AN ASSESSMENT OF THE GENETIC DIVERSITY IN SELECTED WHEAT LINES USING MOLECULAR MARKERS AND PCA-BASED CLUSTER ANALYSIS

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Abstract. A comprehensive germplasm evaluation study of wheat elite lines was conducted at Wheat Research Institute Faisalabad-Pakistan to identify new sources of leaf, stripe and stem rust resistance and high yield potential during crop seasons 2015-2017. The parent lines were selected on the basis of phenotypic characteristics and slow rusting history for race non-specific resistance genes by the selection of desirable parents used in filial generation (F1-F5). In primary evaluation, 112 lines were selected on the basis of rust reaction and high phenotypic uniformity for further testing against rust resistance and high yield potential. Among these, 32 lines exhibited *Lr34/Yr18*, 22 lines showed *Lr46/Yr29*, and 30 lines indicated the combination of *Sr2/Yr30*. Principal component analysis (PCA) based cluster analysis exhibited that, cluster I and III had clear separation compared to cluster II, IV and V. It was concluded that seven elite lines i.e. V-70003, V-70034, V-70054, V-70070, V-70085, V-70103 and V-70104 exhibited both the linkages of three slow rusting genes (*Lr34/Yr18, Lr46/Yr29* and *Sr2/Yr30*) and high yield characteristics and are expected to contribute toward food security at national and global levels.
Keywords: *breeding, grain yield, rust resistance, SSR markers, Triticum aestivum*

Introduction

Wheat (*Triticum aestivum* L.) along with maize and rice is a strategic crop for worldwide food security. The estimated global wheat production for the year 2015-2016 is 734.2 MT which is slightly higher than the demand of 716.2 MT (FAO, 2016). The demand for wheat continues to rise at an annual rate of 1.6% and some estimates indicate that 60% more wheat will be needed by 2050 (FAO, 2016).

Wheat is mainly hit by three types of rusts stripe/yellow, leaf/brown and stem/black that reduce its produce (Roelfs et al., 1992). Evolution of two high temperature tolerant yellow rust races caused severe epidemics in main wheat growing regions of the world since 2000 (Hovmoller et al., 2008). Recent identification of various virulent races of Ug99 i.e. TTKSK, TTKSF, TTKSF+, TTKSP, PTKST and three virulent brown rust races CCPS, MCDS and FBPT are significant threat to wheat production worldwide necessitating integrated and collaborative management strategies of the diseases (Terefe et al., 2014; Pretorius et al., 2015; Patpour et al., 2016).

In Pakistan yellow and leaf rust have been a constant risk to its sustainable production. The reason behind rapid collapse of the assortments is associated to the evolution of new virulent races in assortment due to race specific genes of presentation. The recent and last trend of genomic fight in wheat assortment is “resistance based on preservative effects of accumulation of race non-specific genes” (Singh et al., 1998).

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The race non-specific yellow and leaf rust resistance appearing in several assortments is based on durable genes that have additive effects (Singh et al., 2005). The economic, most effective, environmentally friendly, and easy to use method to reduce losses caused by the rusts is cultivation of resistant assortments (Cheng and Chen, 2014; Kalappanavar et al., 2008). In current era of scientific research main focus is to achieve race non-specific slow rusting resistance by combining several minor or adult plant genes (Singh et al., 2000).

Continuous breeding results in narrow genetic variation in gene pool of wheat advance lines and also lead to problems regarding adaptation as well as biotic and abiotic stresses (Zhang et al., 2005). Highest genetic variation among parentage is necessary to achieve transgressive segregation (Joshi et al., 2004). Selection of genetically different parentage through breeding results in maximum variation in progenies. Therefore, there is an urgent need to exploit the existing elite lines to evolve high yielding lines that have extensive adoptability under changing meteorological conditions (Baranwal et al., 2012). The use of molecular markers for the assessment of genetic variation is receiving much attention. Many wheat researchers have studied the genetic diversity in bread wheat using different molecular markers such as RFLPs (Kim and Ward, 2000), ISSRs (Nagaoka and Ogihara, 1997), STS (Chen et al., 1994), AFLPs (Burkhamer et al., 1998) and RAPDs (Joshi and Nguyen, 1993). Though, the most of these molecular marker systems (Devos and Gale, 1992) exhibit a low level of genetic diversity in the selected wheat lines, especially among cultivated lines/cultivars.

The simple sequence repeats (SSRs), also termed as microsatellites, have been proposed as the most-suitable markers for the evaluation of diversity and genetic variation among wheat cultivars/lines, as they are chromosome-specific, multiallelic and consistently distributed along chromosomes (Roder et al., 1998). The SSRs markers have been applied widely for genetic stability of gene bank accessions (Borner et al., 2000), marker-assisted selection in wheat (Huang et al., 2000), identifying QTLs (Kandel et al., 2017), and tagging resistance genes (Mutari et al., 2018). Such molecular markers have also demonstrated a high level of genetic diversity among diploid species (Hammer et al., 2000). Such markers also revealed a high level of polymorphism among diploid species (Hammer et al., 2000), in the accessions of tetraploid wild wheat *Triticum dicoccoides* (Fahima et al., 2002), and as well as in hexaploid wheat varieties (Stachel et al., 2000; Prasad et al., 2000). Cluster and principal component analyses are main genomic diversity tools having comparative differences with each other. PCA based cluster analysis is robust technique to assess family linkage (Mellingers, 1972). Hence, the main goal of present study were to evaluate (1) wheat advanced lines having race non-specific rust resistance through DNA molecular markers (2) high yielding lines through cluster and Principal component analyses.

Materials and methods

For genetic evaluation plant material comprised 855 wheat elite lines (F6 generation) of 45 diverse crosses based on 8-10 year wheat rust history and high yield characteristics (*Table 1*) were selected from gene pool of Wheat Research Institute Faisalabad. The trial was sown during 2nd week of November, 2015-2016 at Wheat Research Institute (WRI) Faisalabad through hand drill following augmented design with single replication split with 9 blocks having five plots per block containing 19 genotypes with one check (Morocco). Each plot comprises of 20 rows 2.5 m long and

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25 cm apart. Morocco was inoculated using spraying, dusting and hypodermal needle injection methods twice during month of January and February to develop high rust inoculum pressure (Roelfs, 1988). Disease severity percentage and field response were observed following modified Cobb's scale (*Table 2*) for five consecutive observations after every 7 days interval when morocco became 50-60% susceptible.

Table 1. *Detail of parents used in crossing*

S/N	Name of line/cultivar	Leaf rust resistance status	Stripe rust resistance status	Acceptable yield kg ha^{-1}	Maximum yield kg ha^{-1}
1	INQ.91	Moderately resistant	Moderately resistant	4800	6700
2	WBLLI	Resistant	Resistant	4250	6850
3	AS-2002	Moderately resistant	Susceptible	4550	6655
4	FSD.08	Partially resistant	Moderately resistant	4453	6650
5	AUQAB 2000*2/LAKTA-1	Resistant	Resistant	4775	6900
6	V-87094	Partially resistant	Partially resistant	4850	6900
7	V-09014	Slow rusting	Susceptible	4700	6850
9	SH.88/PAK.81	Partially Resistant	Resistant	4611	6800
10	SHAFAQ-06	Partially resistant	Partially resistant	4100	6400
11	MILAN/KAUZ	Resistant	Susceptible	4011	6100
12	BABAX	Partially resistant	Partially resistant	4100	6700
13	ALTAR	Moderately resistant	Resistant	4310	6850
14	MAYA 74'S'/MON'S'	Susceptible	Partially resistant	4300	6500
15	MAYA/PVN	Resistant	Resistant	4011	6430
17	PB96/V87094//MH97	Moderately resistant	Resistant	4204	6100
18	TRAP#1	Resistant	Resistant	4500	6600
19	SH88/2*ATTILA	Moderately resistant	Moderately susceptible	4800	6700
20	CNDO/R143	Resistant	Resistant	4250	6850
21	SERI.1B	Moderately resistant	Susceptible	4550	6655
22	PBW343*2/KUKUNA	Partially resistant	Moderately resistant	4453	6650
23	C80.1/3*BATAVIA	Susceptible	Resistant	4475	6800
24	WH576	Partially resistant	Partially resistant	4750	6700
25	PASTOR	Resistant	Resistant	3800	6450
26	MEXI_2	Partially resistant	Partially resistant	4200	6600
27	KRONSTADF2004	Moderately resistant	Resistant	4310	6760
28	ROLF07*2/KIRITATI	Susceptible	Partially resistant	4200	6650
29	KIRITATI	Resistant	Resistant	4050	6050
30	WAXWING	Slow rusting	Susceptible	4000	6150

The genotypes recorded to be resistant through primary evaluation (112) along with five checks were subjected to further screening for rusts resistance and high yield potential at WRI Faisalabad during second week of November, 2016-2017. The genotypes were planted by Norvigion in experimental area of Wheat Research Institute in Augmented design. Each test entry was planted in a plot (six rows of five meter

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length). In order to facilitate development of rust epidemics two rows of Morroco were planted around each side of experimental material. Artificial inoculation of experimental material was done in the morning from first week of January to end of February using spraying, dusting and hypodermal needle injection method (Rao et al., 1989), twice a week until a heavy inoculum develops (Roelfs et al., 1992). The applied inoculum consisted of yellow (80E85) and mixture of leaf rust (PHTTL, PGRTB, KSR/JS, TKTPR and TKTRN) races collected from Murree, Kaghan and Faisalabad. High humidity was maintained by frequent irrigations.

Table 2. *Disease rating scale used for rust resistance/susceptibility of wheat elite lines*

Reaction	Code	Symptoms
Immune	0	No visible infection
Resistant	R	Visible necrotic or chlorosis with or without uredia
Moderately resistant	MR	Small uredia surrounded by necrotic areas
Mixed (intermediate)	M	Small uredia present surrounded by necrotic areas as well as medium uredia with no necrosis but possible some distinct chlorosis
Moderately susceptible	MS	Medium uredia with no necrosis but possible some distinct chlorosis
Moderately susceptible-susceptible	MSS	Medium uredia with no necrosis but possible some distinct chlorosis as well as large uredia with little or chlorosis present
Susceptible	S	Large uredia are present with little or no chlorosis

Cobb's scale (Peterson et al., 1948)

Molecular evaluation and yield testing of 112 selected wheat advance lines through molecular marker and PCA based cluster analysis

The putatively selected 112 wheat advance lines through primary evaluation were further assayed to molecular characterization to identify race non-specific resistance genes *Lr34/Yr18, Lr46/Yr29* and *Sr2/Yr30* using a set of 3 DNA molecular markers viz. X-barc-352, XWMC-44, and Xgwm-533 respectively (William et al., 2003; Suenaga et al., 2003; Hussain et al., 2015). This present study work was carried out at Integrated Genomics Cellular Developmental and Biotechnology Laboratory, Post Graduate Agricultural Research Station (PARS) Campus, University of Agriculture Faisalabad.

DNA extraction and quantification

The fresh leaf samples from 30 day-old seedling were collected from the Wheat Research Institute Faisalabad. After tagging, samples were washed with purified water and frozen immediately in liquid nitrogen (LN_2) chamber available in PARS campus University of Agriculture-Faisalabad and stored at -80 °C in deep freeze for DNA extraction by using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Bansal et al., 2014). Leaves were crushed in CTAB buffer to release DNA from the cell. Samples were incubated in water bath at 65 °C for 25–30 min. Tubes were centrifuge at 4000 rpm for 5 min and the upper aqueous phase was transferred to new tubes. Chloroform: isoamyl alcohol (24:1 v/v) (300-500 µl) was added and vortex 4-5 times to mix the contents properly. For further purification other reagents such as RNase and NaCl were also added and centrifuged for 5 min at 14000 rpm and supernatant was transferred to fresh tubes. DNA was precipitated by adding (500 µl) of chilled isopropanol in the tubes and let it at –20 °C for 25-30 min. The tubes were then

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centrifuged at 14000 rpm for 15 min to precipitate the DNA. The DNA pellet was washed 2-3 times with 500 µl of 70% ethanol and air dried before re-suspension in 20-30 µl ddH_2O. The DNA concentration was measured by spectrophotometer. An aliquot of sample was diluted in water ($1/80^{th}$ or $1/100^{th}$) and its absorbance was measured at 260 nm using a UV spectrophotometer.

PCR-marker assay

PCR amplifications were performed in a total volume of 25 µl containing 50-100 ng/µl of genomic DNA, 2.5 µl of 10X PCR buffer with 2.5 mM (2 µl) of Mgcl2, 0.5 of 10 mM $dNTP_S$, 0.5 µl each forward and reverse primer, 1U of Taq DNA polymerase and 17 µl of ddH20 (Ahmad et al., 2017). Reagents were purchased from Invitrogen (USA). PCR was performed using the Eppendorf Mastercycler, Germany. The amplification parameters used for all primer sets i.e. X-barc-352, Xwmc-44 and Xgwm-533 restricted to specific durable resistance genes are presented in *Table 3*.

Table 3. *Amplification parameters used for all primer sets linked to their specific durable resistance genes*

Resistance genes	Primers	Cycle condition
Lr34/Yr18	X-barc-352	94 °C 5 min, 38 cycles (94°C 30 s, 60°C 30 s-1 min, 72 °C 30 s), 72 °C 5 min
Lr46/Yr29	Xwmc-44	94 °C 5 min, 45 cycles (94 °C 1 min, 55 °C 1 min, 72 °C 2 min), 72 °C 10 min
Sr2/Yr30	Xwm-533	94 °C 5 min, 45 cycles (94 °C 1 min, 60 °C 1 min, 72 °C 2 min), 72 °C 10 min

Electrophoresis

After PCR amplification, electrophoresis was carried out on the Syngene gel documentation system USA for SSR markers (Hussain et al., 2015). An amount of 1.5 g high resolution agarose gel was weighted in the electric balance and dissolved in 100 mL 1 X TAE buffer (acetic acid pH = 7.8; Sodium acetate 2 mM; EDTA 10 mM; Tris HCL 40 mM) in a conical flask. It was heated for about 2-3 min by keeping it in oven and then left to cool under running tap water and mixed gently after adding 2 µl ethidium bromide (fluorescent dye) in this solution. The prepared solution was poured slowly into the gel tank. The combs of required size and teeth were inserted in it and leave it for 10-15 min to allow polymerization of gel. After polymerization, the 1XTAE buffer was poured into the gel tank to submerge the gel to 3-6 mm depth. The first well was loaded with 1 Kb ladder molecular weight marker (Promega) as a size standard. Appropriate amounts of about 8 µL of each PCR samples were loaded into the other wells. The gel tank was closed and the gel was run for 30 min by providing 50 to 100 V current to intercalate ethidium bromide in gel. After electrophoresis, the amplified products were visualized under ultraviolet transilluminator and gel pictures were obtained using Gene Snap version 7.6.03 of Syngene gel documentation system USA.

Yield testing of selected advanced lines on the basis of their genetic traits

For yield testing, data of other genetic traits such as plant height (cm), spike length (cm), the number of spikelet per spike, yield (kg ha^{-1}), thousand grain weight (gram)

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and protein percentage of all the 112 selected advanced lines along with five checks were recorded. The combined data of grain yield and its components were then subjected to analysis to estimate mean, standard error, range, simple correlation and variance. All variables traits were analyzed by PCA based cluster analyses using software program Statistca v. 10 and SPSS v.12. Cluster analysis identifies parameters which are further classified into a number of clusters following Ward's methods (Ali et al., 2008). The lines in each cluster were also analyzed for simple statistics. To show variation pattern among lines Euclidean distance were calculated and their relationship was shown in the scattered diagram.

Results

The current study was planned to achieve durable-type resistance by accumulating designated slow rusting race non-specific genes with high yield characteristics of wheat advance lines. The plant material was selected from 855 heads rows of 45 crosses planted at Wheat Research Institute Faisalabad, during crop 2015-2016, only 112 lines were selected on the basis of grain color, shape, high phenotypic uniformity and rust reactions (*Table 4*).

Table 4. *Selection of single head crosses from F6 generation of 45 crosses during 2015-2016*

Sr. #	Name of crosses	Tested entries	Selected entries
1	CHENAB2000/INQ.91/5/WBLL1*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ	19	3
2	AS-2002/5/FRET2*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAL	19	1
3	FSD.08/6/BABAX/3/FASAN/Y//KAUZ/4/BABAX/5/LU 26/HD2179	19	4
4	KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES/5/KAUZ//ALTAR	19	8
5	SH.88/PAK.81//MH.97//OTUS/TOBA97	19	3
6	SH.88/PAK.81//MH.97//CUMHURIYET/NE	19	3
7	OASIS/5*ANGRA//INQ.91///MILAN/S87230//BABAX	19	4
8	TRM//MAYA 74'S'/MON'S'/3/INQ.91/4/PBW343	19	7
9	87094/ERA//PAK-81/2*V-87094/3/SHAFAQ-06/4/MAYA/PVN	19	5
10	PFAU/MILAN/5/CHEN/A.SQ(TAUS)//BCN/3/VEE#7/BOW/4/PASTOR/6/QINGHAIBRI/WBLLI//BRBT2	19	3
11	INQALAB 91*2/KUKUNA//KIRITATI///V-09014	19	3
12	AUQAB 2000*2/LAKTA-1	19	4
13	FSD.08/6/BABAX/3/FASAN/Y//KAUZ/4/BABAX/5/LU26/HD2179/7/PB.96/87094//MH.97	19	6
14	TAM200/Tui/6/PVN/CRC422/ANA/5/BOW//CROW/BUC/PVN/3/YR/YR/4/TRAP#1/7/*21NQ-91	19	2
15	INQ/AUQAB/3/SH.88/90A204//MH.97	19	1
16	SH88/WEAVER/3/DWL5023/SNB//SNB	19	1
17	SH88/WEAVER/6/LU26/HD2179/5/BABAX/3/MANGO/VEE#10//PRL/4/BABAX	19	0
18	KAUZ//ALTAR84/AOS/3/PASTOR/4/TILHI/7/CNO79//PF70354/MUS/3/PASTOR/4/BAV92/5/FRET2/KUKUNA//FRET2/6/MILAN/KAUZ//PRINIA/3/BAV92	19	0
19	SH88/2*ATTILA/6/ACHTAR*3//KANZ/KS8585/4/MILAN/KAUZ//PRINIA/3/BAV92/5/MILAN/KAUZ//PRINIA/3/BAV92	19	2

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20	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001/8/OASIS/SKAUZ//4*BCN/3/2*PASTOR	19	3
21	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETOF2001/8/CROW'S'/NAC//BOW'S'	19	9
22	PFAU/SERI.1B//AMAD/3/INQALAB91*2/KUKUNA/4/WBLL1*2/KURUKU/5/PVN/YACO/3/KAUZ*2/TRAP// KAUZ	19	3
23	HUW234+LR34/PRINIA//PBW343*2/KUKUNA/3/ROLF07/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ	19	0
24	PRL/2*PASTOR//PBW343*2/KUKUNA/4/CAR422/ANA//TRAP#1/3/KAUZ*2/TRAP//KAUZ	19	0
25	C80.1/3*BATAVIA//2*WBLL1/3/PBW343*2/KUKUNA/4/KAUZ / SITE	19	3
26	INQALAB 91*2/KONK//INQALAB 91*2/KUKUNA/3/INQ-91*2/TUKURU	19	1
27	WHEAR/KRONSTAD F2004/3/CROW'S'/NAC//BOW'S'	19	1
28	WHEAR/KRONSTADF2004/3/PB96/V87094//MH97	19	1
29	FRT/SA42/3/PB96/87094//MH-97	19	1
30	WHEAR/KRONSTAD F2004//KAUZ / SITE	19	7
31	PFAU/MILAN//PBW343*2/TUKURU/3/T.DICOCCON P194625/A.SQ (372)//TUI….	19	1
32	PFAU/MILAN//PBW343*2/TUKURU/3/NR381	19	1
33	CROC_1/AE.SQUARROSA(205)//KAUZ/3/ATTILA/4/BOW/PRL//BUC/3/WH576/5/AMSEL/ATTILA//INQ.91/PEW'S'	19	3
34	CROC_1/AE.SQUARROSA (205)//KAUZ/3/PASTOR/4/THELIN/5/INQ/AUQAB	19	5
35	MINO/898.97/4/INIA66/7C//MAYA/3/PCI/TRM	19	1
36	CHONTE//PBW343*2/KUKUNA/3/CHENAB2000/INQ.91	19	0
37	CHONTE//PBW343*2/KUKUNA/INQ.91*2/TUKURU/3/T.DICOCCOM/P194624/AE.SQ (409)//BCN/4/2*INQ.91/2*/….	19	1
38	PB96/87094/MH-97/3/AMSEL/ATTILA//INQ.91/PEW'S'	19	0
39	PB96/87094//MH-97/3/MILAN/S87230//BABAX	19	1
40	LU26/HD2179//TTR'S'/JUN'S'/3/HP1744//4/MILAN/S87230//BABAX	19	0
41	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/IRENA/6/LERKE/7/TAN/PEW//SARA/3/CBRD	19	5
42	PBW343*2/KUKUNA//KRONSTADF2004/3/PBW343*2/KUKUNA/4/CHENAB2000/INQ.91	19	1
43	PBW343*2/KUKUNA//KRONSTADF2004/3/PBW343*2/KUKUNA/4/CHENAB2000/INQ.91	19	1
44	ATTILA*2//CHIL/BUC*2/3/KUKUNA/4/WAXWING*2/TUKURU	19	1
45	ROLF07*2/KIRITATI/3/SW8688//PBW343*2/KUKUNA	19	2
Total		855	112

All 112 selected wheat elite lines were further characterized on the basis of amplification of SSR molecular markers X-Barc352, Xwmc-44 and Xgwm-533 (*Table 5*). Among these lines, 32 lines exhibited *Lr34/Yr18*, 22 lines showed *Lr46/Yr29*, and 30 lines indicated the combination of *Sr2/Yr3*. Molecular marker X-barc-352 indicated association to *Lr34/Yr18* which was present on chromosomal loci 1BL. Only

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24 advanced lines were amplified by polymerase chain reaction (PCR) in which 19 genotypes were resistant and five advanced lines i.e. V-70001, V-70005, V-70006, V-70008, V-70009 and V-70010 were found susceptible (*Fig. 1*).

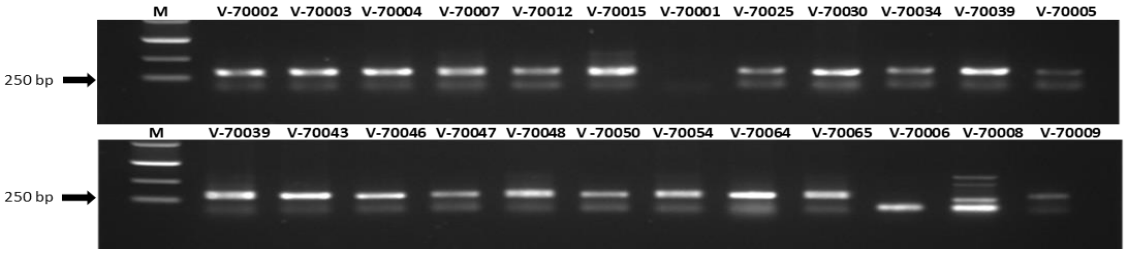

Figure 1. *PCR amplification profile of 24 advanced lines for SSR marker X-barc 352 linked to Lr34/Yr18; M = 1 Kb DNA Ladder Marker*

Table 5. *Detail of selected elite lines showing combination of three designated slow ruster, race non-specific resistance genes*

Plant material		**Genotypic markers**		
V. Code	Name of genotypes	*Lr34/Yr18* (X-barc-352)	*Lr46/Yr29* (XWMC-44)	*Sr2/Yr30* (Xgwm-533)
70002	CHENAB2000/INQ.91/5/WBLL1*2/4/SNI/TRAP #1/3/KAUZ*2/TRAP//KAUZ PB-36259-0A-0A-0A-9A-0A	+	–	+
70003	CHENAB2000/INQ.91/5/WBLL1*2/4/SNI/TRAP #1/3/KAUZ*2/TRAP//KAUZ PB-36259-0A-0A-0A-12A-0A	+	+	+
70004	AS2002/5/FRET2*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAL PB-36109-0A-0A-0A-7A-0A	+	–	+
70007	FSD.08/6/BABAX/3/FASAN/Y//KAUZ/4/BABAX/5/LU 26/HD2179 PB-36121-0A-0A-0A-8A-0A	+	+	–
70012	KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES/5/KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES PB-36189-0A-0A-0A-5A-0A	+	–	+
70014	KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES/5/KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES PB-36189-0A-0A-0A-15A-0A	–	+	+
70015	KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES/5/KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES PB-36189-0A-0A-0A-17A-0A	+	+	–
70025	OASIS/5*ANGRA//INQ.91///MILAN/S87230//BABAX PB-36286-0A-0A-0A-8A-0A	+	–	+
70030	TRM//MAYA74'S'/MON'S'/3/INQ.91/4/PBW 343 PB-36360-0A-0A-0A-11A-0A	+	–	+

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70033	TRM//MAYA74'S'/MON'S'/3/INQ.91/4/PBW 343 PB-36360-0A-0A-0A-19A-0A	−	+	+
70034	87094/ERA//PAK-81/2*V87094/3/SHAFAQ06/4/MAYA/PVN PB-36369-0A-0A-0A-11A-0A	+	+	+
70039	PFAU/MILAN/5/CHEN/A.SQ(TAUS)//BCN/3/VEE#7/BOW/4/PASTOR/6/QINGHAIBRI/WBLLI//BRBT2 PB-36377-0A-0A-0A-3A-0A	+	+	+
70043	INQALAB91*2/KUKUNA//KIRITATI///V-09014 PB-36447-0A-0A-0A-14A-0A	+	−	+
70046	AUQAB 2000*2/LAKTA-1 PB.37077-0A-0A-0A-8A-0A	+	−	+
70047	AUQAB 2000*2/LAKTA-1 PB.37077-0A-0A-0A-14A-0A	+	+	−
70048	AUQAB 2000*2/LAKTA-1 PB.37077-0A-0A-0A-19A-0A	+	+	−
70050	FSD.08/6/BABAX/3/FASAN/Y//KAUZ/4/BABAX/5/LU 26/HD2179/7/PB.96/87094//MH.97 PB.37082-0A-0A-0A-9A-0A	+	−	+
70054	FSD.08/6/BABAX/3/FASAN/Y//KAUZ/4/BABAX/5/LU26/HD217 9/7/PB.96/87094//MH.97 PB.37082-0A-0A-0A-19A-0A	+	+	+
70061	SH88/2*ATTILA/6/ACHTAR*3//KANZ/KS8585/4/MILAN/KAUZ//PRINIA/3/BAV92/5/MILAN/KAUZ//PRINIA/3/BAV92 PB No. 36821-0A-0A-0K-8A-0A	−	+	+
70064	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETOF2001/8/OASIS/SKAUZ//4*BCN/3/2*PASTOR PB No. 36829-0A-0A-0K-15A-0A	+	+	+
70065	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETOF2001/8/CROW'S'/NAC//BOW'S' PB No. 36830-0A-0A-0K-1A-0A	+	−	+
70070	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETOF2001/8/CROW'S'/NAC//BOW'S' PB No. 36830-0A-0A-0K-12A-0A	+	+	+
70072	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETOF2001/8/CROW'S'/NAC//BOW'S' PB No. 36830-0A-0A-0K-14A-0A	+	−	+
70076	PFAU/SERI.1B//AMAD/3/INQALAB91*2/KUKUNA/4/WBLL1*2/KURUKU/5/PVN/YACO/3/KAUZ*2/TRAP// KAUZ PB No. 36836-0A-0A-0K-10A-0A	+	+	+
70084	WHEAR/KRONSTADF2004//KAUZ/SITE PB No. 36880-0A-0A-0K-1A-0A	+	−	+
70085	WHEAR/KRONSTADF2004//KAUZ/SITE PB No. 36880-0A-0A-0K-11A-0A	+	+	+

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70086	WHEAR/KRONSTADF2004//KAUZ/SITE PB No. 36880-0A-0A-0K-12A-0A	+	–	+
70087	WHEAR/KRONSTADF2004//KAUZ/SITE PB No. 36880-0A-0A-0K-13A-0A	+	+	–
70088	WHEAR/KRONSTADF2004//KAUZ/SITE PB No. 36880-0A-0A-0K-15A-0A	–	+	+
70092	PFAU/MILAN//PBW343*2/TUKURU/3/NR381 PB No. 36885-0A-0A-0K-13A-0A	+	–	+
70096	CROC_1/AE.SQUARROSA(205)//KAUZ/3/PASTOR/4/THELIN/5/INQ/AUQAB PB No. 36893-0A-0A-0K-3A-0A	–	+	+
70098	CROC_1/AE.SQUARROSA(205)//KAUZ/3/PASTOR/4/THELIN/5/INQ/AUQAB PB No. 36893-0A-0A-0K-5A-0A	+	–	+
70101	MINO/898.97/4/INIA66/7C//MAYA/3/PCI/TRM PB No. 36894-0A-0A-0K-15A-0A	+	–	+
70103	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/IRENA/6/LERKE/7/TAN/PEW//SARA/3/CBRD PB No. 36976-0A-0A-0K-4A-0A	+	+	+
70104	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/IRENA/6/LERKE/7/TAN/PEW//SARA/3/CBRD PB No. 36976-0A-0A-0K-10A-0A	+	+	+
70107	CNDO/R143//ENTE/MEXI_2/3/AEGILOPSSQUARROSA(TAUS)/4/WEAVER/5/IRENA/6/LERKE/7/TAN/PEW//SARA/3/CBRD PB No. 36976-0A-0A-0K-18A-0A	+	+	–
70108	PBW343*2/KUKUNA//KRONSTADF2004/3/PBW343*2/KUKUNA/4/CHENAB2000/INQ.91 PB No. 36978-0A-0A-0K-8A-0A	+	+	–

+ sign shows the presence of rust resistance genes in wheat genotypes while
– sign shows absence of rust resistance genes in wheat genotypes

SSR marker Xwmc-44 exhibited linkage to *Lr46*/*Yr29* leaf and stripe rust resistance gene located on chromosome arm 7B. Its bands showed the amplification in the range of 242 bp. Eight elite lines were resistant while 16 advanced lines like V-70011, V-70012, V-70013, V-70014, V-70016, V-70017, V-70018, V-70019, V-70020, V-70021, V-70022, V-70023, V-70024, V-70026, V-70027 and V-70028 were found susceptible with *Lr46/Yr29* and the amplification of only 24 elite lines by polymerase chain reaction has been demonstrated (*Fig. 2*). PCR-based diagnostic marker XGWM-533 was linked to *Sr2*/*Yr30* stem and stripe rust resistance gene. *Sr2/Yr30* was exist on chromosomal loci 3BS. All advanced lines indicated the presence of this gene with the band size of 120 bp. Twenty four lines amplified by PCR showed that 13 lines were resistant while 11 genotypes i.e. V-70007, V-70015, V-70029, V-70031, V-70032, V-70035, V-70036, V-70037, V-70038, V-70040 and V-70041 were found susceptible (*Fig. 3*).

From this investigation it was concluded that among 112 advanced lines, only 10 lines V-70003, V-70034, V-70039, V-70054, V-70064, V-70070, V-70076, V-70085, V-70103 and V-70104 demonstrated the association of 3 designated slow rusting/race non-specific genes. This is very significant linkage, as it gives resistance against all 3 types of rust i.e. stripe, leaf and stem rust. Similarly, 15 genotypes Viz. V-70002, V-

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70004, V-70012, V-70025, V-70030, V-70043, V-70046, V-70050, V-70065, V-70072, V-70084, V-70086, V-70092, V-70098 and V-70101 exhibited the linkage of *Lr34*/*Yr18* and *Sr*2/*Yr*30. Linkage of *Lr46/Yr29* and *Sr2/Yr30* was indicated in 5 lines viz. V-70014, V-70033, V-70061, V-70088, V-70096 and the association of *Lr34*/*Yr18* and *Lr46*/*Yr29* was identified in 7 lines including V-7007, V-70015, V-70047, V-70048, V-70087, V-70107, and V-70108. All these brilliant advanced lines having durable type resistance along with low values of area under disease progress curve may be used in future hybridization schemes to enhance level of resistance in the adapted wheat cultivars of Pakistan (Inqilab-91, Uqab-2000, AS-2002, Seher-2006 and Fareed-06 etc).

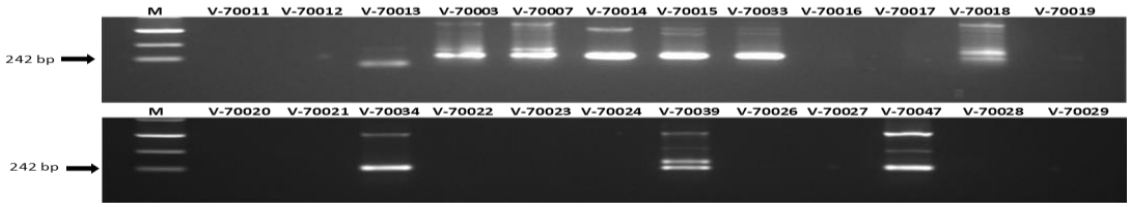

Figure 2. *PCR amplification profile of 24 advanced lines for SSR marker XWMC-44 linked to Lr46/Yr29; M = 1 Kb DNA Ladder Marker*

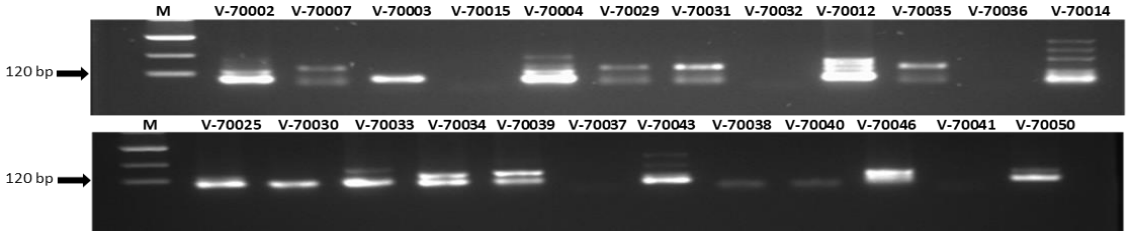

Figure 3. *PCR amplification profile of 24 advanced lines for SSR marker XGWM-533 linked to Sr2/Yr30; M = 1 Kb DNA Ladder Marker*

Yield testing of selected wheat elite lines on the basis of their genetic traits

Basic statistics for all parameter is described in *Table 6* showed a considerable variability among germplasm that were under study. Medium to high variance was determined for plant height (cm), thousand grain weight, number of spikelet per spike and grain yield (kg ha^{-1}) while small variance was determined for spike length (cm) and protein percentage.

Correlation analyses

A matrix of correlation coefficient among grain yield and its component was determined (*Table 7*). Results indicated that plant height exhibited significant correlation with protein (%) while highly significant correlation with spike length. Thousand grain weight exhibited significant relationship with number of spikelet per spike. A highly significant correlation was observed between grain yield (kg/ha^{-1}) and 1000 grain weight indicating the need of more emphasis on these parameters to increase yield in wheat.

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Table 6. *Basic statistics for 6 quantitative variables of 112 advance lines along with five checks*

Sr. no	Parameters	Mean ± S.D.	Minimum value	Maximum value	Variance
1.	PH (cm) [a]	106.479 ± 7.1192	86.000	124.000	50.70
2.	GY (Kg/ ha^{-1}) [b]	3762.829 ± 609.2895	2469.000	4800.000	371233.7
3.	P (%) [c]	11.430 ± 0.8742	9.400	13.600	0.8
4.	TGW (g) [d]	36.921 ± 3.5343	30.000	45.000	12.5
5.	SL (cm) [e]	9.392 ± 1.1790	6.980	12.920	1.4
6.	SSP [f]	45.458 ± 4.8649	36.430	55.980	23.7

[a]Plant height (cm); [b]Grain yield (Kg ha^{-1}); [c]Protein (%); [d]1000 grain weight (g); [e]Spike length (cm); [f]Number of spikelet per spike

Table 7. *Correlation coefficient (r) matrix for estimated six parameters of genotypes*

Parameters	X1	X2	X3	X4	X5	X6
PH (cm) (X1)	1.000					
GY (kg/ha^{-1}) (X2)	0.077 0.406	1.000				
P (%) (X3)	0.193* 0.037	0.152 0.102	1.000			
TGW (g) (X4)	0.044 0.638	0.252** 0.006	0.062 0.504	1.000		
SL (cm) (X5)	0.256** 0.005	0.074 0.429	0.226* 0.014	0.170 0.067	1.000	
SSP (X6)	0.098 0.296	0.079 0.396	0.054 0.565	0.205* 0.026	0.482** 0.000	1.000

Upper values indicated Pearson's correlation coefficient. Lower values indicated level of significance at 5% probability. * = significant (P < 0.05); ** = highly significant (P < 0.01). Abbreviations as in *Table 6*

Cluster analysis categorized 112 wheat lines along with five checks into 5 clusters (*Table 8; Fig. 4*). Distribution pattern of all the genotypes into various clusters exhibited the presence of considerable genetic variability among the genotypes for most of the traits studied. Association among these cluster members showed that clusters V, IV and II showed maximum, while cluster I and III indicated minimum mean values for most of the traits respectively (*Table 9*). Results confirmed that all genotypes formed in cluster V under trial condition exhibited highest mean values for all traits. After testing under different environmental conditions, all 13 lines of cluster V except V-70078 due to lack of resistance genes (*Table 5*) could be used for their direct release as variety. Furthermore, all these outstanding lines might be used in hybridization programs to develop rust resistance and high yield varieties.

Six PCs (PC1-PC6) were made from original statistical data revealing 98% of total variation (*Table 10*). Out of six principal components three PCs (PC1-PC3) have Eigen value greater than 1, accounted for individual variance values of 30.93, 18.44, and 17.84% with 67.21% of cumulative variation of grain yield respectively. The first two PCs were plotted on PC axis 1 and 2 that showed high variability in the existing wheat lines and checks (*Fig. 5*). Traits with largest absolute values closer to unity within the

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PC1 influence the clustering more than those with lower absolute values closer to zero (Chahal and Gosal, 2002). Therefore, in this investigation, differentiation of the advanced lines into different cluster was due to the cumulative effect of a number of traits rather than the contribution of specific few characters. All traits in PC1 showed negative component value whereas, grain yield (kg/ha^{-1}) exhibited great effect in second Principal Component (PC2). Traits having relatively higher value in the PC3 like number of spikelet per spike, thousand grain weight and spike length had more contribution to the total variation and they were the ones that most differentiated the clusters. Plant height (cm), grain yield (kg/ha^{-1}) thousand grain weight (g) in the PC4, plant height (cm), protein (%), thousand grain weight (g) in PC5, spike length (cm), grain yield (kg/ha^{-1}) and thousand grain weight (g) in PC5 were the major contributors to each Principal Components (PC). The current investigation confirmed that advanced wheat lines exhibited wide range of variations for the traits studies and it also proposed that ample prospects for genetic improvement of wheat genotypes through selection directly from bread wheat genotypes and conservation of the germplasm for future utilization.

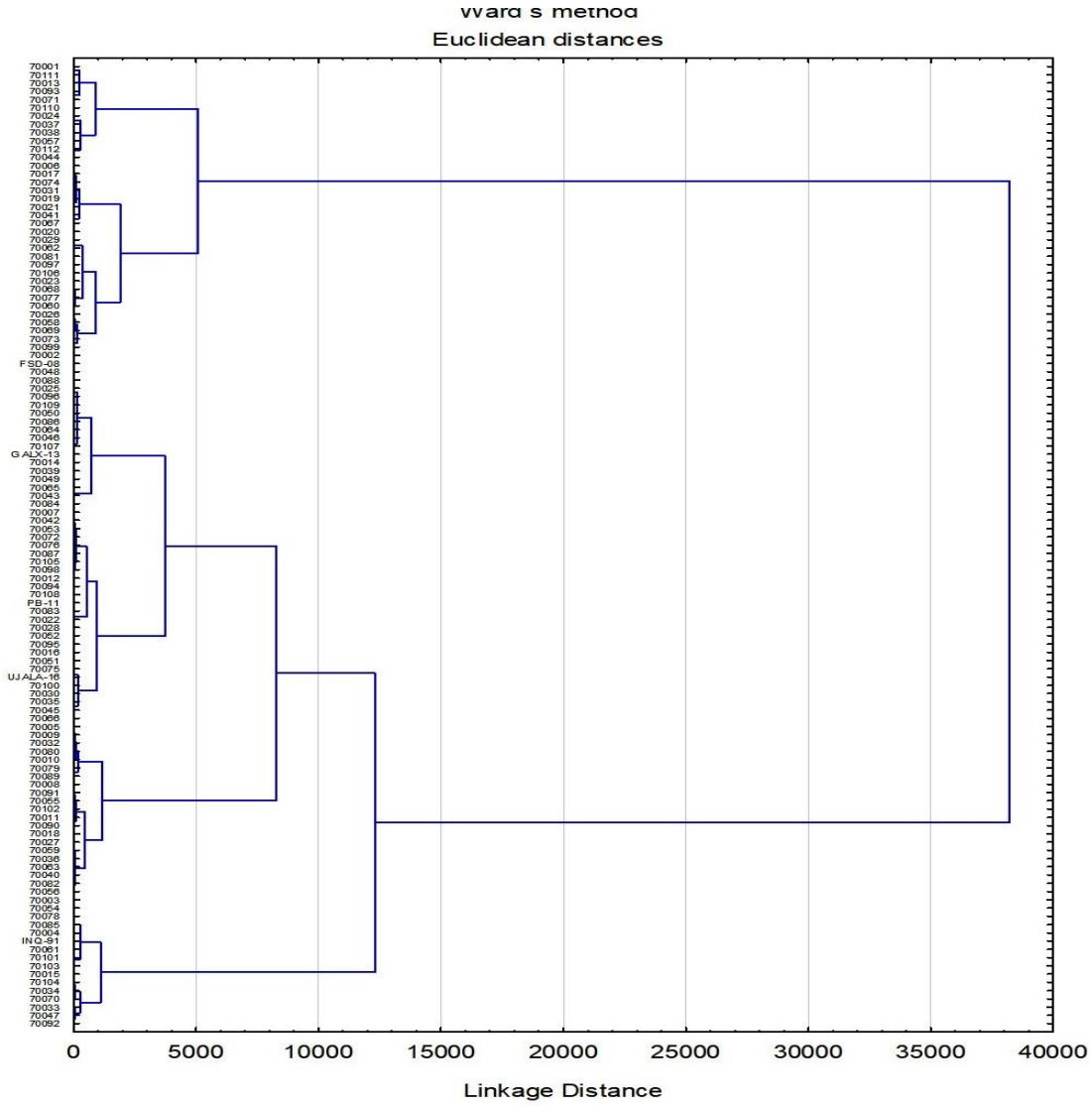

Figure 4. *Cluster diagram of 112 advance lines and varieties based on sic traits under study*

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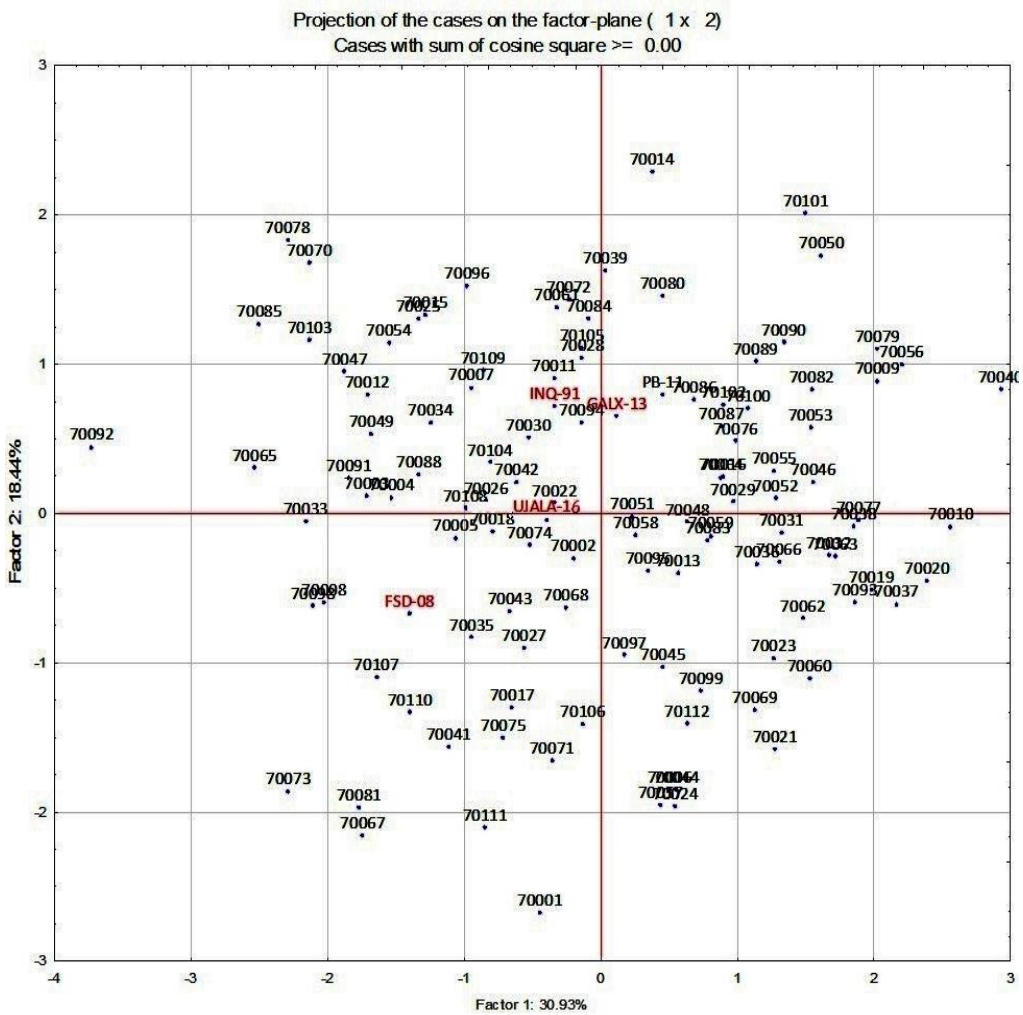

Figure 5. *Scattered diagram of first two PCs (Factor 1 as PC1 and Factor 2 as PC2)*

Discussion

PCR-based DNA markers associated with genes controlling target economic traits have significant role to attain sustained wheat production. Molecular marker-trait combinations give an effective alternative to phenotyping for selecting varieties that have linkage of desirable genes in breeding populations.

Here we used three SSR markers XGWM-533, XWMC-44 and X-barc-352 for effective marker assisted selection of *Sr2/Yr30*, *Lr46*/*Yr29* and *Lr34/Yr18* in selected wheat elite lines. This investigation exhibited, among all tested genotypes only 10 advanced lines showed a tight linkage to *Sr2/Yr30*, *Lr46*/*Yr29* and *Lr34/Yr18* having durable type resistance under the severe disease epidemics. According to Singh and Bowden (2011), resistance near immunity can be achieved by combining 4-5 race non-specific resistance genes in a cultivar. Though, slow level of resistance can be attained by combining 2 to 3 race non-specific/minor genes in a genotype (Lagudah et al., 2009). International Maize and Wheat Improvement Centre (CIMMYT) and Ayub Agriculture Research Institute (AARI) planned a technique of combining race non-specific resistance genes alone or in linkage with some other genes to control recently evolved strains of wheat rust (Rehman et al., 2013).

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Table 8. *Five cluster grouping wheat lines based on six parameters under study*

Cluster	Frequency	Cluster membership
I	22	V-70005, V-70009, V-70010, V-70011, V-70018, V-70026, V-70027, V-70032, V-70036, V-70040, V-70056, V-70058, V-70059, V-70063, V-70069, V-70073, V-70079, V-70080, V-70082, V-70089, V-70090 and V-70099
II	29	V-70007, V-70008, V-70012, V-70016, V-70022, V-70028, V-70030, V-70035, V-70042, V-70045, V-70051, V-70052, V-70053, V-70055, V-70066, V-70072, V-70075, V-70076, V-70083, V-70087, V-70091, V-70094, V-70095, V-70100, V-70102, V-70105, V-70108, UJALA-16 and PB-11
III	30	V-70001, V-70006, V-70013, V-70017, V-70019, V-70020, V-70021, V-70023, V-70024, V-70029, V-70031, V-70037, V-70038, V-70041, V-70044, V-70057, V-70060, V-70062, V-70067, V-70068, V-70071, V-70074, V-70077, V-70081, V-70093, V-70097, V-70106, V-70110, V-70111 and V-70112
IV	21	V-70002, V-70014, V-70025, V-70033, V-70039, V-70043, V-70046, V-70048, V-70049, V-70050, V-70064, V-70065, V-70084, V-70086, V-70088, V-70096, V-70098, V-70107, V-70109, GALX-13 and FSD-08
V	15	V-70003, V-70004, V-70015, V-70034, V-70047, V-70054, V-70061, V-70070, V-70078, V-70085, V-70092, V-70101, V-70103, V-70104 and INQ-91

Table 9. *Mean and standard deviation for five clusters based on six parameters under study*

Traits	Mean ± SD				
	Cluster – I	**Cluster –** II	**Cluster –** III	**Cluster –** IV	**Cluster –** V
PH (cm)	104.55±9.64	106.41±5.17	106.33±7.88	106.81±6.49	109.27±4.92
GY (g)	3583.3±148.9	3969.0±92.0	2911.2±235.0	4246.7±72.8	4653.5±100.0
P (%)	11.21±0.96	11.39±0.76	11.40±0.90	11.34±0.96	12.00±0.59
TGW (g)	36.62±3.81	36.62±3.46	35.80±2.64	37.48±3.83	39.41±3.51
SL (cm)	8.73±1.10	9.29±0.88	9.50±1.41	9.69±1.02	9.93±1.16
SSP	43.46±4.55	45.18±4.72	45.55±4.96	46.58±5.09	47.18±4.58

Abbreviations as in *Table 6*

Table 10. *Eigen values, percent individual variance and percent cumulative variance for 6 characters studied on 112 advanced wheat lines along with five checks*

Parameters	PC1	PC2	PC3	PC4	PC5	PC6
PH (cm)	-0.47	-0.33	-0.47	0.65	0.04	-0.12
GY (kg ha^{-1})	-0.40	0.71	-0.25	0.047	-0.53	0.05
P (%)	-0.46	-0.06	-0.64	-0.56	0.21	-0.14
TGW (g)	-0.49	0.59	0.24	0.16	0.57	0.01
SL (cm)	-0.77	-0.34	0.18	-0.10	-0.06	0.49
SSP	-0.67	-0.19	0.53	-0.11	-0.19	-0.43
Eigen value	1.85	1.11	1.07	0.79	0.70	0.47
Individual variance (%)	30.93	18.44	17.84	13.25	11.68	7.85
Cumulative variance (%)	30.93	49.37	67.21	80.46	92.14	98.00

Abbreviations as in *Table 6*

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These genotypes are important source of rust protection with high yield potential. The resistance in determined genotypes seems to be durable in nature. The race specific resistance controlled by the parent lines was vulnerable as the single line V-70001 exhibited severe disease outbreaks ranging from 50-60% in the disease screening plots. Combination form these parent lines against common stripe and leaf rust races proved very effective with lower disease severity in the country (Hussain et al., 2006).

Many new released cultivars have been banned for general cultivation only because of disease vulnerability against novel stripe and leaf rust races (Khan et al., 2002). Combining 2-3 or more genes in a wheat genotype for race non-specific resistance has remained the main emphasis of researchers to combat the changing nature of novel virulent races (Roelfs, 1988). To contest this problem, DNA molecular marker technique was used for improving rust resistance through combining various race non-specific resistance genes in selected wheat lines. Genotypes possessing slow rust linkage illustrated lower area under disease progress curve at adult-plant stage have durable resistance as also indicated by various researchers (Bariana et al., 2001; Singh et al., 2005; Singh and Bowden, 2011). Because the race non-specific resistance like partial and durable rust resistance is polygenic as observed elsewhere, therefore, it remains effective for longer time period, even if the pathogen change its virulence pattern through mutation or recombination (Dehghani and Moghaddam, 2004). Thus, in present study, genotypes having low rust intensity could be considered as durable lines carrying high level of rust resistance to *Sr2, Lr46,* and *Lr34* virulences, that might be used in future hybridization schemes to protect crop stability. For its relative ease, productivity and specificity, many researchers have examined the robustness of these molecular markers to identify the occurrence of stripe and leaf rust resistance in wheat germplasm (Dakouri et al., 2013; Lagudah et al., 2006; Mustafa et al., 2013).

In order to evaluate, maintain and use advanced lines effectively it is necessary to study the extent of genetic variability available. Morphological characterization has been successfully used for determination of genetic variability and variety development (Fufa et al., 2005). Analysis of genetic variability through cluster and PCA analyses among germplasm collection help in sorting and core collection of genotype that used for specific breeding purpose (Muhammadi and Prasana, 2003). The cluster analysis classified lines into clusters that showed high intra cluster homogeneity and inter cluster heterogeneity (Jaynes et al., 2003). Considering the significant correlation between grain yield and thousand grain weight and also that the average values of these two parameters for Cluster V are greater than the average of all elite lines. Member of this cluster may be used to increase yield in breeding schemes. The results of this investigation are in line with the findings of Leilah and Al-Khateeb (2005), Ali et al. (2008), Hendawy et al. (2011), and Hristov et al. (2011) who demonstrated the significant correlation between grain yield and other quantitative variables. Spike length and plant height were positively correlated. Same was observed in case of thousand grain weight and number of spikelet per spike i.e. as it decreased the grain yield (kg ha^{-1}) also decrease and as it increased the grain yield also increase (Kamyab et al., 2009).

The principal component analysis showed that, all six PCs had 98% of total variation in the data (Hailegiorgis et al., 2011). Principal component and cluster analyses allowed natural clustering of wheat germplasm. Accordingly, the various measurement methods can be properly used for clustering of wheat germplasm (Kraic et al., 2009). Thus results demonstrated that PCA based cluster analyses is more precise indicator of difference among wheat advance lines than cluster analyses not based on PCA.

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However, increased yield potential is stated goal for researchers. Progress in yield characteristics results from the progressive accumulation of minor genes possessing high yield potential (Ajmal et al., 2013). In present investigation, 32 lines showed the linkage of *Lr34/Yr18*, 22 lines demonstrated *Lr46/Yr29* and 30 lines exhibited the linkage of *Sr2/Yr30*. Determining the existence of *Sr2*, *Lr46*, and *Lr34* genes in current elite lines is useful to predict field response. The stability of these selected elite lines helps decision in selecting parentage for future hybridization and to develop new varieties with improved yellow and leaf rust resistance. Thus, the scheme of combining race non-specific genes through breeding is the best way to attain durable resistance in wheat germplasm under continuously changing virulence pattern in the country.

Conclusion

The advanced lines V-70003, V-70034, V-70039, V-70054, V-70064, V-70070, V-70076, V-70085, V-70103 and V-70104 exhibited the combination of all three slow rusting genes (*Lr34/Yr18*, *Lr46/Yr29* and *Sr2/Yr30*). In principal component analysis was demonstrated that six principal components PC1, PC2, PC3, PC4, PC5 and PC6 accounted for 98.00% of the total variation. The first two principal components PC1 and PC2 with values of 30.93 and 18.44%, respectively, contributed more to the total variation indicating hybridization breeding program can be initiated by the selection of genotypes from the PC1 and PC2. Among all traits evaluated plant height (cm), Plant height (cm), grain yield (kg ha^{-1}), protein (%), thousand grain weight (g), spike length (cm), and number of spikelet per spike in each principal component contributed more to the total genetic variations. In conclusion, the crosses between advanced lines selected from cluster-V are expected to produce better genetic recombination and segregation in their progenies. Therefore, these advanced lines need to be crossed and selected to develop high yielding pure line variety.

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