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Molecular characterization of wheat (*Triticum aestivum* L.) genotypes using ISSR marker system

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Abstract

A study was conducted to examine the genetic diversity of 14 wheat (*Triticum aestivum* L.) genotypes, using 10 ISSR (Inter-simple sequence repeats) markers. A total of 74 alleles were produced and majority of ISSR markers showed a high level of polymorphism. The polymorphic information content (*PIC*) values ranged from 0.32 to 0.81 with an average of 0.56. The results showed that the number of polymorphic bands per assay unit was 6.6 for ISSR markers and the average number of the loci per assay unit was 7.4. The expected heterozygosity (H_{ep}) obtained was 0.56. The estimates of the effective multiplex ratio (6.58) and marker index (3.29) were observed for the ISSR marker system. Therefore, ISSRs could be suitable for studying genetic diversity among wheat genotypes because of their ability to produce more number of bands. A dendogram discriminated the 14 wheat genotypes into 4 clusters. Results indicated that wheat cultivars had high genetic diversity that can be used in wheat breeding programs. More primers should be used for saturation of different regions in further studies.

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Introduction

Wheat (*Triticum aestivum* L.) is the world's prime cereal crop which is cultivated widely across the globe. Wheat is one of the most abundant sources of energy and protein for the world population. Wheat production increased to 25,286 thousand tons in 2013-14 in Pakistan (Anonymous, 2014).

Loss of genetic diversity has become a problem for agriculturally important species like wheat. Ancient cultivars or landraces and wild relatives of domesticated species are being vanished as modern varieties become adopted by farmers. This had led to appeal for genetic conservation of crop germplasm (Frankel and Bennett, 1970). The use of molecular markers for the estimation of genetic diversity is very common. Assessment of genetic variation level among accessions is essential for germplasm conservation and based on phenotype that posses restriction (Fufa *et al*., 2005). In contrast, molecular markers based on DNA sequence polymorphisms are independent from environmental regimes and can be estimated using DNA from any developmental stage (Tatikonda *et al*., 2009). Molecular markers offered a brilliant tool for attaining genetic information and also their application has been enhanced in the assessment of genetic divergence in wheat (*Triticum aestivum* L.) over the last few years (Jin *et al*., 2008; Al-Doss *et al*., 2009). Molecular markers are valuable supplements to physiological categorization of cultivars because they are abundant, independent of tissue and ecological effects, and permit cultivar documentation early in plant development (Barakat *et al*., 2010). Molecular representation of genotypes is also beneficial to evaluate potential genetic erosion, defined as a decrease of genetic diversity (Manifesto *et al*., 2001). Application of molecular markers and polymorphic nucleotide sequence reformation disseminated during the genome have provided for evaluating genetic diversity (Gostimsky *et al*., 2005). Inter simple sequence (ISSR) are one of the DNA based markers which are extensively used in various areas of plant research. Najaphy *et al*., (2012) showed that for evaluation of genetic variability of wheat genotypes through ISSR markers have provided adequate polymorphism and reproducible fingerprinting profile. The aim of our study was to evaluate the polymorphic information content of 10 ISSR markers, genetic variation among 14 wheat genotypes and selection of parents to develop high-yielding cultivars in breeding programs.

Materials and methods

14 different genotypes of bread wheat (*Triticum aestivum* L.) were obtained from Institute of Agriculture Biotechnology and Genetic Research (IABGR), National Agriculture Research Centre, (NARC), Islamabad, Regional Agriculture Research Institute, Bahawalpur and sown in polythene bags in the green house of Department of Plant breeding and Genetics, Bahauddin Zakariya University, Multan. They were planted in two factor factorial complete randomized design (CRD).

Table 1. Name of accessions along with their origins.

Sr#	Name of accession/ variety	Origin
1	011822	Pakistan
2	011823	Pakistan
3	011936	Pakistan
4	011938	Pakistan
5	011886	Pakistan
6	Fareed-06	Pakistan
7	011890	Pakistan
8	011903	Pakistan
9	011905	Pakistan
10	011935	Pakistan
11	9383	Local
12	013165	Local
13	11B-2043	Local
14	Blue-Silver	Pakistan

DNA electrophoresis

The DNA of 14 genotypes of wheat was extracted from the leaf tissues which were taken from young leaves of eight week old plants. Sterilized scissor was used to cut the healthy portion of youngest leaf of the tiller then washed with distilled water (to remove spore of microorganisms and any other source of foreign DNA) and dried on tissue paper. By using CTAB method genomic DNA of 14 wheat genotype were extracted with the help of following steps:

2-3 pieces of leaf tissues were cut and grinded in pester and mortar with 1ml CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1990). 750µl solution was taken in eppendorf tubes and incubated in hot water bath at 65°c for 45 minutes.

About 900µl chloroform (chloroform-isoamyl alcohol = 24:1, v/v) was added. The samples were centrifuge for 10 minutes at 13000rpm. Supernatant was transferred into new eppendorf tubes, and chilled isopropanol was added to 60% of this solution. This final solution was kept at -70°c for 10 minutes. The mixture was again centrifuge for 10 minutes at 13000rpm and removed supernatant. The pellets were washed with 200µl (70%) ethanol. The samples were again centrifuge at 13000 rpm for 5 minutes. Ethanol was discarded and DNA pellets were allowed to dry for overnight. After that pellets were dissolved in 150µl water and finally stored the DNA samples at -20°c.

DNA quantity and quality estimation

DNA concentration was estimated spectrophotometrically using 0.8% agarose by gel electrophoresis which was stained with ethidium bromide. DNA concentration was calculated using following formula:

$$\text{Concentration of DNA } \mu l/ml = \text{OD at } 260 \times 50 \times DF$$

In TAE buffer samples were loaded on 0.8% agarose gel to examine the quality of DNA.

PCR amplification

A set of ten ISSR primers of UBC (University of British Columbia) series were used for evaluation of genetic diversity among 14 wheat genotypes. Master mix was prepared from the original stock. PCR of 20µl reaction volume was carried out using 1µL of genomic DNA as template, 2µL of 10X PCR buffer, 2.5 µl of 25mM $MgCl_2$, dNTPs (mM) 1.0 µl, 2µL of Primer (pM/µl) and Taq DNA Polymerase (µ/µl) 0.2 µl. PCR was carried out in (MyCycler, Bio Rad, USA) thermal cycler using following profile *i.e.* first denaturation step of 94°C for 5 min followed by 40 cycles of 1 min at 94°C, 1 min at 52°C and 2 min at 72°C and final extension at 72°C for 10 min. The master mix was distributed in the PCR tubes in equal volume. Then required amount of DNA (desired) was added in each PCR tubes. Spin all the PCR tubes for short interval. Place the PCR tubes in thermo cycler machine.

The PCR reaction was carried out using master cycle gradient (Table 5). After PCR completion, the products were stored at 4°C until the gel electrophoresis was done.

Analysis of PCR products by Agarose Gel Electrophoresis

2g agarose added in 100ml of TBE buffer and kept in oven until it was completely dissolved to make final solution transparent. It was cooled for some time and 3µl ethidium bromide was added in the flask, and poured it in gel apparatus after inserting comb in the trough. Sufficient care was taken while pouring, not allowing the air bubble to trap in the gel. The gel was allowed to solidify and after placing the solidify gel into the electrophoresis apparatus so as to cover the well completely, the comb was removed. The amplified products were carefully analyzed before loading the sample in gel apparatus, and 2µl of dye was added in each sample. The samples were loaded cautiously in wells. Electrophoresis was done at 80 volt constant current. Under UV transilluminator ethidium bromide stained DNA bands were viewed and photographed for documentation.

Gel scoring and data analysis

The bands generated by the ISSR were visually scored. Each band was considered as single allele and scored present as (1) or absent (0) for each of ISSR loci to generate the binary data. Similarity matrix based on Nei's coefficients (Nei, 1972) were developed using binary data of EST-SSR and ISSR markers. The NTSyspc 2.0 software was used to construct dendrograms of each marker system using unweighted-pair group method of arithmetic means (UPGMA).

PIC (polymorphic information contents) of ISSRs

PIC (polymorphic information contents) of each primer pair was calculated (Prevost and Wilkinson, 1999; Sharma *et al.*, 2009a).

$$PIC = 1 - j = 1 - \sum_{P} 2\,IJ$$

$P_{ij}$is the frequency of *jth*allele for primer i

Table 2. ISSR markers and their sequence detail.

Sr#	Primer	Sequence (5' -3')
1	UBC-807	AGAGAGAGAGAGAGA GT
2	UBC-808	AGAGAGAGAGAGAGA GC
3	UBC-809	AGAGAGAGAGAG AGA GG
4	UBC-810	GAGAGAGAGAGAGAG AT
5	UBC-811	GAGAGAGAGAGAGAG AC
6	UBC-812	GAGAGAGAGAGAGAGAA
7	UBC-813	CTCTCTCTCTCTCTCTT
8	UBC-815	CTCTCTCTCTCTCTCTG
9	UBC-816	CACACACACACACACAT
10	UBC-817	CACACACACACACACAA

Results and discussion

Analysis of genetic diversity by ISSR marker system

To characterize and identify the fourteen wheat varieties 10 ISSR primers were used (Table 2). For identification of these varieties the amplification results by PCR with 10 ISSR primers indicated distinctive differences. A total of 74 DNA fragments were present, whereas 66 fragments were polymorphic and 8 fragments were monomorphic. Therefore, out of 74 DNA fragments 72.7% were polymorphic. These results are related to Deif *et al*., (2013) who identified 112 amplified DNA fragments of which 17 fragments were monomorphic (15.2%) and 95 fragments were polymorphic (84.8%). For each primer the number of DNA fragments varied from 4 (UBC-809) to 11 (UBC-808). When each primer was used alone PCR analysis succeeded in distinctive most of the studied wheat varieties in a unique binding pattern. To analyze the genetic diversity in wheat varieties, the higher rate of polymorphism was revealed by ISSR markers indicated that the method is efficient. For detecting DNA polymorphism in wheat variable efficiencies of different marker system has been reported. Najaphy *et al.* (2012) observed that for evaluating genetic diversity of wheat genotypes ISSR markers provide sufficient polymorphisms and reproducible fingerprint profiles. Carvalho *et al.* (2009) observed 12.9 polymorphic bands per primer using 8 ISSR primers in 48 wheat accessions. To reveal genetic diversity in case of wheat high level of polymorphisms among the wheat genotypes using ISSR, indicating high efficiency of the marker technique.

The lowest level of polymorphisms (72.7%) present in UBC-808 primer and highest polymorphism value (100%) was observed within the UBC-807, 809, 811, 816, 817 primers (Table 4). The *PIC* values differed between 0.32 and 0.81. Highest *PIC* value was revealed by primer UBC-815 which showed that this primer have more ability to reveal allelic variation and had more tendency to discriminate/distinguished between two genotypes (Sharma *et al*., 2009a). In many genetic diversity studies the *PIC* index has been used widely (Thudi *et al*., 2011). Among all wheat varieties similarity values showed substantial differences (Table 6).

Similarity matrices

The genetic similarity ranged from 0.53 to 0.88 with an average of 71%. High genetic similarity was observed between 9383 and Fareed-06 (0.88%) where as low value of genetic similarity (0.53) was present between 013165 and 011936 (Table_6). The higher genetic distance between 9383 and Fareed-06 showed that they are diverse compare to low genetic distance value. Higher genetic value is more dissimilar then low genetic value. Molecular finger printing can be used to distinguish varieties from one another. Most of the primers that have been used in this study revealed a high polymorphism thus can be used for screening, evaluation of genetic diversity and molecular mapping studies in bread wheat. In general, diversity measurements were higher in the cultivars, such a high level of genetic similarity may be used for the selection of the material in the breeding programs and cultivars with high genetic distance can also be used for this Purpose. It can be conclude that more polymorphic wheat ISSR markers could be used for efficient screening of the germplasm by saturating more regions of the wheat genome (Table 6).

Marker Indices

The effectiveness of marker system is its capability to distinguish multiple polymorphisms amongst gene pool (Powell *et al*., 1996)**.** In this study ISSR markers were used for the analysis of genetic diversity among wheat genotypes. However, the parameters used to examine the strength of ISSR marker system showed the important differences between these marker techniques.

The results showed that the number of polymorphic bands per assay unit was 6.6 for ISSR markers. The average number of the loci per assay unit was 7.4 for the ISSR markers. The expected heterozygosity (H_{ep}) for the ISSR was 0.56. The estimates of the effective multiplex ratio (6.58) and marker index (3.29) were observed for the ISSR markers. This distinct nature of ISSR markers might be due the huge number of alleles generated by each primer than on the existence of allelic heterozygosity amongst genotypes (Maras *et al.*, 2008) (Table 5).

Cluster analysis for ISSRs

To resolve the genetic association among genotypes based on the allele cluster analysis was performed detected by ISSR marker. A dendogram classified the 14 wheat genotypes into 4 clusters (Fig. 2). The first cluster contain genotypes 011822, 011823, Fareed-06, 9383 and 11B-2043 and the genetic similarity between genotypes 011822, 011823 and Fareed-06, 9383 were 0.76 and 0.74. Second cluster include 011886, 011890 and Blue silver and genetic similarity between 011886 and 011890 was 0.77. Third cluster contain 011930 and 011905 with genetic similarity 0.80. Cluster four contains genotypes 011935, 011936 and 011938. The genetic similarity between 011935 and 011938 was 0.85. Ijaz and Khan-(2009) classified the 63 genotype into three clusters. Salem *et al.* (2008) showed the cluster analysis of seven wheat varieties into two major clusters and three sub cluster. Cluster analysis categorized the individuals on the base of the similarity present among them for characteristics they have. In dendogram cluster analysis result represents a number of dissimilar groups with the same entries. Within the same groups individuals are similar but there are significant differences along with the group (Finsten, 1996) (Fig. 2).

Table 3. ISSR markers used amplified products and analysis of genetic diversity of wheat genotypes.

Primer	Total amplified band	No. of monomorphic band	No. of polymorphic bands	Percentage of polymorphic bands	Polymorphism information content (*PIC*)
UBC-807	10	-	10	100	0.55
UBC-808	11	3	8	72.7	0.55
UBC-809	4	-	4	100	0.38
UBC-810	10	1	9	90	0.77
UBC-811	5	-	5	100	0.50
UBC-812	7	1	6	85.7	0.75
UBC-813	6	1	5	83.3	0.32
UBC-815	8	2	6	75	0.81
UBC-816	6	-	6	100	0.55
UBC-817	7	-	7	100	0.46
Total	74	8	66		
Minimum	4	1	4	72.7	0.32
Maximum	11	3	10	100	0.81
Average	7.4	0.8	6.6		

Table 4. Marker indices for ISSR marker system.

Indices with their abbreviations	Units	ISSR marker system
Number of assay unit	U	10
Number of polymorphic bands per assay	n_p/U	6.6
Number of loci	L	74
Number of loci per assay unit	n_u	7.4
Expected heterozygosity of polymorphic loci	H	0.56
Fraction of polymorphic bands	B	0.89
Effective multiplex ratio	E	6.58
Marker Index	MI	3.29

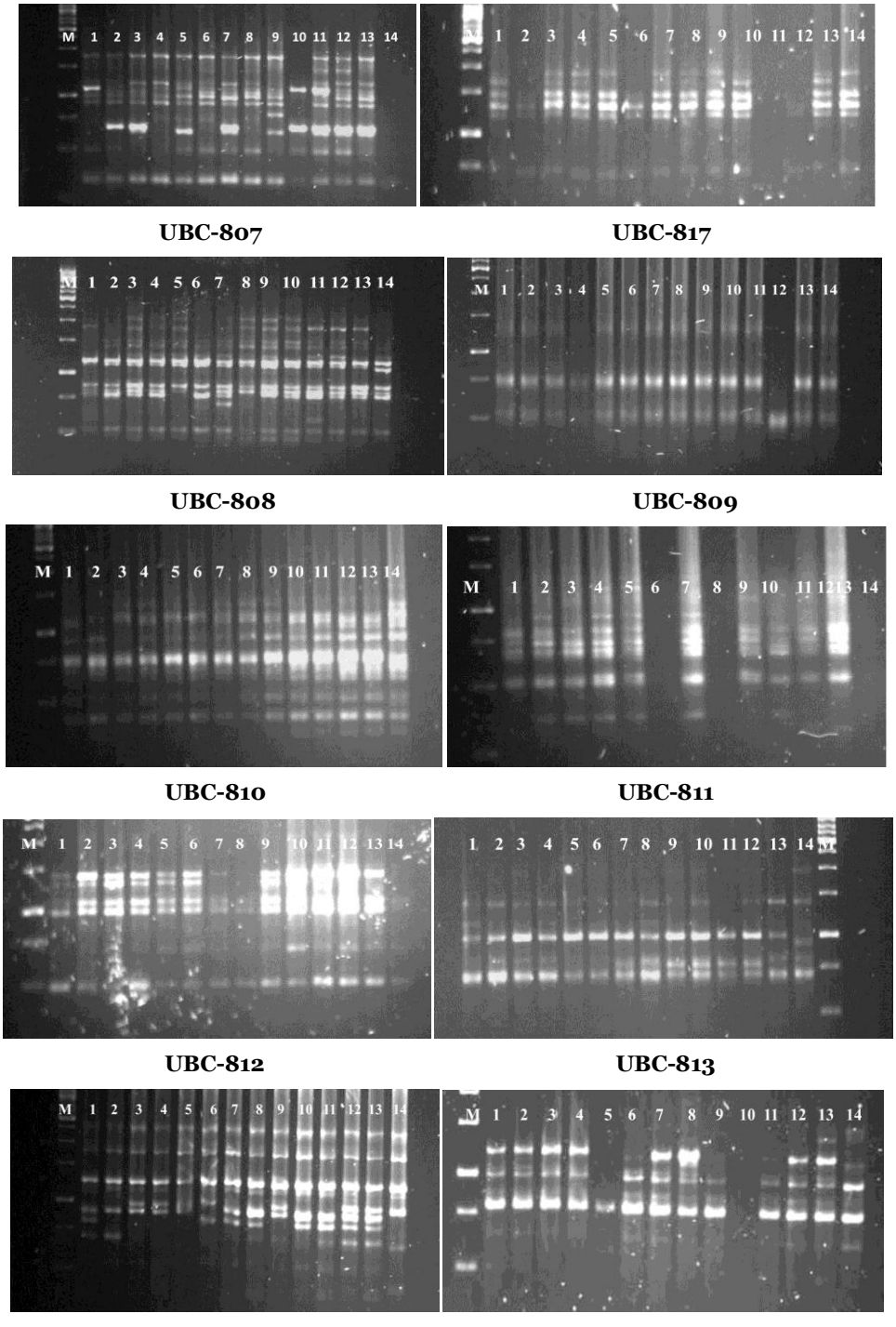

Fig. 1. Electrophoresis profiles of PCR products by using UBC-807, UBC-808, UBC-809, UBC-810, UBC-811, UBC-812, UBC-813, UBC-815, UBC-816, UBC-817 ISSR primer for 14 wheat varieties.

Table 5. Genetic similarity percentages of 14 wheat varieties based on ISSR band patterns.

	011822	011823	Fared-06	9383	11B-2043	Blue Silver	011886	011890	011903	011905	011935	011936	011938	013165
011822	1													
011823	0.8	1												
Fareed-06	0.81	0.84	1											
9383	0.77	0.83	0.88	1										
V11B-2043	0.73	0.80	0.80	0.79	1									
Blue silver	0.70	0.76	0.74	0.72	0.75	1								

	011822	011823	Fared-06	9383	11B-2043	Blue Silver	011886	011890	011903	011905	011935	011936	011938	013165
011886	0.73	0.75	0.80	0.76	0.76	0.75	1							
011890	0.72	0.70	0.76	0.74	0.69	0.76	0.77	1						
011903	0.73	0.69	0.80	0.73	0.73	0.69	0.65	0.69	1					
011905	0.66	0.65	0.70	0.64	0.69	0.59	0.64	0.68	0.80	1				
011935	0.69	0.70	0.68	0.64	0.66	0.68	0.64	0.62	0.74	0.76	1			
011936	0.68	0.69	0.69	0.68	0.62	0.66	0.68	0.66	0.70	0.69	0.85	1		
011938	0.70	0.69	0.74	0.73	0.62	0.66	0.70	0.72	0.70	0.69	0.74	0.81	1	
013165	0.61	0.57	0.62	0.55	0.55	0.59	0.61	0.57	0.64	0.57	0.57	0.53	0.66	1

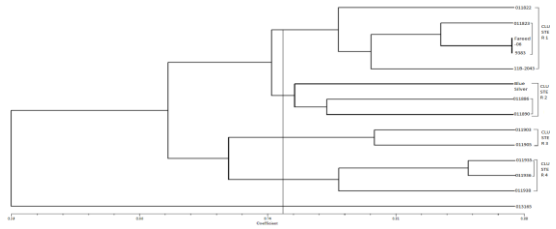

Fig. 2. Dendogram for 14 wheat germplasm showing the genetic similarity.

Conclusion

10 ISSR markers were used for analysis of genetic diversity among 14 wheat genotypes. The *PIC* value ranged from 0.32 to 0.81. Highest *PIC* value was revealed by primer UBC-815 which showed that this primer have more ability to reveal allelic variation and had more tendency to discriminate/distinguished between two genotypes. *PIC* values also showed significant positive correlation with number of alleles and allele's size. A total of 74 fragments were detected. The genetic distance clearly separated 14 wheat genotypes into four distinct clusters. The genotype belonging to same cluster is genetically more or less similar and genotype belonging to different cluster is genetically different from each other. Among the various currently available DNA based markers in the present study, the set of microsatellite markers are used, provide a positive assessment of the ability of ISSR marker in producing unique DNA profiles and establishing direct identity of wheat genotypes which otherwise were not possible using morphological traits. ISSRs could be suitable for studying genetic diversity among wheat genotypes because of their ability to produce more number of bands. This makes genomic divergence estimates a possibly valued predicting source for selecting diverse parent genotypes for favorable heterotic combinations in a wheat improvement program.

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