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RUST RESISTANCE EVALUATION OF ADVANCED WHEAT (*TRITICUM AESTIVUM* L.) GENOTYPES USING PCR-BASED DNA MARKERS

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Abstract

The most effective and environmental friendly approach for the control of wheat rust disease is the use of resistant genotypes. The present study was conducted to explore rust resistance potential of 85 elite wheat genotypes (36 varieties and 49 advanced lines) using various types of DNA markers like STS, SCAR and SSR. DNA markers linked with different genes conferring resistance to rusts (Leaf rust=Lr, Yellow rust=Yr and Stem rust=Sr) were employed in this study. A total of 18 genes, consisting of eleven Lr (lr1, lr10, lr19, lr21, lr28, lr34, lr39, lr46, lr47, lr51 and lr52), four Yr (yr5, yr18, yr26 and yr29) and three Sr genes (sr2, sr29, and sr36) were studied through linked DNA markers. Maximum number of Lr genes was found in 17 advanced lines and 9 varieties, Yr genes in 26 advanced lines and 20 wheat varieties, and Sr genes in 43 advanced lines and 27 varieties. Minimum number of Lr genes was found in advanced line D-97 and variety Kohinoor-83, Yr genes in wheat variety Bwp-97 and Sr genes in 6 advanced lines and 8 varieties. Molecular data revealed that genotypes having same origin, from a specified area showed resistance for similar type of genes. In this study, an average similarity of 84% was recorded among wheat genotypes. Out of 18 loci, 15 were found to be polymorphic.

Introduction

The rust fungi, a monophyletic group and obligate parasites are well known disease causing organisms in crop plants (Kolmer *et al*., 2009). Stripe (Yellow) rust caused by *Puccinia recondita* Roberge, *Puccinia striifornis* and *Puccinia graminis* Pers is the most damaging disease of wheat crop in the world. The pathogen of the disease had short life cycle but new strains of pathogen severely damage wheat crop (Line & Chen, 1995). Leaf rust caused by *Puccinia* spp., is an epidemic disease that may infect hundreds of Kilometer away from its source plant (Bolton *et al*., 2008).

Wheat, the most important staple food of the world, is cultivated on large scale in Pakistan, contributing approximately 14.4% production in agricultural field. Wheat is a complex plant due to large genome size (16000Mb) with high percentage of microsetallites (~80%) having three types of sub-genomes, namely A, B and D (Gupta *et al*., 2008).

Large numbers of approaches are being utilized to control and eradicate rust disease. Cultivation of disease resistant genotypes is one of best strategies to over come the disease and use of gene "pyramids" or "Stacks" having resistance against most of the local strains of pathogen may increase durability of resistance. The use of DNA markers associated with double haploid technology provides a way for the production of homozygous lines for multiple resistance genes (Mago *et al.*, 2011). Further more, marker assisted selection (MAS) is attaining considerable attention of researchers due to exact transfer of gene of interest and improving parent plant genome (Babu *et al*., 2004; Rasheed *et al*., 2012; Yang *et al*., 2013; Zeb *et al*., 2013).

The present study was conducted to explore the rust resistance potential of advanced wheat genotypes and to assess the genetic relationships, distances and relatedness among these genotypes. The information obtained from the present study will be helpful in accelerating the breeding program in future, including pyramiding of different rust resistance genes in high yielding commercial wheat genotypes.

Materials and Methods

Seeds of eighty five wheat *(Triticum aestivum* L.) genotypes (Table 1) were obtained from Wheat Research Institute AARI Faisalabad and these wheat genotypes were grown at the field area of Agricultural Biotechnology Research Institute, AARI, Faisalabad, Pakistan under natural conditions. Fresh leaves from all genotypes were taken and DNA was extracted using modified CTAB method following Iqbal *et al.* (1997). Quality of DNA was checked by loading and running the samples on agarose gel and then compared with DNA standards. DNA quantification was done by using Nano Drop spectrophotometer (Model-1000 3.3.1) and dilutions of 15ng /µL were made from stock solutions. Dilutions were further confirmed by comparing them with DNA quantification standard on agarose gel. A total of 20 SSR (simple sequence repeats), STS (sequenced tagged sites) and SCAR (sequence characterized amplified regions) primers were used in the present study (Table 2).

Amplification reactions were carried out using following temperature profile:

Step 1=94°C-95°C for 5-7min, (initial denaturation)
Step 2= 94°C-95°C for 1min (denaturation)
Step 3=45°C -65°C for 1min (annealing)
Step 4=72°C for 1min (extension)
Repeat steps 2-4 for 35-45 cycles
Step 5=72°C for 7-10 min (final extension)

Table 1. List of advanced wheat (*Triticum aestivum* L.) lines evaluated for their rust resistance potential, during the present study.

S. No.	Genotype	S. No.	Genotype	S. No.	Genotype	S.No.	Genotype
1.	5C003	22.	V-3138 (Lasani)	43.	Wada	64.	C-228
2.	5C009	23.	V-4188	44.	Rohtas-90	65.	C-217
3.	5C011	24.	V-4022	45.	Shakar-95	66.	C-250
4.	5C034	25.	V-4178	46.	Punjab-96	67.	C-271
5.	6C001	26.	AS-02	47.	MH-97	68.	C-273
6.	6C002	27.	Inqlab-91	48.	Kohistan-97	69.	Dirk
7.	6C005	28.	Sehar-06	49.	Uqab-2000	70.	Mexipak
8.	6C006	29.	Uqab	50.	Chenab-2000	71.	Chenab-70
9.	2KC050	30.	Pasban-90	51.	Iqbal-2000	72.	Baxani-70
10.	99FJ03	31.	Tw-76001	52.	SH-02	73.	SA-42
11.	32862	32.	Tw-76002	53.	Bhakkar	74.	Blue silver
12.	45006	33.	Tw-76003	54.	D-97	75.	Lyallpur-73
13.	56123	34.	Tw-76004	55.	GA-02	76.	Sandal
14.	66205	35.	Tw-76005	56.	D-04611	77.	Potowar
15.	99BT022	36.	Tw-76006	57.	Shafaq-06	78.	Pari-73
16.	Fareed-06	37.	Tw-76007	58.	Fsd-83	79.	Sa-75
17.	Punjnad-1	38.	Tw-76008	59.	V-04067	80.	Punjab-85
18.	Manthar-03	39.	Tw-76009	60.	V-03079	81.	Lu-26S
19.	Bwp-97	40.	Tw-76010	61.	V-03094	82.	WL-711
20.	Bwp-2000	41.	Shalimar-86	62.	C-518	83.	Punjab-81
21.	V-1489	42.	Pak-81	63.	C-591	84.	Barani-83
						85.	Kohinoor-83

Table 2. List of primers, linked gene and amplified product size.

S. No.	Molecular marker	Linked gene	Locus	Product size	Reference
1.	Lr-1	Lr1	5DL	570bp	Feuillet *et al*., (1995)
2.	Lr-10	Lr10	1A	310bp	Feuillet *et al*., (2003)
3.	Lr-28	Lr28	4AL	378bp	Blaszczyk *et al*., (2004)
4.	Lr-47	Lr47	7AS	380,450bp	Helguera *et al*., (2000)
5.	Scs-253	Lr19	7DL	736bp	Gupta *et al*., (2006)
6.	Scs-265	Lr19	7DL	130bp	Gupta *et al*., (2006)
7.	S30-13l	Lr51	1S	783bp	Helguera *et al*., (2005)
8.	Sts 7-8	Yr5	1B	500bp	Murphy *et al*., (2009)
9.	Stm773-2	Sr36	2B	199bp	Tsilo *et al*., (2007)
10.	Ksud-14	Lr21	7DL	669bp	Huang *et al*., (2001)
11.	Xwmc-44	Lr46/Yr29	1BL	242bp	Suenaga *et al*., (2001)
12.	Xwmc-477	Sr36	2B	190bp	Tsilo *et al*., (2007)
13.	Xbarc-352	Lr34/Yr18	1BL,7DS	250bp	Suenaga *et al*., (2003)
14.	Xgwm-11	Yr15/Yr26	1B,1BS	213bp	Ali *et al*., (2010)
15.	Xgwm-44	Yr18	7DS	178bp	Imtiaz *et al*., (2004)
16.	Xgwm -234	Lr52	5BS	250bp	Singh *et al*., (2010)
17.	Xgwm-259	Lr46/Yr29	1BL	105bp	Bariana *et al*., (2007)
18.	Xgwm -296	Lr39	2DS	145bp	Raupp *et al*., (2001)
19.	Xgwm -429	Sr36	2B	211bp	Jin *et al*., (2007)
20.	Xgwm- 533	Sr2	3B	120bp	Spielmeyer *et al*., (2003)

Agarose 2% and Polyacrylamide Gel Electrophoresis (PAGE) 0.8% were used to determine and confirm size of amplification products. Data on the presence and absence of DNA fragments of specific size was recorded. Only scoreable reported bands were considered for genetic diversity analysis. The scoring was started from lower band (small fragment size) and continued to upper band (large fragment size).

Each DNA fragment/band was considered single loci for genetic diversity analysis. Similarity matrix was generated following Nei & Li's (1979) coefficients. Software package NTSyspc 2.0 was used for the construction of dendrogram using unweighted pair group method of arithmetic means (UPGMA).

Results

Maximum number of Lr genes was found in 17 advanced wheat lines and 9 wheat varieties Yr genes in 26 advanced lines and 20 varieties and Sr genes in 43 advanced lines and 27 varieties. Minimum number of Lr genes was shown by advanced wheat line, D-97 and wheat variety Kohinoor-83, Yr genes by one variety Bwp-97 and Sr genes by 6 advanced wheat lines and 8 varieties. The genotypes showing complete absence of Lr, Yr and Sr genes are listed (Tables 3, 4, 5).

The dendogram analysis revealed ten clusters among wheat genotypes. Cluster A comprise of eight genotypes and further subdivided into two sub clusters i.e., A1 and A2 having 2 and 6 genotypes respectively (Fig. 1). Cluster B consist of 31 genotypes, among these 29 genotypes are 100% similar and two are only 5% genetically different from other cluster members. This shows that this group of genotypes has same set of rust resistance genes. Cluster C comprised of five genotypes out of which four are 100% similar to each other and remaining one is share 95% similar genes which have been explored in the present study, Cluster D comprised of 18 genotypes which can be further sub divided into five sub-clusters which are only 10% different from each other. Cluster E comprised of 5 genotypes which fall in two sub-clusters. Whereas clusters F, G and H have three genotypes each and have maximum genetic differences of 15% from each other. Cluster I has four genotypes which has maximum genetic distance of 20%. Last cluster i.e., J has five genotypes which has maximum genetic distance from rest of the clusters and genotypes (Fig. 1).

Table 3. Genotypes showing absence of Leaf rust resistance genes (Lr).

S. No.	Primer name	Genotype name	Lr genes
1.	Lr1	5C003, 5C034, Bwp-97, V-4022, Inqlab-91, C-518, C-591, C-217, Mexipak, Chenab-70, Wl-711, Barani-83, Kohinoor-83	-ve
2.	Lr10	V-1489, V-3138 V-4188, V-4022, Tw-76007, Blue silver, Sandal	-ve
3.	Lr28	V-4022, Tw-76002, Tw-76008, Chanab-2000, D-97, Sa-42 Sandal, Kohinoor-83	-ve
4.	Lr47	Tw-76007, Punjab	-ve
5.	Scs253, Lr19	V-1489, V-4178, As-02, Tw-76005, Tw-76008, Pak-81, Wada, Mh-97, Uqab-2000, Chanab-2000, D-97, V-04067 C-518, C-25, Sa-42, C-273, Mexipak, Baxani-70, Sa-42, Potowar, Sa-75, Lu-265, Barani-83	-ve
6.	Ksud14,Lr21	Manthar-03, V-3138, V-4188, Tw-76002, Tw-76005, Wada, Sh-0 Bahakar 2, D-97 Mexipak, Chenab-70, Potowar, Punjab-81, Kohinoor-83	-ve
7.	Xgwm296, Lr39	45006, Tw-76002, Wada, V-04067, Kohinoor-83	-ve
8.	S-30-13l, Lr51	All genotypes showed resistance against leaf rust (Lr51)	+ve
9.	Xgwm-234, Lr52	6C002, GA-02, C-250,	-ve
10.	Xgwm-44, Lr19	V-1489, V-4178, As-02, Tw-76005,Tw-7 6008, Mh-97, Pak-81, Wada, Rohtas-90, Mh-97, Uqab-2000, Chanab-2000, D-97, V-04067, C-518, C-250, C-273, Mexipak, Baxani-70, Sa-42, Potowar, Sa-75, Lu-265, Barani-83	-ve
11.	Xgwm-259, Lr46/yr29	5C003, 5C034, 6C001, 45006, 56123, Manthar-03, Bwp-97, V-4188, V-4022, V-4178, Uqab, Pasban, Tw-76001, Wada, Mh-97, Kohistan-97, D-97, D-04611, C-228, C-271, C-273, Sa-42, Punjab-81, Barani-83	-ve
12.	Scs 265, Lr19	V-1489, V-4178, As-02, Tw-76005, Tw-76008, Pak-81, Wada, Rohtas-90, Mh-97, Uqab-2000, Chanab-2000, D-97, V-04067, C-518, C-250, C-273, Mexipak, Baxani-70,SA-42, Potowar, SA-75, Lu-26S, Barani-83	-ve
13.	Xwmc-44, Lr46/Yr29	All genotypes showed resistance against leaf rust (Lr 46)	+ve
14.	X-Barc 352Lr34/Yr18	All genotypes showed resistance against leaf rust (Lr34)	-ve

-ve sign shows absence and +ve sign show presence of leaf rust resistance genes

Table 4. Genotypes showing absence of stripe rust resistance genes (Yr).

S. No.	Primer name	Genotypes name	Yr genes
1.	STS 7-8 (Yr5)	Bwp-97, Pari-73, Punjab-81, Kohinoor-83	-ve
2.	X-BARC352 (Lr34/Yr18)	All genotypes showed resistance against yellow rust(yr 18)	+ve
3.	XGWM-11(Yr26)	45006, Manthar-03, Bwp-97, Bwp-2000, V-1489, Sehar, Tw-76002, Tw-76004, Tw-76005, Tw-76006, Rohtas-90, MH-97, Kohistan-97, Fsd-83, C-250, Dirk, Cheneb-70, Blue Silver, SA-75, Wl-711	-ve
4.	XGWM-259 (Lr46/Yr29)	5C003, 5C034, 6c001, 45006, 56123, Manthar-03, Bwp-97, V-4188, V-4022, V-4178, Uqab, Pasban, Tw-76001, Wada, MH-97, Kohistan-97, D-97, D-04611, C-228, C-271, C-273, Sa-42, Punjab-81, Barani-83	-ve

-ve sign shows absence and +ve sign show presence of stripe rust resistance genes

Table 5. Genotypes showing absence of stem rust resistance genes (sr).

S. No.	Primer name	Genotypes name	Sr Genes
1.	STM-773-2(Sr36)	5c003, Manthar-03, Uqab, Tw-76002, Kohistan-97, Fsd-83, C-250, Baxani-70, WL711,	-ve
2.	XGWM-429(Sr36)	5c003, Manthar-03, Uqab, Tw-76002, Kohistan-97, Fsd-83, C-250, Baxani-70, WL-711	-ve
3.	WMC-477(Sr36)	5c003, Manthar-03, Uqab, Tw-76002, Kohistan-97, Fsd-83, C-250, Baxani-70, WL-711	-ve
4.	XWMC-44(Lr46/Yr29)	All genotypes showed resistance against stripe rust Yr29	+ve
5.	XGWM-533(Sr2)	Tw-76005, Shahlimar-86, Pak-81, Sandal,	-ve

-ve sign shows absence and +ve sign show presence of stem rust resistance genes

The minimum and maximum Nei & Li's coefficient value ranged from 0.64 to 1.00 indicating that these genotypes were all most genetically identical. Genotype, Barani-83 showed genetically different behavior as compared to other genotypes forming an out-group (Cluster J) with coefficient value of 0.64. Genotypes, SA-42, TW-76001, WL-711, LU-26 and TW-76002 also showed genetically different behavior from other genotypes having coefficient value of 0.70.

As per results of this study an average similarity of 84% was noticed among them, Out of total 18 loci, 15 were found to be polymorphic. Maximum similarity was observed among 75 genotypes and minimum similarity of about 0.55 was recorded in 10 wheat genotypes.

DNA fragment of 380bp, linked with Lr47 was not amplified from two wheat genotypes TW-76007 and Punjab-96 as is evident from gel picture (Fig. 2). Gel picture showed the presence of 199 bp DNA fragment in 14 genotypes out of 15 (Fig. 3) i.e., except genotype No. 72 (barani-70). All the genotypes studied carry genes which confer resistance to Lr34 (Table 3). This was confirmed with the amplification of a 250bp DNA fragment linked with this durable resistance gene (Fig. 4).

Discussion

Results of present study revealed the presence of maximum leaf rust resistance genes in local wheat genotypes/ varieties. However in some cases results were not as per expectations. Two different DNA markers for the same gene/loci as reported in two different studies (Bariana *et al.*, 2007 and Suenaga *et al.*, 2001) produced different results. In this study leaf rust resistance gene Lr46 was tried to identify using two different primers i.e., Xgwm-259 and Xwmc-44 as reported by Bariana *et al.*, 2007 and Suenaga *et al.*, 2001 respectively. Results of both these markers were different. Primer Xwmc-44 showed the presence of resistance gene linked with Lr-46 in all the 85 genotypes/ varieties under study whereas results obtained with primer Xgwm-259 revealed that Lr-46 gene is not present in all genotypes rather missing in 24 genotypes (Table 3). This difference may be explained on the basis of the difference in plant genetic material utilized in those studies. In this respect it is suggested that screening of germplasm on the basis of linked DNA markers identified and reported by scientists using their own local genetic material may only be reliable if validation of DNA markers was accomplished by comparing the results of molecular markers with field/pathological evaluations.

Results of molecular study need to be confirmed through field observations and artificial application of race specific inoculum using gene differentials. As in this study molecular data showed the presence of Lr-51 and Lr-52 genes in almost all genotypes whereas in field all these genotypes are not showing resistance to various races of leaf rusts. Durable rust resistance conferred by minor genes is considered very important now a days. Lr-34 and Lr46 are two most important minor genes which confer resistance to leaf rust. Results of present study revealed the presence of both these genes in all genotypes which donot match with the actual field observations as presence of these genes should confer resistance to leaf rust where as all these are not showing this type of reaction. If valid DNA markers are used to locate the minor genes in local germplasm, it would be very useful in practical wheat breeding and improvement programmes.

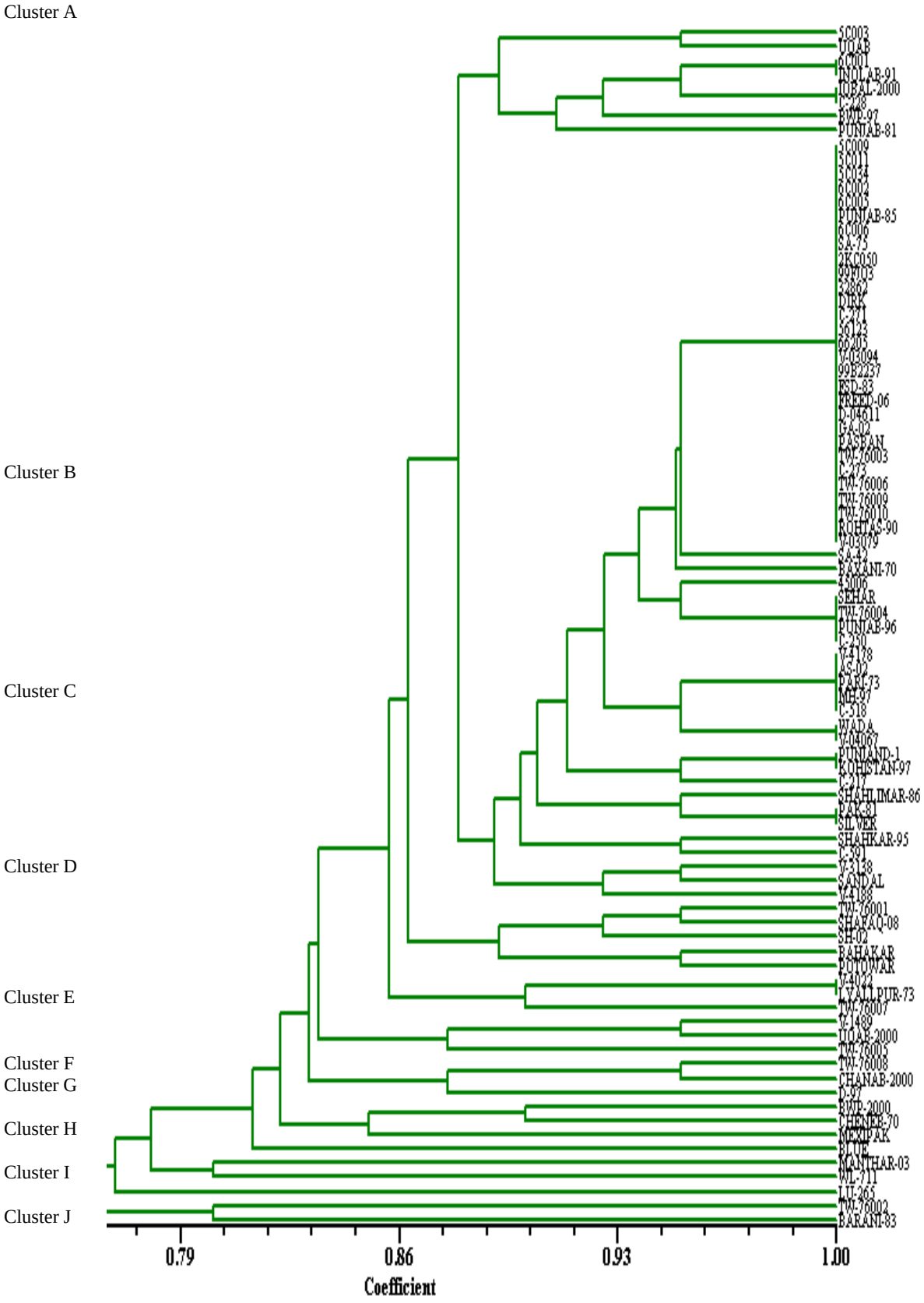

Fig. 1. Dendrogram showing clustering of 85 genotypes based on coefficient of similarity.

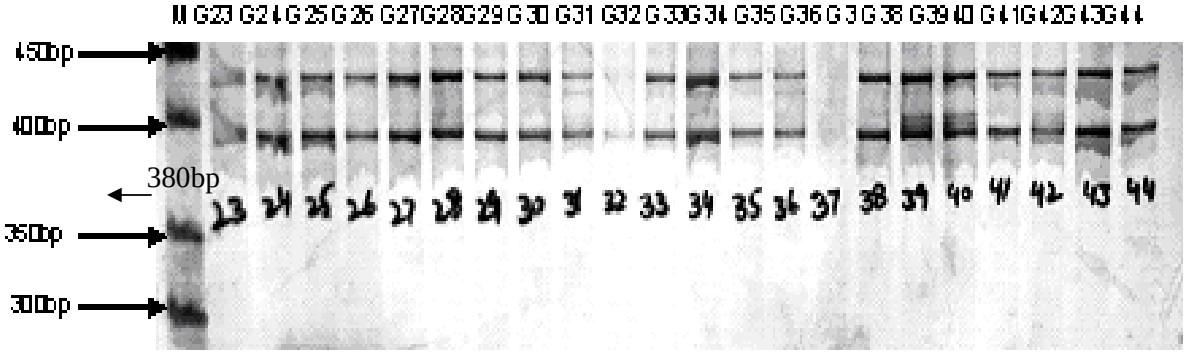

Fig. 2. All genotypes show resistance against leaf rust except Tw-76007 (G37) Primer (Lr47).

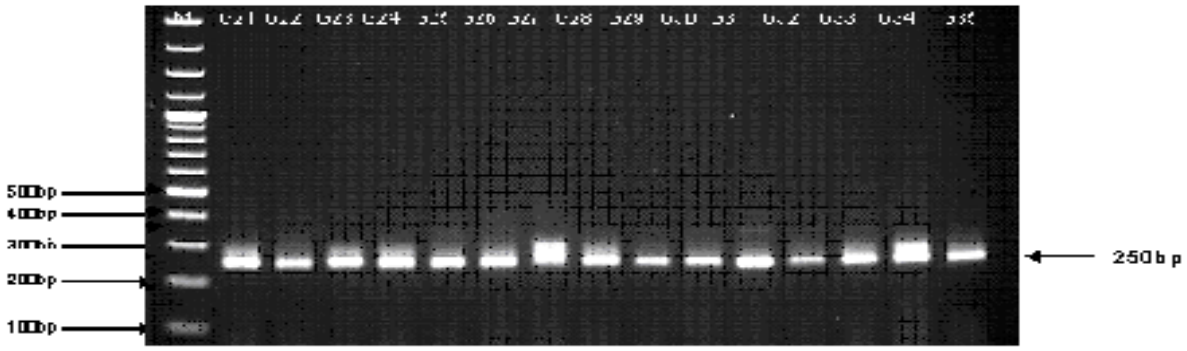

Fig. 3. All genotypes show resistance against stripe rust (Lr34/ Yr18) primer Xbarc352.

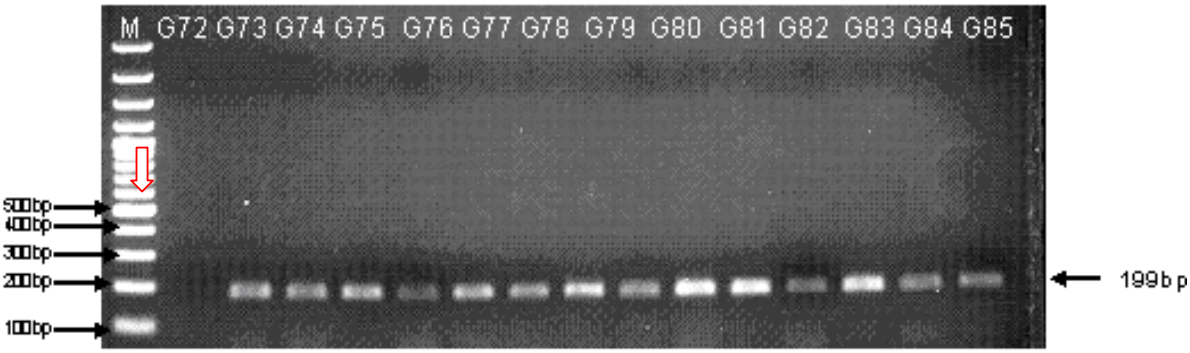

Fig. 4. All genotypes show resistance against Stem rust (Sr36) except Baxani-70 (G72) as revealed by Primer STM 773-2.

Bariana *et al*. (2007) reported microsatellite *STM773-2* and other RFLP markers that showed complete linkage to the *Sr36* locus in a double haploid (DH) population of wheat lines, Our results indicated that the wheat genotypes showed presence of stem rust resistance gene Sr-36, except in nine genotypes out of 85 studied and *STM773-2* marker amplified same bands that can make it easy to distinguish homozygous from heterozygous genotypes. In this study, *Xgwm-429*, *ST*, *M773-2*, and *WMC477* markers were found to be diagnostic for *Sr36* locus. These three DNA markers proved to be very effective for detecting the presence of Sr-36 loci and results of all the markers are same which proved their usefulness and effectiveness in practical DNA marker assisted breeding and pyramiding of rust resistance genes in elite genotypes.

It has been reported that the amplification products from primers S30-13L (Lr51) genes showed better amplification in all genotype with the product size of 783bp (Helguera *et al*., 2005). Our results also showed similar trends, all genotypes showed resistance against Lr51. Our results are in accordance with Hussain & Hussain (2011). They worked on 25 Pakistani Wheat genotypes. A band of 310bp was amplified showing the presence of *Lr10* gene. The polymorphic data revealed that out of the 25 varieties, Lr10 *was* identified as a fragment of 310bp in 18 varieties having while seven genotypes did not show the presence of *Lr10* gene similar results were given by Waheed *et al*. (2010). Jin *et al.* (2007) developed DNA marker for sr36 gene and reported the size of DNA marker as 199bp, which was found present in most of the genotypes under study.

Our results agree with Gupta *et al.* (2006) in case of primer SCS-253, 736bp but different with primer pair SCS-265,512bp. SCAR primer pairs SCS-265 showed critical behavior having amplification product of only 130bp instead of 512bp as reported by Gupta. Thus the genotypes which are to be assorted and selected for further breeding program should be based on results of both studies i.e., morphological/field and molecular data.

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