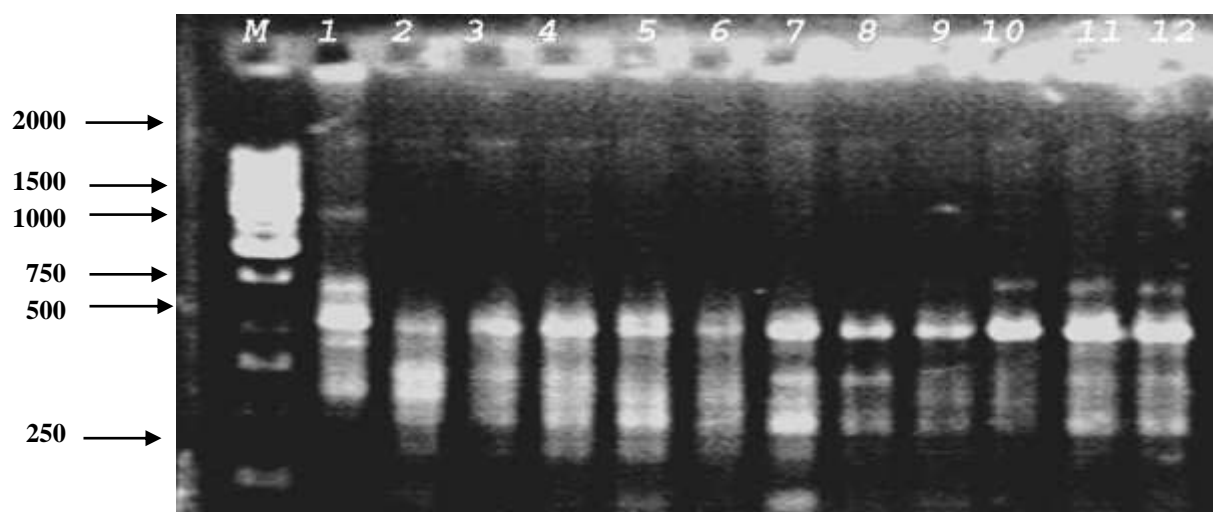
Fig. 1. PCR profiles of 12 genotypes of barley genotypes using RAPD primer GL-C06 (M = Molecular Weight Marker 1k bp; 1-12 = PCR profile for barley genotypes; for detail see Materials and Methods).
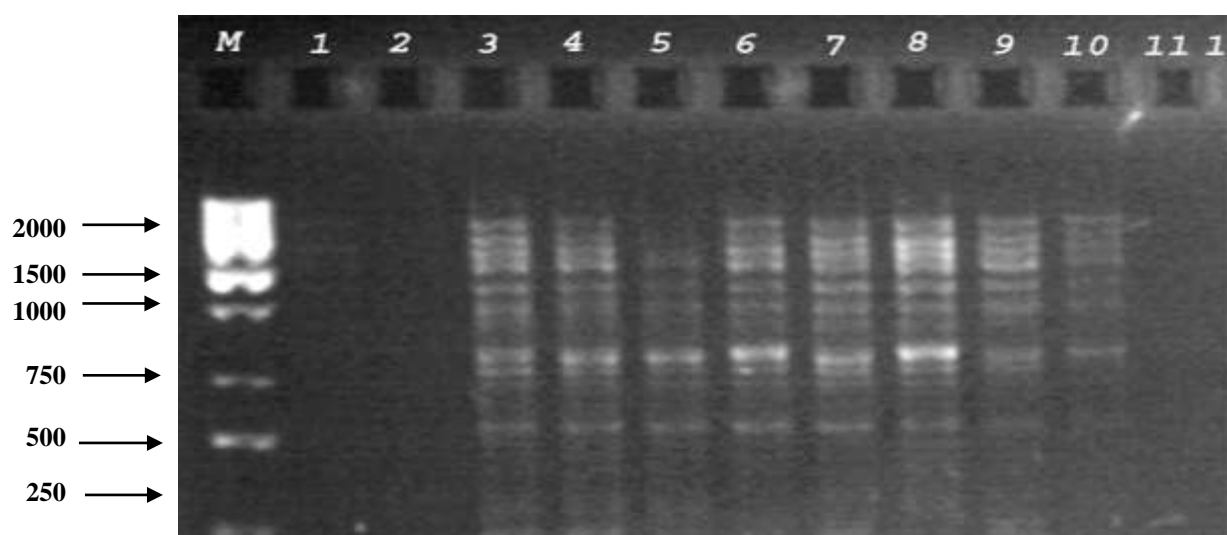

Fig. 2. PCR profiles of 12 genotypes of barley genotypes using RAPD primer GL-D13 (M = Molecular Weight Marker 1k bp; 1-12 = PCR profile for barley genotypes; for detail see Materials and Methods).
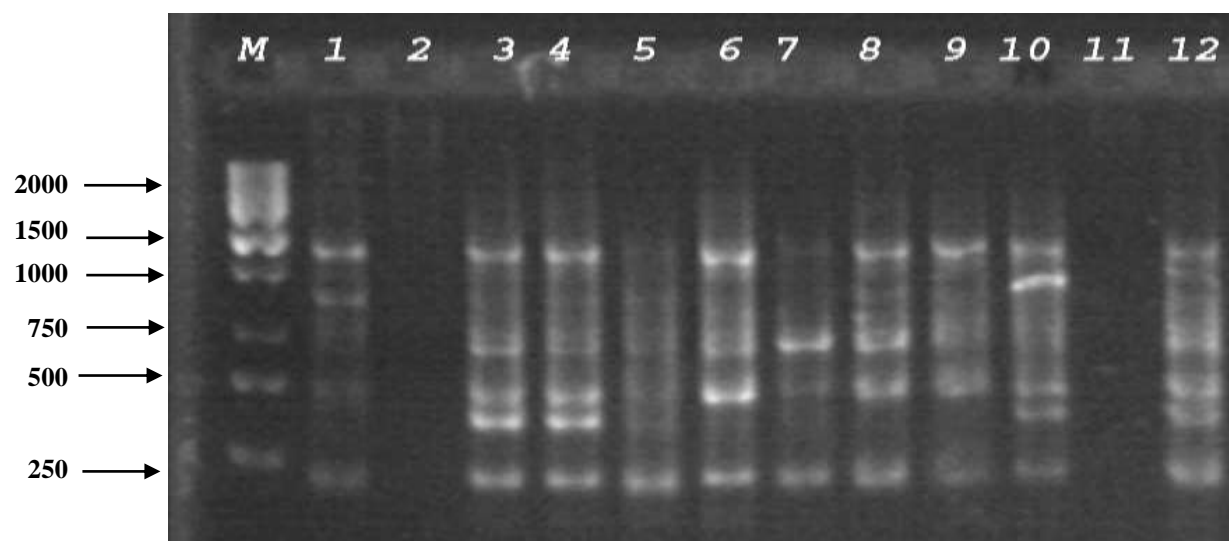

Fig. 3. PCR profiles of 12 genotypes of barley genotypes using RAPD primer GL-G13 (M = Molecular Weight Marker 1k bp; 1-12 = PCR profile for barley genotypes; for detail see Materials and Methods).
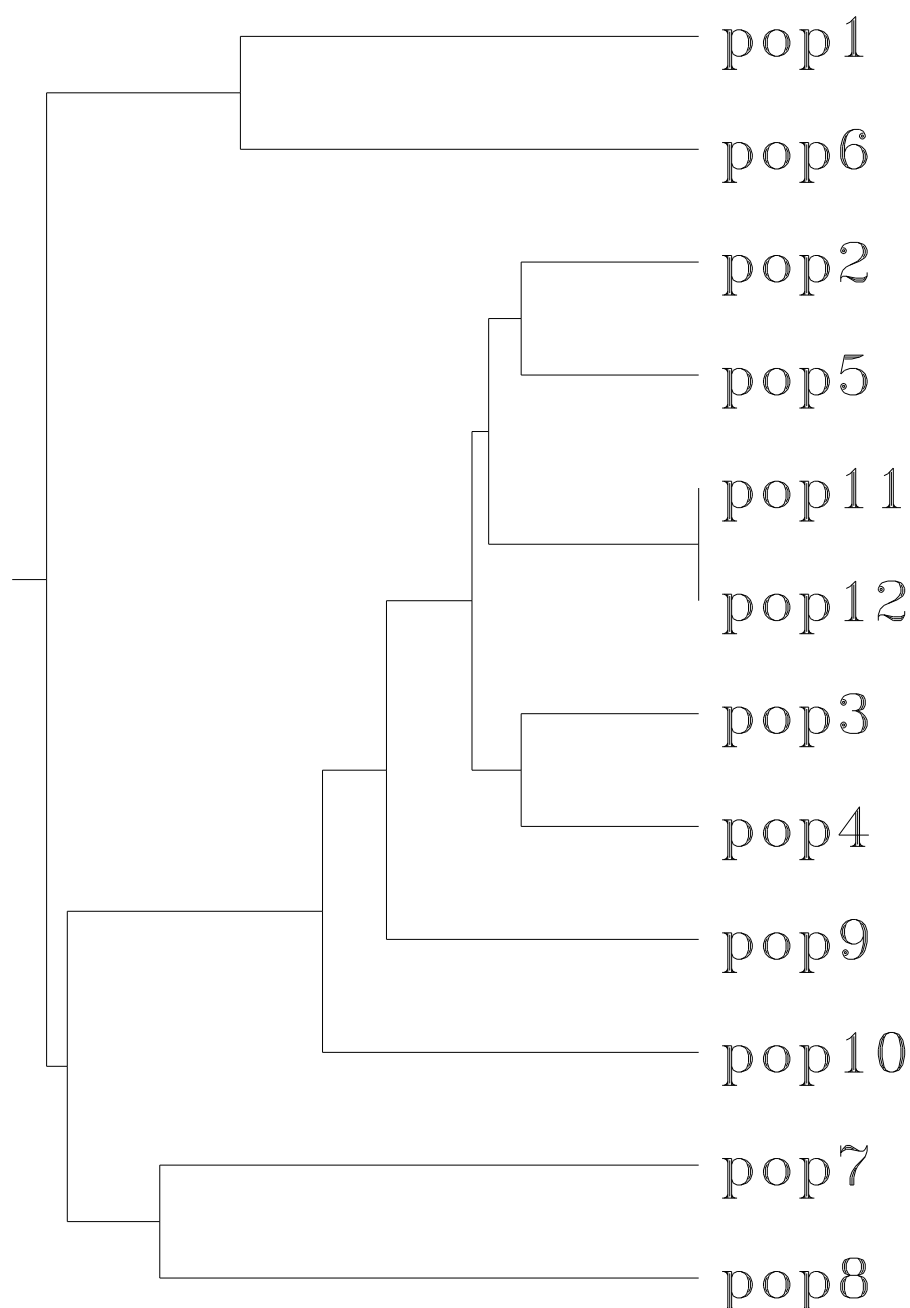
Fig. 4. Dendrograme of 12 barley genotypes grown in Khyber Pakhtun Khwa Province, generated for polymorphic loci of 7 RAPD primers using un-weighted pair group of arithmetic means (UPGMA).   (Pop = Barley genotype serial numbers; for detail see Materials and Methods).

Table 4. Average diversity matrix for dissimimlarities of 12 barley genotypes by pair wise-comparison using RAPD primers.

S.No.	1	2	3	4	5	6	7	8	9	10	11	12
1.	-											
2.	0.43	-										
3.	0.50	0.27	-									
4.	0.53	0.38	0.13	-								
5.	0.52	0.14	0.13	0.18	-							
6.	0.35	0.45	0.29	0.34	0.37	-						
7.	0.58	0.41	0.35	0.39	0,34	0.33	-					
8.	0.40	0.30	0.33	0.29	0.31	0.38	0.33	-				
9.	0.43	0.10	0.24	0.29	0.27	0.40	0.47	0.43	-			
10.	0.40	0.29	0.23	0.30	0.26	0.34	0.28	0.24	0.30	-		
11.	0.33	0.20	0.27	0.60	0.20	0.38	0.30	0.19	0.13	0.19		
12.	0.24	0.20	0.20	0.30	0.24	0.29	0.31	0.22	0.18	0.22	0	-

Column 1-12 = Barley genotypes serial number; For detail see Materials and Methods

Table 5. Average diversity matrix for similarities of 12 barley genotypes developed by pair wisecomparison using RAPD primers.

S.No.	1	2	3	4	5	6	7	8	9	10	11	12
1.	-											
2.	0.57	-										
3.	0.50	0.73	-									
4.	0.47	0.62	0.87	-								
5.	0.48	0.86	0.87	0.82	-							
6.	0.65	0.55	0.71	0.66	0.63	-						
7.	0.42	0.59	0.65	0.61	0.66	0.67	-					
8.	0.60	0.70	0.67	0.71	0.69	0.62	0.67	-				
9.	0.57	0.90	0.76	0.71	0.73	0.60	0.53	0.57	-			
10.	0.60	0.71	0.77	0.70	0.74	0.66	0.72	0.76	0.70	-		
11.	0.67	0.80	0.73	0.40	0.80	0.62	0.70	0.81	0.87	0.81	-	
12.	0.76	0.80	0.80	0.70	0.76	0.71	0.69	0.78	0.82	0.78	1	-

Column 1-12 = Barley genotypes serial numbers; For detail see Materials and Methods

Cluster analysis: Cluster analysis of the data revealed that these genotypes were grouped into three main groups. (1) Group A comprises of genotype serial number 7 and 8 (2) Group B comprises of genotype serial number 2, 3, 4, 5, 9, 10, 10, 11 and 12 (3) Group C genotype serial number 1 and 6. The data further suggested that there was a great diversity among the different barley genotypes under study. Barley genotype number 1 and 8 were found to be the most dissimilar amongst the tested barley genotypes. Average genetic distance was estimated to be from 0 to 60%. Results presented in the dendrogram are in close agreement with the finding of the data presented in Table 4 and 5. Observed genetic variation can be attributed to the individual variation within the tested populations. Fernandez *et al.,* (2002) and Baum *et al.,* (1997) also reported polymorphism among barley cultivars using RAPD analysis.

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