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A N I N T E R N A T I O N A L J O U R N A L

Evaluation of Genetic Diversity amongthe Pakistani Wheat (*Triticumaestivum* L.) Lines through Random Molecular Markers

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ABSTRACT

The presence of geneticdiversity is of great importance in improving wheat traits and developing strategies for optimal conservation of germplasm. Genetic diversity was assessed among common wheat cultivars using RAPD (Random Amplified Polymorphic DNA) markers at the Center of Agriculture, Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad. RAPD primers were used among 14 Pakistani wheat cultivars, to screen the progenies and for the identification of the genes of interest. The polymorphic information content (PIC), was measured as the percentage of polymorphic fragments for all primers. A total of 583 bands(84% polymorphic) in all 14 wheat cultivars was amplified and discriminated all the wheat genotypes. The number of fragments amplified per primer ranged from 35 to 69 with an average of 48.52 fragments per primer averagely was observed. Population structure analysis anddandrogram showed distinct clustering among different wheat genotypes. Millat-11, Punjab-11, PBW-222 generated the maximum level of polymorphism, standing alone in the cluster while others are scatteredin different group.As a result, genetically numerous progenies are known, increasing the quality of sorts collections by broadening the genetic base of wheat cultivars. This study additionally indicates that RAPD markers allow quicker response and provide high throughputprocedure of accessions from a variety assortment to assess genetic diversity among wheat genotypes.

Key words: Wheat, RAPD, genetic diversity, PCoA, Cluster analysis

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INTRODUCTION

Wheat is used as a staple food, being the most important cereal crop all over the world. Wheat is the basis ofhuman nutrition and is economically important worldwide. Common wheat or bread wheat (*Triticumaestivum* L.), club wheat (*T. compactum* L.) and durum wheat (*T.turgidum* L.) are the most commonly used wheat species. About 3.2% GDP of Pakistan depends on wheat [1]. Wheat (*Triticumaestivum* L.) grass belongs to family *Poaceae* and is grown all over the world. The total cultivated area of wheat is more than 200 million hectares and total wheat production is about 733 million tons per year [2]. Wheat has good nutritional value comprised of 58.2% starch, having sufficient amount of sugar and fat. Wheat nourishment is superior in protein 11.2%, 6.8% pentosans, 1.7% ash, and 70% of cases, and having a higher percentage of carbohydrates than other crops [3]. Wheat production facing serious problems in semi-arid regions in the world due to changing environmental conditions [4, 5] several pathogenic diseases [6] and its greater nutritional value [7, 8]. The lower wheat yield in Pakistan is the result of limited diversity in the genome, which is used in breeding programs[56].

Geographically southwestern Asia, asits center of origin and according to earliest historical record wheat was an important cultivated crop in this region. Wild species of *Triticum* are found in Iraq, Syria, Lebanon, eastern Turkey and northern Israel. In Egypt and Greece, wheat was cultivated in pre-historic times at thecenter of diversity for hexaploid wheat is Hindukush [9, 10]. Several Pakistani cultivars are stoked in seed banks and are notdifferentiated efficiently to demand for breeders concerned with their work. Therefore, the genetic diversity in wheat cultivars needs to be categorized.Genetic diversity is one of the key factors for the improvement of many crop plants including wheat. Plant breeders rely on the availability of genetic diversity during selection in cultivar development. Genetic diversity can be assessed from pedigree analysis, morphological traits or using molecular markers [11]. However, genetic diversity based on pedigree selection have generally been found inflated and unrealistic [12]. Genetic diversity estimates based on morphological traits, suffers from the drawback that such traits are limited in number and is influenced by the environment [13].Genetic diversity is more important among all crops for successful production of hybrids and new cultivars. Initially, genetic variations were dependent on pedigree records and co-ancestry [14]. The morphological and agronomic attributes of wheat have been evaluated to measure genetic variation and their close relatives. A large number of field experiments were carried out to evaluate the genetic variance according to different morphological attributes and epigenetic possessions required for gene expression through molecular markers [15]. So, there is a dire need to develop innovative methods to determine the diversity by molecular mean that would substantially beneficial for researchers and breeders [16]. As compared to rice, maize or tomatoes, the development in molecular genetics in wheat has been relatively slow, due to polyploidy difference, complexity and the size of its genome [17], low level of polymorphism and high percentage of repetitive sequence [18].

Molecular marker technology is an important tool of biotechnology because it's not influenced by environment, are abundant and don't require previous pedigree information, which can enhance the efficiency of molecular breeding practices [19].A different kind of molecular markers are used to determine the genetic diversity including labeled probe primers AFLP and SSR [20]. These methods are based on polymerase chain reaction (PCR) with the use of RAPD[21, 22], simple sequence repeat (SSR or microsatellites) analysis [23, 24].Precision and excellence in the selection of germplasm are achieved by the expansion of molecular DNA markers [25].

Genetic diversity analyses by molecular means played an important role in genomic structure composition, figure out important genes for specific traits, and finally preservedthe genetic materials for future use for plant breeding [26].The evaluation of genetic diversity in diploid,tetraploid and hexaploid wheat is effectively measured by the RAPD technique [27]. Due to its simplicity, effectiveness and no necessity of sequence information [28, 29]. RAPD have gained significance among many other DNA based techniques from genomic DNA utilizing random primers of arbitrary sequence[30].The elite cultivars were found helpful for estimation of genetic distance between the actual routine sequences [31] and establish the difference between genetic differences due to extensive breeding programs [32].The morphological attributes can also be used to determine the genetic diversity,

along with molecular markers is a direct genetic approach to figure out the desired gene (s) or gene product that have a positive effect on the crop plant. In short, RAPD markers express a large number of genes with greater accuracy to determine the genetic diversity [33].

This study reveals the genetic relationship and population structure of different Pakistani wheat cultivarsfrom different geographical origin. The information about the genetic differences within and between different populations, which can be efficiently used by breeders for the production of genetically diverse wheat cultivars.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of fourteen wheat cultivars belonging to *Triticumaestivum*L.were collected from Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad. The seeds were planted in pots during normal growing season and after germination 8-10 leaves were obtained from plants and frequently stored at -80^{o}C for DNA isolation. The wheat cultivars used for experiment are listed in

Table 1).

Table 1 Wheat Cultivars and their ploidy level

Cultivars	Ploidy level	Cultivars	Ploidy level
Aas-11	Hexaploid	Minthar-03	Hexaploid
AARI-11	Hexaploid	Inqalab-91	Hexaploid
SH-2002	Hexaploid	FD-85	Hexaploid
Barani-83	Hexaploid	PBW-222	Hexaploid
Koh e Noor-83	Hexaploid	FD-83	Hexaploid
Punjab-11	Hexaploid	MH-97	Hexaploid
Millat-11	Hexaploid	Pak-81	Hexaploid

DNA ISOLATION

DNA from these samples was extracted from leaf tissue by the following modified CTAB procedure [34]. PCR conditions were optimized according to the concentration of genomic DNA, $MgCl_2$ 10X PCR buffer, primer, dNTPs, Taq DNA polymerase and annealing temperature as these factors influence the reproducibility of RAPD technique and the type of thermal cycler used. The RAPD-PCR reaction was performed in 25 ul containing [10x Buffer + $(NH_4)_2$ SO_4 is 2.5 ul, d2h20 8.3 ul, Gelatin (0.025%) is 2.5 ul, $MgCL_2$ (50mM) 3.0 ul, **Table 2**). Amplified products were electrophoresed on a 1.5% agarose gel using TBE buffer 0.5x. 5 ul of each PCR product and visualized using a gel documentation system.

DATA ANALYSIS

The RAPD bands were scored as 0 for their absence and 1 for their presence, generating a matrix. The genetic similarity was computed by UPGMA [35]clustering (Unweighted Pair Group Methods of Arithmetic Average). The polymorphism information content (PIC) for each primer was calculated to estimate its allelic variation according to the formula

dNTPs (2.5mM each) is 4.0 ul, Primer (15ng/ul) 2.0 ul, Taq DNA polymerase (0.2U/ul) 0.2 and DNA template (15 ng/ul) is 2.5 ul]. Amplification was carried out by PCR thermal cycler (AG No. 533300839, Germany) in a reaction as follows: initial denaturation at 94 °C for 5 min followed by forty cycles of denaturation for 1 min, annealing temperature 36 °C for 1 min and elongation time at 72 °C for 2 min and final extension at 72 °C for 10 min. The reaction was performed in a total volume of 25 µl. The RAPD primers and their sequence were presented in

$$\mathrm{PIC} = 1 - \sum_{j=1}^{n} P_{ij}^{\,2}$$

Where *P*ij is the frequency of the *i*th allele for marker j and the summation extends over *n* alleles, calculated for each RAPD marker [36]. Data generated from RAPDanalysis were analyzed using Jaccard similarity coefficient [37]. These similarity coefficients were used to construct the dendrogram using the unweighted pair group method with arithmetic average (UPGMA) employing the SAHN (sequential, agglomerative, hierarchical, and nested clustering) from the *NTSYS-pc* (version 2.1) software[38]. The

correlation and similarity matrix are determined by using a Mantel test.

Table 2. Description of 14 wheat RAPD primers and their sequences.

Primer code	Primer sequence	Primer code	Primer sequence
GL DecamerA-13	5'- TGCTCTGCCC -3'	GL Decamer K-01	5'- TCATCCGAGG -3'
GL DecamerA-14	5'- GGTGACGCAG -3'	GL Decamer K-03	5'- TCGTTCCGCA -3'
GL DecamerB-04	5'- GTCCACACGG -3'	GL Decamer K-12	5'- CCCAGCTGTG -3'
GL DecamerB-09	5'- GTAGACCCGT -3'	GL Decamer K-16	5'- CACAGGCGGA -3'
GL DecamerB-11	5'- CCTTGACGCA -3'	GL Decamer K-17	5'- GTGTCGCGAG -3'
GL DecamerB-13	5'- TTTGCCCGGA -3'	GL Decamer L-05	5'- GACTGCACAC -3'
GL DecamerB-17	5'- AGGGAACGAG -3'	GL Decamer L-11	5'- ACGCAGGCAC -3'

RESULTS

The dendrogram shows 92.5 % genetic similarity among the wheat genotypes (**Fig. 1**). The dendrogram was cut at 0.50 similarity index value, which was averaged value for similarity. The consensus tree was divided into 4 groups and 3 separate groups. Millat-11, PBW-222 and FD-85 is standing alone and not making any cluster with other wheat line. Punjab- 11 showed as the parent line in this cluster. AARI-11 is making group with SH-2002 at similarity 0.50. FD-83 and inqalab-91 is in the same group at similarity 0.72 and is making group with Minthar-03. Group IV has 2 cultivars like Koh e noor and barani-83 at similarity 0.68.

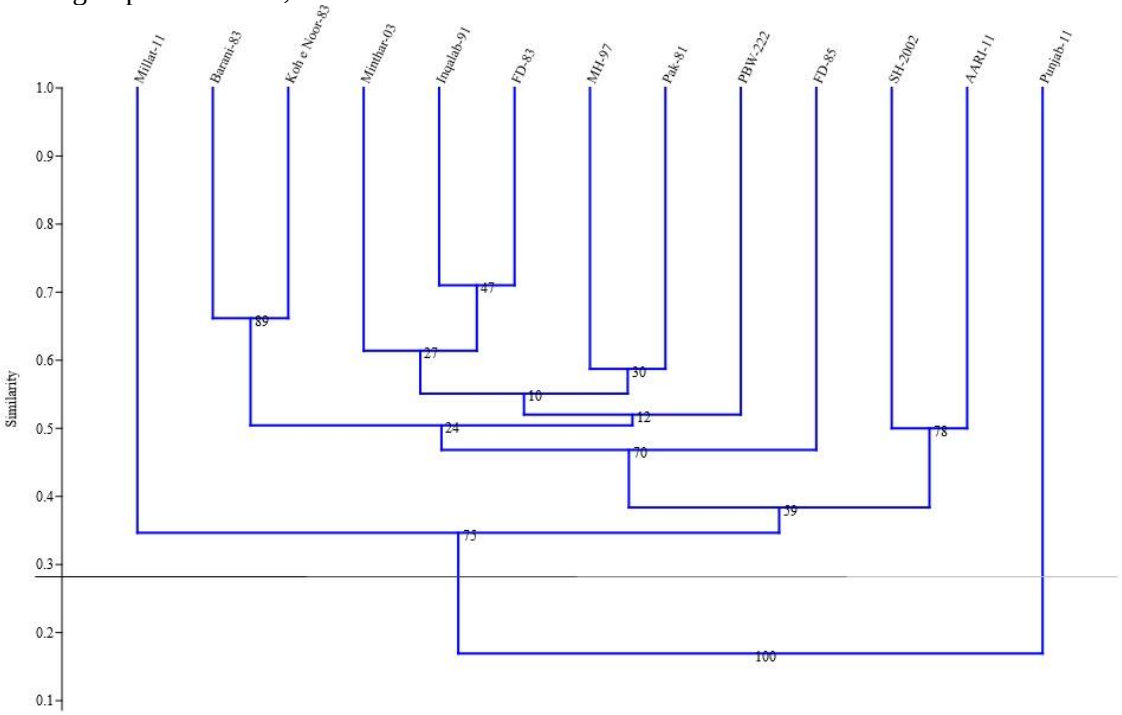

Figure 1 - Dendrogram produced by Jaccard's coefficient and the unweighted pair group method with arithmetic average (UPGMA) clustering method based on RAPD data in 14 wheat genotypes.

Two dimensional biplot results were presented in Figure 2**Fig. 2**. PCoA1 have four cultivars that are more diverse as compared to other such as SH-2002, AARI-11, Millat-11, PBW-222 and Punjab-11, while 2PCoA have Koh e Noor 83, Barani-83, FD 85 and Inqilab-91. All the four cultivars are away from the center, which means that these are diversefrom others. The eigenvalue and % variance data on the basis of RAPD score data using (UPGMA) was presented (figure 2 table 3). It represents that the cumulative Eigen value for six coordinates have 1.91 from all coordinates. The percentage variances or diversity among the wheat cultivars for first six coordinatesare80 % of the total.

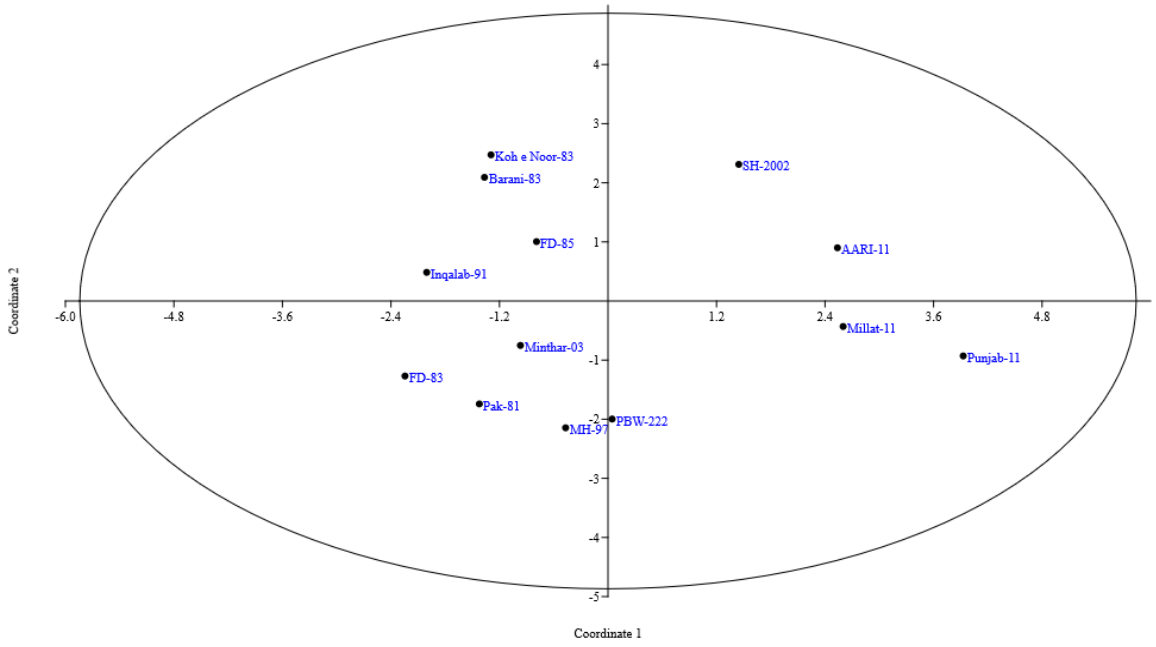

Figure 2 Two-dimensional plot based on Jaccard coefficient with the first two principal coordinates analysis on the basis of RAPD markers for 14 Wheat Varieties.

Table 3. Cumulative Eigenvalue and % variance of all principle components analysis (PCoA) based on RAPD markers

Coordinates	Eigenvalue	Cumulative Eigenvalue	Percent Variance	Cumulative
1	0.63	0.63	26.32	26.32
2	0.37	1.00	15.36	41.67
3	0.29	1.29	12.31	53.98
4	0.25	1.54	10.61	64.59
5	0.20	1.75	8.46	73.05
6	0.17	1.91	6.94	80.00

Table 4. Principal component coordinates (PCoA) analysis results for 14 Wheat cultivars produced by RAPD score data

	Coord 1	Coord 2	Coord 3	Coord 4	Coord 5
AARI-11	0.37	0.16	0.54	-0.28	-0.21
SH-2002	0.21	0.40	0.27	-0.15	0.19
Barani-83	-0.20	0.37	0.02	0.43	0.01
Koh e Noor-83	-0.19	0.43	-0.04	0.41	0.07
Punjab-11	0.57	-0.16	-0.30	0.04	-0.01
Millat-11	0.38	-0.08	-0.30	0.25	-0.27
Minthar-03	-0.14	-0.13	-0.28	0.07	-0.08
Inqalab-91	-0.29	0.08	-0.21	-0.35	-0.47
FD-85	-0.12	0.18	-0.34	-0.56	0.47
PBW-222	0.01	-0.35	0.04	0.10	0.09
FD-83	-0.33	-0.22	0.05	-0.14	-0.26
MH-97	-0.07	-0.37	0.13	0.11	0.56
Pak-81	-0.21	-0.30	0.44	0.06	-0.10

For better understanding the relationship among these cultivars were determined by the principal coordinate's analysis (PCoA). It was used to construct the data set to determine the similarities among the genotypes. The first six coordinates showed 80% cumulative variance of the total which means that RAPD primers showed diversity among the wheat cultivars. The First PCoA(26.31) which was followed by 15.35, 12.30, 10.61, 8.46 and 6.94 in second, third, fourth, five and six coordinates respectively. The principal coordinates for 14 wheat cultivars data was presented (Table 4). The data regarding coordinates represent that PCoA have five cultivars named AARI-11, SH-2002, Punjab-11, Millat-11 and PBW-222, while the second coordinates have six cultivars named AARI-11, SH-2002, Barani-83, Koh e Noor 83, Inqilab-91 and FD-85. The maximum Euclidean distance (0.57, 0.38, 0.37, and 0.21) was observed in Punjab-11, Millat-11, AARI-11 and SH-2002 in PCoA1 respectively. The maximum distance (0.43) was observed in Koh e Noor 83 in PCoA 2 which was followed by 0.40 (SH-2002), 0.36 (Barani-83), 0.15 (AARI-11) and least was 0.08 (Inqalab-91).

Table 5. Total Number of fragments and PIC value for each primer producing diversity among wheat cultivars

Primers name	Total Fragments	PIC value
GL DecamerA-13	38	0.85
GL DecamerA-14	56	0.84
GL DecamerB-04	69	0.87
GL DecamerB-09	35	0.78
GL DecamerB-11	49	0.82
GL DecamerB-13	57	0.87
GL DecamerB-17	57	0.82
GL Decamer K-01	43	0.87
GL Decamer K-03	52	0.88
GL Decamer K-12	49	0.88
GL Decamer K-16	39	0.82

GL Decamer K-17	39	0.79
Total Fragments	583	
	48.52	84

The polymorphic information content (PIC), measured as the percentage of polymorphic fragments for all primer pairs were presented in

Table 5**)** and varied ranged from (79- 87 %).The maximum PIC (polymorphic information contents) was 87% with (GL Decamer B-13) and producing 38 fragments in all 14 wheat cultivars while a minimum PIC value (0.79) was observed in primer (GL Decamer K-17) and producing 39 fragments bands. The average value of each primer is 48.52 among all the primers used in this experiment and producing 84% PIC as an average for each primer. The ability for producing diversity for each RAPD primer varies significantly for all range 38 – 69 loci. The Jaccard's similarity coefficient among 14 wheat cultivars based on RAPD data was presented in **Erro! Fonte de referência não encontrada.**).

Table 6. Jaccard's similarity coefficient among 14 wheat cultivars based on RAPD data

	AARI-11	SH-2002	Barani-83	Koh e Noor-83	Punjab-11	Millat-11	Minthar-03	Inqalab-91	FD-85	PBW-222	FD-83	MH-97	Pak-81
AARI-11	1.00												
SH-2002	0.50	1.00											
Barani-83	0.36	0.48	1.00										
Koh e Noor-83	0.36	0.49	0.66	1.00									
Punjab-11	0.19	0.14	0.15	0.13	1.00								
Millat-11	0.30	0.35	0.34	0.38	0.29	1.00							
Minthar-03	0.31	0.41	0.54	0.53	0.21	0.42	1.00						
Inqalab-91	0.33	0.41	0.55	0.54	0.14	0.33	0.58	1.00					
FD-85	0.29	0.41	0.46	0.49	0.11	0.28	0.50	0.54	1.00				
PBW-222	0.32	0.38	0.43	0.44	0.19	0.39	0.49	0.44	0.41	1.00			
FD-83	0.40	0.45	0.58	0.55	0.18	0.40	0.65	0.71	0.53	0.61	1.00		
MH-97	0.31	0.37	0.46	0.43	0.18	0.31	0.52	0.43	0.42	0.50	0.59	1.00	
Pak-81	0.44	0.39	0.51	0.50	0.12	0.33	0.55	0.52	0.41	0.56	0.69	0.59	1.00

DISCUSSION

Genetic diversity analysis is important to interpret the genetic relationship, including parentage and their management of genotypes and hence used for breeding improvement [39]. Molecular characterization is more valuable than morphological because in molecular data, a large number of data set or alleles are present within the same species, while morphological traits might be affected by environment [40, 41].As a result RAPD primers are used to evaluate genetic diversity among the 14 hexaploid wheat cultivars. The polymorphic information contents (PIC) for all primers combinations were 84 %, which is very high and predicted that the diversity is present among the wheat cultivars. These results are lined with [42, 43,44, 45] reported that 80.52 %, 61 %, 62.5 %, 71.10 % polymorphism intensity was observed respectively. Similar results were obtained

by[46,47,48,49,50] reported that RAPD primers showed 83%, 89%, 79.8%, 81% and 92.5 % diversity respectively for different cultivars.

The average number of amplified fragments for each primer is 48.52 greater that [51] who observed 22.32 fragments bands per primer. Our results are more similar with [45] who discovered 46.37 fragments while [52] and [46] observed 57 bands per primers from wheat cultivars. In another study [49] reported that 53 fragments per primers were reported in the evaluation of common wheat cultivars at different locations. Though, molecular characterization considerably contributes towards phenotypic variation, but cannot be accurately phenotype[42]. So genetic diversity is best studied at the level of arrangement of nucleotide bases in genomic DNA, which is the primary source of all biological information [53]. At this stage, even identical seeming accessions could display vast differences, if only we could use appropriate DNA profiling techniques. Besides, the availability of a number of molecular markers, RAPD is one such method [54, 55] of discovering polymorphism that can be used to elicit information on genetic variations between individuals of a population, among lines or germplasm or any breeding material.

CONCLUSION

The present study was conducted to determine with the aimed to determine the genetic diversity among the wheat hexaploid grown in Pakistan using RAPD molecular markers. Our results revealed that the wheat cultivars SH-2002 and AARI-11 are the more diverse genotypes from the other while Millat-11, Punjab-11 and PBW-222 are closely related to SH-2002 and AARI-11 present in the PCoA. From cluster data it was revealed that Millat-11, Punjab-11 and PBW-222 are the more diverse genotypes and standing alone in the cluster as compared to others. SH-2002 and AARI-11 is making group with each other in one group while FD-83, Inqalab-91 and Minthar-03 is in the second group. RAPD markers also showed diversity among the wheat cultivars by PIC values range (78-87 %). So, finally we can conclude that RAPD markers are a good technique by which we can determine the genetic diversity among and within the wheat cultivars.

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