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Full Length Article

Wheat Genotyping by Karnal Bunt Resistance Associated SSR Markers Depict Connotation with their Phenotypic Response against *Tilletia indica*

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Abstract

Genetic characterization of wheat genotypes and their response to the pathogen is necessary for devising suitable disease management strategies. In this study, 42 wheat genotypes were screened during Rabi season of 2015–2016 and 2016–2017, to explore the existence of Karnal bunt (KB) resistance using seventeen SSR markers followed by their phenotypic response against the virulent isolate (PB-25) of *Tilletia indica* reported earlier from Faisalabad, Pakistan. Among 42 wheat genotypes, 14 exhibited highly resistant (HR), six showed resistant (R) while 22 showed moderately susceptible (MS) reaction during Rabi season of 2015–2016. Second year (Rabi 2016–2017) results showed the same reactions with few exceptions that showed depletion in the resistance. Out of seventeen SSR markers, thirteen were able to amplify bands linked with KB resistance in different wheat genotypes. A total of 40 alleles were detected for the 42 genotypes with an average of 3.1 alleles per SSR. The maximum number of polymorphic bands (8 amplicons) were generated by the SSR primer Xgwm174 followed by Xgwm340 (7 amplicons). The present study showed a strong association between the genotypic and phenotypic response of different wheat genotypes. However, these genotypes (99172, DN-102, NRL-1130, V-11160, V-11005-2013, 12266, 11098, 11C023, PR-111, PR-112, PR-113, NR-423, NR-429, NR-436, 9459-1, KT-338, FSD-08, TW1150, ESW-9525, NIA-CIM-04-10) were found consistently resistant in both years and can be utilized in the breeding program for the incorporation of high yields.

Keywords: SSR Markers; Karnal bunt; Resistance; Wheat; Genotyping; Coefficient infection

Introduction

Wheat (*Triticum aestivum* L.) is a staple diet of many countries and extensively grown in the world (Huang and Roder 2004). It is an important source of fiber, fat, sugar, minerals, protein, and carbohydrates that can partially meet human energy needs (Topping 2007). Various biotic and abiotic factors affect the wheat production quantitatively and qualitatively (Carris *et al*. 2006; Farooq *et al*. 2011, 2014; Kumar *et al*. 2016). Fungal diseases are one of the major biotic factors which affect the wheat yield by causing diseases such as rust, powdery mildew, loose smut and karnal bunt (Bonde *et al*. 1997). Karnal bunt (KB), caused by a fungus *Tilletia indica (T. indica*), is a floret infecting fungus belonging to order Ustilaginales. It is also known as partial bunt as it typically infects the part of kernels and replaced it by powdery black teliospores masses (Carris *et al*. 2006). The infected kernels emit fishy odor due to the production of trimethylamine and affects the quality (Kumar *et al*. 2016). It is a concern because 1 to 4% infection in kernels makes wheat grain unpalatable whereas 5% infection in grain seed lot caused chemical changes in flour quality and gluten content (Rush *et al*. 2005).

The *T. indica* differs from other pathogens as it infects plant during anthesis and sporulates on the same generation of that infected host (Carris *et al*. 2006). Infected sori of KB infected seeds are broken easily during harvesting which further contaminates the healthy seeds, soil and machinery and are the major source of inoculum. These spores can blow by the wind to the long distances contaminating the healthy fields (Bonde *et al*. 2004). The thick-walled, dormant *T. indica* spores can survive in warm, dry and harsh summer conditions during post-harvest period in the soil up to 5 years and germinate at the optimum temperature of 20

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to 25°C (Gill *et al*. 1993).

The KB is a highly alarming issue to wheat grain industry not only in terms of yield losses but also limited international trade due to quarantine regulations (Brar *et al*. 2018). The loss of export market due to KB in any country would lead to a reduction in production, price and ultimately farmer's income (Gupta *et al*. 2019). Total loss due to KB was estimated in North West Mexico to be 7.02 million dollar/year and was 27 million dollars reported in USA (Brennan *et al*. 1990; Vocke *et al*. 2002). In Pakistan, KB prevalence has been increased over time in Punjab and Khyber Pakhtunkhwa (Aasma *et al*. 2012) and has posed a threat to wheat production even in the dry and hot region of Punjab (Khan *et al*. 2010). Raees *et al*. (2013) reported 39% yield losses from Rawalpindi and Chakwal of Punjab. Maximum disease prevalence was observed in Chakwal (50%) as compared to Rawalpindi (35%). Sajjad *et al*. (2018) observed 91.7% KB prevalence in district Lodhran while lowest in Rahimyar Khan (68.2%).

Aasma *et al*. (2019a) surveyed different districts of Punjab and KP and reported the prevalence of KB in Jehlum (18.5%), Chakwal (15.9%), Kohat (40.0%), Charsadda (33.3%) and Mansehra (13.3%) whereas samples from Haripur, Malakand, and Mingora showed no visual infection during 2013. While in another survey conducted during 2014, recorded the prevalence from the samples collected from Gujranwala (70.4%), Muzfarghar (46.7%), Jhelum (35.3%), Peshawar (43.2%), Charsadda (33.3%), Nowshera (25.0%) and Buner (11.57%) which shows an increasing trend of the disease.

Effective management approaches are necessary to reduce crop damage. Being the seed, soil and air-borne sporidial disease, its control is limited through the fungicidal application (Gupta *et al*. 2019); therefore, host plant resistance is considered to be the paramount choice for KB pathogen management. The use of resistant varieties is the most practical and economical method (Sharma *et al*. 2008).

Jafari *et al*. (2000) worked for the exploration of the sources of resistance against KB by inoculating the spikes using the injection method on 26 wheat advanced cultivars/ genotypes at the booting stage. Coefficient of infection and the percentage of infected grains for each genotype were estimated. They found most of the cultivars showed a susceptible reaction; however, the two cultivars exhibited partial resistance to KB. A significant correlation was found between the coefficient of infection and the percentage of infected grain for each genotype. Kumar *et al*. (2014) screened 150 genotypes of wheat for KB resistance by artificial inoculations under field conditions during the consecutive 2009–2010 seasons. The results showed variability in the incidence of disease among the genotypes. The disease incidence ranged from 0.2–63.1 and 12 genotypes exhibited highly resistant (HR) while 6 showed resistant, 6 as moderately resistant reaction, whereas the rest of genotypes were found moderately susceptible to highly susceptible. Several genotypes (HD29, W485, KB 2012–03 (in PBW343 background), ALDAN "S"/IAS 58, and H 567.71/3∗PAR) were screened and it was identified that these genotypes carry stable resistance in multiple environments in India (Sharma *et al*. 2005). Intensive breeding has been carried out for KB resistance. However, breeding is often hindered by the lack of a simple, rapid and environment independent methods of screening host genotypes against pathogens. Moreover, transfer of genes through conventional breeding is laborious and time-consuming. Alternately molecular tools provide a better understanding of the existing genetic variability needed to be considered for further progress in breeding programmes (Fischer and Edmeodes 2010). Simple identification of PCR-based markers associated with KB resistance provides the possibility of using a marker-assisted selection scheme in the development of resistant wheat cultivars (Kumar *et al*. 2007). Hence, detection of disease resistance genes coupled with screening could be helpful in different molecular breeding programs.

Kumar *et al*. (2015) discovered SSR markers linked with KB resistance during the year 2010 and 2011. They tested different inbred genotypes by crossing of resistant and susceptible parent and found a lot of variation among these genotypes based on the coefficient of infection. Out of 70 markers, 42 were polymorphic. They detected, with an average of 2.09 alleles per locus. In addition to the already identified KB resistant primers, they found a new marker *Xgwm* 6 that was linked with KB resistance.

The purpose of this study was to analyze different Pakistani wheat genotypes to detect KB resistance genes, identify the reproducible SSR markers associated with KB resistance and infer the association of wheat genotypes with their phenotypic response against the virulent isolate of *T. indica*. Thus, it could be effectively utilized in the breeding program to combat the disease.

Materials and Methods

Plant material

Forty-two wheat genotypes were used in this study (Table 1). The seeds were collected from the National Coordinated Wheat Program, National Agriculture Research Center, Islamabad, Pakistan.

Phenotypic screening of wheat

Forty-two wheat genotypes (Table 1) were sown in the pots during November 2015 and the same set of genotypes were repeated for screening during November 2016 to confirm the disease resistance sustainability in the glasshouse at NARC using CRD design. Each genotype was replicated thrice in different pots. These pots were kept under controlled condition i.e. temperature 18–20ºC and misting system of automatic sprinkling water 5 times per day to maintain humidity (approx. 80%); (Brar *et al*. 2018). Recommended agronomic practices of fertilizers and irrigation were followed as and when required (Tandon and Sethi 1991).

Inoculum preparation

Fresh sporidial suspension derived from teliospores of an aggressive isolate PB-25 found to be aggressive in a previous study conducted by Aasma *et al*. (2019b) was used as a reference isolate, and inoculum was prepared according to the method of (Brar *et al*. 2018). The concentration of inoculum was adjusted to 4–5 × 10^4 spores mL^{-1} with a haemocytometer (Tandon *et al*. 1994).

Boot inoculation

The plants were inoculated by boot inoculation method at booting stage following the procedure of Singh and Krishna (1982) and Aujla *et al.* (1983). Three spikes per genotype were inoculated just before the emergence of awns. 1 mL of *T. indica* inoculum (Prepared above) was injected into the boot with the aid of a hypodermic syringe. The inoculated spikes were covered with glycine bags to maintain the maximum moisture content for fungus proliferation and later were tagged and labelled (Aujla *et al*. 1989).

Harvesting and scoring

The harvesting of the individually inoculated spikes was done at maturity (58–60 days) after inoculation. Each spike was collected in paper bags and then threshed manually. The infected seeds were divided into different degrees of infection depending on the extent of the damage on the severity/scoring scale 0–5 (Aujla *et al*. 1989; Aasma *et al*. 2019b); (Table 2; Fig. 1). These infection grades have numerical values (Table 2) therefore to obtain a *gross total; the numeric value was multiplied with the number of grains present in each grade (Warham 1986; Aujla *et al*. 1989; Riccioni *et al*. 2008; Aasma *et al*. 2019b). The CI was calculated as follows:

$$\text{Coefficient of infection} = \text{Gross total} / \text{Total no. of seeds} \times 100$$

Percent infection of each genotype was calculated by using the following formula (Jafari *et al*. 2000).

$$\text{Percent infection} = \text{infected seeds in a sample} / \text{Total no. of seeds in a sample} \times 100$$

Molecular genotyping/ screening for KB resistance

Seeds of 42 wheat genotypes (Table 1) were surface sterilized with 1% Clorox (Giri *et al*. 2001) and sown in test tubes containing moist cotton swab at the bottom. The tubes were incubated at 22°C until the seedlings established with 2–3 leaves (Giri *et al*. 2001). These leaves were harvested for DNA extraction.

Extraction of DNA

Genomic DNA was extracted from fresh leaves of wheat using the CTAB method as described by Doyle and Doyle (1987). Fresh leaves (200 mg) of each genotype were ground with a mortar and pestle having 2–3 mL of CTAB 2% solution. Then, 750 μL of the leaf tissue was placed in a 1.5 mL Eppendorf tube and incubated in a water bath for 30 min at 65°C. Subsequently, 750 μL of a chloroform mixture: isoamyl alcohol was added in 24:1 ratio. The supernatant was placed in a new Eppendorf tube after it was centrifuged at 12000 rpm, 4°C for 10 min. For DNA precipitation, 600 μL of ice-cold isopropanol was added and incubated at 4°C for 20 min. To discard the supernatant, the tubes were centrifuged at 12,000 rpm for 10 min. The DNA pellet was washed with ethanol (70%). After adding ethanol (70%), the tube was centrifuged at 12,000 rpm for 10 min at room temperature. The DNA pellet was then dried overnight and then dissolved in 50–100 μL ddH2O. Next, 1 μL of RNase (10 mg/mL) was added to remove RNA and stored at -20°C for further use. The DNA was electrophoresed on agarose gel (1%) by applying 100 volts for 40–50 min and observed under UV light using a gel documentation system (Biometra). Seventeen SSR markers (Table 5) linked with KB used to amplify the resistance genes.

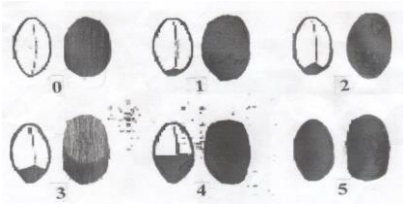

Fig. 1: Disease severity rating scale of Karnal Bunt (Warham 1986; Aujla *et al.* 1989)

A PCR reaction consisted of buffer (1X), primers (2 μM), dNTPs (0.4 μM) Taq polymerase (0.2 U), $MgCl_2$ (2.4 m*M*), DNA (20–25ng) was amplified on automated Thermal Cycler (Applied Biosystems) with the following cycling conditions; initial Denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 40 sec, 50–55°C for 40 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified products were electrophoresed on 1.8% Agarose gel at a constant voltage of 100V for 1hr. The gel was visualized under UV light to observe the presence and absence of bands (Kumar *et al*. 2015).

Statistical Analysis

The incidence of disease and the coefficient of infection (CI) were calculated to verify the level of resistance and susceptibility of the genotypes (Bonde *et al*. 1996). The data for both years was calculated and all the genotypes were categorized as highly resistant, resistant, moderately susceptible, susceptible and highly susceptible based on their coefficient of infection means (Aujla *et al*. 1989; Aasma *et al*. 2019b). Analysis of variance for CI and PI were calculated. LSD was applied on all pairwise comparisons of CI and PI and was calculated by using Statistix software. Design of the experiments was completely randomized with three replications. Band frequency was calculated based on the presence (1) and

Table 1: List of wheat genotypes and their pedigrees used in this study

Code	Genotypes	Pedigree
V-1	99172	KAUZ/PASTOR//V.3009
V-2	99346	MH-97/FAREED-06
V-3	112802	NOT AVAILABLE
V-4	DN-102	CHAM6/ATTILA//PASTOR
V-5	CT 09137	SERI.1B*2/3/KAUZ-2/BOW//KAUZ/4/
V-6	SRN 09111	PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07
V-7	NRL-1123	PSN/BOW//MILAN/3/2*BERKUT
V-8	NRL-1130	SOKOLL/EXCLAIBUR
V-9	V-12001	WAXWING/4/SNI/TRAP#1/3/KAUZ/*/TRAP//KAUZ
V-10	V-10110	KAUZ/CMH77A-308///BAU/3/INQ-91
V-11	V-11160	KAUZ//ALTAR 84/AOS/3/MILAN/KAUZ/4/HUITES/5/ T.DICOCCON P194624/AE.SQUARROSA(409)// BCN /6 /2*KAUZ// ALTAR84 / AOS/3/MILAN/KAUZ/4/HUITE
V-12	12266	ATTILA/3*BCN//BAV92/3/TILHI/5/BAV92/3/PRL/SARA//TSI/VEE#5/5/CROC_1/AE.SQUARROSA(224)//2*OPATA
V-13	11098	BABAX/LR43//BABAX/6/MOR/VEE#//DUCULA/3/DUCCULA/4/MILAN/5/BAU/MILAN/7/SKAUZ/BAV92
V-14	11138	WHEAR/KRONSTAD F2004
V-15	12304	WAXWING/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ/5/TECUE#1
V-16	11C022	SOKOLL//SUNCO/2*PASTOR
V-17	11C023	SOKOLL/EXCALIBER
V-18	AUR-08010	WBLL1*2/4/YACO/PBW65/3/KAUZ*2/TRAP/KAUZ
V-19	TW11510	93T347/V94105
V-20	HD-29	NOT AVAILABLE
V-21	ESW-9525	KAUZ/Gen
V-22	DANI-1313	Kiran/Khirman/Bhittai
V-23	NIA-CIM-04-10	PBW343*2/KONK
V-24	PR-106	MTRWA92.161/PRINIA/5/SERI*3//RL06010/4*YR/3/PASTOR/4/
V-25	PR-110	KAUZ//ALTAR0B84/AOS/3/MILA/KAUZ/4/HUITIES/7/CAL/NH/H567.71/3/SERI/4/CAL/NH//H567.71/5/5*KAUZ/6/PASTOR
V-26	PR-111	TOB/ERA//TOB/CN067/3/PLO/4/VEE#5/5/KAUZ/6/
V-27	PR-112	KAUZ//ALTAR 84/AOS/3/MILAN/KAUZ/4/HUITES/7/ CAL/NH//H657.71/3/SERI/4/CAL/NH//H567.71/5/5*KAUZ/6/PASTOR
V-28	PR-113	WHEAR//INQLAB91*2/TUKURU
V-29	NR-423	MTRWA92.161/PRINIA/5/SERI*3//RL6010/4*YR/3/PASTOR/4/BAV92
V-30	NR-429	CNO79//PF70354/MUS/3/PASTOR/4/BAV92/5/FRET2/KUKUNA//FRET2/6/MILAN/KAUZ//PRINIA/3/BAV92
V-31	NR-436	NAC/TH.AC//3*PVN/3/MIRLO/BUC/4/2*PASTOR/5/T.DICOCCON PI94624 / AE.SQUARROSA (409)// BCN/6/ WBLL4// BABAX.1B.1B*2 /PRL/3/PASTOR
V-32	NR-449	SOKOLL//FRTL/2*PIFED
V-33	9459-1	LU26S x 8120
V-34	KT-338	SOKOLL/WBLLI
V-35	09-FJ-34	ERAF 2000/4/ FONCHAN#3/ TRT" S"// VEE# 9/3/COOK/VEES// DOVE"S/ SERI
V-36	SKD-11	Pb96/V87094//MH-97
V-37	V-11005-2013	NOT AVAILABLE
V-38	FSD-08	PBW-65/2*PASTOR
V-39	WL-711	S308/CHRIS//KAL
V-40	Aas-11	KHP/D31708//CM74A370/3/CIAN079/4/RL6043/*4NAC
V-41	TW96018	BK2002 X LU26S/AE. cylindricaD/PAK-81
V-42	Pak-13	MEX94.27.1.20/3/SOKOLL//ATTILA/3*BCN

V= variety, KT= Kohat, Pak-Pakistan, FSD= Faisalabad, NR= NARC, PR= Pir Sabak, SKD= Sarkand, AUR= University of Arid agriculture, FJ= Fateh Jang, TW= Thal wheat, DN= Dera Ismail Khan, Aas = Released variety, WL-711= Susceptible variety, HD-29= Resistant variety, NRL= NIFA Rain fed lines, NIA= Nuclear institute of Agriculture, ESW= Elite Spring Wheat

Table 2: Rating scale used to assess wheat genotypes for Karnal bunt (*T. indica*)

Infection Category/Grade	Symptoms	The assigned numerical value for the calculation (CI)
0	Healthy	0
1*	Inconspicuous point infection (trace), (5% seed bunted).	0.25*
2*	Well-developed point infection (25% seed bunted)	0.25*
3	Infection spreading along the groove (50% seed bunted)	0.5
4	Three-quarters of seed converted to sorus (75% seed bunted)	0.75
5	Seed completely converted to sorus (100% seed bunted)	1.0

*Categories combined to calculate Coefficient of Infections for comparison

absence (0) (Ghosh *et al*. 1997). Polymorphic information content (PIC) was calculated as follows PIC= 1-(p/q) 2 where p is total alleles detected at a given marker locus, q is a collection of genotypes studied. Cluster analysis was done to estimate the genetic similarity between wheat genotypes using R software. Minitab 16 software was used for cluster analysis of combined data of percent infection and marker data. The association between percent infection and wheat genotypic marker data was estimated using R software.

Results

Screening of wheat germplasms for resistance against *T. indica*

A total of 42 genotypes were screened during the years 2015 and 2016 (Table 1). The results during the year 2015 revealed, thirteen genotypes (NRL-1130, V-11160, 12266, 11C023, TW1150, ESW-9525, NIA-CIM-04-10, PR-111,

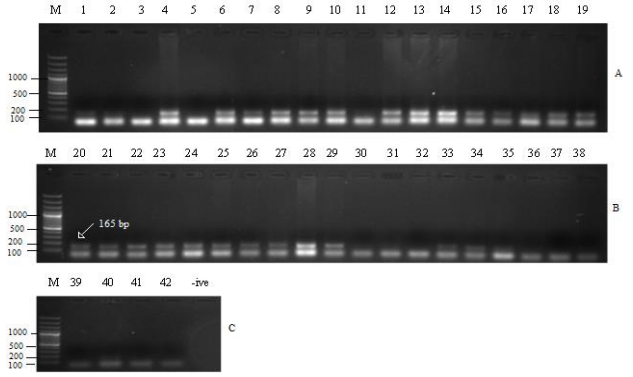

Fig. 2: PCR amplification of Pakistani wheat genotypes associated with Karnal bunt resistance in Wheat using Xgwm-149 marker: lane 20- HD-29 (resistant): lane 39- Wl-711 (susceptible); Lane M-100 bp ladder

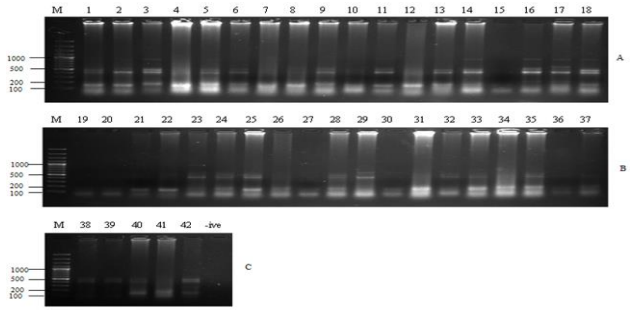

Fig. 3: PCR amplification of Pakistani wheat genotypes associated with Karnal bunt resistance in Wheat using Xgwm-174 marker: lane 20- HD-29 (resistant): lane 39- Wl-711 (susceptible); Lane M-100 b ladder

PR-112, NR-423, NR-429, KT-338 and FSD-08) exhibited HR reaction. Seven genotypes (99172, DN-102, 11098, PR-113, 9459-1 NR-436 and V-11005-2013) showed R reaction while, twenty two genotypes (99346, 112802, CT 09137, SRN 09111, NRL-1123, V-12001, V-10110, 11138, 12304, 11C022, AUR-08010, TW96018, DANI-1313, PR-106, PR-110, NR-449, 09-09-FJ-34and SKD-11, Pak-13, Aas-11, WL-711 and HD-29) showed MS reaction. The screening of the same set of genotypes was repeated during 2016, the results revealed almost the similar reactions except for few genotypes that showed the trend of depletion in the resistance. These genotypes included, SRN09111, NRL-1130, V-12001, V10110, 12266, 11C023, TW1150 PR-106, PR-111 and FSD-08, WL-711 (Table 3).

Amplification of Karnal bunt resistant genes in wheat genotypes

13 out of 17 SSR primers were selected based on their reproducibility, level of polymorphism and numbers of the amplicons (Table 5). A total of 40 alleles were detected for the 42 genotypes with an average of 3.07 alleles per primer. PIC value ranged from 0.14–0.99. Results of this study revealed that the size of the amplified DNA product ranged from 100–1000 base pairs (bp). The maximum number of polymorphic bands was generated by primers Xgwm174 resulting in 8 amplicons followed by Xgwm340 that gave 7 amplicons (Table 6). Banding patterns of 42 wheat genotypes generated by primers Xgwm149, Xgwm174 are presented in Fig. 2 and 3.

Cluster analysis of wheat genotypes based on SSR markers

The Euclidean similarity coefficient was calculated using data from 13 SSR primers. The value of the similarity coefficient (Sm) ranged from 1.0–4.5. A phylogenetic tree generated using UPGMA cluster analysis showed all the 42 wheat genotypes were separated. Wheat genotypes were clustered into two major groups (I & II). These groups were further classified into sub-groups, (the genotypes showing further minor similarities within the group). Group, I was further divided into two groups (IA & IB). The group, IA comprised of 6 genotypes whereas Group I B has 12 genotypes. Group II was subdivided into three groups (IIA, IIB and IIC). IIA comprised of 9 genotypes whereas IIB was comprised of 4 genotypes. Group IIC carried 11 genotypes that were split into many small groups and showed varying degrees of similarity (Fig. 4).

Infection of wheat genotypes against *T. indica* PB-25

Wheat genotypes were screened with an aggressive isolate PB-25 (Aasma *et al*. 2019b) during the years 2015 and 2016. The results revealed that in 2015, Pak-13 showed maximum PI (78.9%) while NRL-1130 (V-8), V-11160 (V-11), 12266 (V-12), 11C023 (V-17), TW11510 (V-19), ESW-9525 (V-21), NIA-CIM-04-10 (V-23), PR-112 (V-27), NR-423(V-29), NR-429 (V-30), KT-338 (V-34), FSD-08 showed least percent infection (10%). During the year 2016, WL-711 showed maximum PI (90.4%) followed by AUR-08010 (86.6%) whereas TW11510 (V-19), ESW-9525 (V-21), NIA-CIM-04-10 (V-23), PR-111 (V-26), PR-112 (V-27), NR-423(V-29), NR-429 (V-30), KT-338 (V-34) showed minimum PI (10%); (Table 4). Analysis of variance of the means of PI of these 42 genotypes was found statistically significant.

Cluster analysis based on infection and marker data

Based on PI during 2015 and 2016, marker data analysis of UPGMA clustering tree of 42 wheat genotypes using Minitab-16 was performed (Fig. 5). PI ranged from 80.6–100% in 2015 and 85.9–100% in 2016. While the coefficient ranged from 77.4–100% respectively. Wheat genotypes were clustered into 2 groups I & II. Group, I consisted of 21 genotypes while Group II was further divided into three major groups II A, II B and II C. Group II A comprised of 8, while Group II B comprised of 5 and II C represented 8 genotypes respectively (Fig. 5).

Table 3: Mean value of aggressiveness analysis and disease response of 42 wheat genotypes based on the coefficient of infection during 2015 and 2016

LSD and Reaction of 42 wheat genotypes based on mean CI during 2015-16			LSD and Reaction of 42 wheat genotypes based on mean CI during 2016-17		
Wheat genotypes	Mean	Reaction	Wheat genotypes	Mean	Reaction
Aas-11	19.57^{A}	MS	WL-711	21.31^{A}	S
112802	19.137^{AB}	MS	PR-106	22.06^{AB}	S
11C022	19.007^{ABC}	MS	SRN-09111	21.61^{ABC}	S
TW96018	18.883^{A-D}	MS	V-10110	20.82^{A-D}	S
NR-449	18.717^{A-D}	MS	V-12001	20.51^{A-D}	S
09-FJ-34	18.673^{A-E}	MS	Pak-13	19.727^{A-E}	MS
Pak-13	18.65^{A-E}	MS	Aas-11	18.643^{A-F}	MS
HD-29	18.627^{A-E}	MS	AUR-08010	18.547^{A-F}	MS
99346	18.617^{A-E}	MS	CT 09137	18.333^{A-F}	MS
12304	18.497^{A-E}	MS	PR-110	18.037^{A-G}	MS
SKD-11	18.347^{A-E}	MS	NR-449	17.83^{A-H}	MS
WL-711	18.22^{A-E}	MS	09-FJ-34	17.763^{A-H}	MS
DANI-1313	18.21^{A-E}	MS	SKD-11	17.433^{A-H}	MS
NRL-1123	18.063^{A-E}	MS	TW96018	16.667^{A-I}	MS
SRN-09111	17.183^{A-F}	MS	99346	16.49^{A-I}	MS
PR-106	16.86^{A-F}	MS	12304	16.41^{A-I}	MS
V-10110	16.712^{A-F}	MS	11C022	16.32^{A-I}	MS
AUR-08010	16.603^{A-G}	MS	11138	16.25^{A-I}	MS
PR-110	15.953^{A-G}	MS	DANI-1313	16.207^{A-I}	MS
V-12001	15.902^{A-H}	MS	112802	16.09^{B-J}	MS
CT 09137	15.66^{B-H}	MS	HD-29	15.38^{B-K}	MS
11138	15.143^{B-H}	MS	NRL-1123	15.157^{B-K}	MS
DN-102	15.07^{C-H}	R	12266	14.487^{C-K}	R
99172	14.427^{D-H}	R	FSD-08	13.703^{D-K}	R
NR-436	14.387^{D-H}	R	11098	13.41^{D-K}	R
11098	13.993^{E-H}	R	V-11005-2013	12.72^{E-K}	R
PR-113	13.653^{E-H}	R	99172	12.5^{E-K}	R
V-11005-2013	12.02^{F-H}	R	PR-113	12.477^{E-K}	R
9459-1	12.00^{GH}	R	NRL-1130	12.43^{E-K}	R
PR-111	10.00^{H}	HR	PR-111	12.08^{F-K}	R
NRL-1130	10.00^{H}	HR	9459-1	11.923^{G-K}	R
V-11160	10.00^{H}	HR	11C023	11.597^{H-K}	R
12266	10.00^{H}	HR	DN-102	10.927^{I-K}	R
11C023	10.00^{H}	HR	NR-436	10.52^{JK}	R
TW1150	10.00^{H}	HR	V-11160	10^{K}	HR
ESW-9525	10.00^{H}	HR	TW1150	10^{K}	HR
NIA-CIM-04-10	10.00^{H}	HR	ESW-9525	10^{K}	HR
PR-112	10.00^{H}	HR	NIA-CIM-04-10	10^{K}	HR
NR-423	10.00^{H}	HR	PR-112	10^{K}	HR
NR-429	10.00^{H}	HR	NR-423	10^{K}	HR
KT-338	10.00^{H}	HR	NR-429	10^{K}	HR
FSD-08	10.00^{H}	HR	KT-338	10^{K}	HR
LSD value at 0.05= 6.10			LSD value at 0.05= 5.39		

Column means followed by a common letter are not statistically different from each other at 5% level of probability

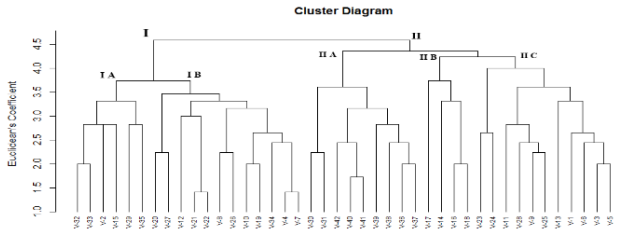

Fig. 4: Dendrogram of 42 wheat genotypes on the basis of 13 SSR markers linked to Karnal bunt resistance

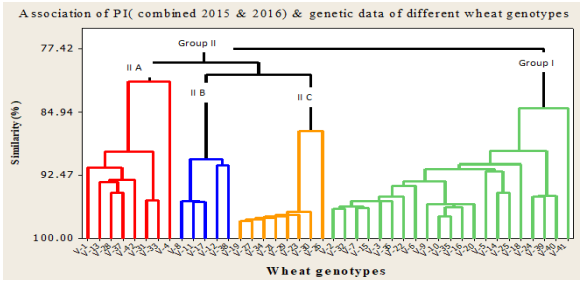

Fig. 5: Dendrogram distributions of 42 genotypes in groups on the basis of combined percent Incidence of both years (2015 & 2016) and marker data of 13 SSR markers linked to Karnal bunt resistance

Association of genetic data with percent infection of 42 wheat genotypes

PI of wheat genotypes was found significantly associated with genetic bands data. Out of 40 bands, 21 bands showed association with the PI of wheat genotypes calculated in 2015. The total association was found at 52.5%. Eighteen bands were highly significant with percent infection at $p<0.001$, while two were significant at $P < 0.01$ and one at

Table 4: Mean value of aggressiveness analysis of 42 wheat genotypes based on Seed infestation (PI) during 2015 and 2016

LSD and PI of 42 wheat genotypes (2015-16)		LSD and PI of 42 wheat genotypes (2016-17)	
Wheat genotypes	Mean (PI*)	Wheat genotypes	Mean (PI*)
PR-106	78.55 A	WL-711	90.42 A
WL-711	76.87 AB	AUR-08010	86.58 AB
Aas-11	76.06 AB	PR-110	81.61 ABC
Pak-13	70.88 ABC	11138	78.81 A-C
V-12001	69.95 A-C	V-10110	76.67 A-C
09-FJ-34	69.85 A-C	09-FJ-34	76.08 A-C
V-10110	68.98 A-C	TW96018	75.20 A-C
11C022	68.24 A-C	11C022	74.82 A-C
TW96018	66.59 A-D	CT 09137	74.17 A-C
HD-29	66.14 A-D	Pak-13	74.05 A-C
DN-102	64.47 A-D	V-12001	72.10 A-D
99346	64.22 A-E	SRN-09111	70.65 A-D
DANI-1313	64.17 A-E	DANI-1313	69.35 A-E
NR-449	63.89 A-E	NRL-1123	66.55 A-F
112802	60.95 A-F	PR-106	65.98 A-F
12304	60.85 A-F	99346	65.56 A-F
NRL-1123	59.90 A-F	SKD-11	65.47 A-F
PR-110	57.41 A-G	12304	64.06 A-G
AUR-08010	57.14 A-G	NR-449	63.19 A-H
SKD-11	56.72 A-G	112802	61.03 B-H
SRN-09111	54.42 A-G	Aas-11	58.41 C-I
11138	53.14 B-G	FSD-08	53.98 C-J
99172	49 C-G	12266	45.34 D-K
CT 09137	46.35 C-H	11098	41.94 E-L
9459-1	43.33 D-H	HD-29	41.30 F-L
11098	42.52 D-H	PR-113	37.03 G-M
NR-436	39.52 E-H	NRL-1130	35.83 H-M
PR-113	38.67 F-H	99172	33.33 I-M
V-11005-2013	35.05 GH	11C023	32.99 I-M
PR-111	10.00 I	V-11160	30.22 J-M
NRL-1130	10.00 I	NR-436	24.29 K-M
V-11160	10.00 I	9459-1	23.33 K-M
12266	10.00 I	DN-102	21.11 K-M
11C023	10.00 I	V-11005-2013	14.67 LM
TW1150	10.00 I	TW1150	10.00 M
ESW-9525	10.00 I	ESW-9525	10.00 M
NIA-CIM-04-10	10.00 I	NIA-CIM-04-10	10.00 M
PR-112	10.00 I	PR-111	10.00 M
NR-423	10.00 I	PR-112	10.00 M
NR-429	10.00 I	NR-423	10.00 M
KT-338	10.00 I	NR-429	10.00 M
FSD-08	10.00 I	KT-338	10.00 M
LSD value at 0.05= 24.8		LSD value at 0.05= 27.6	

Column means followed by a common letter are not statistically different from each other at 5% level of probability

$p<0.05$. In the present study, the statistical t-value is relatively far from zero and is relatively large to the standard error, which indicated a relationship between molecular results and percent infection of 42 wheat genotypes (Table 7).

Significant association of band data with a PI of wheat calculated in 2016 was also observed. Out of 40 bands, 20 bands were found to be associated with the PI. Fifty percent association was found and the coefficients were not equal to zero. Seventeen bands showed high significance at $P < 0.001$, one was at $P < 0.01$ while two were significant at $P < 0.05$. It was further observed that t-values were also high in the association of bands and PI (2016) (Table 8). In the present study, we found two models 33~ aa and 1~ bb (Table 8 and 9) with statistically significant model $P < 0.05$ and relatively high R-square (0.10, 0.099) and adjusted R-square (0.0796, 0.077) value respectively. While other models were not fit model as p values of model were higher than the predetermined value.

Discussion

Karnal bunt is a very important disease in Pakistan; however, relatively very little work is done. The major work in Pakistan is focused on a few surveys, disease screening for resistance and chemical control. The current study was designed to identify the resistance in wheat genotypes and their association with percent infection of *T. indica* isolate. For this study, an aggressive isolate PB-25 was used for screening which was previously studied by Aasma *et al.* (2019a, b).

Table 5: List of SSR markers linked to Karnal Bunt resistance in wheat genotype

Primer Name	Sequence 5´…….3´	Ann Temp °C	Expected Size	Chromosomal Location	Reference
	5'CAA CTC AGT GCT CAC ACA ACG 3'	60	168,176	6D	Kumar *et al.* (2007)
Xgwm469	5'CGATAACCACTCATCCACACC 3'				
	5'GCA CCC ACA TCT TCG ACC 3'	58	195	1A	
Xgwm666	5' TGCTGCTGGTCTCTGTGC 3'				
	5'GAG CCC ACA AGC TGG CA 3'	58	120	2A	
Xgwm425	5' TCGTTCTCCCAAGGCTTG 3'				
	5'AAG ATG GAC GTA TGC ATC ACA 3'	60	112,122,	1AL	Sehgal *et al.* (2008)
Xgwm99	5' GCCATATTTGATGACGCATA 3'		136		
	5'CAT TGT TTT CTG CCT CTA GCC 3'	58	185	4BL	
Xgwm149	5' CTAGCATCGAACCTGAACAAG 3'				
	5'GGG TTC CTA TCT GGT AAA TCC C 3'	58	180,370	5DL	
Xgwm174	5' GACACACATGTTCCTGCCAC 3'				
	5'GCA ATC TTT TTT CTG ACC ACG 3'	58	110	3BL	
Xgwm340	5' ACGAGGCAAGAACACACATG 3'				
	5 CGT ATC ACC TCC TAG CTA AAC TAG3'	60	290	4B	
Xgwm6	5' AGCCTTATCATGACCCTACCTT 3'				
	5'-ACTGTTCCTATCCGTGCACTGG-3'		225 bp	5B	
Xwmc235	5'-GAGGCAAAGTTCTGGAGGTCTG-3'	58			
	5'-TATATAGTTCAATATGACCCG-3' ;		160 bp	1B/5B	
Xgwm604	5'- ATCTTTTGAACCAAATGTG-3'	50			
	5'-CGCCAACACCTACCATT -3';		140 bp	2B/5B/5D	
Xbarc140	5'-TTCTCCGCACTCACAAAC-3'	52			
	5'-GCGTTGGCTAATCATCGTTCCTTC-3'		180 bp	2D/5B	Kumar *et al.* (2015)
Xbarc 59	5'-AGCACCCTACCCAGCGTCAGTCAAT-3'	55			
	5'-CGCATCCAACCATCCCCACCCAACA-3'		200 bp	5A/5B/5D	
Xbarc232	5' CGCAGTAGATCCACCACCCCGCCAGA-3'	58			
	5'-ACTTGTATGCTCCATTGATTGG-3'		120 bp	5B	
Xgwm499	5'-GGGGAGTGGAAACTGCATAA-3')	60			
	5'AAA GAG GTC TGC CGC TAA CA 3'				
Xgwm637	5' TATACGGTTTTGTGAGGGGG 3'	58	120	4A	
	5'GCA TTT CGG GTG AAC CC 3'				
Xgwm538	5' GTTGCATGTATACGTTAAGCGG 3'	60	250	4B	
	5'CCT CTT CCT CCC TCA CTT AGC 3'				
Xgwm337	5' TGCTAACTGGCCTTTGCC 3'	60	175	1D	

Table 6: Number of alleles generated by using 13 SSR markers

Sr. No.	Marker	No. of alleles generated	Size range bp	PIC
1	Xgwm-637	1	120	0.70
2	Xgwm-469	2	100-140	0.55
3	Xgwm-538	1	120	0.14
4	Xgwm-337	4	100-600	0.78
5	Xgwm-99	2	112-120	0.89
6	Xgwm-149	1	165	0.52
7	Xgwm-174	8	150-1000	0.89
8	Xgwm-340	7	100-600	0.99
9	Xgwm-425	1	120	0.18
10	Xwmc-235	3	100-350	0.59
11	Xgwm-604	2	100-140	0.77
12	Xbarc-59	2	200-500	0.53
13	Xbarc-232	6	200-500	0.84

PIC: Polymorphic information content: The highest and lowest PIC values are indicated by bold, underlining

Among total 42 wheat genotypes tested, thirteen genotypes showed HR reaction, seven genotypes showed R reaction while twenty-two showed MS reaction during 2015–2016. Same pattern of resistance and susceptibility was observed during 2016–2017 with few exceptions. However, few genotypes lost resistance in 2016 which indicated the complication in managing this disease even with the resistance sources. This advocated that a continuous screening program at the national level needed to be initiated as it is being followed in the case of rusts (Nagarajan *et al*. 1997). Results on percent seed infection revealed that Pak-13 showed a high percentage of infection. In comparison during 2016, WL-711 exhibited high percent of infection (90.42%). Results of a few genotypes showed inconsistency in the reaction even though the same isolate was used for inoculation. Aujla *et al.* (1989) recorded a similar trend in genotype HD 2967 which showed 1S reaction, was found to be 3S against a particularly virulent isolate. This may be attributed to these genotypes do not confer stable resistance against the pathogen and such genotypes should be discouraged in the breeding program for KB resistance. A similar trend of resistance of some of the genotypes has been reported by Ullah *et al.* (2012). Synthetic genotypes showed resistance and few tend towards moderate susceptibility similar findings were reported by Villareal *et al*. (1995, 1996).

Table 7: Association of wheat genotypes bands with percent infection of wheat inoculated with *T. indica* inoculum during 2015-2016

Sr. No.	Model	Multiple R Squared	Adjusted R square	Std. Error (Estimate)	Std. Error	t-value	P-value
1	1 ~ aa	0.080	0.06	0.81	0.15	5.29	4.38×10^{-06}***
2	2~ aa	0.004	-0.02	0.72	0.15	4.82	1.98×10^{-05}***
3	3~ aa	0.007	-0.02	0.95	0.09	10.24	7.31×10^{-13}***
4	4~ aa	0.005	-0.02	0.60	0.16	3.74	0.000567 ***
5	5~ aa	0.004	-0.02	0.82	0.11	7.40	4.59×10^{-09}***
6	6~ aa	0.001	-0.02	0.87	0.10	8.43	1.72×10^{-10}***
7	7~ aa	0.024	0.00	0.96	0.11	8.70	7.37×10^{-11}***
8	9~ aa	0.045	0.02	1.03	0.05	21.84	$< 2\times10^{-16}$***
9	10~ aa	0.001	-0.02	0.86	0.12	7.22	8.06×10^{-09}***
10	11~ aa	0.019	-0.01	0.56	0.15	3.75	0.000551***
11	13~ aa	0.004	-0.02	0.72	0.14	5.30	4.22×10^{-06}***
12	14~ aa	0.045	0.02	1.03	0.05	21.84	$< 2\times10^{-16}$***
13	15~ aa	0.010	-0.01	0.96	0.09	10.33	5.56×10^{-13}***
14	17~ aa	0.024	0.00	0.96	0.11	8.70	7.37×10^{-11}***
15	19~ aa	0.000	-0.02	0.59	0.16	3.74	0.000572***
16	22~ aa	0.024	0.00	0.40	0.16	2.51	0.0163*
17	28~ aa	0.011	-0.01	0.72	0.13	5.50	2.21×10^{-06}***
18	32~ aa	0.001	-0.02	0.72	0.14	5.17	6.46×10^{-06}***
19	33~ aa	0.102	0.08	0.34	0.11	3.20	0.00263**
20	35~ aa	0.005	-0.02	0.59	0.15	3.87	0.00038***
21	37~ aa	0.026	0.00	0.30	0.12	2.43	0.0197*

***= Significant at 0.001, **= Significant at 0.01, *= Significant at 0.05

Table 8: Association of wheat genotypes bands with Percent infection of wheat inoculated with *T. indica* inoculum during the Year 2016

Sr. no.	Model	Multiple R Squared	Adjusted R square	Std. Error (Estimate)	Std. Error	t-value	P-value
1	1~ bb	0.10	0.08	0.86	0.16	5.42	2.93×10^{-06}***
2	2~ bb	0.00	-0.02	0.74	0.16	4.69	3.03×10^{-05}***
3	3~ bb	0.00	-0.02	0.91	0.10	9.37	9.56×10^{-12}***
4	4~ bb	0.01	-0.01	0.64	0.17	3.83	0.000431***
5	5~ bb	0.01	-0.02	0.81	0.12	6.95	1.91×10^{-08}***
6	6~ bb	0.01	-0.02	0.94	0.11	8.76	6.19×10^{-11}***
7	7~ bb	0.02	0.00	0.96	0.11	8.39	1.95×10^{-10}***
8	9~ bb	0.07	0.04	1.05	0.05	21.49	$< 2\times10^{-16}$***
9	10~ bb	0.01	-0.02	0.78	0.12	6.35	1.39×10^{-07}***
10	11~ bb	0.01	-0.02	0.59	0.16	3.78	0.000502***
11	13~ bb	0.02	0.00	0.66	0.14	4.68	3.16×10^{-05}***
12	14~ bb	0.07	0.04	1.05	0.05	21.49	$< 2\times10^{-16}$***
13	15~ bb	0.01	-0.02	0.95	0.10	9.78	2.79×10^{-12}***
14	17~ bb	0.02	0.00	0.96	0.11	8.39	1.95×10^{-10}***
15	19~ bb	0.02	-0.01	0.73	0.16	4.49	5.74×10^{-05}***
16	28~ bb	0.00	-0.02	0.78	0.14	5.70	1.14×10^{-06}***
17	32~ bb	0.01	-0.01	0.65	0.15	4.49	5.78×10^{-05}***
18	33~ bb	0.05	0.02	0.28	0.11	2.50	0.0165*
19	34~ bb	0.07	0.05	0.24	0.09	2.53	0.0153*
20	35~ bb	0.03	0.00	0.50	0.16	3.17	0.0029**

***= Significant at 0.001, **= Significant at 0.01, *= Significant at 0.05

Molecular markers have been a very powerful tool for characterizing and assessing the genetic diversity of wheat genotypes (Edwards and Caskey 1991). It is important to know the usefulness of the markers before they can be used extensively for crop improvement programs. However, it is not guaranteed that the markers identified in the population will be suitable in another group (Kumar *et al*. 2015). In this study, 17 SSR markers were tested for the presence of KB resistance genes on 42 wheat genotypes. Among 17 SSR markers, 13 markers gave the amplified product in the present study, 9 SSRs (Xgwm 469, Xgwm-337, Xgwm-99, Xgwm174, Xgwm-340, Xgwm- 235, Xgwm-604, Xbarc 59 and Xbarc-232) were found to be multi-allelic. Since micro-satellite primers are locus-specific, each pair of primers must amplify one locus. In previous studies, many scientists reported relatively higher numbers of alleles per locus in bread wheat (Roder *et al*. 1998; Stephenson *et al*. 1998; Vasu *et al*. 2000). Prasad *et al*. (2000) also observed multiple loci for each pair of primers. Ahmed (2002) identified 156 allelic variations at 43 SSR loci, ranging from 2 to 8 alleles per locus with an average of 3.6 alleles per locus. Kumar *et al*. (2007) found 179 alleles at 46 SSR loci with an average of 3.9 alleles per locus with a range of 1 to 8 alleles on each locus. The results of the present study and other studies clearly showed that SSR markers are very informative and confirmed the presence of multiple alleles per locus (Roder *et al*. 1995; Singh *et al*. 2003; Kumar *et al*. 2007).

In the present study, the microsatellite markers Xgwm-637, Xgwm-469, Xgwm-538, Xgwm 337, Xgwm-99, Xgwm-149, Xgwm-174, Xgwm-340, Xgwm- 425, Xgwm-

235, Xgwm-604, Xbarc-59 and Xbarc-232 were found to be associated with resistance. In earlier studies, similar findings were reported by Sehgal *et al*. (2008) that these four markers Xgwm99 (1AL), Xgwm149 (4BL), Xgwm174 (5DL) and Xgwm340 (3BL) are associated with resistance genes in BC5F4 progenies. On the contrary, Kumar *et al*. (2007) reported two markers (Xgwm 337-1D and Xgwm 637-4A) had an association with the resistance of wheat against KB. In another study, Kumar *et al*. (2015) also confirmed the association of the primers Xgwm 337, Xgwm 637, Xgwm 538, Xgwm-469 and Xgwm-425 with the resistance of KB in different populations. Vasu *et al*. (2000) discovered a microsatellite locus associated with the gene of KB resistance, Xgwm637 that has been assigned to 4AL. Recently, Brar *et al*. (2018) identified QTL for KB resistance and some QTL on chromosomes 4A, 7A and 6A are likely to be associated with SSR markers used in this study.

In this study, DNA fragments ranged in size from about 100 bp to 1000 bp compared to Manifesto *et al*. (2001) reported amplified DNA fragments ranging in size from 115 bp to 285 bp, whereas Abbas *et al*. (2008) detected amplified DNA fragments ranging in size from 250 bp to 1000 bp while Kumar *et al*. (2015) observed range from 100 bp to 300 bp. It has been previously reported that KB resistance was controlled by major and minor genes (Singh *et al*. 1996) and DNA polymorphism between genotypes has been achieved from microsatellite marker amplification. It is a useful application for assessing genetic diversity and polymorphism (Lang *et al*. 2001).

Development of resistant varieties was difficult due to the confusing impact of the environment and limited sustainable genetic sources to the nature of quarantine resistance to KB. Therefore, the identification of markers associated with KB resistance using marker-assisted selection (MAS) could help in complementing phenotypic selection. Kumar *et al*. (2016) reported SSR markers Xbarc59, Xgwm232, Xgwm235, and Xgwm604 were linked with KB resistance gene. Similar results were found in our study. A dendrogram was constructed using allelic diversity based on SSR marker data, to determine the genetic relationship between selected wheat genotypes. The dendrogram revealed that all genotypes fall into two main categories with a similarity factor of 4.5. Genetic diversity and population structure information of breeding genotypes of wheat will benefit breeders to make improved usage of their genetic resources and management of genetic variation in their breeding programs. In the present study, the significant genetic divergence of wheat genotypes was observed with SSR markers.

Association of genetics data and percent infection of two years of 42 wheat genotypes were found to be highly significant. An association of 52.5% was recorded in 2015–16 while 50% in 2016–2017. Furthermore, R-square and adjusted R were found to have low value. R-square reflects the goodness of fit of the model to the population taking into account the sample size and the number of predictors used. As we were looking for the association not for prediction, so R-square was not importantly considerable in our results. The sample size was small in our study as 42 wheat genotypes were studied for the molecular and field data. The small sample size was also affected by the model goodness fit.

Conclusion

Results suggested that molecular markers can explain the phenotypic variations for KB resistance in wheat. The use of SSR markers in these populations showed significant results and had the potential for detecting resistance in wheat. Further work is needed to narrow down the environmental factors (*i.e*., humidity and temperature) along with PI as predictors to estimate the KB status on wheat.

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Author Contributions

AA and SA, MF planned the experiments, AA interpreted the results and did write-up of the manuscript, and MA statistically analyzed the data. SA, MN, US, SB checked and improved the write-up of manuscript.

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