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Full Length Research Paper

Polymerase chain reaction (PCR) based molecular characterization of popular wheat varieties of Khyber Pukhtunkhwa (KPK) region of Pakistan

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Wheat breeders in Pakistan relied upon phenotypic traits as indices of genetic variation among different wheat varieties which may genotypically be closely related or vice versa. The main objective of the current study was to determine the genetic diversity among different popular wheat cultivars grown in the Khyber Pukhtunkhwa (KPK) region of Pakistan, which was previously not fully understood. 13 wheat cultivars namely Zarghoon, Haider-2000, Ghaznavi-98, Nowshera-96, Khyber-79, Dera-98, Tatara, Khatakwal, Saleem-2000, Fakhre Sarhad, Pirsabak-85, Takbeer and Inqilab-91 were used in the current study. Deoxyribonucleic acid (DNA) was isolated using standard protocols and procedures. Polymerase chain reaction (PCR) was carried out using randomly amplified polymorphic DNA (RAPD) primers to amplify DNA of all wheat genotypes. PCR product were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide under UV light. Unweighted pair group of arithmetic means (UPGMA) function was used to estimate genetic distance using bivariate data set generated from all loci of RAPD primers. Cluster analysis carried out and dendrogram was generated using computer program PopGene-32 on similarity matrix. The RAPD markers used in this study identified high rate of genetic polymorphism. Of these primers GLA-08 showed highest genetic diversity (43.42%), while GLA-02 showed lowest genetic diversity (31.73%). Four major groups were identified amongst wheat genotypes. Fakhre Sarhad-Tatara and Khatakwal-Takbeer were found to be genetically closely related with a genetic distance of 6.4 and 9.7%, respectively. Zarghoon and Khatakwal are genetically very distant with a genetic distance of 64.8%. Molecular markers used in this study show high rate of genetic diversity that can be used to assist a breeding program for the improvement of wheat in KPK-Pakistan.

Key words: Wheat, molecular characterization, KPK, genetic distance.

INTRODUCTION

Wheat (*Triticum aestivum* L.) locally known as Ghanum is an annual, self-pollinated, long-day cereal crop, and is one of the most important food items in the world. It has played a vital role in the development of many civilizations. It was cultivated throughout Europe in pre-historic times and was one of the most valuable cereals of ancient Persia, Greece and Egypt. It was an important cultivated crop at the beginning of recorded history.

According to International Grain Council (IGC, 2009-2010), total wheat production in the world in the year 2009-2010 was 679 million tons. China and India are the world's leading wheat producing nations while Pakistan is ranked sixth (Agri. Corner, 2011). According to agriculture statistics of Pakistan (2009-2010), the total area under wheat cultivation in Pakistan was 8.9 million

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hectares while total production was 24.2 million tons. The expected area under wheat cultivation in Pakistan for the year 2010-2011 is 9.045 million ha with the total production of 25 million tons. In 2007-2008 total area under wheat cultivation in KPK was 0.747 million hectares while the total production was 1.072 million tons with an average yield of 2116 kg/ha while the national average yield in Pakistan was 2451 kg/ha.

Several high yielding and tolerant verities have been developed through the breeders efforts and are being extensively grown throughout the country including irrigated and non-irrigated areas of Khyber Pukhtunkhwa (KPK). The phenotypic diversity of these varieties and cultivars are known on limited basis, however, elite wheat varieties grown in KPK lack the information on genetic diversity that is important to improve the crop species.

The genetic improvement of wheat has received considerable attention over the years from plant breeders with the purpose of increasing the grain yield and to minimize crop loss due to unfavorable environmental conditions, and attack by various pests and pathogens (Patnaik and Khurana, 2001). The targets of genetic improvement has shifted from improving the farm management practices of early 60's to reducing yield variability caused by various biotic and abiotic stresses and increasing the input-use efficiency. Biotechnology offers a possible solution to the ever changing global food policy by lowering the farm level production costs by making plants resistant to various abiotic and biotic stresses, and by enhancing the product quality in terms of the appearance of the end product, nutritional content or processing or storage characteristics of the end product.

Bread wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD) is an allo-hexaploid which is believed to have evolved from a hybridization event between *Aegilops tauschii* (2n =2x =14, DD) and tetraploid (2n =2x = 28, AABB) wheat (Sears, 1954; Gedye et al., 2004). This hybridization event may have occurred more than once though the exact number is unclear (Talbert et al., 1994). These hybridization events have occurred in a restricted geographical region and have resulted in a narrow genetic diversity for hexaploid wheat (Lagudah et al., 1991; Talbert et al*.,* 1994). The D genome of hexaploid wheat has remained largely unchanged from that of its wild relative *Ae. tauschii*, to the point that *Ae. tauschii* can be considered as an extension of the wheat gene pool (Kimber et al., 1981). Close relationship between the D genome of wheat and *Ae. tauschii* has reduced the genetic recombination barriers, and have allowed the introgression of several new genes into wheat cultivars, including a number of disease and pest resistance genes that have improved the agronomic and quality traits of these cultivars (Villareal et al*.,* 1995*,* 2001*;* Eastwood et al., 1991; Pena et al., 1995*,* 1996).

Over thousands of years, landraces of hexaploid wheat, with a genome composition of AABBDD, have developed under a variety of different edaphic and climatic environments (Strelchenko et al., 2004). This has led to the evolution of a large number of ecotypes adapted to specific local environments. In the past, attempts have been made to describe the eco-geographical differentiation of wheat using morphological and agronomical traits without knowing the genetic makeup that have resulted in the development of these ecotypes. Recent developments using polymerase chain reaction (PCR) based methods have allowed fast and effective approaches for examining phenotypic polymorphism at the DNA level (Devos et al., 1992; Shah et al., 2000) that will enable us to dissect this polymorphism into genetic (that is, G) factor, environmental (that is, E) factor and G X E thus providing a detailed picture of what is happening at the molecular level. Knowing the genetic factor is essential as polymorphism based on phenotypic data alone; that might be an outcome of a single or a few genes might be of little or no importance to a modern breeder.

Modern breeding programs aiming at the development of wheat varieties that are high yielding have resulted in reducing the natural genetic variability by replacing highly variable landraces with that of high yielding pure line varieties (Frankel, 1970; Sears, 1981). This narrowing genetic background poses a challenge to the wheat breeders who are constantly engaged in either maintaining or improving the yield to cope with emerging biotic and abiotic stresses which are constant and ongoing challenges. The use of molecular markers have made it possible to study the phenotypic variation in wheat varieties at DNA level that have originated in different geographical localities and provide an opportunity to design and implement a breeding program based on the genetic difference rather than solely relying on the phenotypic differences which might mislead. Thus, identifying the genetic level of diversity is of great importance for modern breeding program designed to take advantage of the existing variations amongst different species for wheat improvement.

Several PCR-based systems, like random amplified polymorphic DNA (RAPD), SSR, AFLP, among others, are available that differ in complexity, reliability and information generating capacity with each having its own advantages and disadvantages. Development of high genetic density maps of wheat, tobacco, maize, rice has proved a valuable resource in the characterization and evaluation of genetic diversity within and amongst species and populations. But in absence of specific information on nucleotide sequences, RAPD (Williams et al., 1990) or AP-PCR arbitrarily primed–PCR (Welsh and McClelland, 1990), allowed DNA analysis using the PCR.

Different methods are available that can be employed for assessing genetic variability, relatedness and diversity amongst germplasms as well as for molecular fingerprinting. Molecular markers, both biochemical and morphological are influenced by the environment, while

Table 1. Description of the wheat verities grown in KPK.

S/N	Variety	Pedigree	Developed by	Rain fed/irrigated
1	Dera-98*	CM76688-9Y-03M-02Y-2B-OY	ARI-D.I. Khan	Rain fed
2	Fakhre Sarhad	PFAUS/SERI/BOW	NIFA-Peshawar	Irrigated
3	Ghaznavi-98*	98 JUP/Bjyy/Ures	AUP-Peshawar	Irrigated
4	Haider-2000*	CHIL/WUH3	CCRI-Pirsabak	Rain fed
5	Inqilab 91*	WL 711/CROWS	Ayub agri. research station Faisalabad	Irrigated/Rain fed
6	Khatakwal	Land race of Southern KPK, Pakistan	---	Rain fed
7	Khyber-79*	WREN//CNO/GLL/3/C271		Irrigated
8	Nowshera-96*	---	CCRI-Pirsabak	Irrigated
9	Pirsabaq-85*	KVZ/BUSHS/KAL/BB	CCRI-Pirsabak	Irrigated/ Rain fed
10	Saleem-2000*	CHAM6//KITE/PGO	CCRI-Pirsabak	Irrigated, late Planting
11	Takbeer	ATTILA	NIFA-Peshawar	Irrigated
12	Tatara	JUP/ALDS??KLTS/3VEES	NIFA-Peshawar	Rain Fed
13	Zarghon-79*	CC/INIA/3/TOB/CTFN//BB/4/7C	Balochistan	---

* represents the year of release; --- Information not available

DNA based markers provides a reliable tools as they are not influenced by the environment and thus provide an accurate assessment of diversity at the genetic level. But the use of DNA based markers in hexaploid wheat is complicated because of its large genome size, highly repetitive DNA sequence, continuous inbreeding caused by self-pollination coupled with narrow genetic base (Joshi and Nguyen, 1993). Different molecular marker techniques such as AFLP (Paull et al., 1998; Shah et al., 2000), SSR (Plaschke et al., 1995), sequence tagged site (Talbert et al., 1994) and AFLP (Barrett and Kidwell, 1998) have been used for the estimation of genetic diversity in wheat. The RAPD technique, regardless of its sensitivity to reaction conditions and problems with repeatability and amplifying of non-homologous sequences (Devos and Gale, 1992), has been successfully used for the genetic diversity assessment of diploid, tetraploid and hexaploid wheat (Shah et al., 2000; Liu et al., 1999; Sivolap et al., 1999; Myburg et al., 1997; Dweikat et al., 1994; Devos et al., 1992; He et al., 1992).

MATERIALS AND METHODS

Most commonly cultivated wheat varieties of KPK were selected for this study (Table 1). The varieties selected for this study represents a broad spectrum of climatic zones of KPK. The seeds of these varieties were kindly provided by Cereal Crop Research Institute (CCRI), Pirsabak.

DNA preparations

The seed of all varieties were grown in pots. For isolating total genomic DNA, 10 cm long piece of fresh leaf material from 3 to 4 weeks-old seedlings was cut in the screen house and dipped in 1.5 ml Eppendorf tubes, which were then put in liquid nitrogen. The frozen plant material was ground to a fine powder with the help of a sterilized glass rod. 500 µl DNA extraction buffer (1% SDS, 100 mM NaCl, 100 mM tris base, 100 mM Na_2EDTA, pH: 8.5; Weining and Langridge, 1991) was added to each Eppendorf tube containing the crushed leaf material and mixed well with the glass rod and then an equal volume (500 µl) of phenol: chloroform: isoamyl-alcohol (25:24:1) was added to it. The tubes were shaken well until a homogenous mixture was obtained. Samples were then centrifuged at 10000 rpm for 5 min. The supernatant was transferred to a fresh tube. Then 1/10 volume of 3 M-sodium acetate (pH = 4.8) and 500 µl isopropanol was added to each tube and mixed gently to precipitate DNA. Samples were then centrifuged at 6000 rpm for 4 min to pellet DNA. The pellet was then washed with 70% ethanol, dried and dissolved in 50 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0., DNA was then treated with 40 µg RNAase-A (0.2 µl of commercially supplied RNAase-A of Fermantas Company) at 37°C for 1 h. After RNAase treatment, DNA samples were then run on 1% gel to check the quality of DNA and then stored at 4°C. For PCR 1: 5 dilution of DNA was performed in de-ionized and autoclaved doubled distilled water.

Polymerase chain reaction

RAPD primers (Genelink Technology USA) were used to detect level of genetic polymorphism at DNA level among the 13 genotypes used in the present study. These RAPD primers were selected arbitrarily. The sequences and amplification conditions of the primers are given in Table 2. PCR reaction were carried out in 25 µl reaction containing 50-100 ng total genomic DNA template, 0.25 µM of each primer, 200 µM of each dATP, dGTP, dCTP, dTTP, 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase (Dweikat et al., 1993). Amplification conditions were as follows: An initial denaturation step of 4 min at 94°C followed by 40 cycles each consisting of a denaturation step of 1 min at 94°C, followed by an annealing step of 1 min at 36°C and an extension step of 2 min at 72°C. The last cycle was followed by 7 min extension step at 72°C to ensure that primer extension reaction proceeded to completion. All amplification reactions were

Table 2. Sequence information and amplification conditions.

S/N	Primer	Sequence (5´-3´)	Size(bp)	Molecular weight (MW)	Melting temp (tm) (ºC)	% GC
1	GL DecamerA-02	TGCCGAGCTG	10	3044.01	33.6	70
2	GL DecamerA-05	AGGGGTCTTG	10	3099.04	29.5	60
3	GL DecamerA-08	GTGACGTAGG	10	3108.04	29.5	60
4	GL DecamerA-10	GTGATCGCAG	10	3068.02	29.5	60

GL = Genelink Technology USA

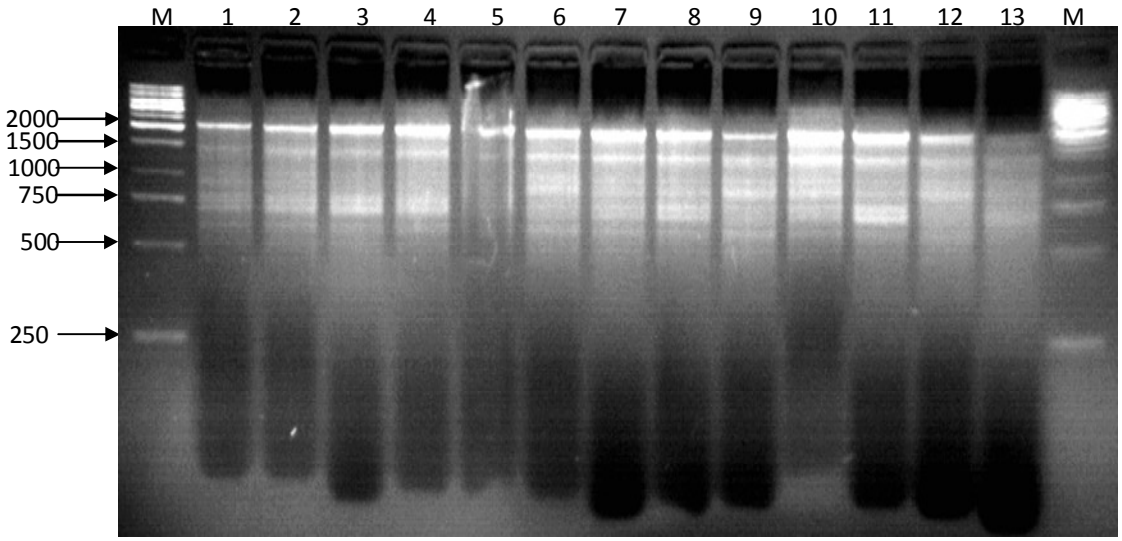

Figure 1. PCR profile of 13 genotypes of wheat varieties using RAPD primer GLA02. Lane 1= Zarghon, lane 2=Haider-2000, lane 3= Ghaznavi-98, lane 4= Nowshera-96, lane 5= Khyber-79, lane 6=Dera-98, lane 7=Tatara, lane 8= Khatakwal, lane 9=Saleem-2000, lane 10=Fakhre Sarhad, lane 11=Pirsabaq-85, lane 12= Takbeer, lane 13= Inqilab-9, M= Molecular weight marker (1Kbp ladder)

performed using GeneAmp PCR system 2700. The amplification products were electrophoresed on 2% agarose/TBE gel and visualized by staining with ethidium bromide and viewing under U.V light.

Data analysis

Data was analyzed using Nei and Lei formula for the calculation of genetic distance, while dendrogram was constructed using PopGene-32 software program. Bands detected by each primer pair were interpreted as loci at that primer. Only the score able bands were included in the analyses. Every single band was considered as a single locus/allele for all analyses. The loci were scored as present / absent. Bivariate data (1-0) were used to estimate genetic distances (Ds). Unweighted pair group of arithmetic means (UPGMA) function (Nei, 1978) was used to estimate genetic distances between the genotypes using following Nei equation that results to a dissimilarity matrix for each primer.

$$GD = 1 - d_{xy}/d_x + d_y - d_{xy}$$

Where, GD is the genetic distance between two genotypes; d_{xy} is the total number of common loci (bands) in two genotypes d_x is the number of loci (bands) in genotype 1 and d_y is the number of loci (bands) in genotype 2.

RESULTS AND DISCUSSION

RAPDs is widely used for detecting genetic polymorphism between genotypes at molecular level in many crop species like wheat (He et al., 1992; Myburg et al., 1997; Liu et al., 1999; Sivolap et al., 1999; Dweikat et al., 1992), maize (Bennetzen and Feeling, 1993; Zhang et al., 1998; Bernardo et al., 1997) and brasssica (Crockett et al., 2000; Yuan et al., 2004; Rajcan et al., 1999; Das et al., 1999; Sharma and Tewari, 1998).

In the present study, all genotypes showed various levels of genetic polymorphism for the loci detected by using all four primers. A total of 255 alleles (bands) were observed in 13 genotypes for the four primers giving an average of 19.69 bands per genotype (Figures 1 and 2). Among the four primers used in the current study, primer A10 yielded on an average maximum number of bands (6.85) per genotype, while primer A02 yielded lowest number of bands on the average. Results of these analyses are presented as genetic similarity matrix in Table 3. Range of genetic distances observed was 6.4 to 64.8%. Maximum genetic distances (64.8%) was

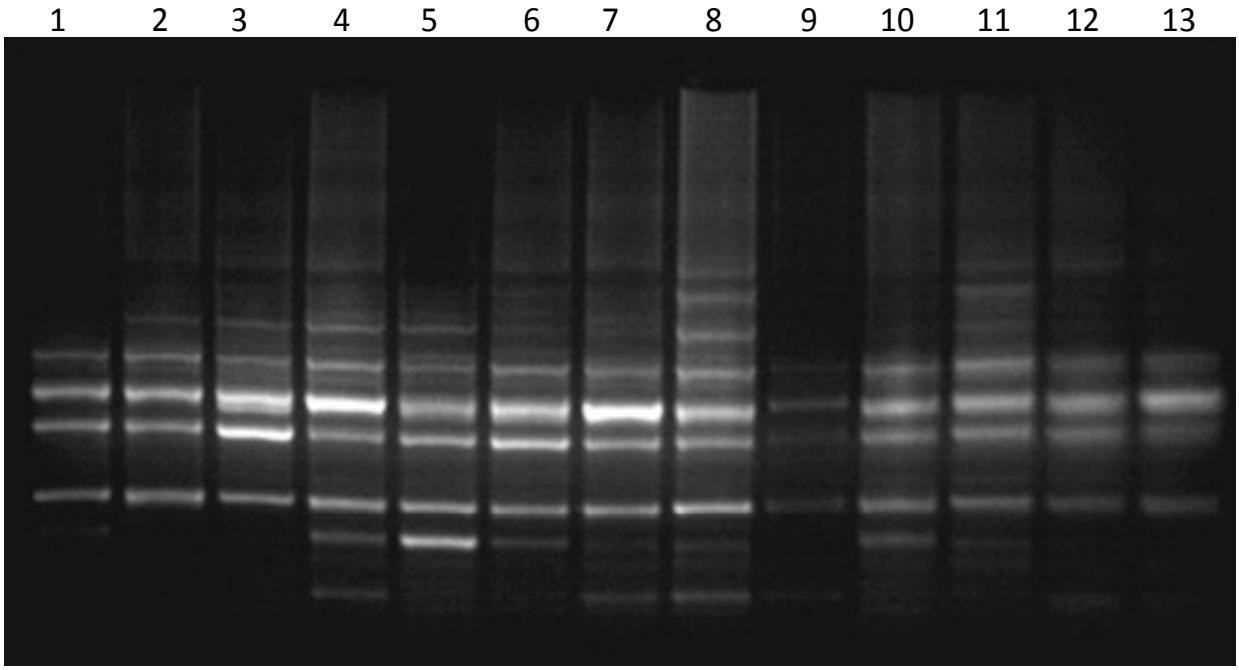

Figure 2. PCR profile of 13 genotypes of wheat varieties using RAPD primer GLA10. Lane 1= Zarghon, lane 2=Haider-2000, lane 3= Ghaznavi-98, lane 4= Nowshera-96, lane 5= Khyber-79, lane 6=Dera-98, lane 7=Tatara, lane 8= Khatakwal, lane 9=Saleem-2000, lane 10=Fakhre Sarhad, lane 11=Pirsabaq-85, lane 12= Takbeer and lane 13= Inqilab-91.

Table 3. Nei's genetic identity table.

S/N	1	2	3	4	5	6	7	8	9	10	11	12	13
1	-												
2	0.774	-											
3	0.742	0.645	-										
4	0.677	0.581	0.806	-									
5	0.742	0.645	0.677	0.806	-								
6	0.645	0.548	0.774	0.774	0.839	-							
7	0.516	0.484	0.710	0.774	0.645	0.806	-						
8	0.452	0.484	0.516	0.581	0.516	0.613	0.742	-					
9	0.516	0.484	0.516	0.645	0.645	0.677	0.548	0.806	-				
10	0.581	0.548	0.710	0.774	0.710	0.871	0.936	0.742	0.613	-			
11	0.839	0.806	0.774	0.645	0.645	0.613	0.613	0.548	0.484	0.677	-		
12	0.484	0.516	0.484	0.548	0.613	0.645	0.645	0.903	0.839	0.645	0.516	-	
13	0.613	0.581	0.613	0.806	0.742	0.645	0.774	0.774	0.645	0.774	0.645	0.677	-

1= Zarghon, 2=Haider-2000, 3= Ghaznavi-98, 4= Nowshera-96, 5= Khyber-79, 6=Dera-98, 7=Tatara, 8= Khatakwal, 9=Saleem-2000, 10=Fakhre Sarhad, 11=Pirsabaq-85, 12= Takbeer, 13= Inqilab-91.

observed for the combination of Zarghoon and Khatakwal, while Tatara and Fakhre Sarhad showed a close homology at DNA level with an average genetic similarity of 93.6% (Table 3). The average genetic distance calculated was 33.33%. The dendrogram was generated using PopGene-32 software identified four groups amongst the thirteen verities used in the present study (Figure 3). Group-A includes Zarghon, Pirsabaq-85 and Haider-2000, Group-B includes Ghaznavi-98, Nowshera-96 Khyber-79 and Dera-98, Group-C includes Tatara, Fakhre Sarhad and Inqilab-91 and Group-D includes Khatakwal, Takbeer and Saleem 2000.

Our findings are in contrast to the earlier concluded findings by Shahid et al. (2002) where they observed genetic distance of approximately 20%, while the current study revealed genetic distance of 33.33%. This

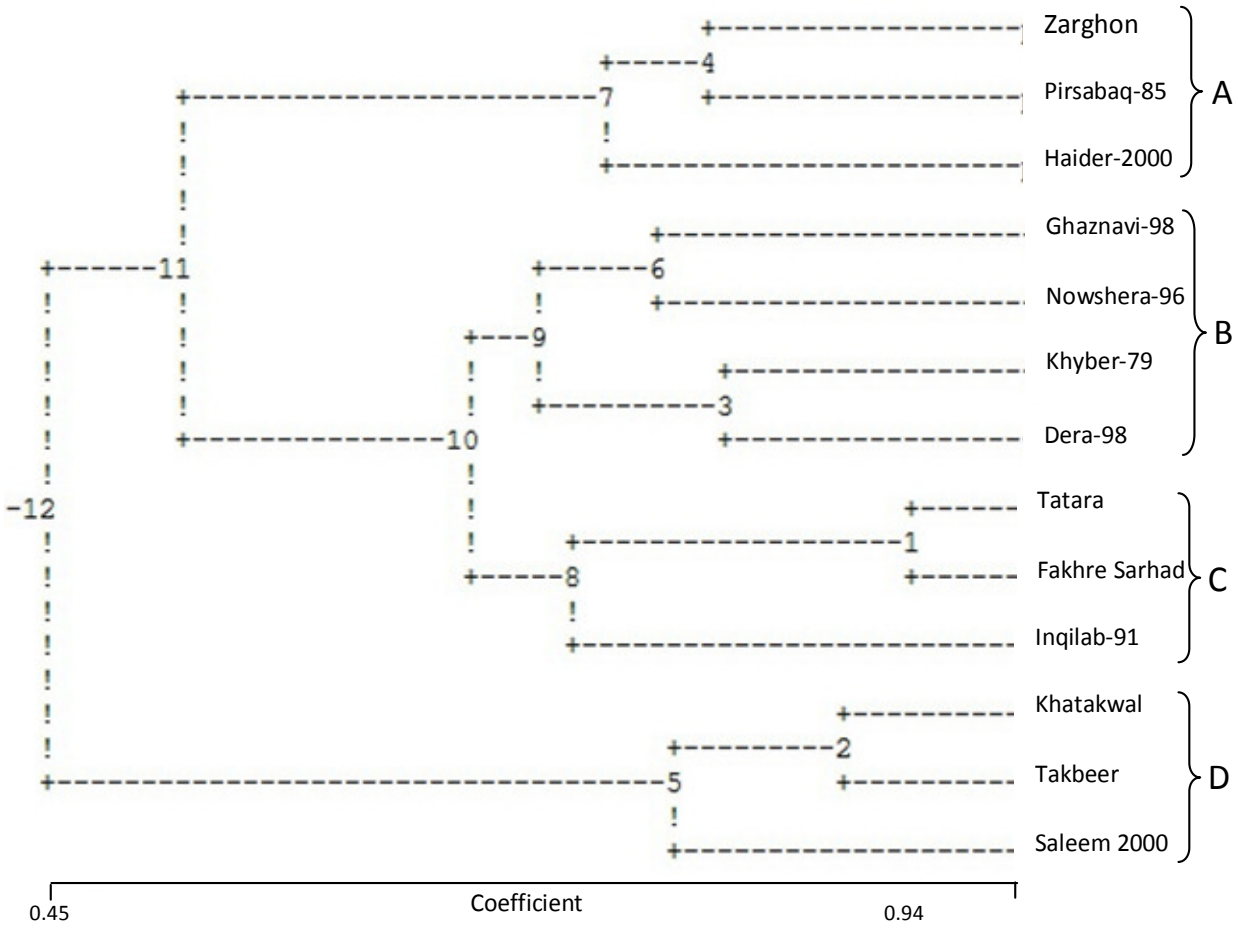

Figure 3. Dendrogram of all 13 wheat varieties grown in NWFP, generated for polymorphic loci of 4 RAPD primers using un-weighted pair group of arithmetic means (UPGMA).

difference can be attributed to the fact that cultivars selected for the current study came from diverse backgrounds with different pedigree.

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Abbreviations

KPK, Khyber Pukhtunkhwa; **PCR,** polymerase chain reaction; **RAPD,** randomly amplified polymorphic DNA; **UPGMA,** unweighted pair group of arithmetic means.

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