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# Identification of rust resistance genes *Lr10* and *Sr9a* in Pakistani wheat germplasm using PCR based molecular markers

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Wheat (*Triticum aestivum* L.) rusts are the most widespread and destructive among all other diseases of wheat because of their wide distribution, and their capacity to form new races that can attack previously resistant cultivars which result in serious yield losses. Stem rust caused by *Puccinia graminis* f. sp. *tritici* and leaf of orange rust caused by *Puccinia recondita* are more effective and durable when several rust resistance genes are pyramided into a single line. Molecular survey was conducted to screen altogether 44 Pakistani wheat germplasm lines consisted of 23 cultivated varieties for the presence of *Lr10* and *Sr9a* genes by using co-dominant STS and SSR primers specific for respective alleles. The polymorphic survey revealed that out of 44 germplasm lines obtained from Ayub Agriculture Research Institute, 31 genotypes were observed with *Lr10* gene (including C-228, C-245, C-288), while 13 germplasm lines did not show the presence of *Lr10* gene. Of the 23 cultivated varieties, 21 varieties observed *Lr10* gene (including Auqab 2000, Chenab-70, Punjab-76, Kohsar-95, Parvaz-94, Pasban-90, Chenab-2000, Satluj-86, Shahkar-95), while only Kohsar 95 and PARI 73 did not show the presence of *Lr10* gene. Molecular survey for *Sr9a* gene depicts that 33 genotypes were observed for stem rust resistance like C-248, LR26, C-250, C-271, C-273, C-288 and C-518, while 11 genotypes (including C-228, C-245, C-247 and C-271) showed no such fragment. Of 23 conventional cultivated varieties, 21 varieties showed the presence of *Sr9a* stem rust resistance gene like Punjab-76, Pak-81, Kohistan-97, Kohsar-95, Parvaz-94, Pasban-90 etc and 4 varieties showed the absence of *Sr9a* stem rust resistance gene like Shahkar-95, Parvaz-94, Chenab-2000 and Chenab-79. The identification of *Lr10* and *Sr9a* in Pakistani wheat germplasm will help in accelerating the breeding program in future, including pyramiding of different wheat resistant genes in wheat varieties.

**Key words:** Stem rust, leaf rust, *Sr9a*, *Lr10*, wheat, molecular markers.

## INTRODUCTION

Wheat is the most widely used staple food grain of the world. The world wheat output declined from 591.9 to 589.1 million tons in the last couple of years. Pakistan

and India account for 20% of the annual world wheat production. In Pakistan, wheat being the staple diet is the most important crop and cultivated on the largest acreages in almost every part of the country. It contributes 14.4% to the value added in agriculture and 3.0% to GDP. Pakistan has been divided into ten production zones because of great agro ecological areas where wheat is grown. The zoning is mainly based on cropping pattern,

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disease prevalence and climatological factors. Improved semi-dwarf wheat cultivars available in Pakistan have genetic yield potential of 6-8 ton/ha whereas the national average yields are about 2.5 ton/ha. A large number of experiment stations and on-farm demonstrations have repeatedly shown high yield potential of the varieties. There are progressive farmers of irrigated area who are harvesting 6 to 7 tonnes yield per hectare. However, farmers yield ranges from 0.5 to 1.3 ton/ha depending on the amount of rainfall in rain-fed areas and in irrigated areas it ranges from 2.5 to 2.8 ton/ha depending upon the amount of water available and other factors.

Diseases, especially rusts (stripe/yellow rust, stem rust, leaf rust and emerging scenario of powdery mildew) are major biotic stresses of wheat crop that inflict heavy losses when in epidemic form. Therefore, breeders and pathologists are given priority to develop disease resistant and high yielding varieties in order to cope with threats created by ever changing rust races by using approaches like durable resistance. In the last few decades, disease epidemic of rust inflicted heavy losses to the wheat production in the country. As a consequence, it was realized that there is need of strengthening the agricultural research in the country.

The rusts caused by *Puccinia* species are some of the most important diseases of wheat and these deadly pathogens affected the balance of its trade in under-developing countries like Pakistan. Leaf rust caused by *Puccinia triticina* (*P. recondita* f. sp. *tritici*) is a major foliar disease of wheat, resulting in severely damaged wheat production and yield loss throughout the world (Eversmeyer and Browder, 1974; Kolmer, 1996). The disease is most damaging when the upper leaves of infected plants become severely rusted. Heavy rusting causes early loss of these leaves, which reduces the grain filling period and results in smaller kernel size. Stem rust of wheat caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks and E. Henn is another destructive disease of wheat, reaching devastating epidemic levels in most wheat-growing areas of the world (Knott, 1989). To date, more than 45 stem rust resistance (*Sr*) genes have been identified against different races of this fungus (McIntosh et al., 2003).

As the name implies, it infects the stem but is not always confined to the stem. It can infect leaves, sheaths, glumes and awns. The host epidermis is ruptured by the elongate, reddish brown (brick-red) pustule with ragged margins, giving the stems and sheaths a rough-textured surface. Stem rust pustules are larger than leaf rust pustules and often erupt on both upper and lower leaf surfaces.

Knowledge of genes for resistance to diseases is important to estimate the risk of the disease according to the virulence in the population of the pathogen. Although, single gene resistance may overcome new races of pathogens, the use of resistant varieties is still the most commercial and economic method to reduce yield losses (McIntosh, 1988). To avert rust crisis, it is essential for

plant breeders to use rust resistant genes, for the choice of the most suitable resistance genes and their combinations for the breeding process. Pyramiding of several rust resistant genes in a single line after following molecular markers could dramatically improved yield losses. Moreover resistant varieties are the best control for biotic stress. So for this reason, it is necessary to screen Pakistani wheat lines that are considered effective against all races of pathogen and provide broad spectrum resistance. Marker assisted selection provides an indirect selection of a genetic determinant(s) of a trait of interest. Microsatellite markers (SSR) are PCR-based markers and are located in repetitive deoxyribonucleic acid (DNA) sequences (Roder et al., 1998). Only a few SSR markers are sufficient to discriminate among even closely related wheat breeding lines (Roder et al., 1995; Plaschke et al., 1995; Bryan et al., 1997). Thus, it is important to investigate, in wheat, whether mapped microsatellite markers are randomly distributed or clustered in certain regions of chromosomes (Roder et al., 1998).

The wheat genome is large, hexaploid, ( $1.6 \times 10^{10}$  bp) and (80%) highly repetitive sequences. Diversity of resistance to leaf rust caused by *P. triticina* can be enhanced in wheat cultivars through a better knowledge of resistance genes that are present in important cultivars and germplasm. To date, more than 45 stem rust resistance (*Sr*) markers (genes) (McIntosh et al., 2003) and nearly 58 leaf rust resistance markers have been identified against different races of this fungus. Leaf rust resistance genes are designated as *Lr1* through *Lr58* (McIntosh et al., 2005; Kuraparthi et al., 2007), but most varieties have only a few *Lr* genes. *Lr10* is a single-copy gene on chromosome 1AS (Feuillet et al., 1997). *Lr10* confers enhanced resistance to leaf rust; *Lr10* has similarities to *RPM1* in *Arabidopsis thaliana* and to resistance gene analogs in rice and barley. A collection of 68 cultivars of common wheat has been screened for leaf rust resistance genes with the use of molecular markers (Urbanovich et al., 2006).

The potential benefits of molecular marker-assisted selection (MAS) have been widely discussed (Melchinger, 1990; Paterson et al., 1991; Young, 1996; Mohan et al., 1997; Anderson, 2003), especially to provide solutions to overcome some of the problems faced by classical phenotypic screening approaches in plant breeding programs. For example, to facilitate breeding for durable resistance to stem rust, molecular markers are useful tools in developing resistant cultivars and especially, pyramiding several disease resistance genes (Anderson, 2003). Marker-assisted selection can be used at an early stage of plant development when multiple DNA markers are used to screen several genes simultaneously.

The objective of this work was to address this issue by using SSR markers and to verify the application of the molecular marker for the determination of the gene cluster *Sr9a* and *Lr10* in Pakistani wheat germplasm to develop wheat lines that are considered effective against

**Table 1.** Primer sequences of *Lr10* and *Sr9a*.

Locus name	Location (Chromosome #)	Primer sequence	Reference
<i>Lr10</i>	1AS	Frw: 5'GTGTAATGCATGCAGGTTCC3' rev: 5'AGGTGTGAGTGAGTTATGTT3'	Stepien et al. (2003).
<i>Sr9a</i>	2BL	SSR marker Xgwm47 Frw: 5'TTGCTACCATGCATGACCAT3' rev: 5'TTCACCTCGATTGAGGTCCT3'	Tsilo et al. (2007).

all races of stem and leaf rust pathogens. This information will be directly useful to wheat breeders and plant pathologists to help develop wheat cultivars with high levels of leaf and stem rust resistance.

## MATERIALS AND METHODS

### Plant materials

Seeds of 44 wheat genotypes obtained from the Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan comprising 23 conventional cultivars were grown in pots at Institute of Biotechnology (IBT), Bahauddin Zakariya University (BZU), Multan Pakistan. List of all these lines along with their parentage is given in Table 1. All these were tested with PCR-based DNA markers for, leaf rust and stem rust resistance genes *Lr10*, *Sr9a*, respectively.

### DNA extraction

Total genomic deoxyribonucleic acid (DNA) from individual genotype was extracted from young leaves at seedling stage by CTAB (cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1990). Fresh leaves (five plants of each accession) were ground in mortar with a pestle with continuous addition of liquid nitrogen and transferred to a 50 ml falcon tube. The 15 ml of hot 2x CTAB was added and incubated for 30-45 min at 65°C with occasional swirling. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inverting the tube to form an emulsion, then spun at 11,000 rpm for 10 min. The supernatant was collected into a new 50 ml tube and a nucleic acid was precipitated with 0.6 volume of chilled isopropanol and left in the refrigerator for 2 h. Nucleic acid was pelleted at 12,000 rpm for 5 min and supernatant was discarded and the pellet was washed with 70% ethanol and air dried before resuspending in 0.5 ml 0.1x TE buffer. The suspension was transferred to 1.5 ml Eppendorf tube, added 7 µL of RNase and incubated at 37°C for 1 h. DNA concentration was measured by fluorometer DyNA Quant™200 and the DNA were diluted to 10 ng/µL using sterilized distilled water and stored in microfuge tubes at 4°C for further use.

### PCR Amplification of *Lr10* and *Sr9a*

To screen rust resistance genotypes, polymerase chain reaction (PCR) was performed as described by Chen et al. (1997) in a 96-well plate of Gene Amplification PCR System 9700 Applied Biosystems.

Amplification reaction were carried out with a 15 µL of final reaction mixture containing 50 ng genomic deoxyribonucleic acid, 1 µL of each of primer, 100 µM dATP, dCTP, dGTP and dTTP, 1 unit

of Taq polymerase (fermentas), 1x Taq polymerase buffer and 2.5 Mm magnesium chloride. DNA amplification reaction was performed in a Gene Amplification PCR System 9700 of Applied Biosystems programmed for *Sr9a* as under; initial denaturation of 5 min at 94°C, 45 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 2 min and for *Lr10* as under; initial denaturation of 5 min at 94°C, 35 cycles of 94°C for 1 min (denaturation), 55°C for 1min (annealing), 72°C for 2 min. The amplified products were resolved at 8% denaturing polyacrylamide gels followed by staining with AgNO<sub>3</sub> (Heukeshoven and Dernick, 1985; Budowle et al., 1991) and scored for the presence and absence of *Lr10* and *Sr9a* linked DNA fragments.

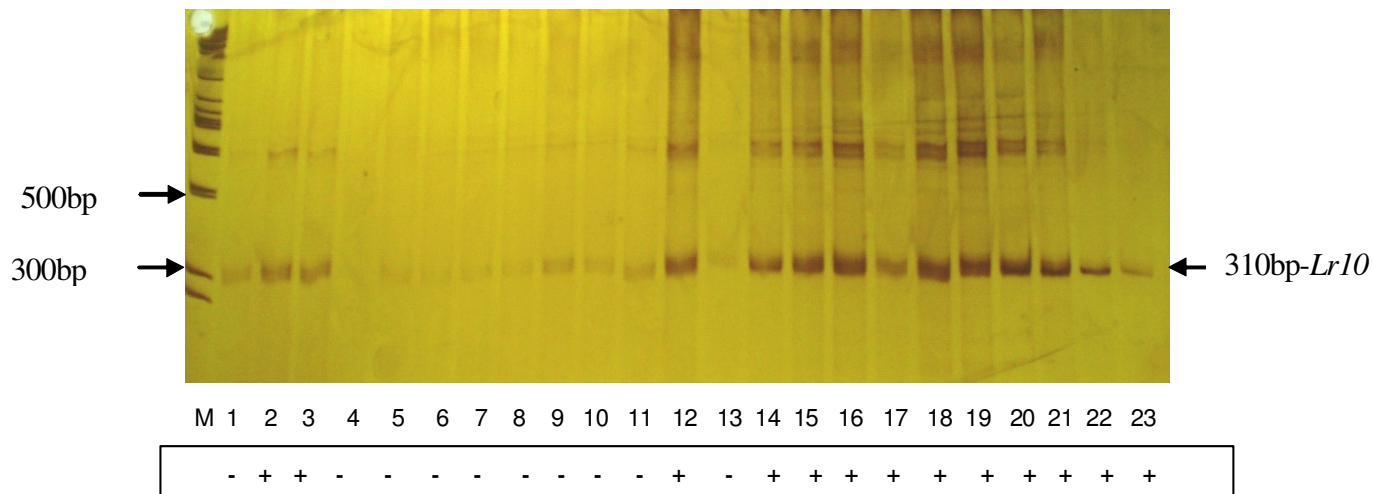
### Data analysis

The amplified fragments of all wheat genotypes for *Lr10* and *Sr9a* were observed and compared with band size describe by Stepien et al. (2003) and Tsilo et al. (2007).

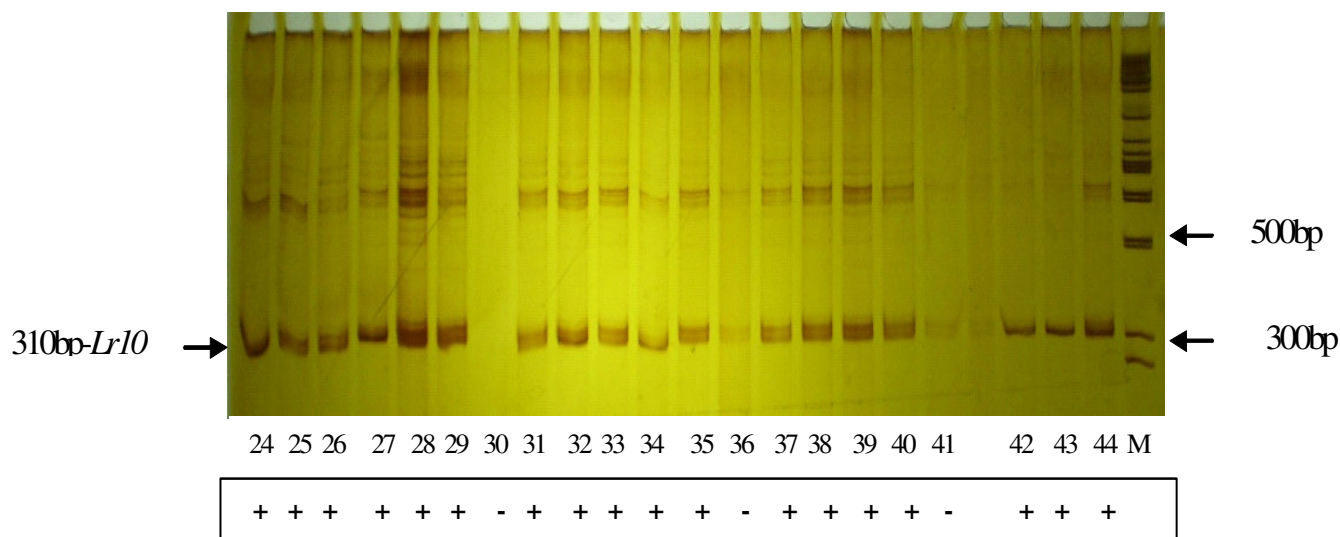
## RESULTS AND DISCUSSION

The wheat cultivars become susceptible to rusts due to their narrow genetic base for resistance and the rapid rate of evolution of the pathogen, making it necessary to search for new source(s) for resistance. Genetic resistance is important to control many phytopathogenic epidemics. Thus, the wheat production has been largely dependent on the development and the use of resistant cultivars having diverse and well characterized genes. Resistance based on single major gene is often considered short-lived due to the genetic shifts or the emergence of new virulence in the pathogen population in response to selection imposed by the host. It is believed that, in wheat, certain gene combinations give better and long lasting resistance to rust diseases than given by any of the genes individually (Dyck and Samborski, 1982). Molecular marker SSR offers an effective way of assessing the resistance of a set of genotypes and these markers are widely distributed throughout plant genomes and provide discrete data about morphological traits. Similar kind of work was conducted by Arif et al. (2008) on screening of bacterial blight resistant line in rice and practically showed the significance of molecular markers.

Amplification of DNA using *Lr10* F primer and R primer has been described as a way to detect *Lr10* gene and the amplified fragments corresponded to leaf rust resistance,



**Figure 1.** Banding patterns showing the presence and absence of *Lr10* gene in germplasm of wheat line amplified 310 bp size fragment. + sign represent presence of band whereas - sign indicates absence of *Lr10* gene. Arrow is showing amplified gene (310 bp). M = 1000 bp size marker.

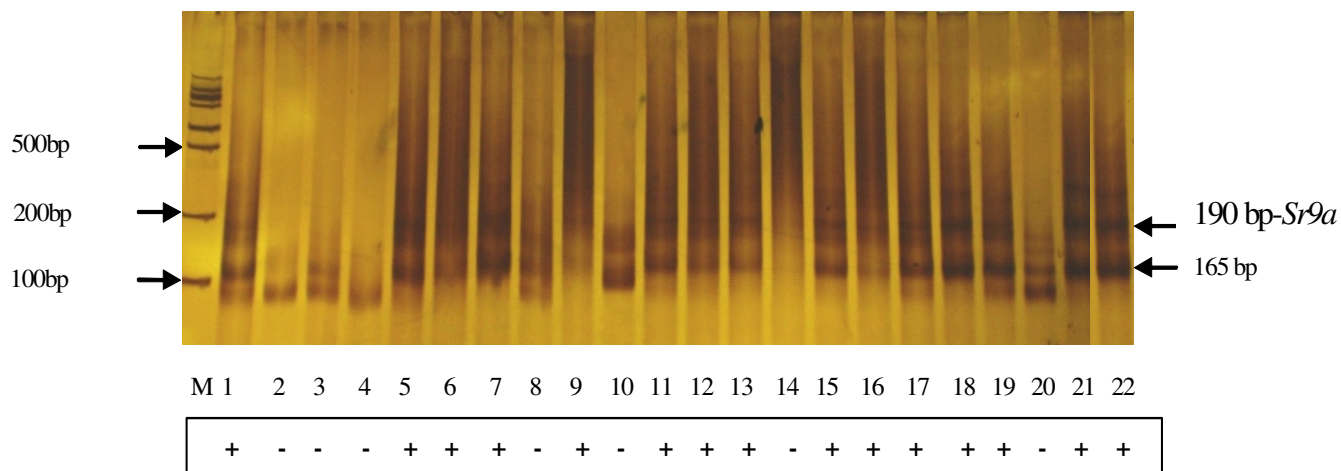


**Figure 2.** Banding patterns showing the presence and absence of *Lr10* gene in germplasm of wheat line amplified 310 bp size fragment. + sign represent presence of band whereas - sign indicates absence of *Lr10* gene. Arrow is showing amplified gene (310 bp) M = 1000 bp size marker.

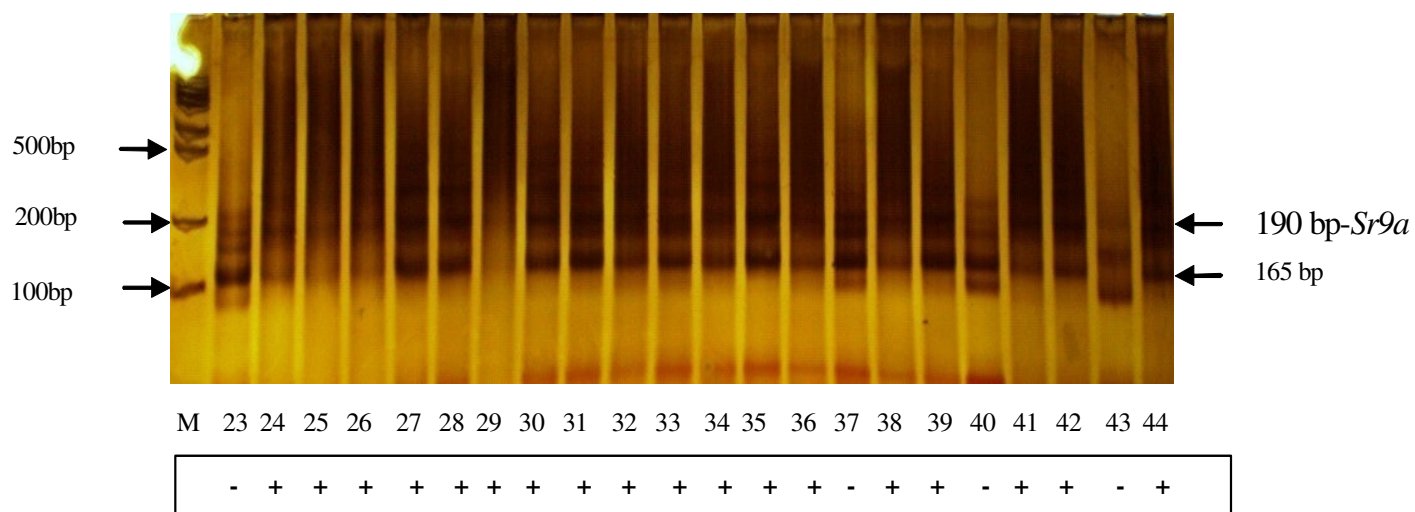
results were compared with the work which has already been done on *Lr10* (Stepien et al., 2003). The size of the band corresponds to *Lr10* was 310 bp. Varieties possessed 310 bp size of band thought to be resistant against leaf rust whereas lines which lack 310 bp of band which are susceptible to leaf rust carry no *Lr10* gene (Figures 1 and 2). The amplified fragments corresponded to stem rust resistance and the results were compared with the work which has already been done on *Sr9a* (Tsilo et al., 2007). The size of the band corresponding to *Sr9a* was 190bp and varieties having 190 bp size of band are found to be resistant against stem rust whereas lines having

165 bp of band which are susceptible to stem rust carry no *Sr9a* gene. The presence and absence of *Lr10* has shown in Figures 1 and 2 and *Sr9a* in Figures 3 and 4 while the screening of 44 wheat genotypes with *Lr10* and *Sr9a* markers whose loci are already mapped, provide information about presence and absence of resistant genes in Pakistani germplasm and varieties (Table 2).

This polymorphic survey revealed that out of 44 germplasm lines obtained from Ayub Agriculture Research Institute, 31 genotypes were observed with *Lr10* gene, namely: C-228, C-245, C-288, while 13 germplasm lines did not showed the presence of *Lr10* gene. Of the 23



**Figure 3.** Banding patterns showing the presence and absence of *Sr9a* gene in germplasm of wheat line amplified 190 bp and 165 bp size fragments respectively. + sign represent presence whereas - sign indicates absence of *Sr9a* gene. Arrow is showing amplified gene (190 bp) M = 100 bp size marker.



**Figure 4.** Banding patterns showing the presence and absence of *Sr9a* gene in germplasm of wheat line amplified 190 bp and 165 bp size fragments, respectively. + sign represent, presence whereas - sign indicates absence of *Sr9a* gene. Arrow is showing amplified gene (190 bp) M = 100 bp size marker.

cultivated varieties, 21 varieties observed *Lr10* gene which includes: Auqab 2000, Chenab-70, Punjab-76, Kohsar-95, Parvaz-94, Pasban-90, Chenab-2000, Satluj-86, Shahkar-95, etc, and only Kohsar 95 and PARI 73 did not show the presence of *Lr10* gene. Molecular survey for *Sr9a* gene depicts that 33 genotypes were observed for stem rust resistance like C-248, LR26, C-250, C-271, C-273, C-288 and C-518, while 11 genotypes showed no such fragment like C-228, C-245, C-247 and C-271, etc. Of 23 conventional cultivated varieties, 21 varieties showed the presence of *Sr9a* stem rust resistance gene like Punjab-76, Pak-81, Kohistan-97, Kohsar-95, Parvaz-94, Pasban-90, etc, and 4 varieties showed the absence of *Sr9a* stem rust resistance gene like Shahkar-95, Parvaz-

94, Chenab-2000 and Chenab-79 (Table 2). The identification of *Lr10* and *Sr9a* in Pakistani wheat germplasm will help in accelerating the breeding program in future, including pyramiding of different wheat resistant genes in wheat varieties.

**Abbreviations:**

**MAS**, molecular marker-assisted selection; **SR**, stem rust resistance; **CTAB**, cetyl trimethyl ammonium bromide; **PCR**, polymerase chain reaction; **LR**, leaf rust resistance; **DNA**, deoxyribonucleic acid; **SSR**, microsatellite markers; **Sr**, stem rust resistance gene; **Lr**, leaf rust resistance gene.

**Table 2.** List of wheat germplasm along with their parentage showing presence and absence of *Lr10* and *Sr9a* genes.

S/N	Variety code	Pedigree / Parentage	Lr10	Sr9a
1	IBT-1	C-217 (C-516 x C-591)	-	+
2	IBT-2	C-228(Hard Federation x 9D)	+	-
3	IBT-3	C-245	+	-
4	IBT-4	C-247	-	-
5	IBT-5	C-248,LR26, 14A	-	+
6	IBT-6	C-250Hard Federation x 9D	-	+
7	IBT-7	LR30, 14A	-	+
8	IBT-8	C-256 LR10, 23, 30	-	-
9	IBT-9	C-258	-	+
10	IBT-10	C-271(C220xIP165)	-	-
11	IBT-11	C-273,(C209 x C-591)	-	+
12	IBT-12	C-288	+	+
13	IBT-13	C-518, (T9 x 8A)	-	+
14	IBT-14	C-591(T9 x 8B)	+	-
15	IBT-15	AS-2002 = WD-97603KHP/D.31708//CMH74A.370/ 3/CNO79/4/RL6043/*4NAC.PBD.795-23A-1A-0A	+	+
16	IBT-16	AUQAB 2000CROW'S'/NAC//BOW'S' PB 22138-3A-0A-0A-234A-0A	+	+
17	IBT-17	BHAKKAR-2000P102/PIMA//F3.71/TRM/3/PVN	+	+
18	IBT-18	BLUE SILVER = SONALIKAI53.388/AN//YT54/N10B/3/LR/4/	+	+
19	IBT-19	CHENAB 70	+	+
20	IBT-20	CHENAB79,PB76/CH70	+	-
21	IBT-21	CHAKWAL86,FORLANI/ACC//ANA 75	+	+
22	IBT-22	CHAKWAL 97,BUC'S'/FCT'S'	+	+
23	IBT-23	CHENAB-2000, CBRD (CHUM18/BAU), CM92991-59M-0Y-0M-5Y-0B	+	-
24	IBT-24	FAISALABAD83, FURY//KAL/BB, CM 37138-48Y-1M-5Y-1M-4Y-5Y-0A	+	+
25	IBT-25	FAISALABAD85,MAYA'MON'S'//KVZ/TRM,CM 44083-N-3Y-1M-1Y-1M-1Y-0B	+	+
26	IBT-26	GA-2002, DWL 5023/SNB//SNB, CM 84986-H-1M-3M-2B-0Y	+	+
27	IBT-27	INQILAB 91	+	+
28	IBT-28	IQBAL2000,BURGUS/SORT1213//KAL/BB/3/PAK.81,PB21912-11A-0A-0A-59A- 0A,KOHINOOR 83,OREF1158/FDL//MFN/2*TIBA63/3/COC	+	+
29	IBT-29	KOHISTAN97,V-1562//CHRC'S'/HORK/3/KUFRA-1/4/CARP'S'/BJY'S',PB.24883B-1A-0A	+	+
30	IBT-30	KOHSAR 95	-	+
31	IBT-31	LU 26,BLS/KHUSHAL 69	+	+
32	IBT-32	LYP 73,BB/NORTENO.67	+	+
33	IBT-33	MEXIPAK 65,PJ62/GB55,II.8156-0PAK	+	+
34	IBT-34	MH 97 = ATTILA, ND/VG 9144//KAL/BB/3/YACO/4/VEE#5,CM. 85836-50Y-0M-0Y-3M 0Y	+	+
35	IBT-35	PAK 81,KVZ/BUHO//KAL/BB= VEE#5	+	+
36	IBT-36	PARI 73,BLUEBIRD,CNO//SN64/KLRE/3/8156	-	+
37	IBT-37	PARWAZ 94, V 5648/PRL'S' (V-87189), PB. 20089-7A-4A-0A	+	-
38	IBT-38	PASBAN90,INIA66/A,DISTT//INIA 66/3/GEN 81	+	+
39	IBT-39	PUNJAB76,NAI 60/CB 151//S 948/3/MXP	+	+
40	IBT-40	SA 42,C 271/LR 64//SON 64	+	-
41	IBT-41	SA 75, NAI 60/CB 151//S 948/3/MXP	-	+
42	IBT-42	SATLUJ 86,CMT/YR//MON'S'	+	+
43	IBT-43	SHAHKAR95,WL711//F3.71/TRM,PB 20371-20A-4A-0A	+	-
44	IBT-44	INQ.91/FINK'S', SH-2002, PB. 25553-1A-0A-1A-0A	+	+

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