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Evaluation of yellow rust resistance in backcross populations of wheat

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Abstract

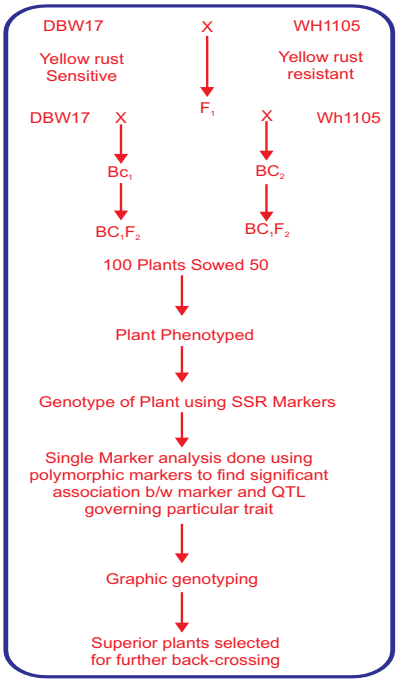
Aim: To screen wheat populations derived from cross DBW17 × WH1105 for loci imparting yellow rust resistance and selection of plants using polymorphic SSRs.

Methodology: The study for yellow rust resistance was carried out on two populations, *i.e.*, BC_1F_2 and BC_2F_2. Stress was provided by planting infector rows between the blocks and by artificial inoculation using a mixture of races 46S102, 47S103 and 78S84 of stripe rust pathogen. DNA isolated from young leaves was checked for the presence of yellow rust resistance genes using gene specific primers.

Results: Fifteen primers were found to be polymorphic among parents DBW17 and WH1105. Fifteen polymorphic SSR markers were dispersed over the wheat genome (AABBDD), with allele range 2-5. These polymorphic SSR markers were used to produce molecular diversity among progeny lines. Cluster analysis of parents and both the populations, showed that two parents were diverse genetically and in both backcrosses progeny lines resembled their respective recurrent parent. Single marker analysis using data revealed that primers on nine chromosomes were associated with grain yield per plant, other yield attributes and yellow rust resistance in both populations.

Interpretation: This study showed that a linked marker like Xgwm582 could be a promising tool for breeding wheat with enhanced tolerance to yellow rust resistance. However, growth rates and biomass production provide reliable criteria for assessing the degree of yellow rust resistance and the ability of a plant to withstand it.

Key words: SSR markers, Wheat, Yellow rust



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Introduction

Wheat (*Triticum aestivum* L.) is the third most important food crop in the world after maize and rice. It is an important staple food crop and has been cultivated in major civilizations of Europe, West Asia and North Africa. Today, wheat is grown on land area, covering about 29.32 million ha with annual production of 103.6 million tons in 2019-20 (FAOSTAT, 2020). It continues to be the most important food grain source contributing about a fifth of total calories consumed by humans. To meet the increasing food demand of a growing population, wheat crop has been constantly improved for increased resistance to biotic and abiotic stresses using various genetic improvement programmes but still its production is challenged by several diseases; among them, rusts are most prominent. Yellow rust is the most widely distributed and one of the most destructive diseases of wheat, caused by the fungus *Puccinia striiformis f.* sp. *tritici* severely threatens the wheat worldwide. The yield losses from stripe rust infection vary from 10 to 100% depending on the susceptibility of cultivar, infection stage and environmental conditions (Tahir *et al.,* 2020).

Presently, two methods are being followed for managing the problem of yellow rust; first is use of chemicals and secondly cultivation of rust resistant varieties. The erstwhile can prevent the disease but the application of these chemicals adds a significant extra cost to farmers and cause adverse effects to environment also. Therefore, cultivating rust resistant varieties is recommended for better management of crop. In this scenario, it would be difficult to succeed with conventional breeding as the resistance is frequently broken and the role of plant sciences and biotechnology becomes crucial for the future of humankind and provides better understanding of the existing genetic diversity that should be considered for raising the yield frontier in wheat (Bigini *et al.,* 2021). DNA markers are highly effective in identifying genes and selecting multi-genic traits and genes which are mainly influenced by environmental conditions (Yashveer *et al.,* 2020). The rapidly evolving technology of DNA markers helps to open a real possibility for developing functional markers as reliable genetic markers for use in plant breeding. Molecular markers, specifically simple sequence repeats (SSRs), play a major role in marker-assisted wheat breeding programs.

They have become the preferred markers as they are widely distributed in plant genomes, including coding and non-coding genomic regions (Tyagi *et al.,* 2021) and are being used for investigation of genetic divergence, genome and QTL mapping for yellow rust resistant genes such as *Yr5, Yr10, Yr15, Yr26, Yr45, Yr53, Yr64* and *Yr65* (Yaniv *et al.,* 2015) and are useful tools for gene pyramiding to speed up the development of resistance in wheat cultivars. Therefore, the identification of molecular markers closely associated with yellow rust resistance is a promising approach to accelerate the breeding process by discovering novel resistance genes and pyramiding multiple genes in a robust and simplified manner. The present paper describes the use of microsatellite markers for genetic analysis as they co-segregate with the trait and are therefore candidate markers for yellow rust resistance genes. Thus SSRs help in screening the plants with linked markers for confirming the presence of introgressed genes. In view of the above, the aim of the study was to identify SSR markers that are tightly linked to stripe rust genes and other yield related traits in the present study.

Materials and Methods

BC_1F_2 and BC_2F_2 populations of a cross between well-known cultivar, DBW17 with good agronomic traits but susceptible to yellow rust and cultivar having wide adaptability, excellent biological characteristics and higher resistance to stripe rust, WH1105 were sown in 1 row of 2m length. Infector rows were planted between the blocks and artificial inoculation using a mixture of races 46S102, 47S103 and 78S84 of stripe rust pathogen was carried out under field conditions. Infected leaves containing uredospores were also directly rubbed with healthy leaves to spread the infection. The disease severity on leaves was evaluated by Modified Cobb's Scale in which rust severity was recorded as a percentage of leaf area infected with values ranging from 0 to 100%. The field response of the genotypes to the rust infection was scored on the basis of selected plants classified as highly resistant (HR), moderately resistant (MR), moderately susceptible (MS) and highly susceptible (HS) (Fig. 1).

Genomic DNA isolation and SSR screening: Genomic DNA was isolated from young leaf tissues of the BC_1F_2 and BC_2F_2 progeny plants and parents using CTAB method (Saghai-Maroof *et al.,* 1984). Quantitative estimation of isolated genomic DNA was done on a UV spectrophotometer at a wavelength of 260 nm as well as 280 nm. Using the Beer-Lambert Law of 1.0 O.D. as 260 nm equivalent to 50 ng DNA per ml, the quantity of DNA was estimated by the following formula:

$$\text{DNA (ng/µl)} = \text{O.D. A260} \times \text{Dilution factor} \times 50$$

Quality of DNA samples was checked by both UV spectrophotometer as well as by agarose gel electrophoresis. Parental DNA was amplified using 99 SSRs to study polymorphism mentioned in Table 1. All these primers were custom synthesized from Sigma Chemicals Co. USA.

Polymerase Chain Reaction Amplification: PCR amplification reaction was carried out in applied biosystem thermocycler with reaction volume of 20 µl containing 10X PCR buffer, 10 mM dNTPs, 0.4 µM of each primer, 1 unit Taq DNA polymerase and 50–80 ng template DNA. Amplified DNA products were resolved by submerged horizontal electrophoresis in 2.5% agarose gels, viewed under UV light fluorescence using Labnet Ultra violet trans-illuminator and image was taken by GenoSens Gel Doc system. The presence of DNA band on agarose gel was taken as one and the absence was read as zero. The size (in nucleotides base pairs) of the amplified bands was determined based on its migration relative to standard DNA marker (100 bp DNA ladder).

Fig. 1: (a) Wheat genotypes showing response to rust infection: Traces of Yellow Rust (HR); (b) Resistant Reaction to Yellow Rust (R); (c) Moderately Resistant Reaction to Yellow Rust (MR); (d) Showing Moderately Susceptible Reaction to Yellow Rust (MS) and (e) Showing Highly Susceptible Reaction to Yellow Rust (HS)

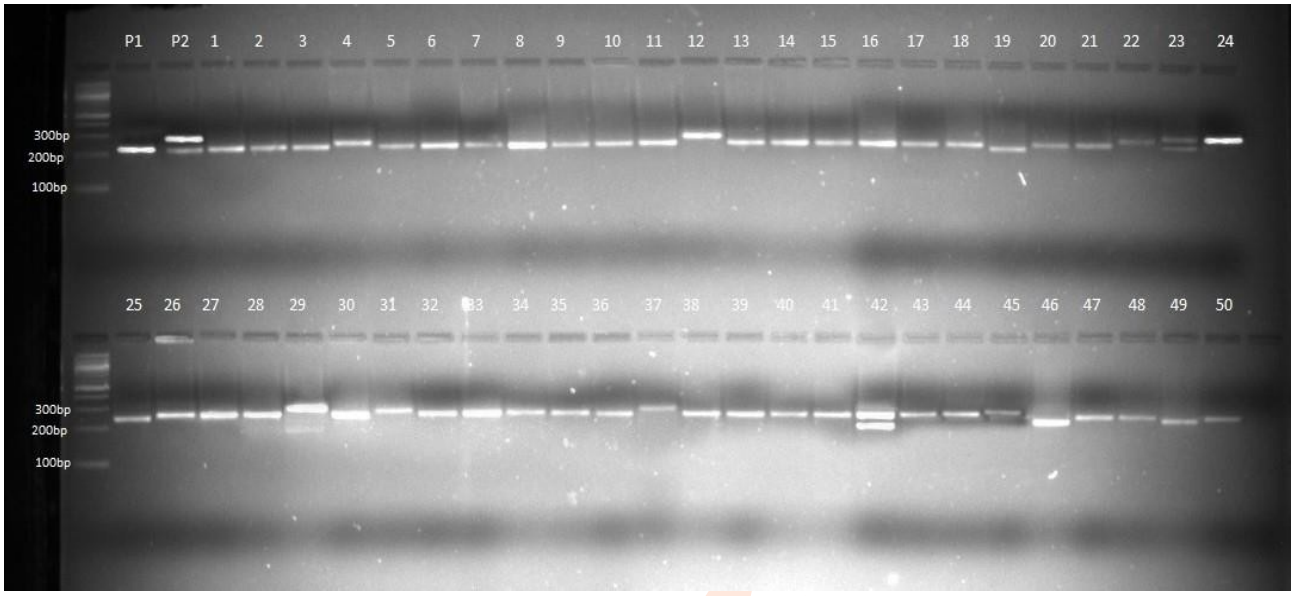

Fig. 2: Polymorphism in BC_1F_2 population using marker Xgwm 429.

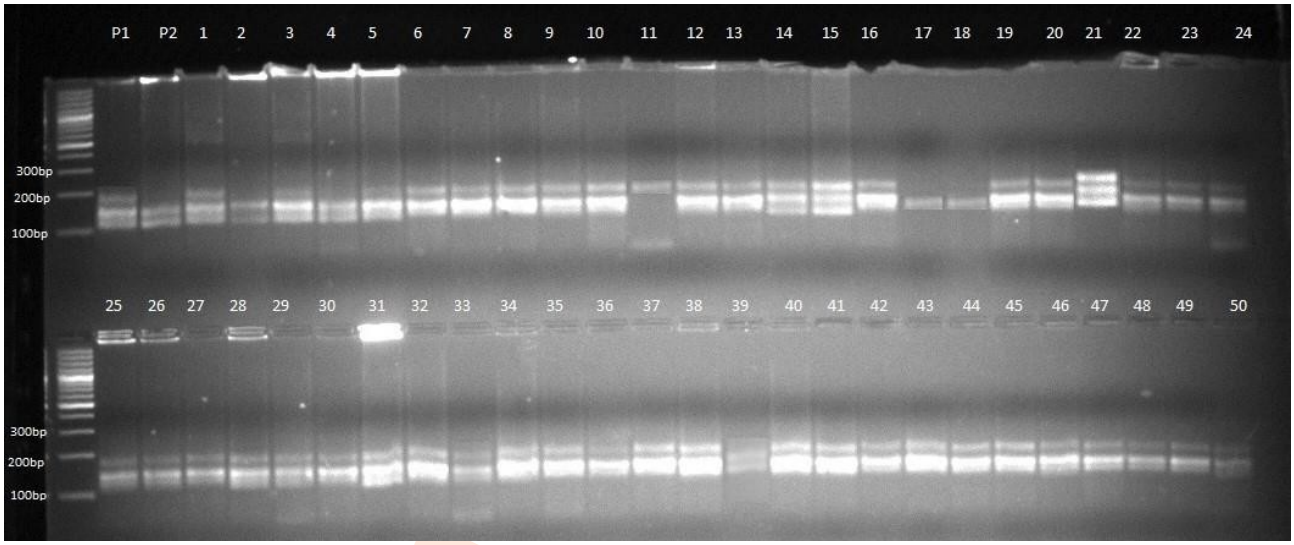

Fig. 3: Polymorphism in BC_2F_2 population using marker Xgwm408.

Statistical Analysis: Binary data was used to calculate similarity genetic distance using 'simqual' sub-program of software NTSYS–PC (Rohlf, 1992) and dendrogram was constructed on similarity bases. Single marker analysis was done using 15 polymorphic primers. The A, B, H scoring data of BC_1F_2 and BC_2F_2 populations along with yellow rust reaction and other morpho- physiological traits were analyzed using software Win QTL-Cartographer. Graphic genotyping was done using software such as GGT 2.0 which helps the breeders to visualize the genotypes of individuals and populations.

Results and Discussion

Goodness of fit test was also used by Arora *et al*. (2021) to determine the nature and number of leaf rust and yellow rust resistance genes in the segregating population. In BC_1F_2 out of 100 plants, 9 plants showed 5-10 percent severity, 35 plants were on 20 scale, 32 plants showed 40 percent severity, 18 showed 60 percent and 6 plants showed 100 percent infection and in BC_2F_2 population out of 50 plants, 7 were on 5-10 scale, 16 plants

Table 1: List of 99 SSR markers (including Yr specific markers) used for screening polymorphism among parents

Maker	Position	Tm	Maker	Position	Tm
Xgwm140	Yr29	57.3	Xbarc137	1B	57.0
Xgwm574		56.8	Xbarc187	1B (Yr24)	59.0
Xgwm537	7B	62.0	Xbarc59	2D, 5B	64.0
Xgwm644	6B	59.0	Xbarc352	4D, 7D	70.4
Xgwm130	Yr7	58.7	Xbarc76	7D, 2A, 6B (Yr18)	67.0
Xgwm247	2B	59.1	Xbarc182	7B	70.0
Xgwm382	Yr1	58.1	Xbarc167	2B (Yr5)	61.0
Xgwm46	7B	55.0	Xbarc353	2D (Yr17)	61.3
Xgwm319	2B	61.0	Xbarc72	7B	65.0
Xgwm120	Yr5	51.0	Xbarc136	6B	69.5
Xgwm273	1B (YrH52)	50.5	Xbarc101	3B (Yr36)	56.0
Xgwm181	3B	55.0	Xbarc7	2B	56.0
Xgwm6	4B	51.5	Xbarc147	3B	56.0
Xgwm186	5A	61.0	Xbarc80	1B	56.0
Xgwm16	2B/5D/7B	63.5.	Xbarc146	6B, 6D, 6A	56.0
Xgwm192	5D	64.0	Xbarc124	2A	56.0
Xgwm140	Yr29	60	Xbarc240	1B, 1D	64.5
Xgwm359	2A/Yr32	49.5	Wmc44	1B (Yr29)	57.0
Xgwm374	2B	49	Wmc120	1A	62.0
Xgwm429	2B	50	Wmc198	2A (Yr32)	57.0
Xgwm437	7D	54.2	Wmc43		60.0
Xgwm539	2D	59	Wmc167	2D	56.0
Xgwm582	1B(Yr9)	49	Wmc25	2B	53.0
Xgwm674	3A	55	Xwmc407	2A	58.0
Xgwm349	2D(Yr5)	61.0	Xwmc631	3D	61.0
Xgwm268	1B	60.2	Wmc170	2A	56.0
Xgwm501	2B (Yr5)	63.5	Wmc559	3A	56.0
Xgwm325	6B	59.0	Wmc398	6B (Yr17)	56.0
Xgwm261	2D	62.0	Xwmc175	2B	59
Xgwm630	2B	61.0	Xwmc215	5D	66.5
Xgwm146	7B	56.0	Xwmc216	1D	60.5
Xgwm70	6B	59.0	Xwmc273	7A	53.5
Xgwm264	1B, 3B (Yr15)	56.0	Xwmc276	7B	52.5
Xgwm498	1B (Yr26)	57.5	Xwmc332	2B	60.7
Xgwm295	7D (Yr18)	58.0	Xwmc406	1B	58.5
Xgwm170		56.0	IAG95	1D (Yr9)	51.0
Xgwm259	1B (Yr25)	56.0	Gwm11	Yr15/Yr24	57.0
Xgwm631	7A	63.0	Xcfd12	5A	58.0
Xgwm413	1B (Yr15)	62.5	Cfd2	7A	56.0
Xgwm47	2B	47.0	Cfa2185	Yr36	56.0
Xgwm611	7B	56.0	Xcfa2040	7B	58.0
Xgwm437	7D	56.0	Xwgp78		58.5
Xgwm190	5D	56.0	Xwgp82		56.0
Xgwm302	7B	56.0	Xwgp45		56.0
Xgwm630	2B	56.0	S19m93	Yr5	56.0
Xgwm408	5B	56.0	yrSTS7	Yr5	47.0
Xgwm297	2D	56.0	XSPS3000	IBS/ Yr10	47.5
Xgwm95	2A	56.0	Maker	Position	Tm
Xgwm249	2A (Yr16)	56.0	CSLV34	Yr18/Lr26/Sr39	47.0
Xbarc181	1B (Yr26)	55.0	Cfd23	Yr46	58.5

showed 20 percent severity, 20 plants showed 40 percent infection, 5 showed 60 percent and 2 plants showed 100 percent severity (Table 2). Considering the inheritance pattern of resistance to yellow rust, in both the backcross populations 1:1 (resistant: susceptible) ratio was observed which was confirmed by Chi-square test ($\chi 2$ cal. = 1.54 and 0.32). Today, molecular

Table 2: Incidence of yellow rust disease on parents and backcross populations for its inheritance

Parents/ Genotypes	Screened	Resistant	Susceptible	Resistant to Susceptible Ratio	χ2 tabulated	χ2 calculated	p Value
DBW17	5	0	5				
WH1105	5	5	0				
BC1F2	100	44	56	1:1	3.841	1.54	0.215
BC2F2	50	23	27	1:1	3.841	0.32	0.572

Table 3: Allelic diversity in primers used to screen parents, BC_1F_2 and BC_2F_2 populations

Number of markers used	99
Number of markers that show amplification	87
Number of markers that did not show amplification	12
Number of polymorphic markers	15
Number of monomorphic markers	72
Total number of alleles in polymorphic markers	32
Average number of alleles	2.13

Table 4: Band size, allele number and PIC value of polymorphic markers in BC_1F_2 and BC_2F_2 populations

SSR Primer Name	Linkage Group	No. of Alleles	Amplified Fragment size of parents (bp)	PIC value (BC_1F_2)	PIC value (BC_2F_2)
Xgwm95	2A	2	130-180	0.50	0.34
Xgwm190 (*YrAC*)	5D	2	190-260	0.49	0.49
Xgwm268 (*YrH52*)	1B	2	150-180	0.46	0.49
Xgwm297 (*YrMY37*)	7B	4	150-190	0.47	0.44
Xgwm374 (*YrCN19*)	2B	2	300-500	0.66	0.66
Xgwm408	5B	2	150-210	0.50	0.45
Xgwm429 (*YrP81*)	2B	2	250-300	0.70	0.60
Xgwm437 (*Yr33*)	7D	2	100-130	0.41	0.50
Xgwm582 (*Yr9*)	1B	2	120-200	0.44	0.38
Xbarc76 (*Yr18*)	7D	2	200-240	0.40	0.50
Xbarc240(*YrSN104*)	1A,1B,1D,5B	2	200-230	0.66	0.65
Xbarc353 (*Yr17*)	2A	2	210-250	0.50	0.47
Xwmc175 (*Yr5*)	2B	2	210-290	0.35	0.43
Xwmc215	5D	2	200-240	0.49	0.45
Xwmc216 (*YrCH42*)	1D	2	100-160	0.50	0.49

markers are the best tools used for detailed characterization of genetic resources which increase the precision of selection for transgressive segregants in segregating generations. Selection of plants using linked DNA markers for indirect selection of quantitative traits is expected to be more effective as markers are not influenced by the environment and can be scored at all stages of plant growth, thus are more reliable (Nadeem *et al.,* 2018).

Molecular markers also allow gene pyramiding for characters like disease resistance which is very difficult in conventional breeding. Among the DNA markers, simple sequence repeats (SSRs), covers the entire genome and show high levels of polymorphism (Röder *et al.,* 1998) and are suitable for tagging and mapping agronomically important genes in wheat (Rahimi *et al.,* 2021). In the present study, 99 SSR primers were used for genotyping of BC_1F_2 and BC_2F_2 populations. Out of 99 primers 87 were amplified and 15 primers were found to be polymorphic among parents *i.e.,* these 15 markers showed some degree of variability in both the populations, thereby confirming variability among the parents for crossing programme. Similarly, Dale *et al.* (2017) and Sunil *et al.* (2020) have also reported high polymorphism in backcross population. Fifteen polymorphic SSR markers were dispersed over the wheat genome (AABBDD), with allele range 2-5 (Table 3). SSR markers differed in the number of alleles when observed in diverse group of germplasm (Yadav *et al.,* 2018). Polymorphic Information Content (PIC) index is used to evaluate the level of gene variation (Farhangian Kashani *et al.,* 2021) and it ranged from 0.35 (Xwmc175) to 0.70 (Xgwm429) in

Table 5: Linkage to days to heading, number of tillers, spike length and other yield attributes of polymorphic primers in BC_1F_2 population

Trait/Marker	Map Distance	Linkage group	R^2 Value	F Value
Days to Heading				
Xbarc76	235.10	7D	0.0270	0.046*
No. of Tillers				
Xgwm268	148.90	1B	0.0685	0.009 **
Spike Length				
Xgwm95	87.50	2A	0.0121	0.042 *
Xbarc353	100.30	2A	0.0621	0.012 *
Xgwm374	74.00	2B	0.1927	0.000 ****
Spike Weight				
Xbarc353	100.30	2A	0.0979	0.002 **
Xwmc175	127.60	2B	0.0542	0.020 *
Xbarc76	235.10	7D	0.0231	0.027 *
No. of Spikelets per spike				
Xbarc353	100.30	2A	0.0661	0.010 **
Xwmc175	127.60	2B	0.0400	0.046 *
Xbarc76	235.10	7D	0.0271	0.049 *
No. of grains /spike				
Xbarc353	100.30	2A	0.0609	0.013 *
Xgwm190	11.90	5D	0.0430	0.038 *
Biological Yield				
Xbarc353	100.30	2A	0.0870	0.003 **
Xgwm268	148.90	1B	0.0713	0.007 **
Grain yield				
Xbarc353	100.30	2A	0.0887	0.003 **
Xgwm268	148.90	1B	0.0768	0.005 **
Harvest Index				
Xwmc175	127.60	2B	0.0505	0.025 *

Significance at 5%, 1% and 0.01% levels are indicated by *, ** and ****, respectively

Table 6: Linkage to Yellow Rust and different yield attributes of polymorphic primers on BC_2F_2 population

Trait/Marker	Map Distance	Linkage group	R^2 Value	F Value
Yellow rust				
Xgwm582	50.00	1B	0.0685	0.025 *
Spike Length				
Xgwm408	103.20	5B	0.0791	0.044 *
Xgwm297	94.70	7B	0.1342	0.009 **
Xwmc216	55.00	1D	0.1251	0.012 *
Spike weight				
Xgwm190	11.90	5D	0.1183	0.014 *
100 grain weight				
Xgwm190	11.90	5D	0.1048	0.022 *
Grain Yield				
Xwmc216	55.00	1D	0.0814	0.045 *
Harvest Index				
Xgwm582	50.00	1B	0.0541	0.038 *

*Significance at 5%; ** Significance at 1%

BC_1F_2 population and in BC_2F_2 population value ranged from 0.34 (Xgwm95) to 0.66 (Xgwm374) (Table 4). In the present study, Xgwm374, Xgwm429 and Xbarc240 had PIC value greates than 0.5 in both the populations showing that the loci were of high variability and can be considered highly useful for differentiation of wheat genotypes. Sharma *et al.* (2010) showed the marker Xgwm285 having PIC value 0.65 was the most informative one. Correspondingly, Gangwar *et al.* (2019) while evaluating

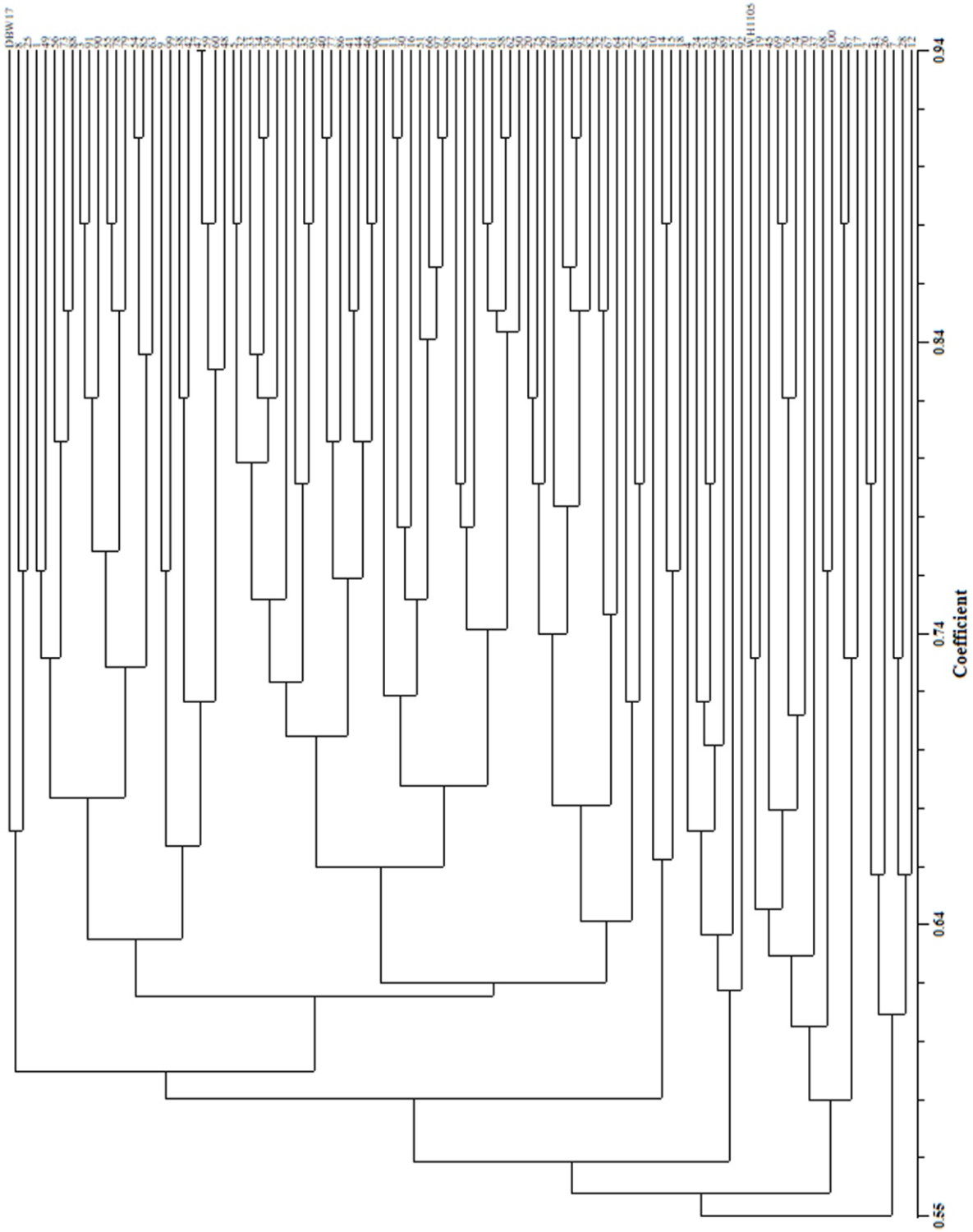

Fig. 4: Dendrogram showing the clustering pattern of BC_1F_2 population on the basis of SSR markers.

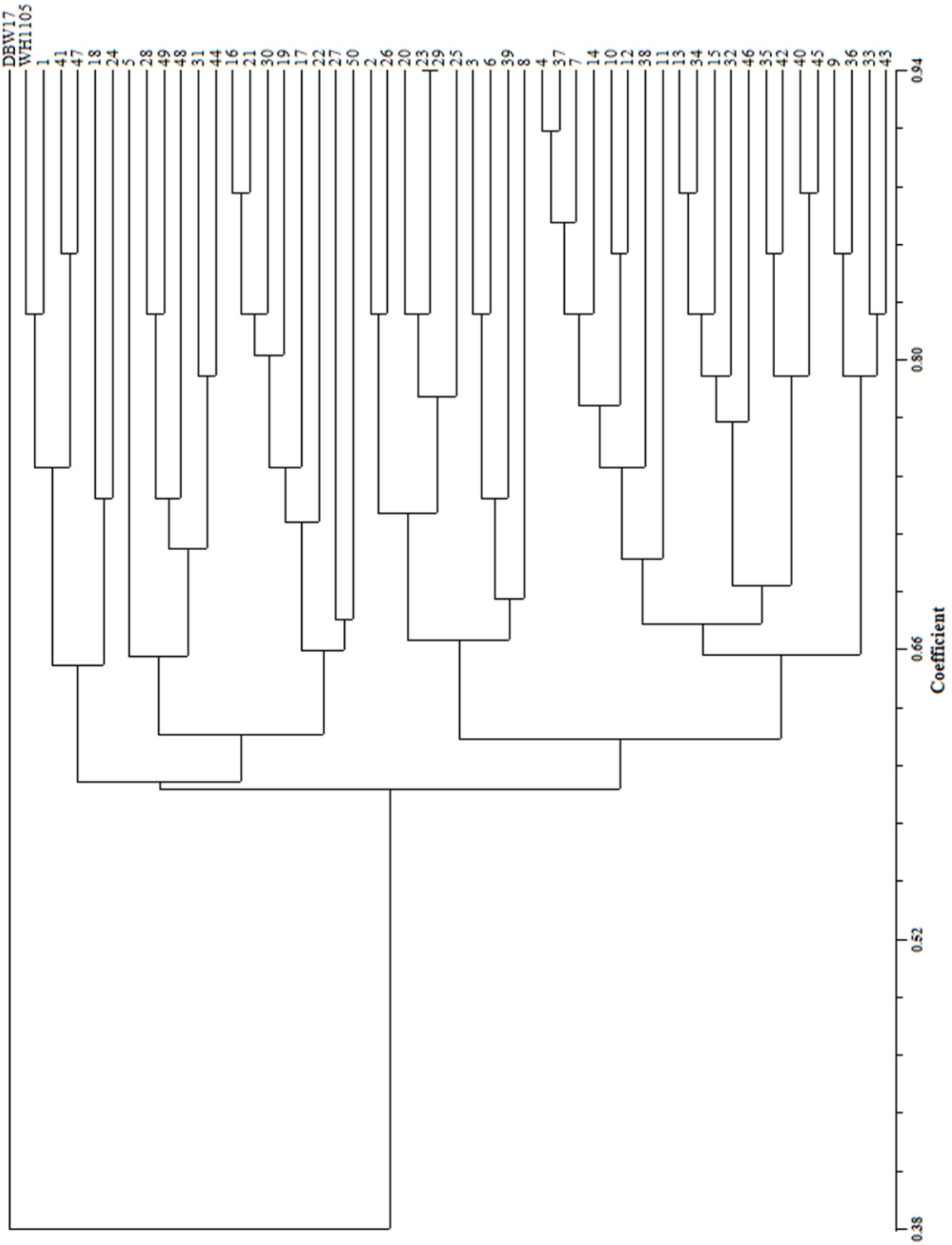

Fig. 5: Dendrogram showing the clustering pattern of BC_2F_2 population on the basis of SSR markers.

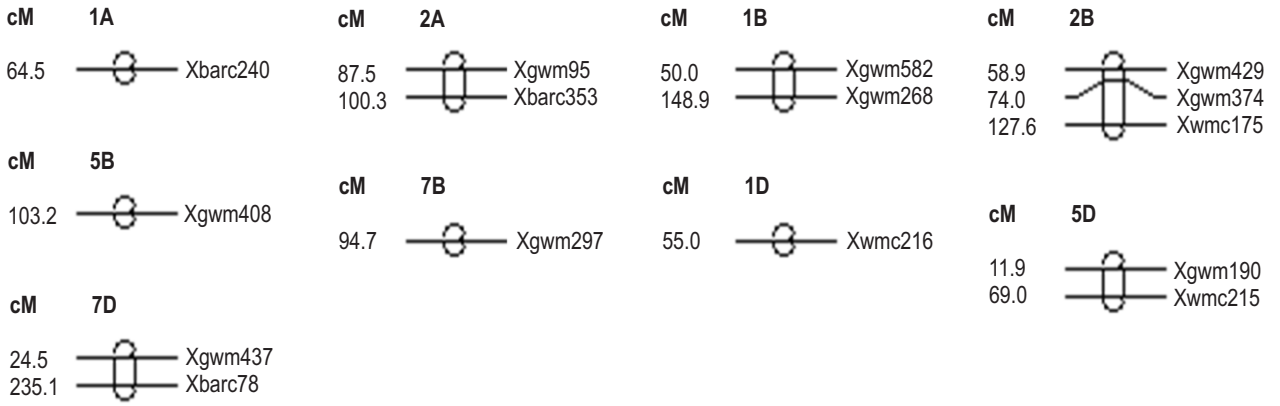

Fig. 6: Position of polymorphic primers on different chromosomes of wheat.

important quantitative traits found six markers were highly informative as their PIC values were >0.69. The overall size of PCR amplified products in the present investigation ranged from 100 bp (Xgwm437, Xwmc216) to 500bp (Xgwm374) as shown in Table 3. The range of major allele, PIC and gene diversity in both the generations showed that selected plants were significantly variable at genetic level.

Dendrograms were prepared separately for both generations by UPGMA method. Hierarchical cluster analysis of BC_1F_2 generation along with their parent showed that most of the progeny in BC_1F_2 resembled parent 1 (DBW17), as in BC_1F_2 generation parent 1 was the recurrent parent (Fig. 4). In BC_2F_2 generation progenies were mainly divided into two major clusters, cluster I and II. Cluster I included parent 1 (DBW17), while cluster II included parent II (WH1105) and is further divided into five sub-clusters (Fig. 5). All the progeny in BC_2F_2 resembled parent 2 (WH1105) as in BC_2F_2 generation parent 2 was the recurrent parent. Thus both the backcross progenies recovered the genetic background of their respective recurrent parents. The earlier workers also reported that gene-specific markers assist selection in backcross progenies in wheat for recovering the genetic background of recurrent parent (De Bustos *et al.,* 2001). The findings of Yashveer *et al.* (2020) and Todkar *et al.* (2020) for background selection and accelerated genome recovery of recurrent parent also suggest the use of polymorphic SSR markers for recovery of recurrent parents. These parents can be used for developing RIL's that could be used for QTL mapping to identify the genes for yellow rust resistance.

Single-marker analysis is based on the idea that if there is an association between a marker genotype and trait value, it is likely that QTL is close to that marker locus. The data set generated using polymorphic markers was subjected to single marker analysis. The linkage group and position of these markers are shown in Fig. 6. In BC_1F_2 population marker, Xbarc76 was found associated with days to heading and marker Xgwm268 showed linkage with number of tillers, grain yield and biological yield. The markers Xgwm95, Xgwm374 and Xbarc353 were found to be associated with the spike length. In this study, the marker Xbarc353 was showed association with spike weight, number of spikelets per spike, biological yield per plant grain yield per plant and number of grains per spike indicating that the loci affecting these traits might be closely present. Marker Xwmc175 and Xbarc76 were linked with spike weight, number of spikelets per spike and harvest index (Table 5). The linkage generated in BC_2F_2 population showed Xgwm582 to be more tightly linked to yellow rust and harvest index at 5% significance (Table 6).

Association of traits with single marker analysis was authenticated by finding a major QTL near Barc1120 marker and WMC 10, 74 and WMC 76 were significantly linked with staygreen trait (Kumari *et al.,* 2012). Saeed *et al.* (2017) showed that the marker Xpsp3123-7D was associated with multiple traits. Similarly, the single marker analysis were done for characterization of different linkage groups for different markers and identification of candidate lines for stripe rust resistance genes Yr5, 10 and Yr18 (Mukhtar *et al.,* 2015; Yang *et al.,* 2016; Yao *et al.,* 2017). So these microsatellite markers showing association can be used in marker assisted selection for breeding of stripe rust resistance and other yield related traits in wheat and also can be used for screening of important traits in crosses in variety of diverse backgrounds. Graphic genotyping help breeders to visualize and identify desirable individuals based on their genotype. GGT allows sorting for allele content through UPGMA analysis. GGT bar diagrams allowed graphical illustration of genotyping data for each line of wheat (Grewal *et al.,* 2018).

Availability of large number of fragments defining independent genetic loci with highly reproducible polymorphism detection enables the efficient evaluation of genetic diversity. Graphic genotyping analysis of BC_1F_2 and BC_2F_2 population was done using 15 polymorphic primers. This analysis indicated the contribution of both the parents (DBW17 and WH1105) on nine

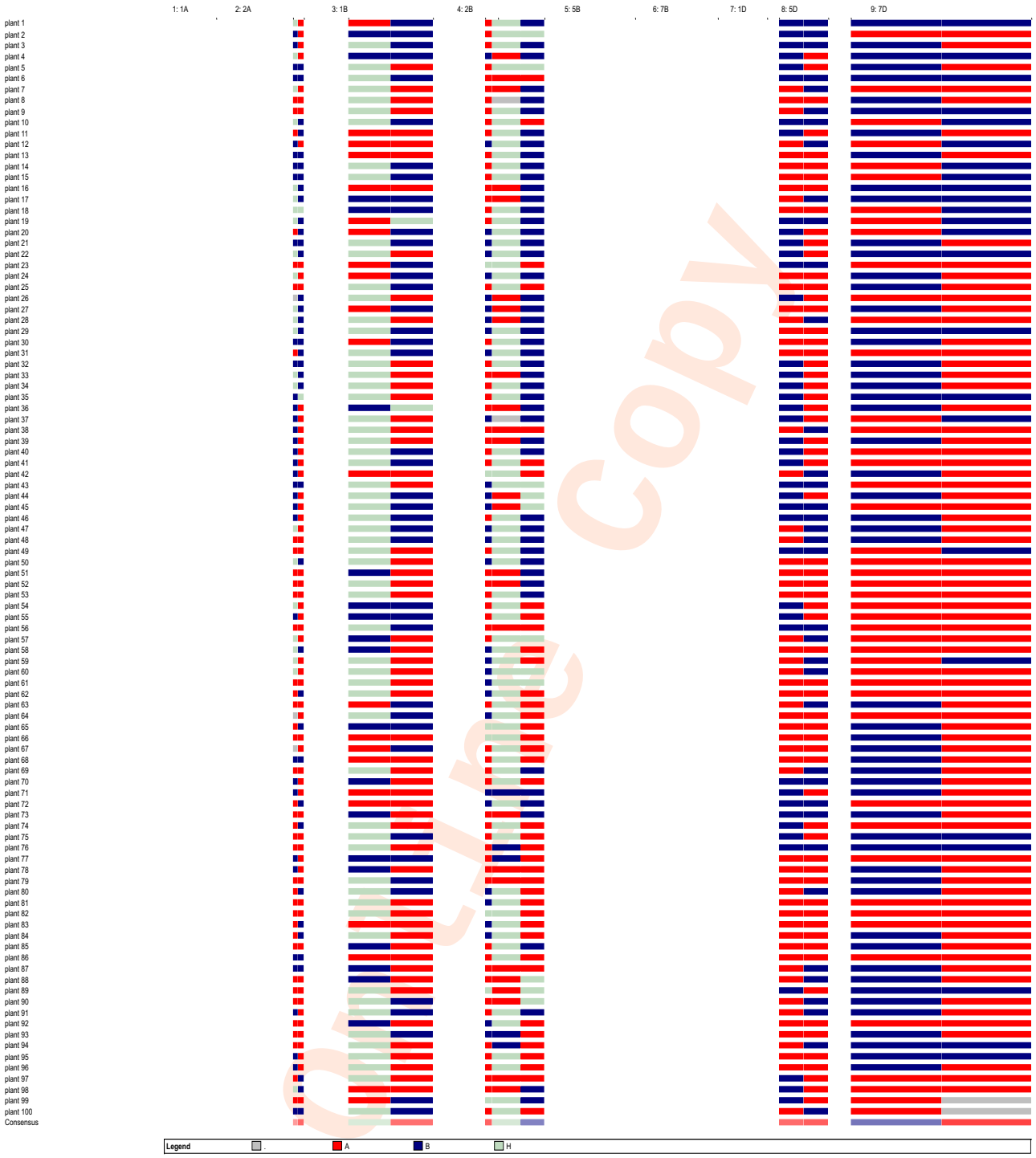

Fig. 7: BC_1F_2 population showing contribution of both parents based upon genotypic and phenotypic data.

chromosomes in BC_1F_2 (Fig. 7) and BC_2F_2 (Fig. 8) progenies. Red color indicated parent A (DBW17), blue color indicated parent B (WH1105), grey color indicated the heterozygotes, and green color indicated the unknown sequences. So this analysis indicated more contribution of respective recurrent parent on nine chromosomes in respective BC_1F_2 and BC_2F_2 progenies, and thus introgressions of desired genes, *i.e.,* rust resistant genes could be trailed from the donor parent. Breeding of resistant varieties is a key measure to

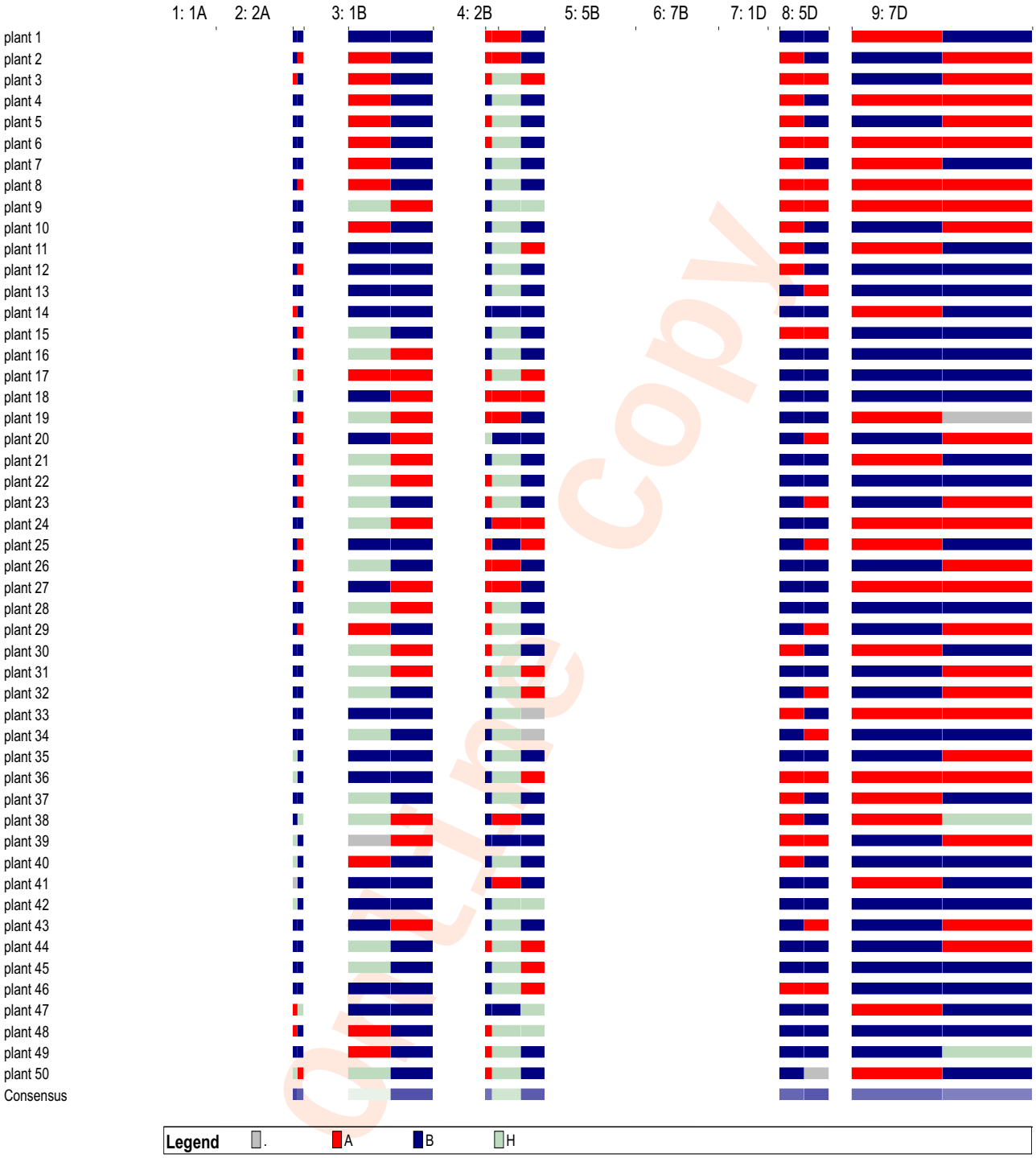

Fig. 8: BC_2F_2 population showing contribution of both parents based upon genotypic and phenotypic data.

control yellow rust disease, but conventional breeding method was low efficiency. Markers can be used to better characterize parental material, thereby improving the efficiency and effectiveness of parental selection for crossing and to track genes in segregating progenies through the selection process. The results based on screening data derived from DBW17 × WH1105 and backcross populations with Xgwm582, may provide an insight into the genetic control of yellow rust resistance in wheat

cross between highly resistant and highly susceptible wheat genotypes. Marker enrichment around the Xgwm582 would assist in resolving the map locations and distances for our future linkage mapping studies, thus improve the possibilities for marker-assisted selection and for recovering the genetic background of recurrent parent. Plants having loci associated with yellow rust resistance and more contributing recurrent parent can be selected for further backcrossing with the recurrent parent to develop stripe rust tolerant short stature wheat lines.

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Add-on Information

Authors' contribution: V. Gupta: Conducting study and data collection. **M. Kumar:** Planning and guidance in research. **V. Singh:** Editing of manuscript. **R.N. Sheokand:** Analysis of data. **L. Chaudhary:** Preparing figures and tables.

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