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Assessment of Molecular Diversity Using ISSR Markers for Characterization of Wheat Varieties

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ABSTRACT: In the present investigation, ten wheat (*Triticum aestivum*) varieties with varying end-product quality were analyzed to assess the genetic diversity using inter simple sequence repeat (ISSR) primers. Ten ISSR primers produced a total of 74 bands out of which 71 were polymorphic with 93.92 % polymorphism. Primer AY46 generated the maximum number (13) of bands while primer AY31 produced minimum bands (9). The Similarity matrices of genotypes were varied from 0.63-0.86. Dendrogram cluster analysis based on similarity indices were constructed and classified the genotypes into two diverse groups.

KEYWORDS: Genetic diversity, ISSR, Polymorphism, End-product quality, Cluster analysis, genotypes.

I. INTRODUCTION

Wheat possesses the prime importance worldwide amongst all cereal crops due to the dependence of huge population for consumption, area under cultivation, production and trading in international market. Wheat is a cereal grass which belongs to the genus *Triticum* and family *Poaceae* (*Gramineae* previously). The species *Triticum aestivum* is grouped into diploids ($2n=2x=14$), tetraploids ($2n=4x=28$) and hexaploids ($2n=6x=42$) genotypes. *Triticum aestivum* is composed of three distinct genetically related genomes with a total genomic size of 1.7×10^{10} base pairs [1]. Wheat is the most appropriate for producing varied range of products such as breads, flat breads, chapatti, breakfast cereals and cookies etc. because of discriminations in the genetic determinants of genotypes [2-5]. A number of studies have been concentrated on the genetic analysis of wheat quality which are generally governed by the relationship between processing characteristics and wheat storage protein alleles [6, 7]. For the genetic development of the crop plants, the investigation of genetic diversity between genotypes is advantageous. Improvement in the finished product quality has been the ultimate objective for such work in several breeding programs [8]. Sustenance of variation is also a substantial aim of breeding programs by contemplating the significance of genetic diversity in minimizing genetic vulnerability [9]. Assessment of genetic diversity is initial yet prime tool for managing genetic resources. Among the number of various methods used for assessing the genetic diversity, use of DNA markers is the most competent and reproducible method [10]. DNA based molecular markers give the provision of the absolute measure and surpasses the ambiguous diversity measures that lay upon geographic origin and agronomic attributes as well as environmental variation. Application of molecular markers and acknowledgement of polymorphic nucleotide sequences distributed around the genome have administered novel avenues for evaluating genetic diversity and describing the inter and intra specie genetic relationship [11]. To establish the genetic polymorphism in higher plants, ISSR marker system is exquisite in all [12]. The discriminating capacity of ISSR markers was investigated amongst wheat genotypes to establish genetic relationship. Zhu, Hu [13] examined the 27 genotypes of wheat using ISSR markers which consisted bread wheat varieties of India released for quality, yield and abiotic stress and demonstrated that the known origin of genotypes was

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in accordance with the cluster analysis of molecular data. The efficacy of ISSR markers was also illustrated by Khaled, Motawea [14] to determine genetic diversity and discriminate durum wheat cultivars.

Mostly the grain quality traits are administered by Quantitative Trait Loci (QTL) due to their polygenic inheritance. Ultimately, it turns difficult to recognize and isolate the genes regulating these oligo/polygenic characters via traditional procedures of plant breeding. ISSR molecular markers have considerably acknowledged in recent years and have been largely utilized for genetic diversity analysis, phylogenetic and evolutionary investigations, and mapping and tagging of genes of agronomic interest in various plant species. The present study was subjected to investigate the potency of ISSR primers to appraise the genetic diversity among wheat genotypes containing different physicochemical profile for prospective breeding programs.

II. MATERIAL AND METHODS

A. Plant Materials

The present study was carried out at Centre for Plant Biotechnology, Hisar. Total ten wheat varieties viz. C306, WH283, HW2004, WH1025, HI977, WH542, WH147, PBW343, DBW16 and PBW373 were procured from various Wheat Research Directorates, Research Institutes and Agriculture Universities. The varieties were chosen on the basis of diverse array of end-product quality and physicochemical properties [2-5, 15]. **Table 1** is illustrating the pedigree and classification of various Indian wheat varieties. The grains were cleaned and sorted manually. The grains of each variety were grown in pots in the green house under controlled conditions and 10-15 days old fresh leaves sample was used for DNA extraction.

Table 1: Indian wheat genotypes, their pedigree and classification

Sr. no.	Variety	Pedigree	Wheat
1	C306	REGENT1974/3*CHZ//*2C591/3/P19/C281	Chapatti
2	WH283	HD1981/RAJ821	Bread
3	HW2004	C 306 *7 //TR 380-14 #7 /3 AG14	Cookies/chapatti
4	WH1025	C 591/PBW 231	Chapatti/cookies
5	HI977	GLL/AUST 61.157/CNO/NO 66/3/Y50E/3/KA	Bread
6	WH542	JUP /BJY"S" //URES	Noodle/bread
7	WH147	E 4870/C286/C273 /4/S339/PV18	Bread
8	PBW343	ND/VG1944//KAL//BB/3/YACO'S'/4/VEE#5'S'	Bread
9	DBW16	RAJ 3765/WR 484//HUW 468	Noodle/ bread
10	PBW373	ND/VG1944//KAL/BB/3/YACO'S'/4/VEE#5'S'	Bread

B. DNA extraction

Young leaf tissue collected from 10-15 days plants grown in green house was used for extraction of genomic DNA using the modified cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson [16]. Leaf samples were grind to fine powder in liquid nitrogen using sterilized pestle and mortar. Three to five grams of ground leaf tissue powder was mixed with 20-25 ml of pre-warmed CTAB extraction buffer (65°C) in sterilized 50 ml polypropylene tubes. 400µl β-mercaptoethanol was added to all the samples. The samples were thoroughly mixed with the extraction buffer by inverting the tubes gently several times and were incubated in water bath at 65°C for 60-120 minutes. Contents of the tubes were mixed gently at an interval of 15 minutes by inverting them several times. After incubation, the samples were cooled to room temperature and then 20-25 ml of chloroform: isoamylalcohol (24:1) mixture was added. Samples were mixed by gently inverting the tubes several times. The samples were centrifuged for 15 min. at 10,000 rpm in centrifuge at 4°C. The upper aqueous phase was transferred to a pre-sterilized clean centrifuge tube and again extracted with 10 ml of chloroform: isoamyl alcohol mixture (24:1). The extracted DNA was precipitated with ice cold iso-propanol and kept at -20 °C for 45 minutes and then centrifuge for 10 minutes at 10,000 rpm at 4°C for precipitation of DNA. After that, DNA samples were washed with 70 % ethanol. The DNA samples were dried at room temperature for 2-3 hours and subsequently dissolved in an appropriate volume of TE buffer. DNA samples were then treated with 5 µl of RNase solution (10mg /ml) and incubated in water bath at 37°C for 3-4 hours to

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 6, June 2016

remove RNA contamination from DNA samples. DNA was again extracted using above method and supernatant was transferred to eppendorf tubes. The DNA was then precipitated using 1 vol. of 3M sodium acetate (pH 6.8) and 2 vol. of chilled absolute alcohol followed by centrifugation to pellet down the DNA. Pellet was then washed with 70% alcohol, air-dried and finally dissolved in TE buffer and stored at -20°C till further use.

B. Quantitative and qualitative estimation of DNA

When DNA was completely dissolved in TE, its quality and concentration were checked by running DNA sample on 0.8 % agarose gel, and viewed in a UV- trans-illuminator. The DNA concentrations were rechecked by visual assessment of band intensity in comparison with uncut Lambda DNA of known concentration using 0.8% agarose gel.

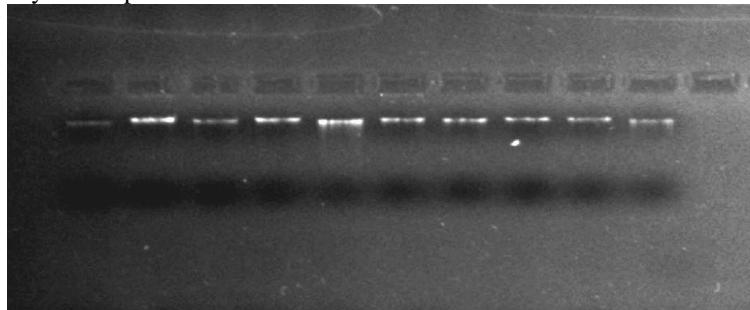


Figure 1 Purified Genomic DNA envisioned on 0.8 % agarose gel.

C. Polymerase chain reaction (PCR) amplification

PCR amplification was carried out in DNA thermal cycler GenePro (BIOER, China). PCR amplification conditions viz. concentration of template DNA, primers, MgCl₂, Taq DNA polymerase and annealing temperature (Tm°C) of primer were optimized initially. Finally the ISSR-PCR reactions were carried out in 25µl of reaction mixture containing 50 ng templates DNA, 250 µM of each of the dNTPs, 1x PCR buffer, 1.5 mM MgCl₂, 0.6 µM of primer and 1.0U of Taq DNA polymerase. A total of 18 ISSR primers (Sigma Aldrich Pvt. Ltd) were screened out of which only 10 primers were amplified, which were then used the assessment of genetic diversity. PCR amplification was performed with an initial denaturation step of 4 min at 94°C, followed by 40 cycles of denaturation (94°C, 45 sec.), annealing (50-56°C, 1 min) and extension (72°C, 2 min) followed by a final extension at 72°C for 7 min and a hold temperature at 4°C at the end of reaction. The amplified products were stored at -20°C till further use.

Amplified DNA fragments were resolved by submerged horizontal agarose electrophoresis in 1.4 % (w/v) agarose gel and visualized by staining with nucleic acid stain (5µl/100ml, gold View Stain, Merck) which was added in the gel and then mixed. Unambiguous and clear amplification profiles of ISSR markers were scored based on presence and absence of bands. Size of amplicons was recorded based on their migration relative to molecular size marker (100 bps ladder and 1 kbps ladder).

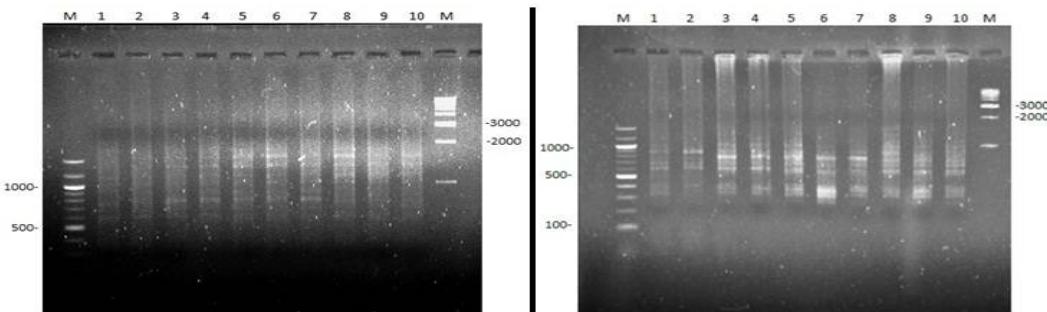


Figure 2 ISSR banding profile of wheat genotypes with primer AY14 and AY46 (Lanes 1-12 M- 100bp DNA ladder; 1-C306, 2- WH 283, 3- HW 2004, 4- WH1025, 5- HI977, 6- WH542, 7- WH147, 8- PBW343, 9- DBW16, 10- PBW373, M- 1kb DNA ladder)

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 6, June 2016

D. Band Scoring and analysis of data

The data obtained by scoring the ISSR profiles with different primers was subjected to the construction of similarity matrix using Jaccard's [17] coefficients. The similarity values were used for cluster analysis. The frequency of polymorphism between different varieties of wheat for each type of marker was calculated based on presence (taken as 1) or absence (taken as 0) of common bands. The 0 /1 matrix was used to calculate similarity genetic distance using 'simqu' sub-program of software NTSYS-PC.

III. RESULTS AND DISCUSSION

E. Diversity analysis

A total of 18 ISSR primers were screened by evaluating their amplification on ten wheat varieties, out of them ten primers evolved markable bands which were used for later analysis. The amplification profile of primer AY14 and AY46 in all wheat varieties are shown in **Figure 2**. Ten selected ISSR primers a total of 74 bands during PCR amplification, amongst them 71 were polymorphic and 3 were monomorphic. The total number of amplified bands varied between 4 and 13 with an average of 7.4 bands per primer. The average polymorphism percentage was 93.92 (**Table 2**). Overall size of PCR amplified products ranged between 100 bp to 3000 bp. Similarly, Abou-Deif, Rashed [18] demonstrated that the eight ISSR primers with 20 wheat distinct varieties evolved 112 amplified DNA fragments ranging in size from 127 to 1857 bp, in which 17 fragments were monomorphic (15.2%) and 95 fragments were polymorphic (84.8%) with an average of 11.87 polymorphisms per primer.

Under this study, the maximum numbers of 13 bands were amplified using primer AY46 and minimum of 4 bands were obtained from primer AY31. The percentage of polymorphism was least for primer AY46 (69.23 %) followed by AY45 (70 %) while 100 % polymorphism was observed for rest of the primers (AY2, AY3, AY5, AY11, AY13, AY14, AY28 and AY31). The genetic variability and yield stability were successfully tested by Motawea, Said [19], in order to better understand the relationship among genotype in 20 wheat varieties using phenotypic and ISSR markers under drought conditions. The percent of polymorphism among varieties ranged from 25 to 80% with an average of 52.08%.

Table 2: The oligo names and the sequences of primers used for ISSR assay including number of Total bands (TB), number of polymorphic bands (PB), and percentage of polymorphism (PP)

Sr. no.	Oligo Name	TM	Sequence	Molecular weight range (bp)	TB	PB	PP
1	AY2	35	CACACACACACAAC	800-1500	5	5	100
2	AY 3	35	CACACACACACAGT	900-3000	7	7	100
3	AY 5	37	GTGTGTGTGTGTCC	100-1200	9	9	100
4	AY 11	52.1	CACACACACACACAG	300-1000	6	6	100
5	AY 13	54.9	ACACACACACACACAG	800-2000	6	6	100
6	AY 14	57.1	ACACACACACACACAG	600-1700	8	8	100
7	AY 28	52.1	CACACACACACACACAG	600-1200	6	8	100
8	AY 31	49.8	AGAGAGAGAGAGAGAGYT	1000-1500	4	4	100
9	AY 45	50.6	CACACACACACACACARG	100-2000	10	7	70
10	AY 46	57.1	ACACACACACACACACYG	300-2000	13	11	69.23
Total					74	71	--
Average					7.4	7.1	93.92

Some primers produces unique banding pattern. AY2 differently produced two bands of 2500 bp and 3000 bp, respectively. Banding patterns of primers AY45 (100 bp and 200 bp) and AY46 (500 bp and 2000 bp) evolved monomorphic bands in varieties C306 and PBW373. On the contrary, among all the varieties HW2004 could not amplified 1000 bp band with primer AY2. PBW373 did not amplify the band of 1200 bp with primer AY5 and similar

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 6, June 2016

kinds of results were generated by primers AY11, AY14, AY28 and AY46 with HI977, WH147, HI977 and WH542, respectively.

F. Genetic similarity and cluster analysis

ISSR data generated from PCR amplification of 74 bands was analyzed for cluster analysis based upon similarity coefficients (**Table 3**). Based on ISSR similarity matrix data, the value of similarity coefficient ranged from 0.63 (in between C306 and PBW373) to 0.86 (in between WH1025 and HW2004; and PBW371 and DBW16). The NTSYS-pc UPGMA cluster tree analysis led to the distribution of all the wheat varieties into two major groups at similarity coefficient 0.63, clusters I and II (**Figure 3**). The varieties C306, WH283, HW2004, WH1025, HI977, WH542 and WH147 were clustered under first major group and the second cluster was occupied by PBW373, DBW16 and PBW343.

Table 3 ISSR similarity matrices of wheat varieties

	C306	WH283	HW2004	WH1025	HI977	WH542	WH147	PBW343	DBW16	PBW373
C306	1									
WH283	0.81	1								
HW2004	0.74	0.85	1							
WH1025	0.79	0.85	0.86	1						
HI977	0.73	0.75	0.74	0.82	1					
WH542	0.68	0.79	0.75	0.80	0.79	1				
WH147	0.67	0.78	0.77	0.79	0.78	0.85	1			
PBW343	0.67	0.73	0.68	0.77	0.67	0.71	0.75	1		
DBW16	0.69	0.73	0.70	0.75	0.69	0.70	0.71	0.80	1	
PBW373	0.63	0.68	0.67	0.75	0.69	0.70	0.69	0.80	0.86	1

ISSR primers based cluster tree analysis demonstrated that similarity coefficient of DBW16 with PBW373 and PBW343 depicts the excelling values 0.86 and 0.80, respectively which suggests the close relationship of these varieties whereas C306 and PBW373 were found most diverse among all as reflected from their least similarity coefficient of 0.63. In addition, similarity coefficient of WH1025 with WH283 and HW2004 was observed maximum of 0.85 and 0.86, respectively. The interpretation from the data suggest that the ISSR technique could be applicable for analyzing genetic diversity among closely related varieties which are complicated or challenging to estimate from other techniques. Recently, Abou-Deif, Rashed [18] analyzed the genetic diversity and relationships between 20 wheat genotypes involving hexaploid, tetraploid and diploid varieties by using eight ISSR markers. Similarity matrices results clearly exhibited considerable differences among the wheat varieties, wherein genetic similarity ranging from 47 to 94%, with an average of 71%. The dendrogram based upon UPGMA projected that the ISSR markers successively distinguished most of the 20 wheat varieties in terms of their genetic background and geographical origin.

Choudhary, Kajla [20] investigated to monitor somaclonal variation of banana cv Robusta during micropropagation using 26 RAPD and 20 ISSR primers. These primers concluded the study very well. 108 scorable and different bands were produced by 26 RAPD primers with an average of 4.15 bands per primer and the bands were amplified in a range of 100-1150 bps. A number of 20 ISSR primers 81 diverse bands containing an average of 4.05 bands primers in the range of 100-1000 bps. A lots of similar studies have demonstrated the prominent role of ISSR and RAPD molecular markers for assessing genetic stability and diversity [21]. It has been well intimated that the specific end-use quality wheat are constantly losing its genetic diversity predominantly because of the selection of elaborated high yielding

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 6, June 2016

varieties by farmers for repeated production. Present investigation dispensing an outline of the genetic diversity of hexaploid wheat that would be applicable for the admittance of these varieties in different breeding programs. The accessions with advantageous characteristics and genetically distant from each other can be selected by this data for selective and diverse breeding.

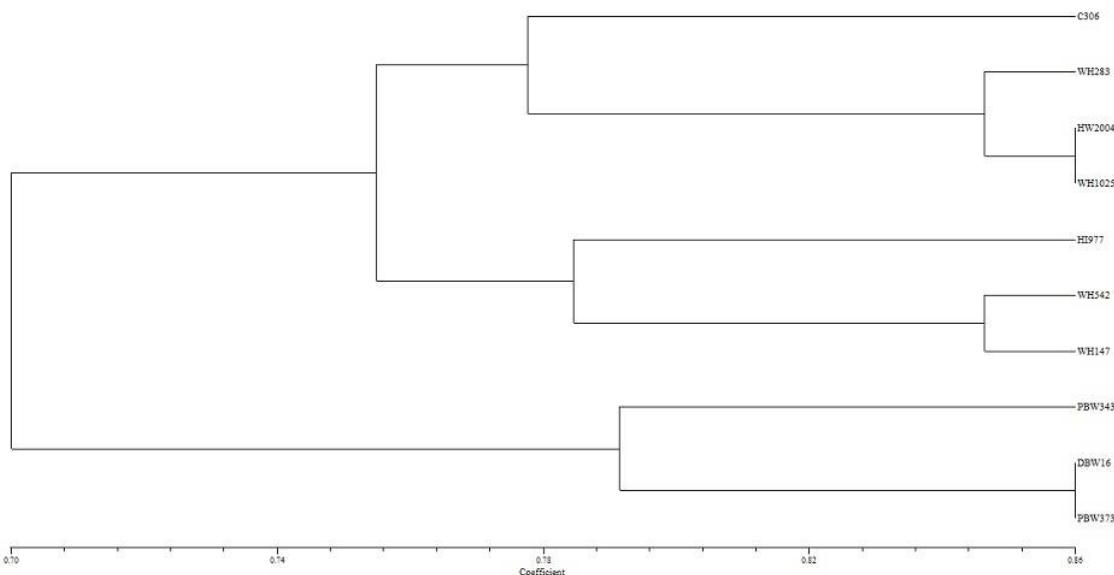


Figure 3 Dendrogram tree clustering pattern using UPGMA

IV. CONCLUSIONS

These results demonstrated that the ISSR markers proves their suitability for DNA fingerprinting and provide necessary information which can be further used to evaluate genetic variation in the wheat genotypes having different purposes to consume. The study also revealed that there is a noticeable genetic diversity among tested genotypes. Similarity matrices and dendrogram tree cluster analysis revealed that the studied varieties have different genetic traits as their pedigree and classification based on physicochemical properties and end-product quality. Eventually, this genetic diversity analysis can be used further for breeding wheat varieties with specific end-product quality.

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International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 6, June 2016

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