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GENETIC VARIABILITY ESTIMATION IN WHEAT USING RANDOM AMPLIFIED POLYMORPHIC DNA BASED MARKERS

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Favorable variation within the genome is an important aspect to modify the crops. A huge range of molecular markers are available to dissect the genome of different crops. The data generated by these markers is more reliable as compare to pedigree analysis. To evaluate the diversity of durum genotypes at molecular level, almost 40 RAPD primers were selected on the basis of their polymorphism reported in previous studies. These markers are cost effective and minute quantity of DNA is required for this analysis. In this study, DNA extraction protocol was modified for maximum recovery of DNA from small samples. Both local and exotic wheat accessions were obtained from different sources for this study. On the basis of similarity matrix the genotypes were distributed into three groups. This classification was also in agreement with the already reported pedigree data of these genotypes. Inqilab-91 (9436) and PKV-1600 (9247) proved to be genetically different as compare to other genotypes. A huge amount of dissimilarity between the genomes was also observed which make these accessions an important tool for enhancing breeding of certain characters. Shortly the characterization of genetic variation among plants specie can lead to widen the selective breeding and genome wide analysis.
Keywords: Markers, diversity, Wheat, RAPD, Genetic similarity.

INTRODUCTION

Wheat is the basic staple food which is most widely produced in modern world. Today's wheat has undergone several genetic modifications which resulted in its increased production and made it invulnerable to different harsh environmental conditions. In Pakistan, the average yield of wheat is about 2883 kilogram per hectare (Economic Survey of Pakistan, 2018-19) and area under wheat crop reduced by 0.6 percent (8,740,000 hectares as compared to 8,797,000 hectares last year).The decrease in areas is due to shifting of farmers from wheat to other crops (FAO Statistics, 2019). Understanding the genomic arrays of wheat with the help of genetic markers can enhance its breeding suitability and can also lead to increased production (Anna *et al*., 2005; Abbas *et al*., 2008). The variations and similarities of the genome is the basic need for breeding program (Gashaw *et al*., 2007; Naghavi *et al.,* 2009; Colomba and Gregorini, 2011). Not only different cereal crops have been made resistant to different disorders due to genomic dissimilarity for a number of years, many incoming selective breeding tasks also depend upon these variations. Therefore, the knowledge about genomic variations is of great importance for high yielding and biotic and abiotic stress tolerant crop varieties (Buerstmayer, 2002). Research work on genetic diversity studies in durum wheat has already been reported by Al-Fares and Abu-Qaoud (2012) and Abouzied *et al*. (2013).

Understanding of evolutionary relationship between varieties of a specific plant species also depends on the knowledge of patterns of variations. Conventionally the differences among a number of wheat varieties were based on their structure and ancestral information (Gegas *et al*., 2010). Currently the focus is to get knowledge about the molecular variation that exists among different crop cultivars. A number of genome arranging methods are used for this purpose utilizing different molecular markers. Markers including Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) Amplified Fragment Length Polymorphism (AFLP) etc. are used according to the objectives and type of variation we want to determine. RAPD DNA markers are extensively used for amplification of random non coding sequence within genome (Zhu *et al*., 2000; Schuster *et al*., 2006; Babu *et al*., 2014). RAPD molecular markers have been used in many crops to identify the variations due to their simple, quick and easy use (Demek *et al*., 1996; Liu *et al*., 2003; Bertini *et al.,* 2006). The RAPD markers based on the polymerase chain reaction (PCR) are also of great importance which are used to identify different

characteristics associated with particular gene locus (Edward, 2017). The knowledge about the genetic diversity of wheat is the basic requirement of genetic modification for enhanced traits. Diverse genotypes are prerequisite for new varieties. Novel variety can be pure as well as a hybrid (Whitford *et al.*, 2013). With the help of distance and similarity estimation of different parent varieties new cultivars have been developed with better and enhanced traits. Wheat breeding via hybridization additionally requires the choice of diversified genotypes, irrespective of whether or not the stop product is a pure line or a hybrid variety. New varieties which have resulted from the crossing over between existing varieties are basically used to measure the diversity (Weising *et al*., 1995; Baird *et al*., 1997; Dje *et al*., 2000).

Genomic variance for ramification of structural characteristics is further analyzed by cluster analysis method of evaluation (Gaudreau, 2013) but this process is affected by the trending morphology. Therefore, genetic markers are a better option that are not affected by other environmental conditions and are widely available. RAPD and RFLP are two important methods to detect diversity at low level in wheat (Khan *et al*., 2000). Although other molecular markers like SSR are also widely present in nature and have the potential to detect more genetic variations (Shen *et al*., 2004; Yildirim *et al*., 2009; Zhang *et al*., 2005; MacRitchie and Sun, 2004). Molecular markers used for the analysis of genetic diversity are an effective method in a number of crop plants including wheat.

MATERIALS AND METHODS

***Plant material and DNA extraction*:** A RAPD analysis was carried out to estimate the genetic variation among commercially grown lines (Table-1). Genomic DNA was extracted with improved extraction method (Han *et al*., 2012). For extraction of DNA seeds were germinated in dark and at 3 leaf stage they were crushed in liquid nitrogen. Extraction buffer (200mM Tris-HCl (pH 7.5), 250mM NaCl,25mM EDTA, 0.5%SDS) was added in tube (400µL) and mixed at maximum speed with mixer for 15min. Placed at room temperature for 15 min and centrifuged at 15000rpm for 10 min. Ice cold isopropanol (300µL) was added in the supernatant and mixed gently. Centrifuged at 15000rpm for 10 min. Pellet was separated and 70% ethanol was added. Centrifuged at 15000rpm for 5 min. Supernatant was discarded and pellet was dried at room temperature. Tris EDTA buffer was added to the pellet and stored at 4°C. DNA was quantified on spectrophotometer (Bio-mate 3), at absorbance of 260/280nm. DNA quantity was further analyzed on agarose gel.

Table-1. Wheat genotypes/accessions with their codes

Sr. No.	Genotype	Code
1	Sarsabz	9444
2	Kiran-95	9877
3	Marvi-2000	8121
4	Bhitai	9452
5	Khirman	9479
6	ESW-9525,	9428
7	Abadgar 93	9189
8	Inqilab-91	9247
9	SARC-1	9459
10	PKV-1600	9436
11	Chakwal-86	9481
12	CM 24/87	9451

Table- 2. List of RAPD primer used for the estimation of genetic diversity in wheat genotypes.

Sr. No	Primer Name	Sequence	Sr. No	Primer Name	Sequence
1	GL DecamerB-07	GGTGACGCAG	21	GL Decamer J-04	CCGAACACGG
2	GL DecamerB-10	CTGCTGGGAC	22	GL Decamer J-05	CTCCATGGGG
3	GL DecamerB-11	GTAGACCCGT	23	GL Decamer J-06	TCGTTCCGCA
4	GL DecamerB-13	TTCCCCCGCT	24	GL Decamer J-13	CCACACTACC
5	GL DecamerB-17	AGGGAACGAG	25	GL Decamer J-14	CACCCGGATG
6	GL DecamerD-06	ACCTGAACGG	26	GL Decamer J-19	GGACACCACT
7	GL DecamerD-12	CACCGTATCC	27	GL Decamer J-20	AAGCGGCCTC
8	GL DecamerD-13	GGGGTGACGA	28	GL Decamer K-07	AGCGAGCAAG
9	GL DecamerD-19	CTGGGGACTT	29	GL Decamer K-11	AATGCCCCAG
10	GL DecamerD-20	ACCCGGTCAC	30	GL Decamer K-13	GGTTGTACCC
11	GL Decamer I-02	GGAGGAGAGG	31	GL Decamer K-15	CTCCTGCCAA
12	GL Decamer I-05	TGTTCCACGG	32	GL Decamer K-17	CCCAGCTGTG
13	GL Decamer I-06	AAGGCGGCAG	33	GL Decamer K-18	CCTAGTCGAG
14	GL Decamer I-07	CAGCGACAAG	34	GL Decamer K-19	CACAGGCGGA
15	GL Decamer I-09	TGGAGAGCAG	35	GL Decamer K-20	GTGTCGCGAG
16	GL Decamer I-10	ACAACGCGAG	36	GL Decamer L-07	AGGCGGGAAC
17	GL Decamer I-11	ACATGCCGTG	37	GL Decamer L-09	TGCGAGAGTC
18	GL Decamer I-15	TCATCCGAGG	38	GL Decamer L-10	TGGGAGATGG
19	GL Decamer I-17	GGTGGTGATG	39	GL Decamer L-16	AGGTTGCAGG
20	GL Decamer I-20	AAAGTGCGGG	40	GL Decamer L-19	GAGTGGTGAC

PCR with random primers: Forty primers (Table-2) were used to amplify the DNA. For PCR reaction mixture of 25 µL contained 1 µL (20 ng) of genomic DNA template, 2.5 µL of 10x PCR buffer, 2.5 µL of dNTPs (dATP, dCTP, dGTP, dTTP), 3.8 µL $MgCl_2$, 3.5 µL of 5 picomoles of a single 10-base primer (Operon Technologies, Inc. Alameda, USA), 2.5 µL of gelatin, 0.2 µL of (5 U/µL) *Taq* DNA polymerase (Promega, catalog # M2661) and double distilled and sterile water was added to make up the final volume of 25 µL.

PCR was used for DNA amplification in a thermal cycler (Eppendorf AG No. 533300839). The PCR conditions were optimized for proper amplification of RAPD fingerprints, hot start at 95˚C for 5 min (initial denaturing step), 40 cycles of denaturation at 95˚C for 1 min, annealing at 36˚C for 1 min and extension at 72˚C for 2 min and final extension at 72˚C for 10 min.

To separate the DNA fragments according to their charge to mass ratio, 10 µL of PCR product was electrophorased in 1.2% agarose gel containing 3.0 µL of ethidium bromide (EtBr) solution (10mg of EtBr/100mL of H_2O) in TBE buffer and visualized under UV light using UV transilluminator (Model - M-20E Upland-CA 91786 USA).

***Data analysis*:** Data were scored from photographs of good quality amplifications. The counting pattern of bands was from top to the bottom of each lane. All bands whether visible or unambiguous were counted and gave the name of total score able fragments. Amplifications of all the accessions were compared with each other and bands of DNA fragments if present were scored as (1) or (0) if absent. Coefficient of similarity among cultivars was calculated according to Nei & Li's (1979). Cluster analysis was performed on the basis of similarity estimates using the unweighted pair-group method with arithmetic means (UPGMA). Each primer was evaluated on the basis of data recorded for total amplified bands and number polymorphic bands out of total bands amplified. POPGEN version-1.1 was used for the estimation of (ANOVA) molecular variance among genotypes.

RESULTS AND DISCUSSION

Twelve elite wheat varieties were analyzed after rigorous selection for different quality traits. Forty random and polymorphic primers were used for amplification of repetitive sequence (Figure-1). The PCR amplified data was also used for generation of dendrogram (Figure-2) differences among the genotypes have arranged them into three different groups. The most distinct group was formed by genotype 9247 and 9436. Two other genotypes (9444 and 9189) also showed variation and were unable to form group. Remaining genotypes were sorted out into three distinct groups constituting genetically similar accessions. While calculating these PCR amplified scores several factors were analyzed such as scattering effect between two different fragments. It was observed that the fragments of a particular part was present on the same chromosome and recombination was absent in them therefore they were linked to first loci irrespective of the reasoning of fragment formation.

The effectiveness of these methods can be enhanced with multiple factors such as primer assembly, amount and quality of the template DNA, type of PCR machines and concentration of polymerase enzyme (Hernendez *et al*., 2001). All the primers used in this study were already reported to be polymorphic (Figure 3-7) the potential ability of these molecular markers can also be improved by following their standard protocol. For measuring the genetic similarity of wheat varieties more than one analysis should be used i.e. multivariate analysis was also performed via matrix of similarity (Nei and Li, 1979). On the basis of information obtained by these similarity estimates and dendogram wheat genotypes were further classified. The twelve different varieties were initially screened for their salt tolerance ability and the results obtained through field experiments were further supported by the molecular data generated after statistical analysis.

pop ID	1	2	3	4	5	6	7	8	9	10	11	12
1	****	0.8356	0.7397	0.5890	0.6575	0.6575	0.6438	0.6438	0.6986	0.7260	0.6986	0.6164
2		****	0.8493	0.6986	0.7671	0.7671	0.7260	0.6986	0.7534	0.7808	0.7534	0.6712
3			****	0.7397	0.8630	0.8356	0.8219	0.7123	0.8493	0.8493	0.8493	0.7671
4				****	0.7397	0.7123	0.6986	0.6986	0.6986	0.6986	0.7260	0.6438
5					****	0.8082	0.7397	0.6849	0.7671	0.7671	0.7863	0.7397
6						****	0.7945	0.7397	0.7671	0.7671	0.7371	0.6849
7							****	0.7260	0.7808	0.8082	0.8356	0.7534
8								****	0.7808	0.7534	0.7260	0.6986
9									****	0.8630	0.8630	0.7534
10										****	0.8630	0.7808
11											****	0.8630
12												****

Figure 1. Genetic similarity estimates of 12 wheat genotypes using 40 polymorphic RAPD primers.

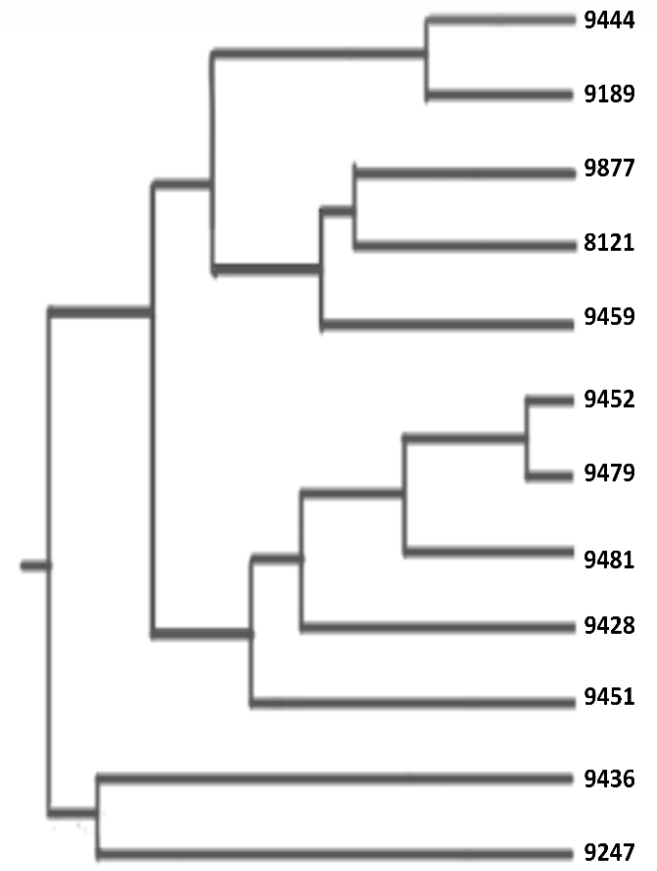
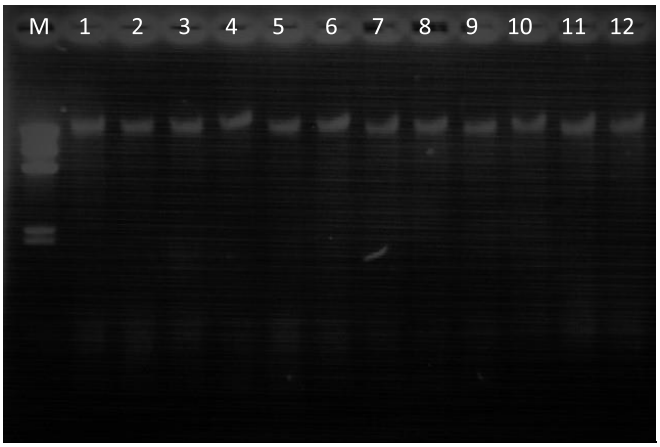


Figure 2. Unweighted pair group method with arithmetic mean (UPGMA).

Genotypes such as 9444 and 9189 were genetically similar and shared seventy seven percent similar genetic makeups. The next group that showed ninety percent genetic similarity contained 9481, 9428 and 9479. The findings in the research discussed above may prove to be another step towards the molecular dissection of the *Triticum* genome. As RAPD is most cost effective marker for its utilization in similarity/diversity estimates of commercially important crop like durum wheat, results may be made reproducible by using more precise protocols for PCR analysis. Characterization of genotypes at molecular level was made quite easy with the invention of molecular markers without assumptions those were critical for statistic based tedious and time consuming pedigree analysis (Brar, 2002; Michael and Appels, 2002). Prior sequence information is not necessary for diversity estimates using these markers which make its use more popular in crops where little sequence information is available (Williams *et al*., 1990; Michelmore *et al*., 1991; Wolfe and Liston, 1998; Babu *et al*., 2014; Edward, 2017)



S. No.	Accession No.	S. No.	Accession No.
1	8121	7	9451
2	9189	8	9452
3	9247	9	9459
4	9428	10	9479
5	9436	11	9481
6	9444	12	9877

Figure 3. Quality of wheat accession DNA used for diversity studies.

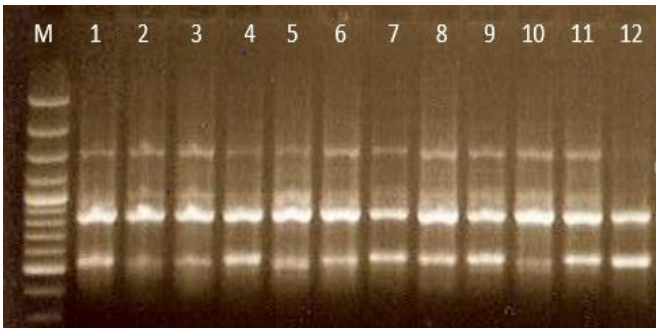


Figure 4. Wheat specific RAPD primer B-07, Lane M: DNA ladder (1kb) Lanes 1-12 PCR amplification of wheat accession DNA.

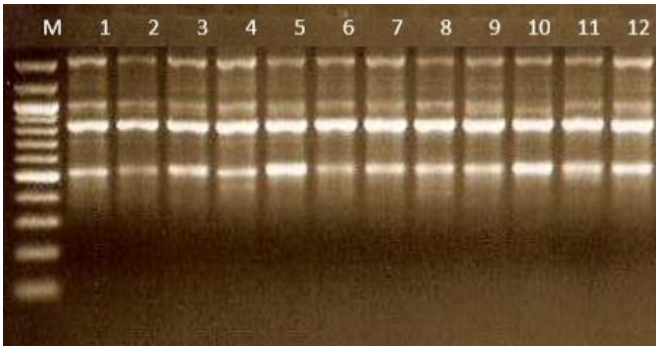


Figure 5. Wheat specific RAPD primer D-12, Lane M: DNA ladder (1kb) Lanes 1-12 PCR amplification of wheat accession DNA.

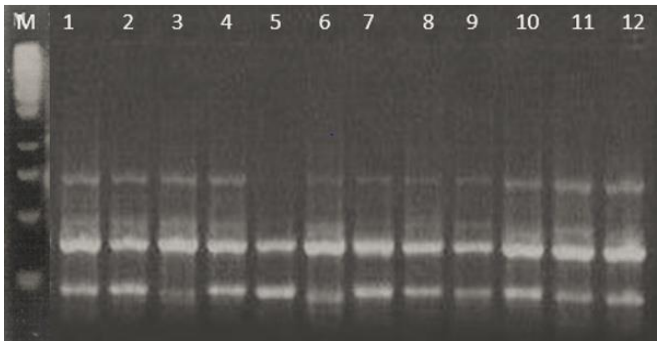


Figure 6. Wheat specific RAPD primer I-07, Lane M: DNA ladder (1kb) Lanes 1-12 PCR amplification of wheat accession DNA.

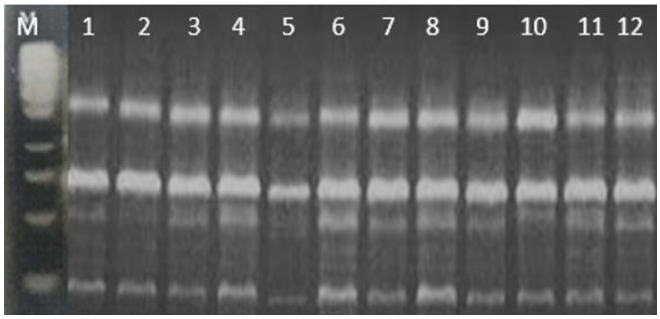


Figure 7. Wheat specific RAPD primer B-17, Lane M: DNA ladder (1kb) Lanes 1-12 PCR amplification of wheat accession DNA.

***Conclusion*:** The data obtained in this study confirmed the efficiency of RAPD as a valuable DNA marker to assess the genetic variability among different plant genotypes. In addition to genetic variability, genotypes may also be evaluated for geographical and morphological relationships using these markers. The data produced during this study using RAPD markers can be helpful to develop strategy for future breeding programs for wheat development. Further, unique RAPD sequences may be used for the development of sequence characterized amplified region (SCAR) markers to eliminate the chances of low reproducibility for evaluation of genotypes.

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