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DNA LANDMARKS FOR GENETIC RELATEDNESS AND DIVERSITY ASSESSMENT IN PAKISTANI WHEAT GENOTYPES USING RAPD MARKERS

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Abstract

DNA profiles from 10 Pakistani wheat genotypes were evaluated for diversity assessment based on RAPD markers. A total of 79 DNA fragments were generated by 10 RAPD primers, with an average of 7.9 bands primer^{-1}. Of these, 64 fragments (81%) were polymorphic among 10 genotypes. Genetic diversity was evaluated *via* UPGMA cluster analysis by constructing dendrogram, which were used for the calculation of similarity coefficients between these genotypes. The greatest similarity (95%) was observed between PR-94 and PR-95, whereas PR-96 with PR-90 showed the lowest similarity (60%). Adoption of this technology would be useful to the plant protection regulatory systems, especially for plant variety identification and registration of new plant varieties, breeding programs and protection purposes.

Introduction

Wheat is the world's most vital cultivated crop and primary staple food of Pakistan. Total area under wheat is 8358 thousand hectares with a total production of 21612.3 thousand tones and an average yield of 2586 kg per hectare (Anon., 2004-2005).

Varieties have been a landmark in the genetic improvement of wheat, as it resulted in increase in its potential for grain yield. Information about genetic diversity and genetic relatedness among elite material is fundamental element in plant breeding (Zhu *et al*., 2000). Cultivar identification is useful for describing a new cultivar, testing genotype purity and speeding up DUS (distinctness-uniformity-stability) test for candidate cultivar (Chan & Susan, 1997). For acquiring Plant Breeder's Rights (PBR), varieties of agricultural importance have to be tested for Distinctness (D), Uniformity (U) and Stability (S) (DUS testing) (Ardley & Hoptroff, 1996). Evaluation of genetic diversity in wheat has been on differences in morphological and agronomic traits or pedigree information (Bernard *et al*., 1998). New varieties have to be shown to be distinct from all existing varieties by the expression of at least one characteristic. As the number of registered varieties increases over time, it becomes increasingly difficult to compare efficiently each newly submitted variety against all existing varieties and this often requires the varieties to be grown to full maturity (Kim & Ward, 2000).

Molecular biology techniques are used for varietal identification, differentiation between species and in resolving many breeding problems. Recently genetic fingerprinting techniques have been used for diversity assessment that rely on simple sequence repeat (SSR), Restriction fragment length polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) and amplified fragment length polymorphism DNA (AFLP) analysis. The use of RAPD for identification of genotypes through DNA fingerprinting is the current method of choice in measuring genetic variation within genotypes (Hernandez *et al*., 2001). The RAPD markers are dominant markers (Chalmers

et al., 2001) and due to simplicity and speed, RAPD technique has been used for diversity analysis in several crops (Zenglu & Randall, 2001). The study of genetic diversity to identify groups with similar genotypes is important for conserving, evaluating and utilizing genetic resources for studying the diversity of pre-breeding and breeding germplasm and for determining the uniqueness and distinctness of the phenotypic and genetic constitution of genotypes with the purpose of protecting a breeder intellectual property rights (Franco *et al*,. 2001).

The main objective of our research was to evaluate the potential of molecular markers (DNA profiling) to assess the genetic diversity and relatedness among 10 Pakistani Wheat genotypes based on RAPD markers and to develop an optimized and efficient operational system for their use.

Materials and Methods

The plant material: A total of 10 Pakistani wheat genotypes were used in this study. A detailed description of the materials used in present investigation is given in Table 1.

Genomic DNA extraction: Total genomic DNA was extracted from dry seed of wheat genotypes according to the method described with minor modifications (Kang *et al*., 1998). Purity and concentration of DNA was monitored spectrophotometrically at a wavelength of 260 and 280 nm using Nanophotometre (IMPLEN, Germany). All the DNA samples were diluted to a working concentration of 20 ng/µl and were used for PCR amplifications.

Primer selection and RAPD analysis: In total, 26 decamers of oligonucleotides from Operon technologies Inc. (Alameda, California, USA) and geneLink were tested as single primers to identify the most promising detectable polymorphisms. After an initial screening, 10 primers (Table 2) were chosen on the basis of their ability to detect the polymorphisms and production of the reliable and scorable banding patterns in wheat genotypes. Sixteen primers failed to amplify the DNA from some genotypes, therefore 10 primers could be used to show the clearly and consistently banding patterns. The best RAPD amplification pattern of each genotype was ultimately chosen for data compilation and examination of the genetic diversity and relationship.

RAPD Amplification: RAPD analysis was performed following the previous protocol with minor modification (Williams *et al*., 1990). Amplification reactions were carried out in a volume of 20 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate (dNTP), 0.4 µM of 10-mer primer (with modification, Operon inc.), 1 unit DNA polymerase (AmpliTaq Gold) and 20 ng of template DNA. Amplifications were performed in a MyGeneTM Series Peltier thermal Cycler (Uniquip, Germany). Thermal cycler was programmed to 1 cycle of 5 min., at 94ºC for initial strand separation. This was followed by 40 cycles of 1 min., at 94ºC for denaturation, 1 min., at 36ºC for annealing and 2 min., at 72ºC for primer extension. Finally, 1 cycle of 10 min., at 72ºC was used for final extension, followed by soaking at 4ºC. The reproducibility of the amplification products was checked twice for each primer.

After amplification, the aliquots of 15 µl of PCR products plus loading dye were loaded into 1.5% agarose gels for electrophoresis in 1xTBE (10 mM Tris-Borate, 1 mM EDTA) buffer containing 0.5 µg ml^{-1} of Ethidium bromide. The 1 kb DNA ladder plus (Fermentas) was used as a molecular size marker. After electrophoresis, the gels were documented using an Alpha Imager HP system (Alpha Innotech, USA).

Table 1. Wheat lines/ Variety and there breeding centre/ institute.

Sr. #	Wheat lines/Variety	Breeding centre/Institute
1.	MSH-14	Nuclear Institute of Agriculture, Tandojam
2.	EWM-9313	Nuclear Institute of Agriculture, Tandojam
3.	Kiran-95	Nuclear Institute of Agriculture, Tandojam
4.	GA-2002	BARI, Chakwal
5.	5-Sarsabz	Nuclear Institute of Agriculture, Tandojam
6.	PR-90	CCRI, Pirsabak, Nowshera
7.	PR-94	CCRI, Pirsabak, Nowshera
8.	PR-95	CCRI, Pirsabak, Nowshera
9.	PR-96	CCRI, Pirsabak, Nowshera
10.	5C-034	BARI, Chakwal

Table 2. List of primers used.

Sl. No.	Primer's name	Sequence (5'~3')
1.	OPJ-13	CCACACTACC
2.	GLE-03	GATGACCGCC
3.	OPK-12	TGGCCCTCAC
4.	0PF-17	AACCCGGGAA
5.	OPJ-10	AAGCCCGAGG
6.	OPK-02	GTCTCCGCAA
7.	OPK-08	GAACACTGGG
8.	OPK-15	CTCCTGCCAA
9.	OPA-15	TTCCGAACCC
10.	OPA-10	GTGATCGCAG

Table 2. Primers used for generating RAPDs in Wheat genotypes from Pakistan.

Sl. No.	Primer's name	Sequence (5'~3')	Amplified Fragments (a)	Polymorphic fragments (b)	Percent polymorphism (b/a x 100)	Fragment size (bp)
1	GLE-03	GATGACCGCC	6	5	83	250 - 1300
2	OPJ-13	CCACACTACC	7	4	57	250 - 2000
3	OPK-12	TGGCCCTCAC	4	3	75	250 - 2000
4	0PF-17	AACCCGGGAA	14	13	92	180 - 1300
5	OPJ-10	AAGCCCGAGG	10	9	90	250 - 2000
6	OPK-02	GTCTCCGCAA	9	7	77	400 - 2000
7	OPK-08	GAACACTGGG	11	10	90	250 - 1500
8	OPK-15	CTCCTGCCAA	6	5	83	250 - 1500
9	OPA-15	TTCCGAACCC	4	2	50	400 - 1000
10	OPA-10	GTGATCGCAG	9	6	66	200 - 1400
Total			**79**	**64**	**81**	

Data analysis: Each DNA fragment amplified by a given primer was treated as a unit character and the RAPD fragments were scored as present (1) or absent (0) of the primer-genotype combinations. Since DNA samples consisted of a bulk sample of DNA extracted from 2~3 seeds, a low intensity for any particular fragment may be explained by the lesser representation of that particular sequence in the bulk sample. Therefore, the intensity of the bands was not taken into account and the fragments with the identical mobility were considered to be the identical fragments. Only major bands consistently

amplified were scored and faint bands were not considered. The molecular size of the amplification products was calculated from a standard curve based on the known size of DNA fragments of a 1 kb plus molecular size weight marker. Pair-wise comparisons of the cultivars based on the presence or absence of unique and shared amplification products were used to generate similarity coefficients. Estimates of genetic similarity (F) were calculated between all pairs of the cultivars according to Nei & Li (1979) based on following formula:

$$\text{Similarity (F)} = 2N_{ab}/ (N_a + N_b)$$

where N_a = the total number of fragments detected in individual 'a'; N_b = the total number of fragments shown by individual 'b' and N_{ab} = the number of fragments shared by individuals 'a' and 'b'.

The resulting similarity coefficients were used to evaluate the relationships among the genotypes with a cluster analysis using an unweighted pair-group method with arithmetic averages (UPGMA). The analysis was plotted in the form of a dendrogram. All computations were carried out using the NTSYS-pc, Version 2.2 package (Rohlf, 2005).

Results

DNA amplification: The genetic diversity and the relationships among wheat genotypes were evaluated by RAPD markers using 26 primers. Fig. 1 shows the amplification profiles generated with the primer OPF-17 across 10 wheat genotypes. A considerable level of variability was observed among different genotypes. The genotype 5-sarsabz displayed unique pattern in comparison to all other genotypes (Fig. 1).

Among the 26 decamer oligonucleotide primers, 10 were chosen. There were 7 primers that did not amplify the DNA in some of the cultivars, 6 primers had resulted in faint bands and 2 primers gave monomorphic banding patterns, hence, they were eliminated from the analysis. Each of the remaining 10 primers varied greatly in their ability to resolve variability among genotypes. Some primers generated several markers, while others generated only a few. A total of 79 reproducible and scorable amplification products were generated across 10 genotypes (Table 2).

The number of amplification products generated by each primer varied from 4 (OPA-15) to 14 (OPF-17) with an average of 7.9 fragments primer^{-1}. A total of 64 (81%) polymorphic bands were observed ranging from 2 to 12 fragments primer^{-1}. The primer OPF-17 gave the highest number of polymorphic fragments (13), while the minimum number of polymorphic bands (2) using OPA-15 primer. The average number of polymorphic fragments per primer among the 10 wheat genotypes was 6. The size of the amplified fragments ranged from 180 to 1500 bp. The study showed that most of the wheat genotypes genetically resembled each other. PR-96 and PR-90 were distinct from all other genotypes.

Similarity matrix: A similarity matrix based on the proportion of shared RAPD fragments was used to establish the level of relatedness between the wheat genotypes. Pair-wise estimates of similarity ranged from 0.60 to 0.95 (Table 3) Genotypes PR-94 and PR-95 were the closest genotypes with the highest similarity index of 95%. The lowest level of similarity (60%) was obtained between PR-96 and PR-90.

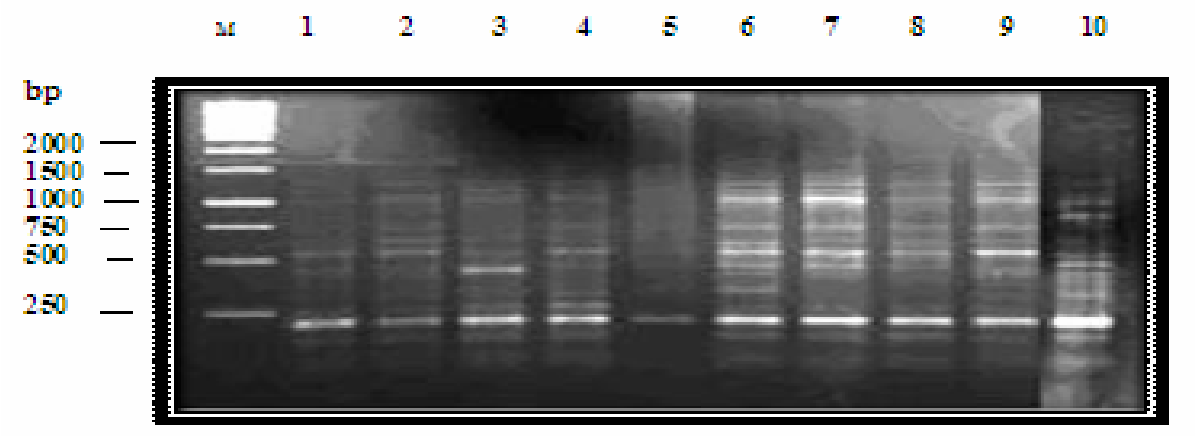

Fig. 1. RAPD amplification profile generated across 10 wheat genotypes using primer OPF-17; M= Marker, 1= MSH-14, 2= RWM 9313, 3= Kiran-95, 4= GA-2002, 5= 5-Sarsabz, 6= PR-90, 7= PR-94, 8= PR-95, 9= PR-96, 10= 5C-034

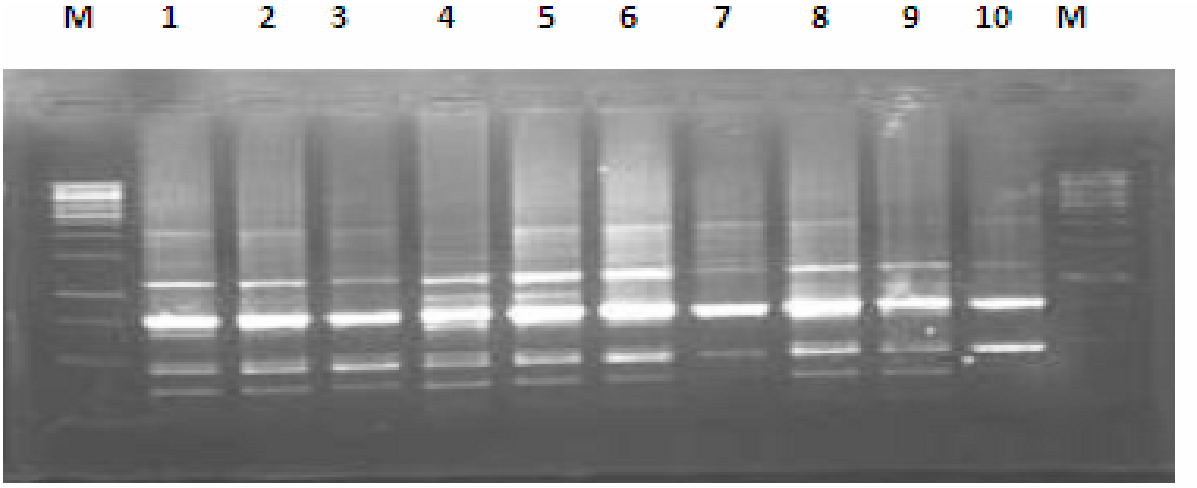

Fig. 2. RAPD amplification profile generated across 10 wheat genotypes using OPJ- 13 marker; M= Marker, 1= MSH-14, 2= RWM 9313, 3= Kiran-95, 4= GA-2002, 5= 5-Sarsabz, 6= PR-90, 7= PR-94, 8= PR-95, 9= PR-96, 10= 5C-034

Table 3. Similarity matrix of wheat genotypes from Pakistan.

Rows/Cols	MSH-14	EWM-9313	Kiran-95	GA-2002	5-Sarsabz	PR-90	PR-94	PR-95	PR-96	5C-034
MSH-14	1.00									
EWM	0.82	1.00								
Kiran-95	0.89	0.80	1.00							
GA-2002	0.80	0.72	0. 86	1.00						
5-Sarsabz	0.81	0.69	0.78	0.84	1.00					
PR-90	0.68	0.70	0.70	0.73	0.69	1.00				
PR-94	0.78	0.80	0.81	0.85	0.81	0.83	1.00			
PR-95	0.77	0.79	0.79	0.85	0.83	0.85	0.95	1.00		
PR-96	0.72	0.72	0.73	0.69	0.61	0.60	0.74	0.72	1.00	
5C-034	0.80	0.69	0.80	0.78	0.81	0.62	0.78	0.75	0.65	1.00

Cluster analysis: Genetic similarities obtained from RAPD data were used to generate a cluster diagram. Cluster analysis based on Nei and Li's similarity coefficients using UPGMA grouped 10 genotypes into 2 main clusters I, II (Fig. 3). The major cluster comprised 9 genotypes MSH-14, Kiran-95, RWM, GA-2002, PR-94, PR-95, 5-Sarsabz, 5C-034 and PR-90. A second cluster comprised only PR-96, which is by far the most dissimilar genotype tested and is 60% related to other genotypes tested in our study.

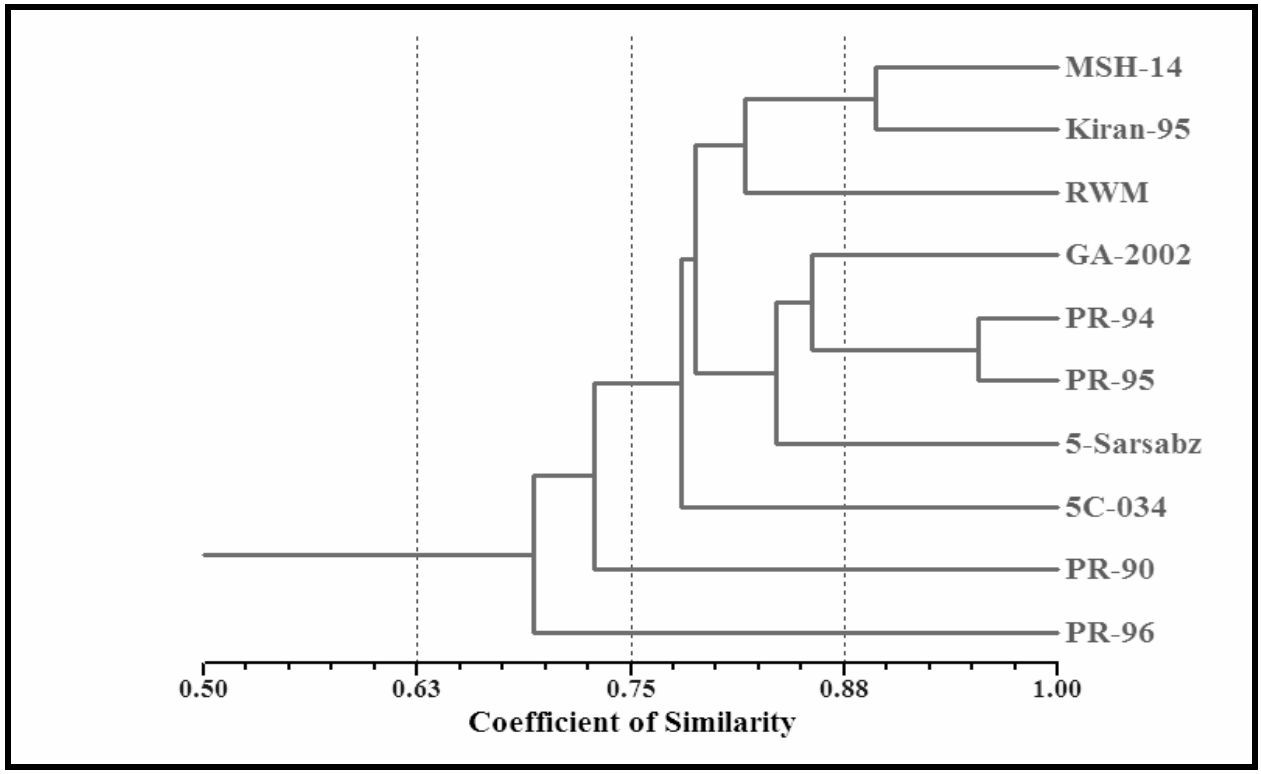

Fig. 3. UPGMA cluster analysis showing the relationship and diversity among wheat genotypes from Pakistan.

Discussion

In the present research, RAPD markers were employed to assess the genetic diversity among 10 wheat genotypes from Pakistan. RAPDs analysis produced a number of bands that were shared among the genotypes. RAPD analysis was quite effective in determining the genetic variation among wheat genotypes and can be used to generate DNA fingerprinting profiles for variety identification (Nebulsi *et al*., 2001).

The clustering of the genotypes might be due to the selection from a single population; the same was observed with other wheat genotypes (Mukhtar *et al*., 2002). Overall broad genetic base was found, with 60% to 95% similarity among the wheat genotypes.

Present study strengthen earlier reports that Randomly Amplified polymorphic DNA can be used for estimation of genetic diversity in crop improvement programs (Czaplieki *et al*., 2000) for cultivars identification (Hu & Quirose, 1991) and for the DNA fingerprinting of genomes (Welsh & McClelland, 1990) and tagging of genes (Kelly *et al*., 1993).

Conclusion

The RAPD assay generated variety-specific products in some of the genotypes. These may be used as DNA fingerprints for variety identification. It would be of immense use for the establishment of proprietary rights and the determination of genotypes purity.

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