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Identification and validation of SSR markers for Karnal bunt (*Neovossia indica*) resistance in wheat (*Triticum aestivum*)

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ABSTRACT

Present study was carried out to screen 107 recombinant inbred lines (RILs) of cross H567.71 (resistant) × WH 542 (susceptible) for Karnal bunt [*Neovossia indica* (Mitra) Mundkar] resistance and to identify and validate SSR markers associated with Karnal bunt resistance in wheat (*Triticum aestivum* L.). There was significant variation among the recombinant inbred lines for percentage and coefficient of infection to Karnal bunt. Most of the RILs (85) and (72) showed up to 5% infection during the year 2010 and 2011 and thus were resistant. The range of percentage of infection (PI) in the susceptible RILs was 5.19 to 22.93 and 5.38 to 36.46 in the year 2010 and 2011 respectively, whereas coefficient of infection on RILs ranged from 0 to 8.28 and 0 to 14.45 in the year 2010 and 2011 respectively. A total of 70 SSR markers were used to screen parental genotypes out of these 42 were polymorphic. Total of 88 alleles were detected, number of alleles per locus ranged from 2-3 with an average of 2.09 alleles per locus. Genetic similarity analysis showed that parental genotypes were quite distinct from each other. The cluster analysis led to distribution of parents and their RILs in to two groups. Major cluster I, included resistant parent H 567.71 with resistant lines and major cluster II had susceptible parent WH 542 and susceptible lines. The three primers (Xgwm 637, Xgwm 337 and Xgwm 538) which were already reported to be associated with Karnal bunt resistance also showed association with Karnal bunt resistance in population under study. A new marker (Xgwm 6) located at 1.6 cM from Xgwm 538 (on chromosome 4B) was identified to be associated with Karnal bunt resistance. These four markers were further used to screen on whole population and results were confirmed. Hence, these markers may be used for marker assisted selection for Karnal bunt resistance in early segregating generations.

Key words: Karnal bunt, SSR Markers, Wheat RILS

Karnal bunt caused by the fungus *Neovossia indica* (Mitra Mundkar) (syn *Tilletia indica*) belonging to order Ustilaginales is a wide spread disease of wheat. It partially damages the seed of bread wheat, durum wheat and triticale (Agarwal *et al.* 1976). Karnal bunt pathogen infects the plant at boot leaf stage, penetrates the individual floret, entering the embryonal end, and proceeds along the ventral crease. The infected parts of kernels are replaced by masses of dark and powdery, fungal teliospores resulting in losses, both in yield and quality (Carris *et al.* 2006).

The yield losses are usually minor but the disease reduces grain quality due to an unpleasant fishy odour of trimethylamine produced by the fungus (Singh and Bedi 1985). Wheat containing 3% bunted grains is considered unfit for human consumption (Mehdi *et al*. 1973). Thus Karnal bunt (KB) of wheat is a disease of great concern by quality conscious countries (United States, Canada, Russia and China etc) of the world which have laid legal restrictions for entry of wheat grains from the infested areas. The disease is difficult to be identified in the field as infected grain shows no symptoms until near maturity. Since the pathogen is seed, soil and air borne, a limited control is achieved through the application of fungicides (Carris *et al.* 2006). Hence, the host plant resistance is regarded as the major option for management of KB pathogen. Though the use of resistant varieties is the most viable and economical approach but truly resistant varieties are not available (Sharma *et al*. 2008). Intensive breeding for Karnal bunt resistance has been carried out but it is greatly hampered by the lack of easy, quick and environment independent methods of screening of host genotypes against the pathogen. Therefore, biotechnological tools, which provide better understanding of the existing and expanded genetic diversity, should be considered to further accelerate the progress of such breeding programs (Fischer and Edmeodes 2010).

MAS are especially valuable for selecting traits such as resistance to Karnal bunt disease of wheat that are difficult to screen. Simple Identification of PCR-based markers linked

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to Karnal bunt resistance offer the prospects of using marker-assisted selection schemes in developing resistant wheat cultivars (Kumar *et al.* 2007). Microsatellite-based markers have been successfully used for mapping studies in hexaploid wheat and to produce a microsatellite map of wheat. High polymorphism detection levels, high throughput capability, and low cost compared to other marker systems enhance the utility of microsatellites for mapping genes associated with agronomically important traits (Dubcovsky 2004).

Keeping in view the importance of these molecular markers in identification of Karnal bunt resistance genes, the aim of the present investigation was to screen recombinant inbred lines for Karnal bunt resistance and to indentify and validate SSR markers associated with Karnal bunt resistance.

MATERIALS AND METHODS

A population of 107 recombinant inbred lines (RILs) of wheat cross, H 567.71 (resistant) × WH 542 (susceptible) was grown along with parents, in a randomized block design in single row plot in three replications in the crop seasons of 2009-2010 and 2010-2011.

Pathogen, Neovossia *indica* was isolated from infected grains of wheat and multiplied on potato dextrose agar (PDA) slants at 19.5±1ºC.

The screening was done under artificial epiphytotic conditions. Inoculum was prepared from 10-12 days old, active culture of *N. indica* containing approximately 10 000 secondary sporidia/ml. Ear heads were inoculated at boot leaf stage by injecting 2 ml of inoculum using hypodermic syringe (Aujla *et al*. 1989). Five to eight spikes of each entry were inoculated. At maturity, the inoculated spikes were harvested individually and threshed manually and percentage of infection and coefficient of infection was worked out as suggested by Aujla *et al.* (1989).

Genomic DNA was isolated using CTAB method of Saghai-Maroof *et al*. (1984) from a small amount of fresh leaf tissue (5.0 g) from each of parental genotypes and their 107 RILs. Agarose gel electrophoresis (0.8%) was used to check quality and quantity of genomic DNA.

The DNA concentrations were estimated by visual assessment of band intensity in comparison with Lambda (λ) DNA of known concentration.

Parental genotypes were screened using a total of seventy SSR markers for the molecular characterization, out of these 42 were polymorphic and used for screeing of RILs and preparation of SSR database in the above population. The PCR amplification conditions were optimized. The PCR reaction was conducted in a reaction volume of 20 µl containing 2ul of 1X PCR buffer, 100 µM dNTPs, 0.5 µl of each primer, 1.5 unit Taq DNA polymerase and 50 ng template DNA. The thermocycling program consisting of an initial denaturation at 95°C for 4 minutes followed by 40 cycles of 95°C for 1 minute, 1 minute and 20 second at annealing temperature (55-63 °C), 1 minute at 72°C and a final cycle of 72C for 10 minutes was used. Amplified products were resolved on 4% polyacrylamide gels using Amersham Biosciences system as described by Chen *et al.* (1997). Gels were pre-run until an adequate temperature (50-60^{0}C) was reached. DNA bands were visualized by using silver staining protocol (SILVER SEQUENCE^{TM} DNA Sequencing System, Promega Inc., Madison, WI, USA) after completion of electrophoresis.

The frequency of polymorphism between different lines of wheat for each type of marker was calculated based on presence (taken as 1) or absence (taken as 0) of bands (Ghosh *et al*. 1997). The 0/1 matrix was used to calculate similarity genetic distance using 'simqual' sub-program of software NTSYSPC (numerical taxonomy and multivariate analysis system programme) (Rohlf 1993). The resultant distance matrix was employed to construct dendrograms by the un-weighted pair-group method with arithmetic average (UPGMA) subprogram of NTSYS-PC.

RESULTS AND DISCUSSION

Disease incidence in RILs

None of the control measures had been proven to be satisfactory for the management of Karnal bunt disease. Seed treatments using fungicides are typically ineffective in reducing the disease because spores are protected with pericarp in the bunted grains. Other approaches used for controlling Karnal bunt including crop rotation, sowing of disease free seeds and adjustment of the time of irrigation only minimize the disease infection (Munjal 1975). Therefore, the most effective and economical method of disease management is through host plant resistance. Targeting genes for improvement of any trait in crop plants may require specific molecular markers, an appropriate population and right choice of parents. In the present investigations 70 SSR markers, well distributed in wheat genome were used to screen hundred and seven recombinant inbred lines of cross H 567.71 × WH 542 for identification and revalidation of markers having association with Karnal bunt resistance genes. The most commonly used other populations for such studies are DH or F2 populations. RIL populations have an edge over the other two by being homozygous while maintaining the variability of F2 population. These107 RILs exhibited significant variation for percentage of infection, coefficient of infection to Karnal bunt, indicating a good population for further investigations.

In the present investigation a wide variation was observed among the 107 RILs evaluated for Karnal bunt infection (Table 1). Most of the RILs, i.e. 69 and 54 showed 0% infection during the year 2010 and 2011 respectively. RILs with percentage of infection up to 5 were taken as resistant and those with more than 5% infection were taken as susceptible. The range of percentage of infection (PI) in the susceptible RILs was 5.19 to 22.93 and 5.38 to 36.46 in the year 2010 and 2011 respectively, whereas, susceptible parent WH 542 had 22.91 and 41.48% infected grains in the year 2010 and 2011 respectively. The range of coefficient of infection (CI) in the RILs was 0 to 8.28 and 0 to14.45 in the year 2010 and 2011 respectively, whereas, susceptible parent WH 542 had 8.76 and 17.18% coefficient of infection in the year 2010 and 2011 respectively (Table 1).

Table 1 Ranges for percentage of infection (PI) and coefficient of infection (CI) on RILs derived from cross H 567.71 × WH 542 during the year 2010 and 2011.

Ranges of infection	2010	2011
No. of RILs showed 0% infection	69	54
Percentage of infection (PI)	5.19 to 22.93	5.38 to 36.46
coefficient of infection (CI)	0.00 to 8.28	0.00 to 14.45
WH 542 (Susceptible parent)	22.91(8.76)	41.48(17.18)
H 567.71 (resistant parent)	0.00	0.00

The two parents were quite diverse in their reaction to Karnal bunt as parent H 567.71 was resistant with 0% infection, and WH 542 susceptible parent was showed a wide range of percent infection. The distribution of Karnal bunt disease severity on the RILs was skewed towards resistant parent in both the years, thereby suggesting the segregation of some major additive effects from the parental wheat line H 567.71. This is consistent with the results of previous studies by Kumar *et al*. (2007). Sharma *et al.* (2005) studied KB resistance in RIL derived from crosses of four resistant stocks (HD 29, W 485, ALDAN 'S/IAS 58, H 567.71/3 PAR) and a highly susceptible cultivar, WH 542 and indicated that two genes in ALDAN S'/IAS 58 were responsible for Karnal bunt resistance. Karnal bunt resistance based on two or more genes with additive effects were also reported by Sirari *et al. (*2008) using two recombinant inbred lines populations. Goel (2010) reported that Karnal bunt resistance is controlled by two dominant genes and the genes interacted with each other in dominant recessive manner.

SSR markers profiling

The DNA polymorphism between both the parental genotypes obtained by using micro-satellite markers, is a useful tool for genotyping and the assessment of genetic diversity (Lang *et al*. 2001). During the present work a total of 70 SSR primers were used to screen parental genotypes and 20 selected RILs out of which ten were most resistant RILs with 0.0% percentage of infected grains and ten were most susceptible RILs (percentage of infection 14.89 to 36.46% (Table 2). Out of 70 SSRs, only 42 primers showed polymorphism among parents. Database of SSRs was generated using above primers.

Similarity coefficient data based on the proportion of shared alleles, using 42 SSR markers, was used to calculate the coefficient values among the selected RILs and parental genotypes. The allelic diversity was used to produce a dendrogram (cluster tree analysis, NTSYS-PC), to demonstrate the genetic relationship among selected RILs and the parental genotypes (Fig 1). Dendrogram showed that all the RILs and their parental genotypes clustered in two major groups at the similarity coefficient of 0.39. Major group I consisted of resistant RILs with resistant parent H 567.71 and major group II consisted of susceptible RILs and susceptible parent WH 542. The level of polymorphism observed in wheat using microsatellite or SSR markers is

Table 2 List of selected RILs their percentage of infection (PI) and coefficient of infection (CI) to Karnal bunt used for SSR marker analysis

Resistant				Susceptible			
Code No.	RIL No.	PI	CI	Code No.	RIL No.	PI	CI
1	1	0.0	0.0	11	7	30.23	12.21
2	2	0.0	0.0	12	18	23.88	11.94
3	3	0.0	0.0	13	44	36.46	14.45
4	16	0.0	0.0	14	57	22.47	9.27
5	38	0.0	0.0	15	59	31.84	13.31
6	42	0.0	0.0	16	63	23.30	10.19
7	54	0.0	0.0	17	80	19.53	9.62
8	70	0.0	0.0	18	86	14.89	8.64
9	74	0.0	0.0	19	88	16.36	7.18
10	92	0.0	0.0	20	101	21.83	8.27

greater than that of RFLPs markers, this makes it more-rapid and economical to map genes of interest. Approximately 80% of the SSR markers tested by Roder *et al*. (1998) were polymorphic between the *T. turgidum-Ae tauschii* synthetic hexaploid × *T. aestivum*. In the present investigation polymorphism was studied among the parental genotypes using wide array of 70 SSR markers, distributed all over the wheat chromosomes. Out of these 42 (60%) detected reproducible polymorphism between the two parental genotypes. The results of the present study and those of other studies thus clearly indicate that SSR markers are quite informative (Roder *et al*. 1995, Singh *et al*. 2003, Kumar *et al*. 2007).

A total of 88 alleles were detected at 42 loci, the number of alleles per locus ranged from 2-3 with an average of 2.09 alleles per locus. Since microsatellite markers are locus specific only one specific locus is expected to be amplified by each primer. In few earlier studies also, more than one locus per microsatellite primer pair were detected and mapped in bread wheat (Roder *et al*. 1998, Vasu *et al*. 2000). This revealed significant differences in allelic diversity among various microsatellite loci. Many earlier studies have reported remarkable differences in allelic diversity (Akagi *et al.* 1997). Prasad *et al*. (2000) also reported relatively higher number of alleles per locus. Ahmed (2002) detected a total of 156 allelic variants at 43 SSR loci, ranging from 2 to 8 alleles per locus with an average of 3.6. In present investigation, size of amplified DNA fragments varied from approx. 100bp to 300bp. Manifesto *et al.* (2001) obtained amplified DNA fragments that varied in size from 115bp to 285bp and Abbas *et al.* (2008) obtained amplified DNA fragments that varied in size from 250bp to 1000bp.

Validation of SSR markers associated with Karnal bunt resistance

Before a marker can find wide application in crop improvement programs, it is important to validate its usefulness for marker assisted breeding in independent mapping populations and different genetic backgrounds.

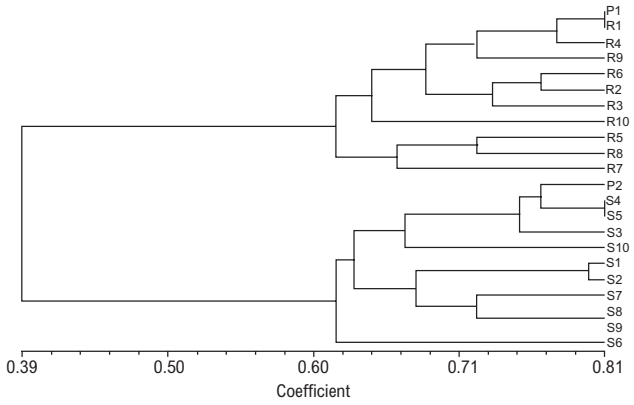

Fig 1 Dendrogram of selected wheat RILs and parental genotypes based on SSR diversity data

However, there is no guarantee that markers identified in one population will be useful in other populations. Three primers namely Xgwm 337, Xgwm 637 and Xgwm 538 were already reported to be associated with Karnal bunt resistance in different populations. Xgwm 337 (present on chromosome 1D) and Xgwm 637 (present on chromosome 4A) were identified in our laboratory by Kumar *et al*. (2007) in the population derived from cross between HD 29 and WH 542. Xgwm 538 was identified by Singh *et al*. 2003 in RILs of HD 29 × WL 711. To test these three markers in our population, initially we screened twenty RILs out of which 10 were most resistant and 10 were most susceptible. The primers characterized resistant parent, resistant bulk and resistant lines differently from susceptible parent, susceptible bulk and susceptible lines. The specific band of each of these markers (Xgwm 637-120 bp, Xgwm 337-175bp, Xgwm 538-250 bp) was present in resistant bulk and resistant lines but the band was not seen in susceptible bulk and susceptible lines. Hence the result clearly indicated that resistant bulk and resistant lines have the resistant allele from resistant parent which was absent in the susceptible bulk and susceptible lines. The recombinant inbred lines were homozygous for both the alleles and the resistant lines had the resistant allele from the resistant parent which is absent in susceptible lines. So the specific band was present only in resistant lines. This indicating the association of these primers with Karnal bunt resistance and validated the results of earlier workers.

After obtaining the association of these primers with KB resistance, these were further used to screen resistant and susceptible bulks and then whole population. Similar results were obtained in case of bulk segregant analysis and all the RILs of present population.

Similarly Parker *et al*. (1998) identified a marker for wheat flour colour on chromosome 7A based on the cross Schomburgk/Yaralinka and later confirmed its usefulness in the Cranbrook/Halberd and Sunco/Tasman crosses (Mares and Campbell 2001). Sharp *et al*. (2001) also identified a marker linked to stem rust *Sr2* gene in Chinese Spring × Chinese Spring (Hope3B) on chromosome 3B, but the polymorphism was not diagnostic when assayed in a wide range of CIMMYT, Australian and other cultivars of known *Sr2* genotypes.

Identification of new marker linked with Karnal bunt resistance

One of the most critically anticipated and most often cited benefits of genetic markers for plant breeding has been their use to facilitate MAS as an indirect selection tool in crop improvement programs (Koebner and Summers 2003). MAS allows breeders to conduct early generation selection for a trait (Koebner and Summers 2003). The advent of molecular markers has revolutionized the genetic analysis of complex traits and identification of chromosomal regions associated with disease, insects, agronomic and grain quality traits in wheat (Liu *et al*. 2001, Huang *et al*. 2006).

In the present investigation out of 42 SSRs screened, a new microsatellite marker Xgwm 6 was also identified in our population which could possibly be linked to Karnal bunt resistance. After observing association with KB resistance on selected RILs, the marker Xgwm 6 was then tested on two bulks, resistant bulk (pooled DNA from 10 selected resistant lines) and susceptible bulk (pooled DNA from 10 susceptible lines) along with selected resistant and susceptible lines. Results obtained for this marker indicated that specific band of Xgwm 6 (290bp) was present in resistant parent and resistant bulk but the same was absent in susceptible parent and susceptible bulk, indicating association of this primer for Karnal bunt resistance. So the bulk segregant analysis also clearly characterized resistant and susceptible RILs. The bands presented in resistant lines were absent in susceptible lines. Xgwm 6 is located on chromosome 4B, at a close vicinity [(1.6 cM) as given in available database] of another SSR marker Xgwm 538 (associated with Karnal bunt resistance). Thus there is co-segregation of these primers in most of the RILs. Similar to our results earlier studies have reported the markers for Karnal bunt resistantce. Microsatellite loci Xgwm 382, Xgwm 369, Xgwm 637, Xgwm 156 and Xgwm 617 mapped on 2AS, 3AS, 4AL, 5AL and 6AL respectively were found to be associated with Karnal bunt resistance (Vasu *et al*. 2000). Micro-satellite marker, Xgwm 538, found to be associated with a (QTL) for Karnal bunt resistance by Singh *et al.* (2003) was further improved by Brooks *et al.* (2006) into a single nucleotide polymorphism (SNP)-based marker.

Singh *et al*. (2007) identified two QTLs, Qkb.ksu-6BS.1 and Qkb.ksu-5BL.1 for Karnal bunt resistance in RILs population-1 (WH 542/HD 29) on the chromosome 4B, 5B and 6B and RILs population-2 (WH 542/W 485) on the chromosome 4B and 6A. Sehgal *et al.* (2008) developed Karnal bunt-resistant near isogenic lines (NILs) and utilized 400 SSR markers for their screening. They found presence of donor alleles of four markers: *Xgwm 99*(1AL), *Xgwm 149*(4BL), *Xgwm 174*(5DL) and *Xgwm 340*(3BL) in the resistant pool.

Confirmation and revalidation of SSR markers

For the further confirmation of the marker, these four

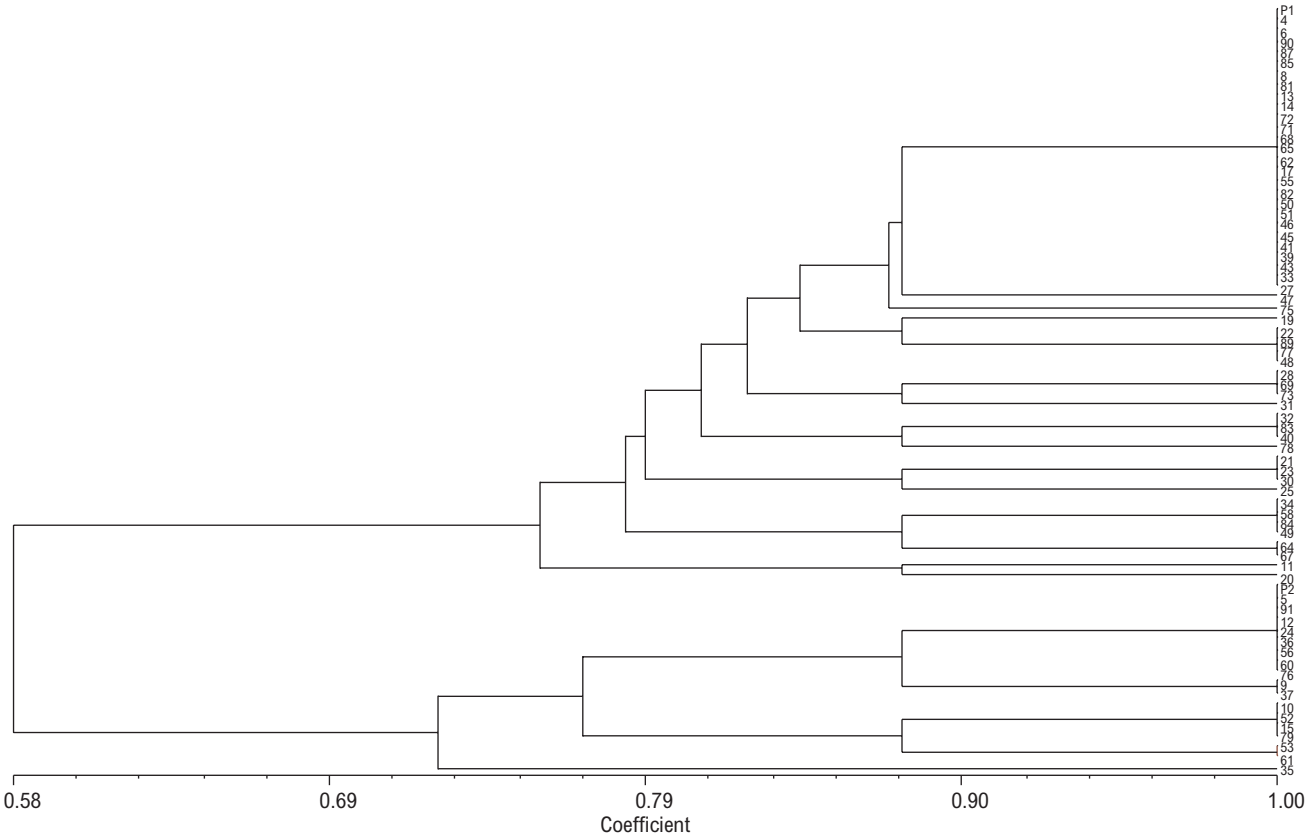

Fig 2 Dendrogram wheat RILs with parental genotypes based on SSR diversity data

primers (Xgwm 538, Xgwm 337, Xgwm 637, and Xgwm 6) with KB resistance, they were further screened on rest of the population for confirming the results. Silver stained gel showed the similar results as obtained on the selected RILs. The band from SSR markers having association with Karnal bunt resistance was present in most of the resistant lines, however the same was absent in susceptible lines. The relationship among the RILs of H 567.71 × WH 542 were further determined with UPGMA cluster analysis (Fig 2). Dendrogram perfectly separated the RILs and parental genotypes into two main groups at about 0.58 genetic similarity. Major group I consisted of large number of lines most of these were resistant lines clustered with resistant parent H 567.71, whereas major group II consisted of less number of lines which were susceptible and clustered with susceptible parent WH 542. So the results validated the association of these primers with KB resistance and confirmed the earlier findings.

Our results together with other reports, suggested that several genes for Karnal bunt resistance are present in diverse wheat lines. These have the potential for pyramiding in desirable genotypes using marker-assisted selection to obtain an appropriate level of resistance to KB in wheat. The primers identified in different populations and validated using our population may have universal application if tested on some more genotypes. Hence, these primers can be used to differentiate resistant and susceptible lines in early segregating generations and can be used for marker assisted selection (MAS) in a diverse group of wheat genotypes.

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