EVALUATION AND MOLECULAR MARKER STUDIES FOR KARNAL BUNT RESISTANCE IN WHEAT

BY

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IN

GENETICS

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2004

To My Beloved Parents

CERTIFICATE – I

This is to certify that this dissertation entitled **"Evaluation and molecular marker studies for karnal bunt resistance in wheat"** submitted for the degree of **Doctor of Philosophy** in the subject of **Genetics** of the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Mr. Mukesh Kumar** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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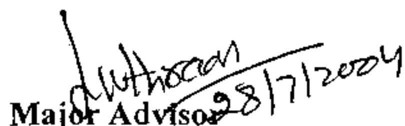

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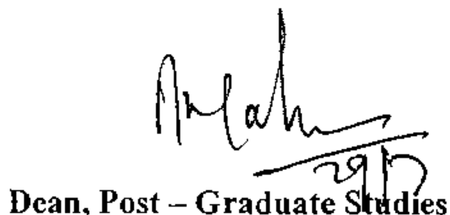

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Mukesh Kumar
6/6/04

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1

INTRODUCTION

Wheat (*Triticum aestivum* L. em thell) occupies a premier place among cereals in the world. It belongs to the sub-tribe Triticinae and the tribe triticeae (= Hordeae) of the family Poaceae. Its tolerance of a wide range of growing conditions has ensured successful production and global exploitation. Thus approximately one sixth of arable land in world is cultivated with wheat. India is the second largest wheat growing country of the world (DWR, 1996). More than half of its population depends on wheat, as it provides major source of energy, protein and dietary fibre in human nutrition. In India wheat has immensely contributed to the advent and flourishment of green revolution transforming India from famine prone to food surplus country.

Some diseases of wheat which were considered to be of minor importance have assumed serious proportions now. One such disease is the karnal bunt caused by the fungus *Neovossia indica* (Mitra) Mundkur. It was first recorded by Manoranjan Mitra in 1930 from the Botanical Research Station, Karnal (Haryana), India (Mitra, 1931). *Neovossia indica* is a non-systemic pathogen and

attacks the susceptible cultivars of wheat via floral infection by seed, air or soil borne sporadia, resulting in partial replacement of kernels with masses of teliospores. These impart a characteristic rotten fish like foul odour to the grain due to trimethylamine and reduce its fitness for consumption. Thus deterioration in the quality characteristics such as appearance, taste and flavour occurs if wheat grain with five per cent infection or more is used (Sekhon *et al.*, 1980; Singh and Bedi, 1985). In India, it has been reported that the disease is prevalent to varying extent in northern states such as Punjab, Haryana, Delhi, Uttar Pradesh, Bihar, Jammu and Kashmir, lower Himachal Pradesh, Madhya Pradesh and Rajasthan. It has also been reported from southern Nepal, Pakistan, Lebanon, Sweden, Syria, Turkey, Afghanistau, Iran, Mexico and USA (Gill *et* al., 1993; Ykema *et al.*, 1996; Torabi *et al.*, 1996; Crous *et al.*, 2001; Haq *et al.*, 2002). To avoid introduction and spread of disease, legal restrictions on seed movement have been enforced by many countries like United States, China, Canada and Russia (Babadoost, 2000). So apart from causing direct quantitative and qualitative losses in terms of yield, seed vigour, quality deterioration and toxicity, huge expenditure is also incurred in meeting the international quarantine measures and non-acceptance of such produce by other countries. Thus, screening and breeding programmes for resistance to this disease has become integral part of research in India and many other countries of the world. Karnal bunt is difficult to control by conventional methods such as by using seed

treatments, fungicides or by planting only certified seeds. Screening and manipulations of different wheat varieties under laboratory conditions using *in vitro* culture techniques may also speed up the resistance breeding programme (Tandon *et al.*, 1999; Kumar *et al.*, 2002, 2003). In recent years, significant progress has been made towards the use of molecular approaches in plant breeding. The techniques of molecular mapping using different types of molecular markers have allowed identification of closely linked traits of economic importance (Caetano-Anolles and Gresshoff, 1997). The molecular markers, so developed may be used for improving the efficiency of conventional plant breeding by facilitating indirect selection for the traits of interest because, these markers are not influenced by the environment and can be scored at all stages of plant growth. This saves time, resources and energy that are needed not only for raising large segregating populations for several generations, but also for estimating the parameters used for selection. It also allows pyramiding of genes like those for disease resistance, which was considered difficult through the use of conventional methods of plant breeding. In addition to these applications, DNA markers can also be used for germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenetic analysis, study of genetic diversity etc. (Rafalaski *et al.*, 1996).

Molecular markers can be broadly classified in the following three groups (Gupta *et al.*, 1999): (i) hybridization based DNA markers- restrictions fragment

length polymorphisms (RFLPs) and oliogonucleotide fingerprinting (in-gel hybridization), (ii) PCR based DNA markers such as random amplified polymorphic DNAs (RAPDs), sequence tagged microsatellite sites (STMS), that are also described as simple sequence repeats (SSRs) or short tandem repeats (STRs), inter-simple sequence repeat amplification (ISSR), amplicon length polymorphism (ALPs), allele specific PCR (AS-PCR), and DNA amplification fingerprinting (DAF), and (iii) sequencing based DNA markers like single nucleotide polymorphisms (SNPs). It has been shown that PCR based molecular markers should be preferred over hybridization based markers like RFLPs (Gupta *et al.*, 1999). Among different type of molecular markers, microsatellite has become the markers of choice in several crops because the other markers detect only a low level of polymorphism (Penner *et al.*, 1995; Paull *et al.*, 1998). In contrast, microsatellites are more abundant, ubiquitous and hypervariable with high polymorphic information content (PIC) (Tautz and Renz, 1984; Gupta *et al.*, 1996). Due to these properties, the microsatellites have recently been used in wheat for mapping (Roder *et al.*, 1998 a, b; Stephenson *et al.*, 1998), genetic diversity studies (Roder *et al.*, 1995; Plaschke *et al.*, 1995; Bohn *et al.*, 1999; Prasad *et al.*, 2000) and gene tagging (Korzun *et al.*, 1997a, b, 1998a; Garry *et al.*,1997; Fahima *et al* ., 1998; Peng *et al.*,1999; Vasu *et al.*, 2000; Khletskina *et al.*, 2002).

Keeping in view the importance of microsatellite markers in disease resistance study, the present investigation was undertaken with the following objectives:

1. Evaluation of recombinant inbred lines for karnal bunt resistance.

2. To study DNA polymorphism among RILs populations using microsatellite markers.

2

REVIEW OF LITERATURE

Karnal bunt [*Neovossia indica* (Mitra) Mundkur], disease of wheat infects the plant at boot leaf stage. The pathogen penetrates the individual floret, enters the embryo end of the kernel and proceeds along the crease. Infected portions of the kernels are replaced with masses of dark, powdery, fishy smelling and fungal teliospores (Joshi *et al.*, 1980). Breeding for karnal bunt resistant cultivars requires a reliable method of selecting plants containing a resistance gene. Greenhouse screening is carried out by creating artificial epiphytotic conditions at boot leaf stage but it is time consuming and labour intensive. An average 10 per cent error rate is typical for the greenhouse screening method. It is highly desirable to employ a screening technique that is based on molecular markers linked to the resistance genes (Ma *et al.*, 1996). Aside from overcoming the problems associated with phenotypic screening, marker-assisted selection (MAS) would enable breeders to combine two or more karnal bunt resistance genes efficiently.

DNA sequences with short repeat motifs (2 to 6 bp) are described either as microsatellites (Litt and Luty, 1989), or as simple sequence repeats (SSRs; Hearne *et al.*, 1992), or else as short tandem repeats (STRs; Edward *et al.*, 1991). The length of microsatellite at a specific locus may vary due to variation in number of repeats at the locus. This locus specific length variation can be detected through PCR amplification using primers designed from the flanking sequences. Consequently, a locus thus identified is described as sequence-tagged microsatellite site (STMS). The co-dominant and multi-allelic nature of STMS loci, detected on the basis of SSR length polymorphism makes them highly informative (Morgante and Oliveri, 1993; Cregan *et al.*, 1994). Since these STMS markers are locus specific (unlike RAPDs, which are random loci), the problem of reproducibility is also largely overcome in this class of molecular markers (Ellsworth *et al.*, 1993). For these reasons, STMS markers have been considered as ideal markers for genetic analysis and marker assisted selection (Gupta *et al.*, 1996; Powell *et al.*, 1996). In bread wheat, a microsatellite map with ~ 300 microsatellite loci has already been published (Roder *et al.*, 1998b). However, related investigations are being reviewed hereafter.

2.1 Prevalence of Karnal Bunt

Karnal bunt disease of wheat caused by *Neovossia indica,* Mitra (Mundkur, 1940) was regarded as minor disease in India until 1968. However, the disease continued to assume a significant importance during 1973, 1975, 1976,

1978, 1979, 1981 and 1982, when its epiphytotics were reported from various parts of the country (Agarwal *et al.*, 1976; Bedi, 1980; Singh *et al.*, 1985; Gill, 1987).

Singh *et al.* (1985) reported gradual spread of the disease to the new areas of West Bengal, Gujarat, northern parts of Bihar and Madhya Pradesh. Aujla *et al,* (1986) reported high incidence of this disease in Punjab during 1973, 1979, 1981 and 1982 due to continuous cultivation of susceptible varieties, agronomical practices and meteorological factors like temperature below 25 ^{0}C and humidity 67-68 per cent. Singh *et al.* (1986) recorded that the disease was present in 9, 34 and 17 per cent samples in Haryana during 1974-75, 1977-78 and 1978-79, respectively, whereas about 60 per cent sample found infected in 1982. Joshi (1988) surveyed in India and reported that the disease was prevalent to varying extents in Punjab, Haryana, Union Territory of Delhi, Eastern and Western parts of Uttar Pradesh, North Bihar and Himachal Pradesh. Gupta *et al.* (1990) reported the overall percentage of infection from 1983-84 to 1987-88 ranged from 0.1 to 0.63 per cent in different cultivars in Haryana. Singh *et al.* (1993a) evaluated 2924 exotic strains of wheat and triticale for resistance to *N. indica* during 1987-90. On the basis of percentage of infected grains, *T. aestivum*, *T. durum*, *T. dicoccum* remained resistant during three years trial. Beniwal *et al.* (1997) carried out survey for 7 crop seasons (1988-89 to 1994-95) and reported the higher incidence of karnal bunt in the humid zone districts than the dry zone districts of Haryana.

Varshney *et al.* (1999) conducted survey in Uttar Pradesh and Haryana during 1990, 1992 and 1993 and reported the incidence of disease ranging from 0.05 to 1.3 per cent in the samples collected from both the states.

Rewal *et al.* (2001) conducted survey in 3 districts, viz. Sangrur, Hoshiarpur and Gurdaspur, in Punjab, India during 1997-99. The incidence of karnal bunt increased marginally during 1998-99 as compared during the year 1996-97. Singh *et al.* (2001) collected wheat grain samples from different markets in Punjab, during the period of maximum wheat production, i.e. from April to mid-May 1999 to determine the occurrence of Karnal bunt. From a total of 552 samples collected, 42.7% samples were affected by Karnal bunt. The maximum bunted samples were found in Faridkot district where the prevalence of the disease was 92.8%, whereas the minimum prevalence of bunt was 3.4% in Amritsar district. The overall disease incidence in Punjab was 0.24%.

Apart from India disease has also been reported from various countries like Nepal (Nath *et al.*, 1981), Iraq (CMI, 1974), Mexico (Duran, 1972), Lebnan, Syria, Turkey (Gill *et al.*, 1993) and Sweden.

Bhutta and Ahmed (1994) reported 58.57 per cent of wheat infected by karnal bunt during 1986-87 and 1991-92 in Pakistan. Ykema *et al.* (1996) reported karnal bunt of wheat in United States, California, New Maxico and in Texas also. On March 8, 1996, the U.S. Department of Agriculture and the Arizona Department of Agriculture jointly announced that Karnal bunt had been found in

Arizona (Release No. 0115.96, Ag News FAX; 42) and on March 21, 1996, the U.S. Secretary of Agriculture implemented an "extraordinary emergency" action to deal with the disease outbreak (*The Washington Post*, Page A23, March 22, 1996). Smiley (1997) reported that weather condition in Pacific North- West, USA during the past 4 decades appeared favorable for infection. Murray and Brennan (1998) reported that in Australia, weather conditions at anthesis were suitable for the infection of susceptible wheat crops by *Tilletia indica* and karnal bunt could develop at many locations in Australia. Bhutta *et al.* (1999) tested 730 wheat seed samples to assess the incidence of karnal bunt (*Tilletia indica*) using the dry inspection method from 1993/94 to 1996/97. High infection percentage (3%) of karnal bunt in various seed lots was found in Central Punjab and northwest areas of Pakistan. Southern parts of the country were found free from 1994/95 to 1996/97. Rong (2000) reported the incidence of karnal bunt in South Africa.

Crous *et al.* (2001) observed that seeds harvested from wheat (*Triticum aestivum*) cultivars SST 876 and SST 825 in Northern Cape Province, South Africa contained a substantial amount of partially bunted kernels with black masses of teliospores and degraded endosperm. Based on kernel appearance, infected grain odour, teliospore morphology and germination characteristics, the pathogen was identified as *Tilletia indica*, the cause of Karnal bunt. Molecular verification via PCR and internal transcribed spacer rDNA sequencing confirmed the isolates as *T. indica*. Haq *et al.* (2002) conducted a survey in 78 localities in

Pakistan to study the prevalence of karnal bunt (caused by *Tilletia indica*) in wheat-producing areas. The highest disease incidence was observed on cv. WL-711 (46.0%) grown in Hathion. High disease incidence was also recorded for Pak-81 (0.47-6.32%) and Pirsabak-85 (0.41-5.57%) and for the districts of Mangora (2.53-23.42%), Mardan (0.41-46.00%), Malakand (6.32%), and Swabi (0.54-5.85%). Wheat-growing areas in Manshera (0.42-1.04%), Peshawar (0.64-2.36%), Abbottabad (0.00-1.37%), and Attock (0.00-1.25%) had low to medium disease incidence.

2.2 Adult Plant Inoculations

Different methods of inoculation have been tried by several workers from time to time to create artificial epiphytotic conditions such as inoculations with syringe, spraying of inoculum, cotton wool inoculation technique, inoculation with Moor's method, dropper, eat dip (drenching of ears) and Go-Go method (Moore, 1936; Chona *et al.*, 1961; Aujla *et al.,* 1982, Aujla *et al.,*1983; Krishna and Singh, 1983; Warham, 1984, 1990; Warham and Cashion, 1984; Royer and Rytter, 1985). The propagules of the pathogen viz., teliospores, germinated teliospores, filiform or allantoid sporidia and mycelial bits were used for inoculations at different concentrations to find out the most vulnerable stage. Wheat plants were inoculated from immature to fully ripened reproductive stages. Syringe inoculations using secondary sporidia at boot leaf stage of the plant was observed to be most

appropriate for disease (Royer and Rytter, 1985; Dhaliwal and Singh, 1988; Singh *et al.* 1988; Dhaliwal and Singh, 1989; Aujla *et al.* 1990; Warham, 1990).

Smiley (1997) reported that inoculation date, crop growth stage and different isolates of *N. indica* affected the development of disease. Teliospore, sporidial germination and infection were highly dependent on climatic conditions. Kaur *et al.* (2000) studied the effects of diurnal temperature and relative humidity variations on disease development by daily inoculating 15 ears of WL 711 at 09.00 to 18.00 h from 9 February to 20 March. Inoculations from 17 January to 13 March produced karnal bunt infected grains. The spike infection ranged from 15-95 and 10-90% in WL 711 and WL 1562, respectively. Incidence of infection was higher when inoculations were performed in the morning than in the afternoon. The highest percentage of infected grains (36-75 and 10-30% in WL 711 and WL 1562, respectively) were detected at 15-16°C. Higher disease incidence in both varieties was favoured by 15-16°C for at least 5-15 days with 65-76% relative humidity, which declined as the temperature or relative humidity increased or decreased.

2.3 Inheritance of Resistance to Karnal Bunt

In vivo screening for resistance and susceptible varieties has been conducted by several workers. Singh *et al.*, (1993a) studied inheritance of resistance to *N. indica* in cross of resistant *Triticum aestivum* line HD 29 with susceptible cv. WL 711 and reported that resistance was controlled by 2 recessive

genes. Inheritance studies conducted at the International Maize and Wheat Improvement Centre (CIMMYT), Mexico (Fuentes-Devila *et al.*, 1995; Singh *et al.*, 1995a; 1995b), indicated that one to six major genes control resistance to karnal bunt. Fuentes-Devila *et al.* (1995) studied the mode of inheritance and allelic relationship among genes conferring resistance to karnal bunt in seven bread wheat genotypes (six resistant and one susceptible) and revealed that two partially recessive genes conferred the resistance to karnal bunt in variety pigeon, whereas partially dominant genes were present in the other genotypes. Resistance was incomplete and stable.

Singh *et al.* (1996b) studied the inheritance pattern of karnal bunt resistance in some wheat crosses and revealed that resistance was dominant to susceptibility and was primarily controlled by a few major gene(s) along with some minor genes. Moreover, the genes governing resistance were not the same in the resistant parents. Singh *et al* .(1999) conducted extensive evaluation of advanced generation (F_8) recombinant inbred lines (RILs) and reported that HD 29 possessed three major genes for resistance to isolate Ni 7 and 2 genes for resistance to Ni 8. One of the two genes containing resistance to Ni 8 was common with one of the genes conferring resistance to Ni 7. Bag *et al.* (1999) conducted studies to determine the nature and number of genes for resistance to karnal bunt and reported that F_1 hybrids from crosses between resistant and susceptible parents were susceptible to the bunt. Segregation in F_3 families of the cross

HD 29 X WL 711 and its reciprocal, confirmed the findings in the F_1 and F_2 that susceptibility was dominant over resistance and that resistance was conferred by a single recessive gene in that cross.

2.4 Microsatellites

2.4.1 Microsatellites in the Nuclear Genome

Microsatellites are abundant and occur frequently and randomly in all eukaryotic genomes (Gupta *et al.,* 1996). The frequency of a specific microsatellite motif varies among different organisms (Wang *et al.*, 1994; Weising *et al.*, 1998). Plant genomes varying in size from 145 Mb in *Arabidopsis* to 18, 000 Mb in *Triticum aestivum* have been shown to contain on an average 10 fold fewer microsatellites than human genome with a genome size of 2800 Mb (Powell *et al.*, 1996). $(CA)_n$ motif is one of the most frequent occurring microsatellites in humans and several other mammals (Beckmann and Weber, 1992), but is comparatively less frequent in plants (Lagercrantz *et al.*, 1993). The frequency and occurrence of the two most common dinucleotide repeats $\{(AC)_n$ and $(GA)_n\}$ has been calculated in different plant species, which suggests that $(GA)_n$ are relatively more frequent (Table 1.). Trinucleotide and tetranucleotide repeats are also found in plant genomes, the most frequent of them being $(AAG)_n$ and $(AAT)_n$ (Gupta *et al.*, 1994). On the basis of published DNA sequences of 54 plant species, Wang *et al.* (1994) observed that the $(AT)_n$ sequences are the most abundant followed by $(A)_n$, $(AG)_n$, $(AAT)_n$, $(AAC)_n$, $(AGC)_n$, $(AAG)_n$,

(AATT) $_n$, (AAAT) $_n$, and (AC) $_n$. On an average, one simple sequence repeat was observed every 64.6 Kb in monocots and every 21.2 Kb in dicots.

Table 1: Frequency and distribution of two microsatellites in plant genomes

Plant material	$(AC)_n$		$(GA)_n$		References
	Total no.	Average distance (Kb) between two SSRs	Total no.	Average distance (Kb) between two SSRs	
Rice (*Oryza sativa*)	1000	450	2000	225	Wu and Tanksley, 1993
	1230	365	1360	330	Panaud *et al.*, 1995
Wheat (*Triticum aestivum*)	23000	704	36000	440	Roder *et al.*, 1995
		292		212	Ma *et al.*, 1996
Maize (*Zea mays*)	12000	110-1100	8000	168-710	Condit and Hubbell, 1991
Arabidopsis	350	430	615	244	Bell and Ecker, 1994
Rapeseed (*Brassica napus*)	15000	300	-	90	Lagercrantz *et al.*, 1993
		440		100	Kresovich *et al.*, 1995
Tobacco (*Nicotiana tabacum*)	-	150	-	170	Lagercrantz *et al.*, 1993

2.4.2 Organization of Microsatellite

Genetic mapping was successfully completed in several crops including rice, barley, maize, soyabean etc. These maps revealed that the mapped micosatellite loci are dispersed and not clustered. For instance, Roder *et al.* (1998 b) showed random distribution of microsatellites in wheat throughout the whole genome except in the centromeric regions of the chromosome 2A, 3A, 3B, 4B, 5B and 6B. More recently in rice, mapping of 312 microsatellite loci also

showed random distribution of microsatellites throughout the genome (Temynkh *et al.*, 2000). In contrast to above reports, there are some reports where genetic mapping showed clustering of microsatellite loci in certain regions. For instance, in tomato, 19 microsatellite markers, that were mapped, though covered all chromosomes, were not uniformly distributed along the length of these chromosomes; these were clustered near centromeres (Areshchenkova and Ganal. 1999). In Soyabean microsatellite map, where 606 SSR loci were mapped, portions of many linkage groups were found to contain clusters of SSR and RFLP loci (Cregan *et al.*, 1999)

Beside the above two type of reports, a third scenario was reported in chickpea, where genetic mapping of 120 STMS markers, covering 613 cM on 11linkage group, showed clustering as well as dispersed distribution of microsatellite loci (Winter *et al.*, 1999).

2.4.3 Strategies for the Detection of DNA Polymorphism using Microsatellites

A number of strategies (both hybridization and PCR based) have been designed to exploit microsatellite sequences for the study of DNA polymorphism in eukaryotes.

2.4.3.1 A Hybridization based approach

The very first effort, which was made use of end labelled oligonucleotides complementary to microsatellites for in-gel hybridization with digested and electrophoresed genomic DNA, revealed multilocus RFLP

fingerprinting in humans (Ali *et al.*, 1986). This technique of in-gel hybridization offers several advantages and has been increasingly utilized to detect hypervariable DNA segments in a variety of plant materials (Weising and Kahl, 1997). The fragments that hybridize with synthetic oligonucleotides range in size from a few hundred base pairs to as many as 8-10 kb. Rarely these fragments may reach a length of more than 20 kb (Weising *et al.*, 1998). It has also been shown that sometimes, more than one type of SSRs are also available within the same restriction fragment. The technique of oligonucleotide fingerprinting using in-gel hybridization gave high level of polymorphism between related genotypes and proved useful in diverse areas of genome analysis including paternity, genotype identification and population genetics (Weising *et al.*, 1995).

2.4.3.2 PCR Based Approaches

Microsatellite polymorphisms are detected by PCR either at individual loci using locus –specific primer sequences flanking the microsatellites or by using as primers the synthetic oligonucleotides representing microsatellite sequences. These assays typically carry out high information content and have been used for a variety of purposes including genetic mapping. gene tagging and genetic diversity studies.

The sequences flanking the microsatellites in the genome are believed to be conserved within a particular species, across species within a genus and rarely even across related genera. These flanking sequences, therefore, can be used for

designing primers to amplify individual microsatellite loci and the technique is described as sequence tagged microsatellite site (STMS) anlysis (Beckmann and Soller, 1990).

Since STMS analysis requires cloning and sequencing, it is very expensive and labour-intensive. In many situations, differences in allele size are also difficult to resolve on agarose gels with ethidium bromide staining (Bell ad Ecker, 1994; Becker and Heun. 1995), so that, high resolutions are achieved through the use of polyacrylamide gel electrophoresis (PAGE) in combination with either ethidium bromide (Scrimshaw, 1992) or silver staining (Klinkicht and Tautz, 1992; Tegelstrom, 1992). Both denaturing and non-denaturing PAGE were used to resolve size differences between alleles (Gay *et al.*, 1999; Brondani *et al.*, 1999). Consequently, despite their being cost/ labour intensive, STMS markers have been used extensively not only for mapping SSR loci in many crop plants, but also for tagging certain genes.

Polymorphism at a given microsatellite locus in a species is due to variation in the length of SSR, and is believed to originate *in vivo* by the polymerase slippage during DNA replication (Levinson and Gutman, 1987) leading to increase and decreases in the number of repeats. Null alleles have also been reported at microsatellite loci and refer to alleles, where no PCR products are obtained using locus specific primers. Null alleles have been reported in many plant species including wheat, where in two separate studies, 13% (Plashchke *et al.*, 1995) and

10% of microsatellite loci (Prasad *et al.*, 2000) gave no PCR products. This showed that although the null alleles generally occur at a relatively low frequency, they occur at a frequency which can not be ignored.

2.4.4 Genome Mapping and Microsatellite Maps

STMS has been extensively used for genome mapping both in animals and plants (Weising *et al.*, 1998). A linkage map of human genome based on the segregation analysis of 814 $(CA)_n$ microsatellite loci was initially constructed which eventually led to the development of saturated map with 5264 microsatellites (Dib *et al.*, 1996). However in plants, mapping with STMS markers did not reach this level of resolution so far, although the very first attempt to map STMS loci in any plant species, was made as early as 1992, in rice using $(GGC)_n$ (Zhao and Kochert, 1992; 1993). This was followed by several attempts to assign STMS loci to linkage groups in a variety of plant genome (Table 2.). Mapping of the whole genome using STMS loci is also currently in progress in many crops viz. *Brassica*, Cucumber and Maize. Microsatellite loci, other than STMS markers, have also been used for mapping in different plant species. In bread wheat, two microsatellite maps, one with 279 loci (Roder *et al.*, 1998b) and another with 50 loci (Stephenson *et al.*, 1998) have been prepared. In tetraploid wheat also, 14 microsatellite loci were mapped on chromosome 5A and 5B, which carry genes for protein content, vernalization response and resistance to Hessian fly.

Table 2: Microsatellite markers for genome mapping in plant systems

Plant material	No. of mapped microsatellite loci	References
Bread wheat	11 *cnl*	Sorrells, 1997(Leroy, 1997a)
(2n=42)	1 *psp*	Devos,1997 (Leroy, 1997a)
	8	INRA, 1997(Leroy, 1997b)
	279 *gwm*	Roder *et al*., 1998b
	50	Stephenson *et al*., 1998
	50 *wmc*	IWMMN, 2000(Leoroy, 2000)
Durum wheat	14 *gwm*	Korzum *et al*., 1998b
(2n=28)	79	Korzum *et al*., 1999
Einkorn wheat (2n=14)	9 (ISSR)	Kojima *et al*., 1998
Rice	1	Zhao and Kochert, 1992;1993
(2n= 24)	10	Wu and Tanskley, 1993
	10	Yang *et al*., 1994
	56	Akagi *et al*., 1996
	86	Coh *et al*., 1997
	110	Mc Couch *et al*.,1997
	121	Chen *et al*., 1997
	300	Temnykh *et al*., 1998
	312	Temnykh *et al*., 2000
Maize	6	Senior and Heun, 1993
(2n=20)	42	Senior *et al*., 1996
	18	Tarmino and Tingley, 1996
Barley (2n=14)	60	Liu *et al*., 1996
Soyabean	7	Morgante *et al*., 1994
(2n=4)	40	Akkaya *et al*., 1995
	540	Cregan *et al*., 1999
Chickpea (2n=16)	120	Winter *et al*., 1999
Tomato	28	Arens *et al*., 1995
(2n=24)	2	Broun and Tanskley, 1996
	19	Areshchenkova and Ganal, 1999

Utilizing ITMI population (150 RILs from Opata 85 x W7984), an integrated map of wheat genome became available to which 279 *gwm* microsatellite loci were added (Roder *et al.*, 1998b). Later, Roder *et al.* (1999) assigned a set of another 70 microsatellite loci to specific chromosomes using nulli-tetrasomic lines. An additional ~ 50 microsatellite loci have been mapped through the efforts of IWMMN (Leroy, 2000).

2.4.5 Gene Tagging and Marker Assisted Selection (MAS)

The first example of a gene linked to microsatellite $(AT)_n$ was a soyabean mosaic virus resistance gene (*Rsv*) (Yu *et al.*, 1994; 1996). Several other resistance genes including those for resistance to peanut mottle virus (*Rpv*), Phytophthora (*Rps3*) and Javanese root knot nematode, were found to be clustered in the same region of the soyabean genome where this $(AT)_n$ microsatellite was found to be associated with *Rsv*. Microsatellite markers, associated with soyabean cyst nematode (SCN) resistance locus, sclerotinia stem root resistance, were also reported (see Table 3. for references). Similarly in wheat. WMS291 marker was closely linked (5.4 cM) to the dwarfing gene *Rht12* (Korzun *et al.*, 1997b) and the marker was closely linked (0.6 cM) to *Rht8* (Korzun *et al.*, 1998a). The stripe rust resistance gene (*Yr15*) has been tagged using WMS33 marker at a distance of 4.5 cM (Fahima *et al.*, 1997, 1998) and the linkage of a WMS marker gene for flour colour and milling yield (Garry *et al.*, 1997) has also been shown. In durum wheat, some microsatellites have been mapped on two regions of 5AL each

carrying a QTL, for high protein content (*Xgwm 186* and *Xgwm693b*) and for heading time (*Xgwm126* and *Xgwm 291*) (Korzun *et al.*, 1999). Vasu *et al.* (2000) used the A-genome specific microsatellite markers for tagging karnal bunt resistance gene (s), microsatellite loci *gwm382, gwm369, gwm637, gwm156* and *gwm617* mapped on 2AS, 3AS, 4AL, 5AL, and 6AL chromosomes, respectively were found to be associated with karnal bunt resistance in different resistant derivatives.

Liu *et al.* (2001) reported that SSR markers WMS67 and WMS213 mapped on 5BL in wheat genome were linked to salt tolerance gene(s). Genetic distance of the two markers to the critical gene(s) is 13.9 cM, 31.0 cM, respectively. Rodriguez and Gustafson (2001) identified the AltBH gene to a 5.9 cM interval between markers *Xgdm125* and *Xpsr914.* Liu *et al.* (2002) identified that two microsatellite markers *Xgwm106* and *Xgwm337* are linked in coupling phase with Dn4 on the short arm of wheat chromosome 1D at 7.4 cM and 12.9 cM, respectively. Two another markers *Xgwm44* and *Xgwm111* are linked in coupling phase with Dn6 near the centromere on the short arm of wheat chromosome 7D at 14.6 cM and 3.0 cM, respectively. Khletskina *et al.* (2002) used SSR markers for mapping red coleoptile colour in wheat (*Rc*1, *Rc*2, *Rc*3). All the three genes map at about 15 to 20 cM distally from the centromere of chromosomes 7AS, 7BS and &7DS, respectively. Buerstmayer *et al.* (2002) found that three genomic regions were significantly associated with Fussarium head blight resistance. The most

prominent effect was detected on the short arm of chromosome 3B and further QTL was located on chromosome 5A and 1B. The QTL regions on 3B and 5A were tagged with SSR markers, the QTL 1B was found to be associated with high molecular weight glutenin locus. Singh *et al.* (2003) mapped markers on chromosomes 2A, 4B and 7B. The genomic region of the largest effect, identified on the long arm of chromosome 4B, reduced karnal bunt disease by half and accounted for up to 25% of the phenotypic variation for karnal bunt resistance. A closely linked SSR marker *gwm 538* may be useful in marker-assisted selection for karnal bunt resistance in wheat.

Table3: Microsatellites for gene tagging and marker assisted selection in crop Plants

Crop	Gene/ QTL	No. of Microsatellite	References
Bread wheat	dwarfing (*Rht 12*)	3	Korzun *et al.*, 1997a
	dwarfing (*Rht 8*)	1	Korzun *et al.*, 1998a
	stripe rust resistance (*Yr 15*)	1	Fahima *et al.*, 1998
	flour colour and milling yield	1	Garry *et al.*, 1997
	salt tolerance	2	Liu *et al.*, 2001
	RWA resistance	4	Liu *et al.*, 2002
	red coleoptile colour (*Rc* 1,2,3)	3	Khletskina *et al.*, 2002
	Karnal bunt resistance	3	Singh *et al.*, 2003
Durum wheat	Protein content QTL	2	Korzun *et al.*, 1998b
	Heading time QTL	2	Korzun *et al.*, 1998b
Rice	*Waxy* gene	1	Bligh *et al.*, 1995
	Yield QTL	2	Xiao *et al.*, 1996
	Bacterial leaf blight resistance	3	Blair & McCouch, 1997

2.4.6 Microsatellite in Variety Identification

In a study on genetic diversity, Prasad *et al.* (2000) found that a set of 12 microsatellite markers could distinguish 48 to 55 elite genotypes. In another study in bread wheat, 42 microsatellite markers (WMS), one for each of the 42 chromosome arms of wheat genome, clearly distinguished varieties bred and/ or grown in three different agroecological areas namely Hungry, Austria and Germany (Lelley and Stachel, 1998). Similarly combinations of four microsatellite markers discriminated 24 genotypes in barley and16 genotypes in tomato (Bredemeijer *et al.*, 1998). In soyabean, most of the 96 different genotypes investigated could be discriminated by a set of 7 loci exhibiting 11-26 alleles per locus (Rongwen *et al.*, 1995).

For variety identification, STMS markers are generally superior to RAPDs, MP-PCR and oligonucleotide fingerprinting because genotyping using STMS alleles can be done unambiguously and data can easily be distributed and reproduced among different laboratories (Weising *et al.*, 1998).

2.4.7 Microsatellites for Genetic Diversity Studies

Microsatellites, have also been considered to be the markers of choice for assessment of genetic diversity among cultivars and their wild relatives although a variety of other molecular markers have also been used for this purpose (Karp *et al.*, 1998). Dograr *et* al. (2000) used SSR for differentiating varieties of winter type durum wheat and found that the genotypes were all distinguished with

each other, with the number of alleles ranging from 5 to 13. The lowest and the highest polymorphic information content were observed to be 0.609 and 0.872, respectively. Three markers alone *WMS6, WMS30* and *WMS120* could distinguish all 16 genotypes. Hayden *et al.* (2001) used selective amplified microsatellite (SAM) to develop informative SSR markers to assist in the construction of an intraspecific wheat map. Three markers were developed for under-represented region in the genetic map and 7 for unassigned linkage groups. Garg *et al.* (2001) reported that the highest number of polymorphic band per assay unit was observed for AFLPs (39.7), followed by RAPDs (12.7) and SSRs (4.0) but polymorphic information content (PIC) range was the highest for SSRs (0.13-0.86). Based on estimation of genetic similarities, more realistic dendrogram was obtained from SSR compared to that obtained from AFLP or RAPD data. Ahmed (2002) analysed 13 wheat genotypes with 43 SSRs. A total of 156 allelic variants were detected at 43 SSR loci, ranging from 2 to 8 per locus with an average of 3.6. The polymorphic information content values of the loci ranged from 0.10 (Xgwm264) to 0.89 (Xgwm471 and Xgwm577). Genetic similarities ranged from 30.1 ('Era' and 'Klasic') to 90.1 ('Neepawa and 'Thatcher') between genotypes.

2.5 Random Amplified Polymorphic DNA (RAPD) Markers

After introduction of polymerase chain reaction (PCR) numerous alternative DNA marker systems have been developed which are comparatively simple. Random amplified polymorphic DNA (RAPD) is also a PCR-based

molecular marker technique independently developed by Welsh and McClleland (1990) and Williams *et al.* (1990). RAPD is simple, fast, requires a small quantity of template DNA and involves no radioactivity or southern blotting and hybridization. It neither requires previous knowledge of any genomic PCR or tedious procedure such as RFLP analysis. Welsh and McClleland (1990) have demonstrated RAPD as useful genetic marker for variety of eukaryotic organism including human. fungi and plants. Devos and Gale (1992) optimized a RAPD protocol in wheat with respect to DNA concentration, Mg^{+2} concentration. polymerase concentration and denaturing temperature and concluded that though RAPD was a sensitive process, it finds many applications in the analysis of genotypes. Vierling and Nguyen (1992) studied polymorphism between 7 genotypes of *Triticum monococcum* and 6 genotypes of *T. urartu* using 60 random primers. RAPD data and unweighted pair group method with arithmetical average (UPGMA) was used in determining genetic relationships among genotypes by constructing a dandrogram. He *et al.* (1992) combined RAPD technique with a denaturing gel gradient electrophoresis system (DGGE) to explore DNA sequence polymorphism among different genotypes of common bread wheat. They concluded that denaturing gel gradient resulted in high level of reproducible polymorphism and to some extent controlled the consistency of PCR products by denaturing artificial hetroduplexes. Menkir *et al.* (1997) studied genetic diversity

and taxonomic relationship in 190 accessions of cultivated races of sorghum using RAPD markers.

Besides finding genetic distances and correlation in different genotypes of a crop, RAPD markers find many applications in mapping studies (Skroch *et al.*, 1992), map based cloning (Martin *et al.*, 1993) and marker assisted selection (MAS) (Kelly, 1995; Mohan *et al.*, 1997). Dweikat *et al.* (1994) has identified a DNA marker associated with Hessian fly resistance (*H9*) gene. The *H9* gene confers resistance against biotype L of the Hessian fly, the most virulent biotype. Hartl *et* al. (1995) tagged wheat powdery mildew resistance gene using RFLP markers. Later on, one RAPD marker was selected from a resistant segregating population. A RAPD marker for downy mildew resistance gene in sorghum has been identified by Gowda *et al.* (1995). Demeke *et* al. (1996) identified a DNA marker for Bt-10 common bunt resistance gene in wheat. A polymorphic marker was identified between resistant and susceptible near-isogenic lines using RAPD technique. Lili *et al.* (1996) identified a powdery mildew resistance gene (*Pm21*). from *Haynaldia villosa* and incorporated into wheat. One RAPD marker, specific to chromosome arm 6VS, OPH17-1900 could be used as a molecular marker for the detection of gene *Pm21* in breeding material. Procunier *et al.* (1997) developed marker linked to a race T10 loose smut resistance gene in wheat using F_6 single seed descent segregating population. The RAPD marker was converted to sequence characterized amplified region (SCAR) marker that represented a single

genetically modified locus in hexaploid wheat. Deweikat *et al.* (1997) while working with near-isogenic lines in wheat has identified 18 RAPD markers linked to the 11 Hessian fly resistance genes in wheat. Seven of these markers were identified by denaturing gradient gel electrophoresis and other by agarose gel electrophoresis. Hu *et al.* (1997) identified 3 RAPD markers closely linked to a gene for resistance to *B. graminis*, causes powdery mildew in wheat. The RAPD marker UBC638 $_{550}$ was converted to a sequence tagged site (STS). Chague *et al.* (1999) identified two RAPD markers linked to *Yr15* gene, which confers resistance to stripe rust in wheat, one in coupling ($UBC199_{700}$) and one in repulsion phase ($UBC212_{1200}$). The two closest markers were shown to be linked to *Yr15* within a distance of about 12 cM. Robert *et* al. (1999) found that RAPD marker OP-Y15 (580) was closely linked to the yellow rust resistance gene (*Yr17*) and converted sequence of this marker into a SCAR. This SCAR was linked at 0.8+/- 0.7 cM to the *Yr17* resistance gene. Cao *et al.* (2001) detected two RAPD markers, UBC521 (650) and RC37 (510) by bulk segregant analysis and located approximately 15.0 and 13.1 cM from the resistance gene *snbTM* (Septoria nodorum blotch) respectively. A SCAR marker was also developed based on the sequence of the RAPD marker UBC521 (650).

Dweikat *et al.* (2002) screened 2000 primers to identify DNA markers that are linked to gene H6 that confers resistance to biotype B of the insect. Six primers were found to be polymorphic associated with resistance to H6. Two of the

markers were tightly linked to the gene with no recombination observed, three were within 2.0 cM, and one was 11 cM from the gene. Three of six were successfully converted to sequence tagged site (STS) markers. Both RAPD and STS primers were used to screen for the presence or absence of the resistance gene in wheat varieties. Cherkuri *et al.* (2003) tagged leaf rust resistance gene *Lr19* with a RAPD marker S73 (728) at 6.4+/- 0.035 cM distance and it was converted to a sequence characterized amplified region (SCAR) marker. Sun *et al.* (2003) used RAPD to characterize the genetic diversity among 35 spring wheat cultivars with different levels of Fusarium resistance. Genetic similarity was ranged from 0.64 to 0.98. Three RAPD markers, H191000, F2500 and B12400 were associated with FHB-resistant genotypes.

3

MATERIALS AND METHODS

The present investigation was conducted in the Department of Genetics, CCS Haryana Agricultural University, Hisar.

3.1 MATERIAL

3.1.1 Plant material

Wheat genotypes *viz.* HD 29, WH 542 and their recombinant inbred lines (104) were used in the present investigations. The pedigree of these genotypes is given in Table 4.

Table 4: Pedigree of wheat genotypes used in the present study

Genotype	Pedigree	Remarks
HD 29	HD 2160- HD 1977 X HD 194- HD 1944 / HD 2136	Resistant to karnal bunt
WH 542	Jupateco / Blueja / Ures	Susceptible to karnal bunt

These were procured from Department of Plant Breeding, PAU, Ludhiana. Seeds of these genotypes and their recombinant inbred lines were sown in the net house of

Department of Genetics, CCS Haryana Agricultural University, Hisar (India) in the crop season of 2001-02 and 2002-03.

3.1.2 Pathogen

The culture of *Neovossia indica* was established from the infected grains and subcultured on Potato Dextrose Agar (PDA) slants.

3.1.3 Glassware

All the glassware used was of borosilicate quality and obtained from Borosil India or Perfit Glass Company.

3.1.4 Chemicals

Only pure chemicals and reagents of Molecular Biology/ Biotechnology/ analytical grade were used (Table 5).

3.1.5 Buffers and Solutions

All buffers and solutions were prepared from above chemicals/ reagents in autoclaved glassware and were sterlised as per need (Table 6.).

3.1.6 Microsatellite Markers

Forty six microsatellite markers representing the locations on all the wheat chromosomes were used for polymorphism studies. The wheat microsatellite primer pairs were custom synthesized based on Roder *et al.* (1998b) linkage map from Lifetech Technologies Inc. Table. 7 givesthe general information about these SSR markers. The map position of the SSR markers on wheat chromosomes are shown in Fig.1.

Table 5: List of chemicals and reagents used in the experiments

Chemical	Molecular formula	Company
Acetic acid glaciel	CH_3COOH	E-Merck
Acetone	CH_3COCH	E-Merck
Acrylamide	C_3H_5NO	Sigma
Agar- agar	-	Hi-Media
Agarose	Agarose 3- glycenohydrolase	Hi-Media
β- mercaptaethanol	C_2H_5OS	Qualigens
Boric acid	H_3BO_3	E- Merck
Bromophenol blue	$C_{19}H_9Br_4O_4SNa$	Hi-Media
Bisacryalamide	$(CH_2{=}CHCONH_2)\ CH_2$	Sigma
Chloroform	$CHCl_3$	E- Merck
Na_2-EDTA	$C_{10}H_{14}N_2O_8Na_2.2H_2o$	E- Merck
Ethanol	C_2H_5OH	E- Merck
Ethidium bromide	$C_{21}H_{20}H_3Br$	Sigma
Formamide	CH_3NO	Gibco BRL
Glycerol	$C_3H_8O_3$	E- Merck
Isoamyl alcohol	$C_5H_{11}OH$	Qualigens
Isopropyl alcohol	$CH_3CHOHCH_3$	E- Merck
Sodium hydroxide	NaOH	E- Merck
Sodium dodecyl sulphate	$C_{12}H_{25}NaO_4S$	Gibco BRL
Sodium chloride	NaCl	Hi-Media
Tris	Tris(hydroxylmethyl) aminomethane	Hi-Media
TEMED	N', N', N', N', Tetramethyl ethylene diamine	Banglore genei
Urea	CH_4N_2O	Sigma
Xylene cyanol	$C_{25}H_{27}N_2O_6S_2Na$	Banglore genei
Taq DNA polymerase	-	Sigma
dNTP's	-	Sigma
PCR buffer	-	Sigma
RNase	-	Sigma
Silver Staining Kit	-	Pro-Omega

Table 6: Composition and preparation of some important solutions, buffers and medium

Solution	Method of preparation/ Composition
40% Acrylamide	In 60 ml distilled water, 38 g acrylamide and 2 g bisacrylamide were dissolved and the volume was adjusted to 100 ml. solution was sterlised by filtration.
10% Ammonium per sulphate (APS)	1 g APS was dissolved in 10 ml distilled water.
0.5 M EDTA	186.1 g disodium ethylenediamine tetra acetate.$2H_2O$ was dissolved in 800 ml distilled water by string vigorously and pH was adjusted to 8.0 with NaOH. Solution was sterlised by autoclaving.
Ethidium bromide (10 mg/ ml)	1 g ethidium bromide was added to 100 ml water and stirred vigorously on a magnetic stirrer for several hours to ensure that the dye has dissolved. The container was wrapped in aluminum foil and stored at 4^0 C.
5M NaCl	292.1 g of NaCl was dissolved in 800 ml of distilled water and volume adjusted to 1L and solution was sterlised by autoclaving.
20% SDS	20 g of SDS was dissolved in 100 ml of distilled water by heating at 70^0C.
1M Tris HCl	121.1 g of Tris base was dissolved in 800 ml of distilled water and the pH to desired value was adjusted by adding concentrated HCl. The volume was made up to 1L and sterlised by autoclaving.
10X TBE	0.9 M Tris base; 0.9 M Boric acid; 0.02 M EDTA
CTAB buffer	2% CTAB; 1.4 M NaCl; 20 mM EDTA; 100 mM Tris Cl; 0.2% β- mercaptaethanol.
TE	10 mM Tris Cl (pH 8.0); 1 mM Na_2 EDTA
Gel loading buffer	0.25% (w/v) Bromophenol blue; 0.25% (w/v) Xylene cyanol; 60% (v/v) Glycerol.
PDA medium	200 g of well peeled potato infusion; 15 g of agar-agar and 15 g of dextrose in one liter.

Table 7: A brief description of Microsatellite markers used in the study

Sr. No.	Locus	Repeat motif	Left primer	Right primer
1	Xgwm 5-3A	$(TC)_{23}$ $(T)_4$ $(GT)_{12}$ $(GA)_{10}$	GCC AGC TAC CTC GAT ACA ACT C	AGA AAG GCC CAG GCT AGT AGT
2	Xgwm 33-1A	$(GA)_{19}$	GGA GTC ACA CTT GTT TGT GCA	CAC TGC ACA CCT AAC TAC CTG C
3	Xgwm 60-7A	$(CA)_{30}$	TGT CCT ACA CGG ACC ACG T	GCA TTG ACA GAT GCA CAC G
4	Xgwm 102-2D	$(CT)_{15}$	TCT CCC ATC CAA CGC CTC	TGT TGG TGG CTT GAC GCA TA
5	Xgwm 124-1B	$(CT)_{27}$ $(GT)_{18}$ imp	GCC ATG GCT ATC ACC CAG	ACT GTT CGG TGC AAT TTG AG
6	Xgwm 146-7B	$(GA)_5$ GG $(GA)_{20}$	CCA AAA AAA CTG CCT GCA TG	CTC TGG CAT TGC TCC TGG G
7	Xgwm 159-5B	$(GT)_{15}$	GGG CCA ACA CTG GAA CAC	GCA GAA GCT TGT TGG TAG GC
8	Xgwm 160-4A	$(GA)_{21}$	TTC AAT TCA GTC TTG GCT TGG	CTG CAG GAA AAA AAG TGC ACC C
9	Xgwm 165-4D	$(GA)_{20}$	TGC AGT GGT CAG AGT TTT CC	CTT TTC TTT CAG ATT GCG CC
10	Xgwm 179-5A	$(GT)_{15}$	AAG TTG AGT TGA TGC GGG AG	CCA TGA CCA GCA TCC ACT C
11	Xgwm 183-3D	$(GA)_{21}$ $(N)_{51}$ $(C)_{25}$	GTC TTC CCA TCT CGC AAG AG	CTC GAC TCC CAT GTG GAT G
12	Xgwm 194-4D	$(CT)_{32}$ imp	GAT CTG CTC TAC TCT CTT CC	CGA CGC AGA ACT TAA ACA AG
13	Xgwm 205-5D	$(CT)_{21}$	CGA CCC GGT TCA CTT CAG	AGT CGC CGT TGT ATA GTG CC
14	Xgwm 210-2B	$(GA)_{20}$	TGC ATC AAG AAT AGT GTG GAA G	TGA GAG GAA GAC TCA CAC CT
15	Xgwm 232-1D	$(GA)_{19}$	ATC TCA ACG GCA AGC CG	CTG ATG CAA GCA ATC CAC C
16	Xgwm 273-1B	$(GA)_{18}$	ATT GGA CGG ACA GAT GCT TT	AGC AGT GAG GAA GGG GAT C
17	Xgwm 282-7A	$(GA)_{38}$	TTG GCC GTG TAA GGC AG	TCT CAT TCA CAC ACA ACA CTA GC
18	Xgwm 285-3B	$(GA)_{27}$	ATG ACC CTT CTG CCA AAC AC	ATC GAC CGG GAT CTA GCC
19	Xgwm 292-5D	$(CT)_{38}$	TCA CCG TGG TCA CCG AC	CCA CCG AGC CGA TAA TGT AC
20	Xgwm 295-7D	$(GA)_{25}$	GTG AAG CAG ACC CAC AAG AC	GAC GGC TGC GAC GTA GAG
21	Xgwm 296-2A	$(CT)_{28}$	AAT TCA ACC TAC CAA TCT CTG	GCC TAA TAA ACT GAA AAC GAG
22	Xgwm 299-3B	$(GA)_{31}$ $(TAG)_4$	ACT ACT TAG GCC TCC C GC C	TGA CCC ACT TGC AAT TCA TC
23	Xgwm 325-6D	$(CT)_{16}$	TTT CTT CTG TCG TTC TCT TCC C	TTT TTA CGC GTC AAC GAC G
24	Xgwm 334-6A	$(GA)_{19}$	AAT TTC AAA AAG GAG AGA GA	AAC ATG TGT TTT TAG CTA TC
25	Xgwm 337-1D	$(CT)_5$ $(CACT)_6$ $(CA)_{43}$	CCT CTT CCT CCC TCA CTT AGC	TGC TAA CTG GCC TTT GCC

Contd..

Sr. No.	Locus	Repeat motif	Left primer	Right primer
26	Xgwm 350-7A	$(GT)_{14}$	ACC TCA TCC ACA TGT TCT ACG	GCA TGG ATA GGA CGC CC
27	Xgwm 368-4B	$(AT)_{25}$	CCA TTT CAC CTA ATG CCT GC	AAT AAA ACC ATG AGC TCA CTT GC
28	Xgwm 382-2A	$(GA)_{26}$	GTC AGA TAA CGC CGT CCA AT	CTA CGT GCA CCA CCA TTT TG
29	Xgwm 383-3D	$(GT)_{27}$	ACG CCA GTT GAT CCG TAA AC	GAC ATC AAT AAC CGT GGA TGG
30	Xgwm 391-3A	$(CA)_{17}$ $(GA)_{9}$	ATA GCG AAG TCT CCC TAC TCC A	ATG TGC ATG TCG GAC GC
31	Xgwm 400-7B	$(CA)_{21}$	GTG CTG CCA CCA CTT GC	TGT AGG CAC TGC TTG GGA G
32	Xgwm 410-2B	$(CA)_{11}$ $(CA)_{10}$ $(CA)_{8}$	GCT TGA GAC CGG CAC AGT	CGA GAC CTT GAG GGT CTA GA
33	Xgwm 425-2A	$(CT)_{21}$	GAG CCC ACA AGC TGG CA	TCG TTC TCC CAA GGC TA
34	Xgwm 427-6A	$(CA)_{31}$ $(CA)_{22}$	AAA CTT AGA ACT GTA ATT TCA GA	AGT GTG TTC ATT TGA CAG TT
35	Xgwm 428-7D	$(GA)_{22}$	CGA GGC AGC GAG GAT TT	TTC TCC ACT AGC CCC GC
36	Xgwm 469-6D	$(CT)_{19}$ $(CA)_{10}$	CAA CTC AGT GCT CAC ACA ACG	CGA TAA CCA CTC ATC CAC ACC
37	Xgwm 495-4B	$(GA)_{20}$	GAG AGC CTC GCG AAA TAT AGG	TGC TTC TGG TGT TCC TTC G
38	Xgwm 538-4B	$(GT)_{6}$ (T) $(GT)_{10}$	GCA TTT CGG GTG AAC CC	GTT GCA TGT ATA CGT TAA GCG G
39	Xgwm 604-5B	$(GA)_{29}$	TAT ATA GTT CAA TAT GAC CCG	ATC TTT TGA ACC AAA TGT G
40	Xgwm 626-6B	$(CT)_{5}$ $(GT)_{13}$	GAT CTA AAA TGT TAT TTT CTC TC	TGA CTA TCA GCT AAA CGT GT
41	Xgwm 637-4A	$(CA)_{18}$	AAA GAG GTC TGC CGC TAA CA	TAT ACG GTT TTG TGA GGG GG
42	Xgwm 639-5A	$(GA)_{19}$	CTC TCT CCA TTC GGT TTT CC	CAT GCC CCC CTT TTC TG
43	Xgwm 639-5D	$(GA)_{19}$	CTC TCT CCA TTC GGT TTT CC	CAT GCC CCC CTT TTC TG
44	Xgwm 644-6B	$(GA)_{20}$	GTG GGT CAA GGC CAA GG	AGG AGT AGC GTG AGG GGC
45	Xgwm 644-7B	$(GA)_{20}$	GTG GGT CAA GGC CAA GG	AGG AGT AGC GTG AGG GGC
46	Xgwm 666-1A	$(CA)_{13}$	GCA CCC ACA TCT TCG ACC	TGC TGC TGG TCT CTG TGC

imp - imperfect repeat.

1A

1B

1D

2A

2B

2D

3A

3B

3D

4A

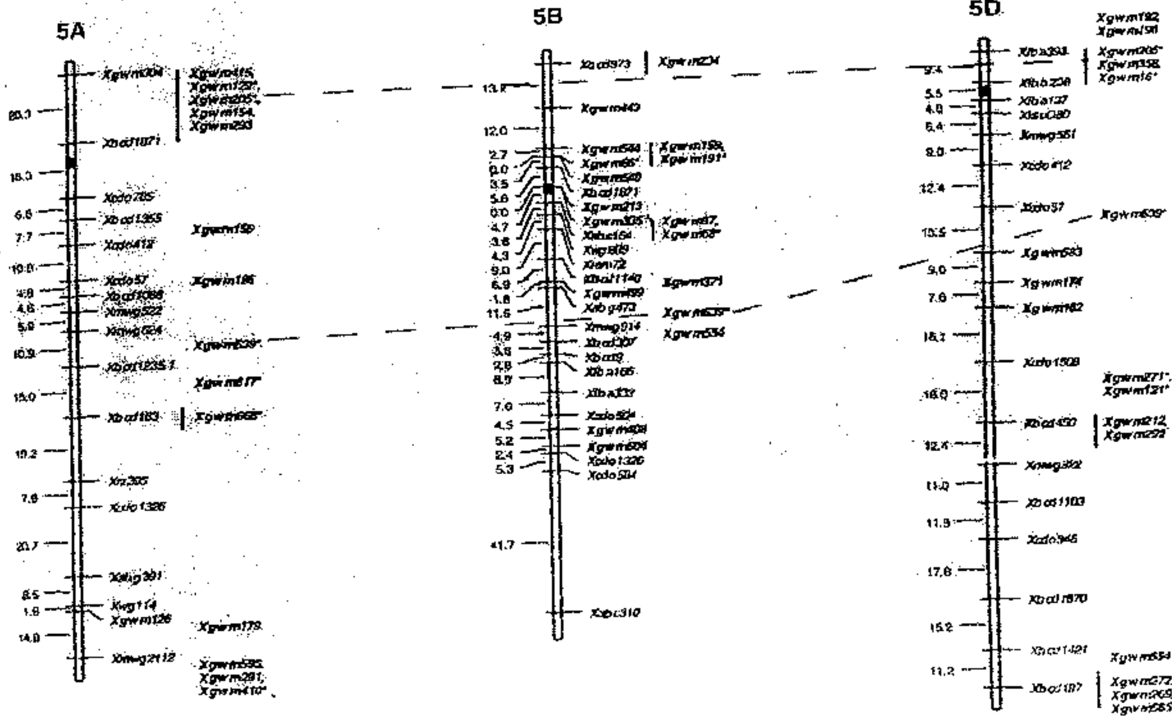
6.0 Xfba78
6.3 Xfba320 | Xgwm4, Xgwm601, Xgwm165*
9.6 XksuF8
11.9 Xbcd402 Xgwm610
Xwg622 Xgwm397
23.4
Xgwm637
15.9
3.0 Xmwg549
6.2 Xbcd1670
4.8 Xcdo475
3.5 XksuD9
2.9 Xbcd130
8.1 Xfba231
Xcdo545 | Xgwm160

4B

Xfba78
6.8 Xfba0
7.2 Xcdo795 Xgwm368
7.7 Xbcd1262 | Xgwm107, Xgwm113, Xgwm66*, Xgwm513, Xgwm495
7.2 Xbcd1265 Xgwm165*
4.5 Xcdo1401 Xgwm149
3.5 Xcdo1312
4.9 Xfbb178 Xgwm251
12.5 Xfbb67
9.7 Xfba177 Xgwm538
Xgwm6

4D

Xmwg634
32.0
Xbcd265
8.6
3.0 Xbcd327 | Xgwm608*
5.4 XksuF8
Xgwm165*
12.8
Xfbb178
10.4
Xcdo1312
11.0
Xbcd1431.1
15.0
Xfbb226
12.8 Xgwm194
Xfba177
11.5
Xcdo949 | Xgwm608, Xgwm624


5A
5B
5D

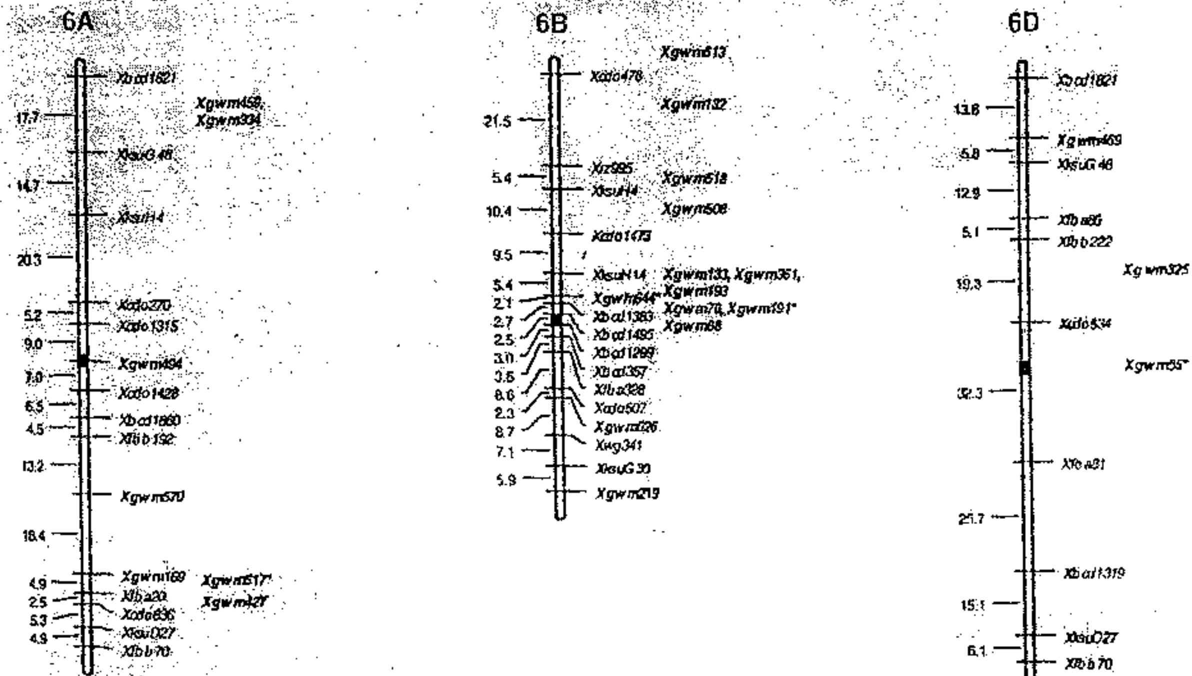

6A
6B
6D

Fig. 1: A microsatellites map of wheat (Roder *et al.*, 1998b).

3.1.7 RAPD Markers

A set of 92 RAPD markers were used in the present study. The 10-mer used as random primers in the PCR were purchased from Operon Technologies, Alameda,USA (Table 8.).

3.2 METHODS

3.2.1 Isolation and Multiplication of *Neovossia indica.*

Neovossia indica, causal organism of karnal bunt of wheat was isolated from bunted grains. After sterilization of the grains with absolute alcohol, the 'teliospores' were dusted directly over the potato dextrose agar (PDA) slants in

Table 8: List of RAPD primers used in the present study

Sr.No.	Name	Sequence (5' – 3')
1	OPA-06	GGTCCCTGAC
2	OPA-09	GGGTAACGCC
3	OPB-09	TGGGGGACTC
4	OPB-14	TCCGCTCTGG
5	OPB-16	TTTGCCCGGA
6	OPD-01	ACCGCGAAGG
7	OPD-02	GGACCCAACC
8	OPD-03	GTCGCCGTCA
9	OPD-05	TGAGCGGACA
10	OPD-06	ACCTGAACGG
11	OPD-07	TTGGCACGGG
12	OPD-08	GTGTGCCCCA
13	OPD-09	CTCTGGAGAC
14	OPE-01	CCCAAGGTCC
15	OPE-02	GGTGCGGGAA
16	OPG-12	CAGCTCACGA
17	OPH-01	GGTCGGAGAA
18	OPH-15	AATGGCGCAG
19	OPI-01	ACCTGGACAC
20	OPI-05	TGTTCCACGG
21	OPI-08	TTTGCCCGGT
22	OPI-17	GGTGGTGATC
23	OPM-01	GTTGGTGGCT
24	OPM-02	ACAACGCCTC
25	OPM-03	GGGGGATGAG
26	OPM-04	GGCGGTTGTC
27	OPM-05	GGGAACGTGT
28	OPM-06	CTGGGCAACT
29	OPM-07	CCGTGACTCA
30	OPM-08	TCTGTTCCCC
31	OPM-09	GTCTTGCGGA
32	OPM-10	TCTGGCGCAC
33	OPM-11	GTCCACTGTG
34	OPM-12	GGGACGTTGG
35	OPM-13	GGTGGTCAAG
36	OPM-14	AGGGTCGTTC
37	OPM-15	GACCTACCAC
38	OPM-16	GTAACCAGCC
39	OPM-17	TCAGTCCGGG
40	OPM-18	CACCATCCGT
41	OPM-19	CCTTCAGGCA
42	OPM-20	AGGTCTTGGG
43	OPN-01	CTCACGTTGG
44	OPN-02	ACCAGGGGCA
45	OPN-03	GGTACTCCCC

46	OPN-04	GACCGACCCA
47	OPN-05	ACTGAACGCC
48	OPN-06	GAGACGCACA
49	OPN-07	CAGCCCAGAG
50	OPN-08	ACCTCAGCTC
51	OPN-09	TGCCGGCTTG
52	OPN-10	ACAACTGGGG
53	OPN-11	TCGCCGCAAA
54	OPN-13	AGCGTCACTC
55	OPN-14	TCGTGCGGGT
56	OPN-15	CAGCGACTGT
57	OPN-16	AAGCGACCTG
58	OPN-17	CATTGGGGAG
59	OPN-18	GGTGAGGTCA
60	OPN-19	GTCCGTACTG
61	OPN-20	GGTGCTCCGT
62	OPO-09	TCCCACGCAA
63	OPO-18	CTCGCTATCC
64	OPQ-01	GGGACGATGG
65	OPQ-07	CCCCGATGGT
66	OPS-06	GATACCTCGG
67	OPS-20	TCTGGACGGA
68	OPY-19	TGAGGGTCCC
69	OPZ-19	GTGCGAGCAA
70	OPAA-16	GGAACCCACA
71	OPAB-13	CCTACCGTGG
72	OPAC-07	GTGGCCGATG
73	OPAD-06	AAGTGCACGG
74	OPAE-10	CTGAAGCGCA
75	OPAF-04	TTGCGGCTGA
76	OPAF-10	GGTTGGAGAC
77	OPAG-04	GGAGCGTACT
78	OPAG-10	ACTGCCCGAC
79	OPAH-03	GGTTACTGCC
80	OPAH-10	GGGATGACCA
81	OPAI-08	AAGCCCCCCA
82	OPAJ-09	ACGGCACGCA
83	OPAK-03	GGTCCTACCA
84	OPAL-03	AAGCGTCCTC
85	OPAM-07	AACCGCGGCA
86	OPAN-10	CTGTGTGCTC
87	OPAO-03	AGTCGGCCCA
88	OPAP-07	ACCACCCGCT
89	OPAQ-07	GGAGTAACGG
90	OPAR-05	CATACCTGCC
91	OPAS-06	GGCGCGTTAG
92	OPAS-10	CCCGTCTACC

test tube by giving a pinprick to the bunted grains in the laminar flow. After that, tubes were kept at 18-20 ± 1^0 C for 15-20 days in BOD incubator. After about 15 days, white powdery shining dot like colonies started appearing. The individual colony was picked up carefully and transferred on the slants for multiplication (Fig. 2.). The slants were kept at 20 ± 1^0 C for 10 days in the upright position so that allantoid sporidia showered down on the slants of culture medium. Sub culturing of the pathogen was done after every 10-12 days on the fresh medium to maintain the pathogen in the sporulating condition.

3.2.2 Evaluation of Parents and RILs for Karnal Bunt Resistance

The screening of both the genotypes and their RIL's against *N. indica* under artificial epiphytotic conditions in green house was carried out. Five plants of both the genotypes and their RIL's were raised in earthen pots in three replications. These were inoculated with the sporidial suspension prepared from freshly sporulating 10-12 days old culture. The sporidial mass was harvested with the help of inoculating needle in sterilized water and homogenized in waring blender for 2-3 minutes. It was filtered through muslin cloth. Concentration of secondary sporidia in the inoculum suspension was adjusted at about 10,000 sporidia per ml. Inoculation was done with the help of hypodermic syringe. About 2 ml of spore suspension was injected in each tiller at boot leaf stage during the evening hours (Aujla *et al.*, 1983). After inoculation, the inoculated tillers were tagged. For successful infection and disease development, optimum temperature (18-22 ^{0} C) and

Fig. 2 : *Neovossia indica* cultured on PDA slants.

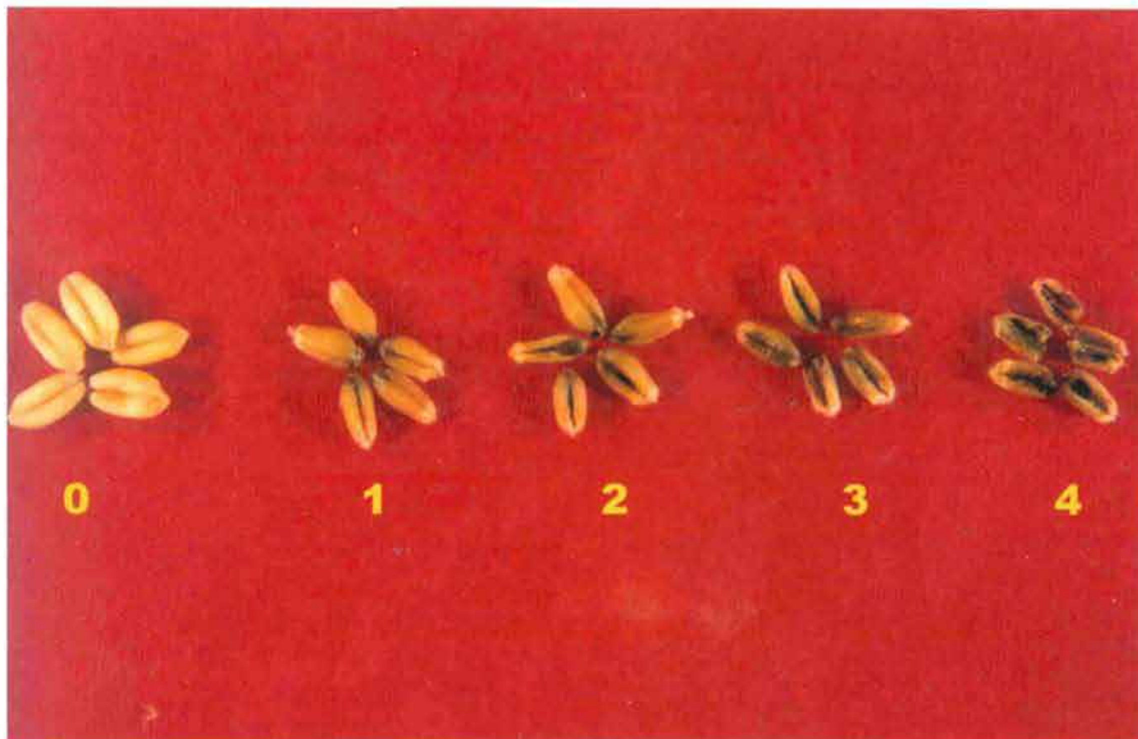


Fig. 3 : Different grading of wheat genotypes.

relative humidity (80 to 100 %) were maintained in the green house. After maturity, the inoculated ear heads were harvested. Grains were removed carefully by hand and were separated into different grades (Fig. 3.). The per cent infected grains and coefficient of infection was worked out.

3.2.3 Isolation and Purification of Plant Genomic DNA

Genomic DNA of both the genotypes and their RIL's were isolated following a modified CTAB (Cetyltrimethylammonium bromide) procedure of Saghai-Maroof *et al.* (1984). It is the method of choice for obtaining good quality total DNA. CTAB is a cationic detergent which solublises membranes and forms a complex with DNA. After cell disruption and incubation with hot CTAB isolation buffer, proteins were removed by chloroform: isoamyl alcohol (CI) and CTAB-DNA complex is precipitated with isopropanol. The DNA pellet resulting after centrifugation is washed, dried and redissolved. RNase treatment was given to remove RNA. The detailed protocol for DNA isolation was described as follows:

Approximately 5 g leaf material was ground to a fine powder using liquid nitrogen in a mortar and pestle. The powder was quickly transferred into 25 ml of prewarmed (60 ^{0}C) isolation buffer in a capped polypropylene tube and incubated for 1 hour at 60 ^{0}C in a water bath and mixed by gentle swirling after every 10 min. To these tubes, equal volume of CI was added and the contents were shaken for 10 min by hand. The tubes were centrifuged for 10 min at 8000 rpm; the upper aqueous layer was extracted twice with fresh CI and the final aqueous layer was

transferred to a centrifuge tube. To these tubes, 0.6 V of ice-cold isopropanol was added and shaken several times. By using a glass hook, DNA was spooled out in the form of whitish fibers and transferred to washing solution and dried. DNA was dissolved in an appropriate volume of 1X TE buffer.

For purification, RNase A was added to the tube (50 μg/ ml) and the mixture incubated for 1 h at 37 ^{0}C. DNA was extracted with CI by centrifuging the tubes at 10,000 rpm for 5 min at room temperature. DNA was precipitated with 2V of ice cold absolute ethanol and was recovered by centrifuging the tubes at 5000 rpm for 10 min; the pellet was washed with 70% ethanol and dissolved in appropriate volume of IX TE.

3.2.4 Detection of DNA Polymorphism between Parents differing for Karnal Bunt Resistance Trait

DNA amplification were carried out in 25 μl reaction mixture, each containing 50 ng of template DNA, 2 μM Microsatellite primers, 100 μM each of dNTPs. 2 μl of Taq DNA polymerase 10X buffer, 1 unit Taq polymerase (Perkin Elmer) and 2.5 mM $MgCl_2$. PCR amplification were performed on a PTC-100 Thermal Cycler (MJ Research, Inc. Watertown, MA, USA) under the following conditions: Initial denaturation at 95 ^{0}C for 5 min, followed by 40 cycles of denaturing at 95 ^{0}C for 1 min, annealing at 50 ^{0}C/ 55 ^{0}C/ 60 ^{0}C for 1 min, extension at 72 ^{0}C for 2 min and final extension at 72 ^{0}C for 10 minutes. The amplification product were resolved on 6% polyacrylamide denaturing gels (PAGE) followed by silver staining (Tegelstrom, 1992). Fragment size was calculated using computer

programme NTSYS-PC/ BIO 1D by comparing with fragments of 100 bp ladder marker DNA.

For RAPD assay, PCR amplification were performed on a PTC 100 Thermal Cycler (MJ Research, Inc. Watertown, MA, USA) under the following conditions: Initial denaturation at 95 ^{0}C for 5 min, followed by 35 cycles of denaturing at 95 ^{0}C for 1 min, annealing at 35.1 ^{0}C for 1 min, extension at 72 ^{0}C for 2 min and final extension at 72 ^{0}C for 10 minutes. The amplification products were resolved on 1.5 % agarose gel.

3.2.5 Bulked Segregant analysis using Polymorphic Primers

For karnal bunt trait, 10 RILs representing the two tails of the normal distribution were selected. DNA from these RILs was pooled and two bulks were prepared. The primers showing polymorphism between parents were used with these two pooled DNA samples (bulks), which is commonly described as bulk segregant analysis (Michelmore *et al.*, 1991).

3.2.6 Selective Genotyping of the RILs belonging to two extreme groups

The primers indicating some association with trait on bulked segregant analysis were used for genotyping individual RILs, used for preparation of bulk (Lander and Botstein, 1989).

3.2.7 Data analysis

3.2.7.1 Per cent infection and coefficient of infection

The per cent infected grains and coefficient of infection was worked out as given below:

$$\text{Per cent infected grains} = \frac{\text{Number of infected grains}}{\text{Total number of grains}} \times 100$$

Coefficient of infection was worked out as suggested by Aujla *et al.* (1989).

The grains obtained from inoculated earheads were separated into different grades as:

Grade	Symptoms of infection
0	Healthy grains, no infection
1	Infection only at the embryonal end and less than 25 per cent of endosperm affected
2	Between 25 to50 per cent of the endosperm affected
3	Between 51 to 75 per cent of the endosperm affected
4	More than 75 per cent or practically whole of the endosperm affected

The χ^2 test was applied to test the goodness of fit of the model or to judge the normality of the population.

Calculations for coefficient of infection

An example of Gill *et al.* (1993)

Total number of grains	=	50			
Total number of infected grains	=	20			
Infected grades	=	1	2	3	4
Corresponding numerical value	=	0.25	0.50	0.75	1.00
Number of infected grains	=	5	5	5	5
Value after multiplication	=	1.25	2.50	3.75	5.00
Total of all ratings	=	1.25 + 2.50 + 3.75 + 5.00 = 12.50			
Coefficient of infection	=	12.50/ 50 x 100 = 25%			

3.2.7.2 Allele scoring and diversity analysis

After silver staining of polyacryalmide gels, size (in nucleotide base pairs) of the most intensely amplified bands for each microsateliite marker was determined based on its migration relative to molecular weight size marker (100 bp ladder). A genotype was assigned a null allele for a SSR locus whenever amplification products were not detected for a particular genotype marker combination.

3.2.7.3 Similarity coefficient

The frequency of both microsatellite and RAPD polymorphism were calculated based on presence (taken as 1) or absence (taken as 0) of common bands

(Ghosh *et al.*, 1997). The binary data were used to compute Pair-wise similarity coefficient (Jaccard, 1908) and the similarity matrix thus obtained was subjected to cluster analysis using the UPGMA (Unweighted Pair-group method with Arithmetic average) algorithm on NTSYS-PC.

4

EXPERMENTAL RESULTS

Experiments were carried out to study genetic diversity among two karnal bunt resistance and susceptible varieties using microsatellite DNA markers. Further studies were carried out to evaluate F_8 segregating population of a cross HD29 (resistant) X WH542 (susceptible) comprising 104 RILs for karnal bunt resistance. A total of 46 SSR markers were tested for molecular diversity analysis of karnal bunt resistant and susceptible plants. The distribution of HD29 and WH542 specific alleles among the selected F_8 RILs has been determined and attempts have been made to identify alleles (electromorphs), which may be linked with the genes for karnal bunt resistance. Polymerase chain reaction based DNA amplification by commercially available decamer random sequence primers, having 60 per cent or more (G+C) content, were also used to generate RAPDs. The results obtained on the different aspects have been presented below:

4.1 Evaluation of Recombinant inbred lines for karnal bunt resistance

4.1.1 Per cent infection and coefficient of infection

Two parents *viz.* HD29 and WH542 and their 104 recombinant inbred lines (RILs) were screened against *N. indica* in green house. Ears at boot leaf stage were inoculated with suspension of secondary sporidia (10,000 sporidia / ml) of *N. indica* and reactions in terms of percentage of infected grains and coefficient of infection were worked out. The data were transformed using angular transformations and analysed following Completely Randomized Design. The two parents differed sharply for mean coefficient of infection in 2001 and in 2002. The coefficient of infection and percentage of infected grains from RILs have been presented in Table 9 & 10. Analysis of variance for coefficient of infection and per cent infected grains showed significant variations among all the recombinant inbred lines (Table 11).

The range of coefficient of infection (CI) on the RILs was 0–81.25and 0–80.80 in years 2001 and 2002, respectively. Among the 104 RILs, there was a wide variation in the response to karnal bunt resistance, as shown in Fig 4. The distribution of karnal bunt disease on the RILs was towards the resistant parent type in both the years.

Table 9: Coefficient of infection (CI) and percentage of infected grains from different RILs derived from cross HD29 x WH 542 during the year 2001

RIL No.	CI (%)	Percentage of infection (%)	RIL No.	CI (%)	Percentage of infection (%)	RIL No.	CI (%)	Percentage of infection (%)
1	33.30	75.0	36	00.00	00.0	71	00.00	00.0
2	00.00	00.0	37	00.00	00.0	72	00.00	00.0
3	00.00	00.0	38	00.00	00.0	73	12.50	29.0
4	00.00	00.0	39	00.00	00.0	74	00.00	00.0
5	20.00	45.6	40	00.00	00.0	75	25.00	46.0
6	00.00	00.0	41	00.00	00.0	76	00.00	00.0
7	00.00	00.0	42	00.00	00.0	77	00.00	00.0
8	00.00	00.0	43	00.00	00.0	78	00.00	00.0
9	00.00	00.0	44	00.00	00.0	79	10.52	23.6
10	06.25	25.6	45	60.00	72.3	80	00.00	00.0
11	11.11	29.9	46	00.00	00.0	81	03.22	06.9
12	20.00	35.6	47	00.0	00.0	82	00.00	00.0
13	00.00	00.0	48	00.0	00.0	83	00.00	00.0
14	00.00	00.0	49	00.00	00.0	84	00.00	00.0
15	00.00	00.0	50	10.53	25.6	85	00.00	00.0
16	00.00	00.0	51	00.00	00.0	86	00.00	00.0
17	00.00	00.0	52	04.54	13.5	87	00.00	00.0
18	00.00	00.0	53	00.00	00.0	88	18.75	33.0
19	10.52	25.6	54	00.00	00.0	89	00.00	00.0
20	00.00	00.0	55	00.00	00.0	90	07.14	15.9
21	81.25	98.8	56	15.00	25.0	91	00.00	00.0
22	00.00	00.0	57	00.00	00.0	92	00.00	00.0
23	00.00	00.0	58	00.00	00.0	93	00.00	00.0
24	25.00	46.6	59	00.00	00.0	94	00.00	00.0
25	00.00	00.0	60	00.00	00.0	95	47.05	78.9
26	00.00	00.0	61	00.00	00.0	96	08.00	12.5
27	00.00	00.0	62	00.00	00.0	97	00.00	00.0
28	00.00	00.0	63	00.00	00.0	98	00.00	00.0
29	18.75	39.5	64	00.00	00.0	99	00.00	00.0
30	30.76	48.5	65	00.00	00.0	100	05.25	12.5
31	00.00	00.0	66	00.00	00.0	101	00.00	00.0
32	00.00	00.0	67	00.00	00.0	102	00.00	00.0
33	09.52	19.8	68	00.00	00.0	103	00.00	00.0
34	00.00	00.0	69	00.00	00.0	104	00.00	00.0
35	09.52	15.5	70	00.00	00.0	HD29	00.00	00.0
						WH542	56.78	70.3

CD = 1.09 1.42

Table 10: Coefficient of infection (CI) and percentage of infected grains from different RILs derived from cross HD29 x WH 542 during the year 2002

RIL No.	CI (%)	Percentage of infection (%)	RIL No.	CI (%)	Percentage of infection (%)	RIL No.	CI (%)	Percentage of infection (%)
1	00.00	00.0	36	00.00	00.0	71	00.00	00.0
2	00.00	00.0	37	00.00	00.0	72	01.00	04.0
3	00.00	00.0	38	00.00	00.0	73	00.00	00.0
4	00.00	00.0	39	00.00	00.0	74	00.00	00.0
5	22.00	39.5	40	00.00	00.0	75	00.00	00.0
6	00.00	00.0	41	00.00	00.0	76	04.68	12.5
7	00.00	00.0	42	00.00	00.0	77	00.00	00.0
8	00.00	00.0	43	00.00	00.0	78	00.00	00.0
9	00.00	00.0	44	00.00	00.0	79	00.86	3.44
10	10.00	30.2	45	00.00	00.0	80	00.00	00.0
11	17.50	29.8	46	00.0	00.0	81	03.57	09.5
12	18.90	33.3	47	00.00	00.0	82	01.92	03.8
13	00.00	00.0	48	00.00	00.0	83	01.13	02.2
14	00.00	00.0	49	00.00	00.0	84	02.50	06.6
15	00.00	00.0	50	21.12	58.9	85	00.00	00.0
16	00.00	00.0	51	00.00	00.0	86	00.86	03.4
17	00.00	00.0	52	00.00	00.0	87	00.00	00.0
18	00.00	00.0	53	00.00	00.0	88	11.11	33.3
19	17.70	32.6	54	00.00	00.0	89	00.00	00.0
20	00.00	00.0	55	00.00	00.0	90	04.31	13.7
21	00.00	00.0	56	00.00	00.0	91	00.00	00.0
22	00.00	00.0	57	00.00	00.0	92	02.50	06.6
23	00.00	00.0	58	00.00	00.0	93	16.07	25.0
24	00.00	00.0	59	00.00	00.0	94	00.00	00.0
25	00.00	00.0	60	00.00	00.0	95	47.05	80.8
26	00.00	00.0	61	00.00	00.0	96	00.00	00.0
27	00.00	00.0	62	00.00	00.0	97	80.80	100
28	00.00	00.0	63	00.00	00.0	98	00.00	00.0
29	22.57	56.6	64	00.00	00.0	99	00.00	00.0
30	00.00	00.0	65	00.00	00.0	100	13.88	30.5
31	00.00	00.0	66	00.00	00.0	101	00.00	00.0
32	00.00	00.0	67	00.00	00.0	102	00.00	00.0
33	00.00	00.0	68	00.00	00.0	103	02.56	05.1
34	00.00	00.0	69	00.00	00.0	104	00.00	00.0
35	15.50	40.8	70	00.00	00.0	HD29	00.00	00.0
						WH542	58.30	72.6

CD = 1.19 1.83

Table 11: Analysis of variance for coefficient of infection and percentage of wheat grains infected with *N. indica*

Source of variation	Degree of freedom	Mean sum of squares	
		Coefficient of infection	Percentage of infection
Genotypes (2001)	105	536.77 **	653.56 **
Error	212	15.86	0.51
Genotypes (2002)	105	573.27 **	539.64 **
Error	212	0.55	0.86

** Significant at 1 per cent

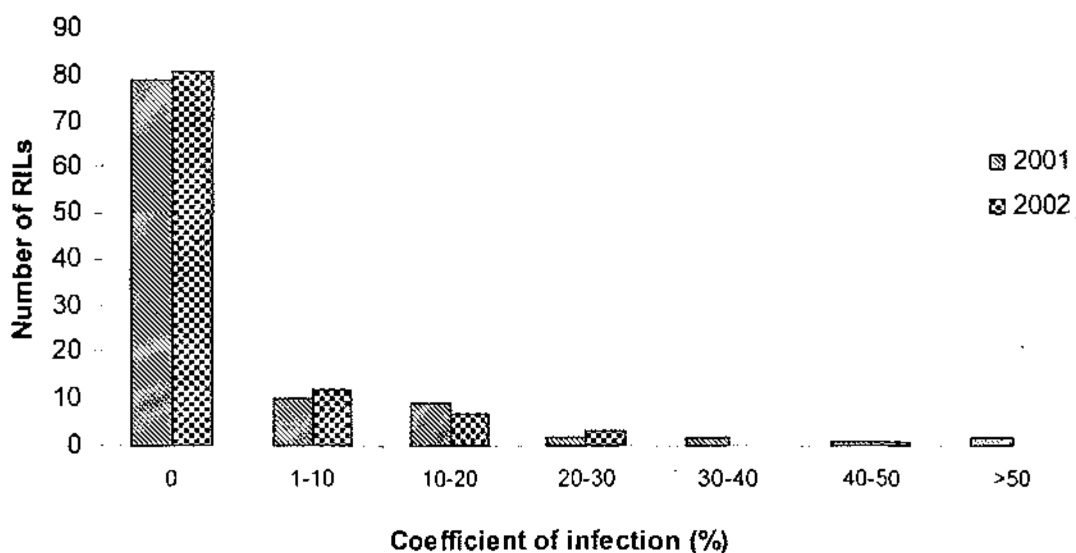


Fig. 4: Histogram showing coefficient of infection on RILs during the year 2001 and 2002

4.1.2 Chi-square test for goodness of fit

Chi-square test for goodness of fit was carried out. The data suggested a good fit ($\chi^2 = 0.05$) and indicated that a single dominant gene is responsible for karnal bunt resistance (Table 12.).

Table 12: Chi-square test for testiug goodness of fit in wheat

R	S	Total	Ratio	χ^2	*P* value
79	25	104	3:1	0.05	0.083 > 0.001

4.2 Optimization of DNA amplification technique

The genomic DNA was isolated from wheat leaves. Results are presented in Fig. 5 & 6 indicated that the DNA was of high molecular weight which resolved as a single band. PCR conditions for SSR and RAPD analysis in wheat were optimized with respect to DNA concentration, Mg^{+2} concentration, dNTPs concentration, duration of denaturing temperature and number of cycles for amplification. Most satisfactory results were obtained in concentration of 35 – 50 ng. Concentration of Mg^{+2} was varied from 1 mM to 3 mM and 2.5 mM Mg^{+2} concentration was found to be optimum concentration. Similarly, 100 mM of each dNTPs concentration was also found as optimum concentration from 100 mM to 250 mM varying concentration tested. Denaturation temperature used was 95 ^{0} C

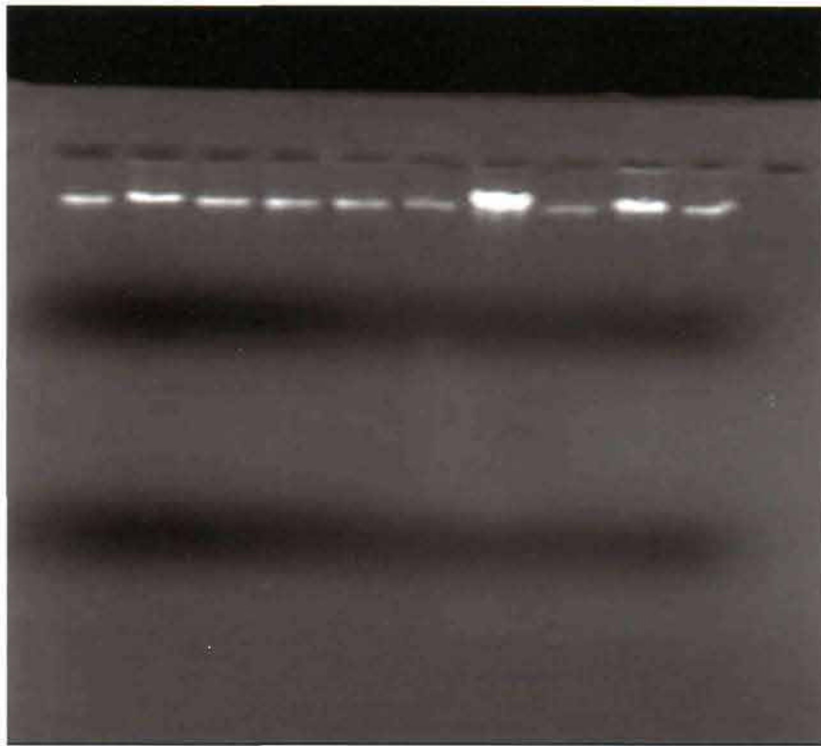


Fig. 5 : Genomic DNA of individual resistant RILs.

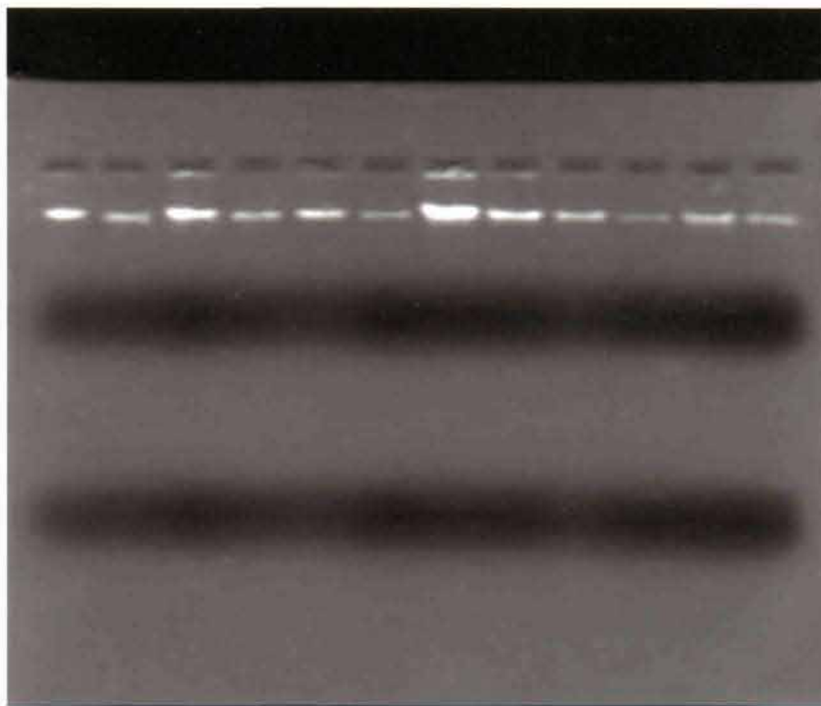


Fig. 6 : Genomic DNA of individual susceptible RILs.

for 3 min or 5 min duration. Better DNA amplification was obtained when 5 min period of denaturation was used. Number of amplification cycles used was 30 to 50. Most satisfactory results were obtained when numbers of cycles were 40 for SSR and 35 for RAPDs. The final optimized conditions obtained for DNA amplification are mentioned in Section 3.2.4 of the chapter on Materials and Methods.

4.3 Microsatellite marker analysis

4.3.1 SSR markers based polymorphism among the parents

Microsatellite markers based fingerprint database was generated using 46 SSR primers for both the parents differing in karnal bunt resistance. Gels displaying allelic polymorphism among the parents for some of the SSR markers have been shown in Figs 7-10. A total of 179 alleles were detected at 46 SSR loci. Data recorded for different alleles for each primer as present (scored as 1) or absent (scored as 0), is presented in Table 13. Out of 46 primers used in the study, both the parents showed polymorphism at 15 SSR loci and 31 primers yielded monomorphic amplified products.

The number of alleles per locus ranged from 1(Xgwm 60, Xgwm 102, Xgwm 160, Xgwm 282 and Xgwm 299) to 8 (Xgwm 205 and Xgwm 383) with an average of 3.9 alleles per locus (Table 14.). The overall size of PCR products amplified ranged from 105 to 290 bp. The molecular size difference between the

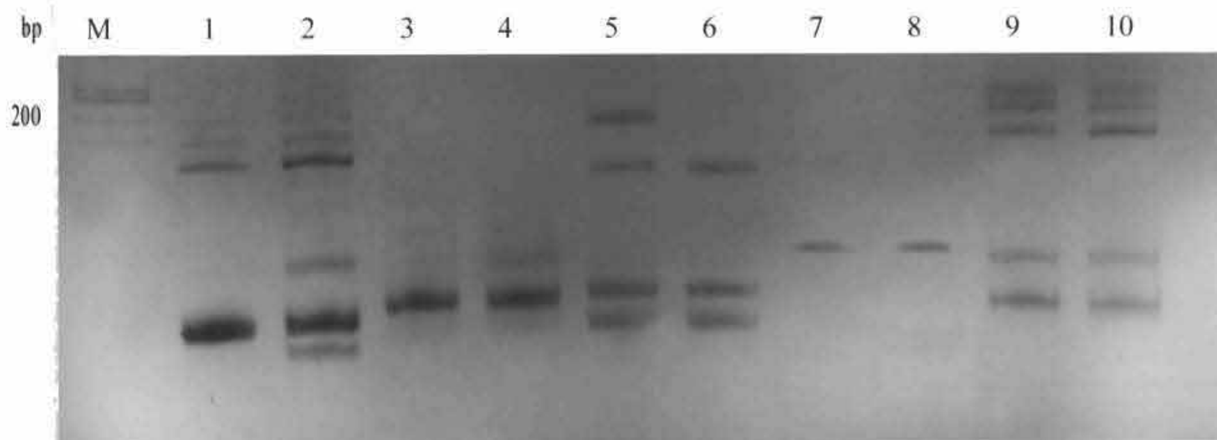


Fig. 7 : A representative sample of SSR polymorphism between parents HD29 (Lanes 1,3,5,7,9) and WH542 (Lanes 2,4,6,8,10) using five primers: gwm 179-5A (Lanes 1,2), gwm 183-3D (Lanes 3,4), gwm 273-1B (Lanes 5,6), gwm 282-7A (Lanes 7,8) and gwm 296-2A (Lanes 9,10).

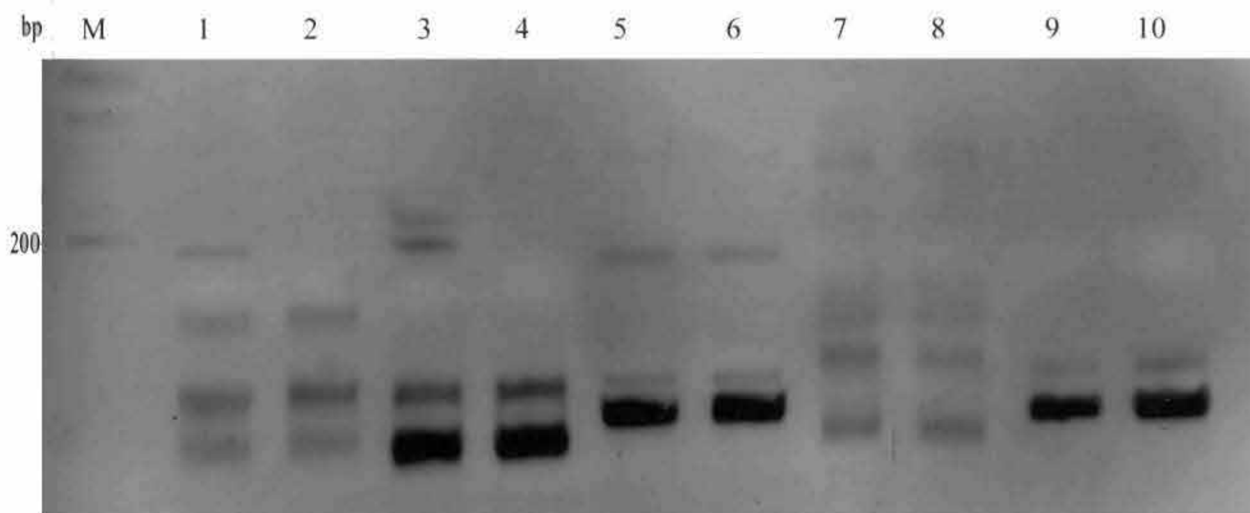


Fig. 8 : A representative sample of SSR polymorphism between parents HD29 (Lanes 1,3,5,7,9) and WH542 (Lanes 2,4,6,8,10) using five primers: gwm 337-1D (Lanes 1,2), gwm 350-7A (Lanes 3,4), gwm 391-3A (Lanes 5,6), gwm 639-5D (Lanes 7,8) and gwm 33-1A (Lanes 9,10).

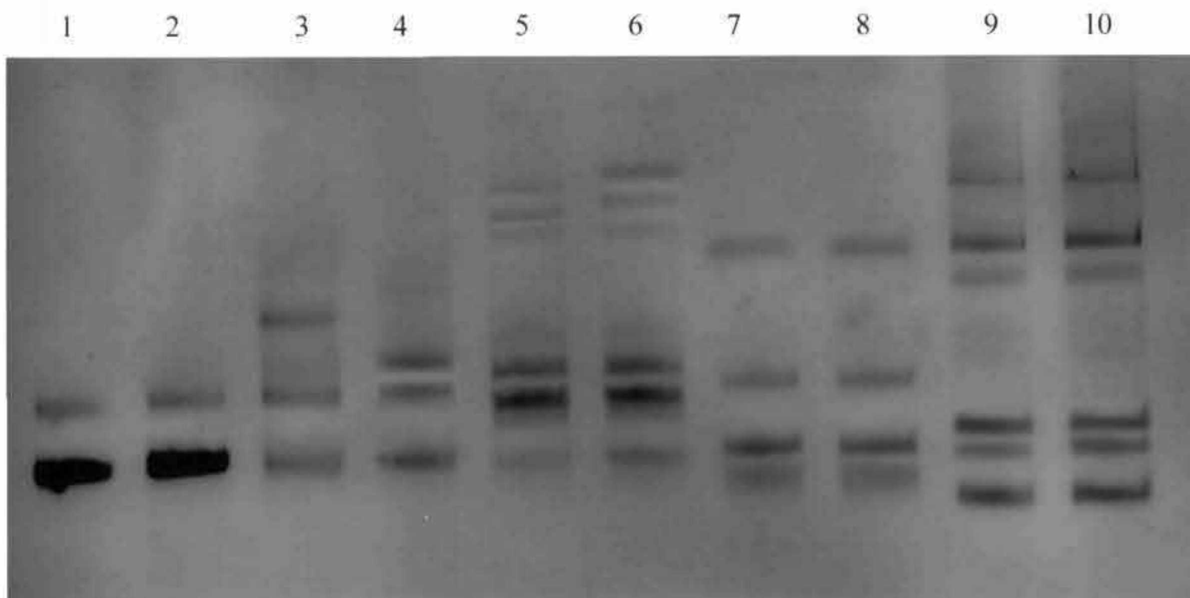


Fig. 9 : A representative sample of SSR polymorphism between parents HD29 (Lanes 1,3,5,7,9) and WH542 (Lanes 2,4,6,8,10) using five primers: gwm 368-4B (Lanes 1,2), gwm 382-2A (Lanes 3,4), gwm 383-3D (Lanes 5,6), gwm 428-7D (Lanes 7,8) and gwm 644-7B (Lanes 9,10).

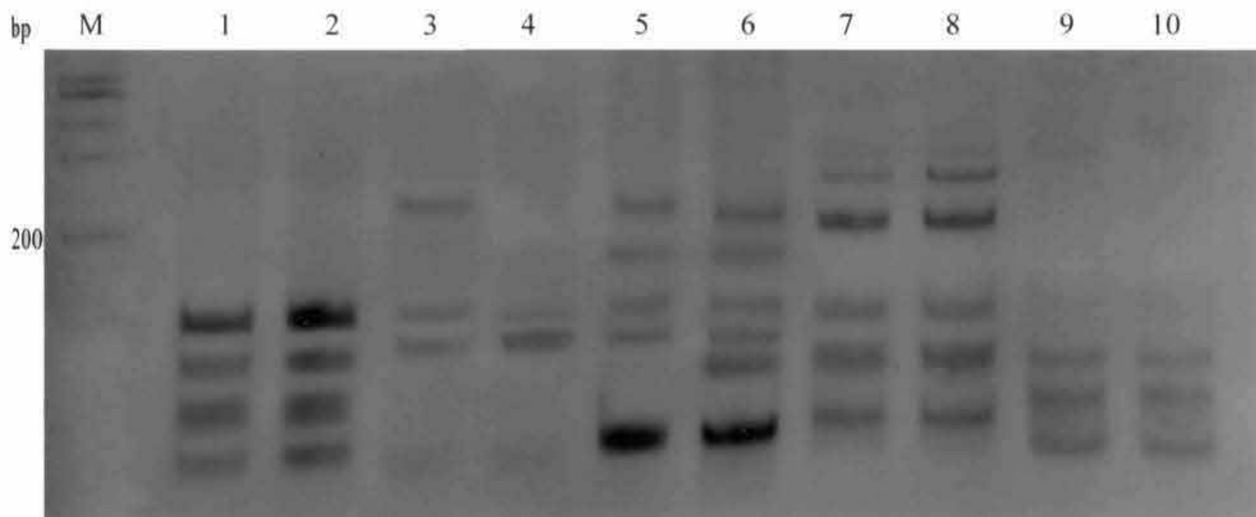


Fig. 10 : A representative sample of SSR polymorphism between parents HD29 (Lanes 1,3,5,7,9) and WH542 (Lanes 2,4,6,8,10) using five primers: gwm 194-4D (Lanes 1,2), gwm 334-6A (Lanes 3,4), gwm 232-1D (Lanes 5,6), gwm 410-2B (Lanes 7,8) and gwm 639-5A (Lanes 9,10).

Table 13: DNA amplification profile as obtained on parents with 46 SSR primers

Marker		HD 29	WH542	Marker		HD 29	WH542
X gwm 5-3A	A1	1	1	X gwm 232-1D	A1	1	1
	A2	1	1		A2	1	1
	A3	1	1		A3	1	1
	A4	1	1		A4	1	1
X gwm 33-1A	A1	1	1		A5	1	0
	A2	1	1		A6	1	1
X gwm 60-7A	A1	1	1	X gwm 273-1B	A1	1	0
X gwm 102-2D	A1	1	1		A2	1	1
X gwm 124-1B	A1	1	1		A3	1	1
	A2	1	1		A4	1	1
	A3	1	1	X gwm 282-7A	A1	1	1
X gwm 146-7B	A1	1	1	X gwm 285-3B	A1	1	1
	A2	1	1		A2	1	1
	A3	1	1		A3	1	1
X gwm 159-5B	A1	1	1		A4	1	1
	A2	1	1	X gwm 292-5D	A1	1	1
	A3	1	1		A2	1	1
	A4	1	1		A3	1	1
X gwm 160-4A	A1	1	1		A4	1	1
X gwm 165-4D	A1	1	1		A5	1	1
	A2	1	1		A6	1	1
	A3	1	1	X gwm 295-7D	A1	1	1
X gwm 179-5A	A1	1	1		A2	1	1
	A2	1	1		A3	1	1
	A3	0	1		A4	1	1
	A4	1	1		A5	1	1
	A5	0	1		A6	1	1
X gwm 183-3D	A1	0	1	X gwm 296-2A	A1	1	1
	A2	1	1		A2	1	1
X gwm 194-4D	A1	1	1		A3	1	1
	A2	1	1		A4	1	1
	A3	1	1		A5	1	1
	A4	1	1	X gwm 299-3B	A1	1	1
X gwm 205-5D	A1	1	0	X gwm 325-6D	A1	1	1
	A2	1	1		A2	1	1
	A3	1	1	X gwm 334-6A	A1	1	0
	A4	1	1		A2	1	1
	A5	1	1		A3	1	1
	A6	1	1	X gwm 337-1D	A1	1	0
	A7	1	1		A2	1	1
	A8	1	1		A3	1	1
X gwm 210-2B	A1	1	1		A4	1	1
	A2	1	1	X gwm 350-7A	A1	1	0
	A3	1	1		A2	1	0
	A4	1	1		A3	1	1

Contd..

Marker		HD 29	WH542	Marker		HD 29	WH542
	A4	1	1		A5	1	0
X gwm 368-4B	A1	1	1		A6	1	1
	A2	1	1		A7	1	1
X gwm 382-2A	A1	1	0	X gwm 495-4B	A1	1	1
	A2	0	1		A2	1	1
	A3	1	1		A3	1	1
	A4	1	1		A4	1	1
X gwm 383-3D	A1	0	1	X gwm 538-4B	A1	1	1
	A2	1	0		A2	1	1
	A3	0	1		A3	1	1
	A4	1	0		A4	1	1
	A5	1	1	X gwm 604-5B	A1	1	1
	A6	1	1		A2	1	1
	A7	1	1	X gwm 626-6B	A1	1	1
	A8	1	1		A2	1	1
X gwm 391-3A	A1	1	1		A3	1	1
	A2	1	1		A4	1	1
	A3	1	1	X gwm 637-4A	A1	1	0
X gwm 400-7B	A1	1	1		A2	1	0
	A2	1	1		A3	1	1
	A3	1	1		A4	1	1
	A4	1	1	X gwm 639-5A	A1	1	1
	A5	1	1		A2	1	1
X gwm 410-2B	A1	1	1		A3	1	1
	A2	1	1	X gwm 639-5D	A1	1	1
	A3	1	1		A2	1	1
	A4	1	1		A3	1	1
	A5	1	1	X gwm 644-6B	A1	1	1
X gwm425-2A	A1	1	1		A2	1	1
	A2	1	1		A3	1	1
	A3	1	0		A4	1	1
	A4	1	1		A5	1	1
X gwm 427-6A	A1	1	0		A6	1	1
	A2	0	1	X gwm 644-7B	A1	1	1
	A3	1	0		A2	1	1
	A4	0	1		A3	1	1
	A5	1	1		A4	1	1
X gwm 428-7D	A1	1	1		A5	1	1
	A2	1	1		A6	1	1
	A3	1	1	X gwm 666-1A	A1	1	1
	A4	1	1		A2	1	0
X gwm 469-6D	A1	1	1		A3	0	1
	A2	1	1		A4	1	1
	A3	1	1		A5	1	1
	A4	1	1				

A – represents allele number

0 - represents absence of band

1 - represents presence of band

Table 14: Allelic diversity among parents HD29 & WH 542 as assessed by 46 SSR markers

Number of primers used	46
Number of alleles	179
Range of alleles	1-8
Average number of alleles	3.9
Number of polymorphic markers	15
Number of monomorphic markers	31

smallest and the largest allele at a SSR locus varied from 0 (Xgwm 60,Xgwm 102, Xgwm 160, Xgwm 282 and Xgwm 299) to 150 bp (Xgwm 637) (Table 15.). The 46 SSR loci used in this study included mostly with di-nucleotide repeat motif, one with tri-nucleotide and one with tetra-nucleotide motif. SSR loci with di-nucleotide motifs were found to be more polymorphic as compared to those with tri or tetra-nucleotide motif. Among the 42 loci with di-nucleotide motifs, 18, 7, 4, 3 and 1 loci had GA, CT, GT, CA and AT repeat motifs, respectively. Loci with GA repeat motif were found to be more polymorphic compared to loci with GT, CT and CA motifs. No null alleles were detected at 46 SSR loci used in the study.

4.3.2 Genetic relationship among both the parents

Similarity coefficient based on DNA amplification using microsatellite primers were estimated using 'Simqual' subprogram of software NTSYS-PC. The similarity coefficients of both the parents are presented in Table 16. The similarity matrix shows a close genetic relationship between both the parents HD 29 and

Table 15: Number of alleles and size range of wheat microsatellite (Xgwm) markers observed on HD29 and WH 542 wheat genotypes

Sr. No.	Locus	Chr. Loc	No. of alleles	Size range (bp)
1	Xgwm 5	3A	4	150-220
2	Xgwm 33	1A	2	125-135
3	Xgwm 60	7A	1	130
4	Xgwm 102	2D	1	150
5	Xgwm 124	1B	3	190-215
6	Xgwm 146	7B	3	140-190
7	Xgwm 159	5B	4	170-220
8	Xgwm 160	4A	1	185
9	Xgwm 165	4D	3	190-250
10	Xgwm 179	5A	5	150-190
11	Xgwm 183	3D	2	105-125
12	Xgwm 194	4D	4	125-160
13	Xgwm 205	5D	8	140-280
14	Xgwm 210	2B	4	190-240
15	Xgwm 232	1D	6	140-230
16	Xgwm 273	1B	4	160-215
17	Xgwm 282	7A	1	160
18	Xgwm 285	3B	4	180-240
19	Xgwm 292	5D	6	190-240
20	Xgwm 295	7D	6	180-260
21	Xgwm 296	2A	5	170-210
22	Xgwm 299	3B	1	210
23	Xgwm 325	6D	2	140-160
24	Xgwm 334	6A	3	110-230
25	Xgwm 337	1D	4	160-195
26	Xgwm 350	7A	4	170-220
27	Xgwm 368	4B	2	150-190
28	Xgwm 382	2A	4	140-175
29	Xgwm 383	3D	8	145-220
30	Xgwm 391	3A	3	150-180
31	Xgwm 400	7B	5	140-205
32	Xgwm 410	2B	5	160-260
33	Xgwm 425	2A	4	135-180
34	Xgwm 427	6A	6	180-220
35	Xgwm 428	7D	4	130-165
36	Xgwm 469	6D	7	160-225
37	Xgwm 495	4B	4	150-240
38	Xgwm 538	4B	4	130-175
39	Xgwm 604	5B	2	150-180
40	Xgwm 626	6B	4	110-135
41	Xgwm 637	4A	4	140-290
42	Xgwm 639	5A	3	140-160
43	Xgwm 639	5D	3	150-180
44	Xgwm 644	6B	6	150-240
45	Xgwm 644	7B	6	140-220
46	Xgwm 666	1A	5	130-145

WH 542 or in other words both the parents were not much diverse for the karnal bunt resistance trait.

Table 16: Similarity matrix among parents as obtained using the allelic diversity at 46 SSR loci

	HD 29	WH 542
HD 29	1.000	
WH 542	0.860	1.000

4.3.3 Selection of resistant and susceptible plants for bulk segregant analysis

The bulk segregant analysis was used to identify SSR markers linked to karnal bunt resistance. Bulked genomic DNA was used as a template for PCR amplification. Two bulks were prepared to obtain molecular marker linked to the karnal bunt resistance. A total of 10 resistant and 10 susceptible RILs were selected from HD29 X WH542 (F_8) segregating population based on their mean coefficient of infection. Extreme RILs 'with CI - 0 (resistant) and CI->12.0 (susceptible) were selected for preparing a bulk for SSR marker analysis. The list of 20 selected RILs, their mean CI and code used are given in Table 17.

4.3.4 Identification of SSR marker for karnal bunt resistance

Fifteen SSR primers, which detected polymorphism between the parent genotypes, were used for conducting bulked segregant analysis using two bulked DNAs. Gels displaying allelic polymorphism among the parents, resistant and

susceptible bulk for some of the SSR markers have been shown in Figs 11 & 12. Data recorded for different alleles for 15 SSR primers as present (scored as 1) or absent (scored as 0), is presented in Table 18. The SSR allelic database for both bulks and the parental wheat genotypes were used for generating similarity matrices data and UPGMA tree cluster analysis. Similarity coefficients of both the parents and bulks are presented in Table 19.

Table 17: List of twenty RILs, their coefficient of infection (CI) and code used for bulk segregant analysis

Resistant			Susceptible		
RIL No.	CI	Code	RIL No.	CI	Code
2	0	1	5	21.00	11
3	0	2	11	14.30	12
4	0	3	12	19.45	13
51	0	4	29	20.66	14
57	0	5	35	12.51	15
62	0	6	56	15.00	16
63	0	7	81	03.40	17
64	0	8	88	14.93	18
65	0	9	95	47.05	19
66	0	10	97	40.40	20

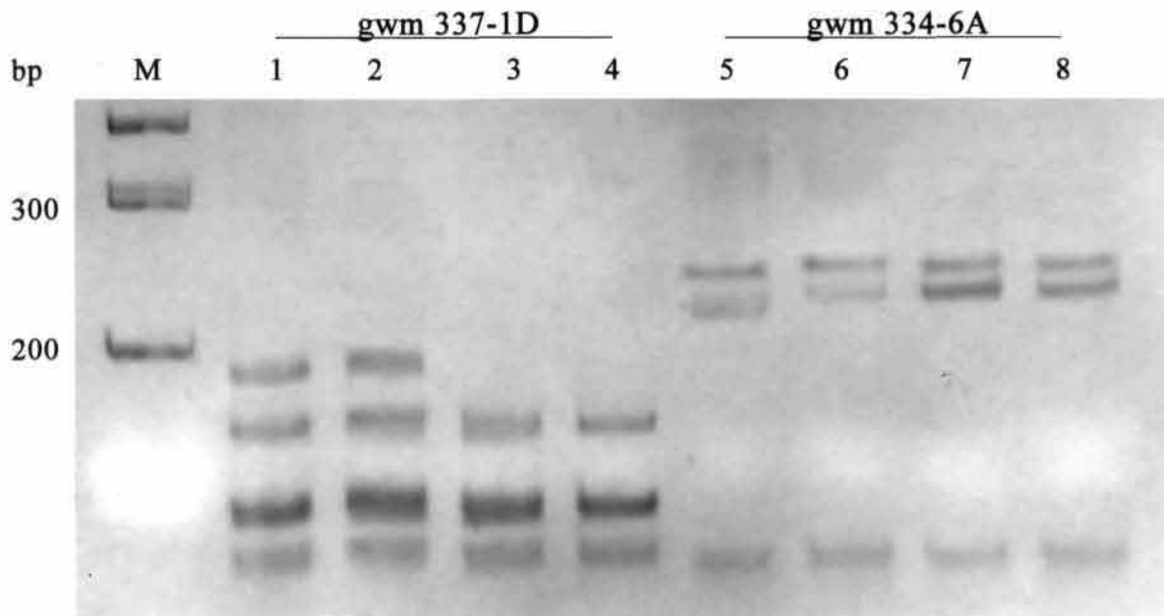


Fig. 11 : Bulked segregant analysis of RILs (representing extreme groups) with gwm 337-1D and gwm 334-6A primers. Lane M : 100-bp ladder marker; 2,6: parent HD29; 3,7: parent WH542; 1,5: bulked segregants for resistant & 4,8: bulked segregants for susceptible to karnal bunt.

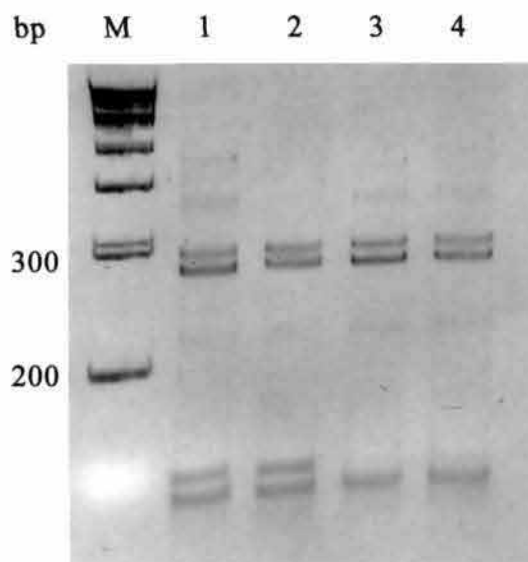


Fig. 12 : Bulked segregant analysis of RILs (representing extreme groups) with gwm 637-4A primer. Lane M : 100-bp ladder marker; 2,3: parents HD29 & WH542; 1,4: bulked segregants for resistant & susceptible to karnal bunt.

Table 18: DNA amplification profile as obtained on parents, resistant and susceptible bulk with 15 SSR primers

Marker	HD 29	Bulk R	Bulk S	WH 542
X gwm 179-5A A1	1	1	1	1
A2	0	0	0	1
A3	1	1	1	1
X gwm 183-3D A1	0	1	1	1
A2	1	1	1	1
X gwm 205-5D A1	1	0	0	0
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	1
A5	1	1	1	1
A6	1	1	1	1
A7	1	1	1	1
A8	1	1	1	1
X gwm 232-1D A1	1	1	1	1
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	1
A5	1	1	1	0
A6	1	1	1	1
X gwm 273-1B A1	1	1	1	0
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	1
X gwm 334-6A A1	1	1	1	1
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	0	0
X gwm 337-1D A1	1	1	0	0
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	1
X gwm 350-7A A1	0	0	0	1
A2	0	0	0	1
A3	1	1	1	1
A4	1	1	1	1
X gwm 382-2A A1	1	1	1	0
A2	1	1	1	1
A3	0	0	0	1
A4	1	1	1	1
X gwm 383-3D A1	1	1	1	1
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	1

Contd..

Marker	HD 29	Bulk R	Bulk S	WH 542
A5	1	1	1	1
X gwm 425-2A A1	1	1	1	1
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	0
A5	1	1	1	1
X gwm 427-6A A1	1	1	1	1
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	1
X gwm 469-6D A1	1	1	1	1
A1	1	1	1	1
A2	1	1	1	1
A3	1	1	1	0
A4	1	1	1	1
A5	1	1	1	1
X gwm 637-4A A1	1	1	1	1
A2	1	1	1	1
A3	1	1	0	0
A4	1	1	1	1
X gwm 666-1A A1	1	1	1	1
A2	1	1	1	1
A3	1	1	1	1
A4	1	1	1	1
A5	1	1	1	1

A - represents allele number
0 - represents absence of band
1 - represents presence of band

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The similarity coefficient between the two parental genotypes was 0.791. The resistant bulk showed similarity of 0.970 with HD 29 which was higher than the similarity (0.820) with WH 542. However, the susceptible bulk also showed more or less equal similarity with HD 29 (0.925) and WH 542 (0.865). The cluster analysis led to distribution of both the bulk and parental genotypes into two groups. Group 1 was further divided into two subgroups. Subgroup-I had HD 29 and resistant bulk. Subgroup-II had susceptible bulk and the two subgroups merged at similarity coefficient of 0.94 (Fig. 13). Group 2 had a lone WH 542 which merged with group 1 at similarity coefficient of 0.82. Among all the primers used with the two bulk DNAs, three primer pair (Xgwm 334-6A, Xgwm 337-1D and Xgwm 637-4A) exhibited apparent association with resistance to karnal bunt.

4.3.5 Specific SSR markers probably linked to karnal bunt resistance

Three SSR markers were detected from studies of 15 SSR markers on bulks, which could possibly be linked to karnal bunt resistance. These Xgwm 334-6A, Xgwm 337-1D and Xgwm 637-4A primers, exhibited amplification profiles characteristic of resistant and susceptible parents in the corresponding bulks following bulk segregant analysis. This indicated an association of these markers with karnal bunt resistance. To further confirm this association, selective genotyping of 5 individual RILs belonging to extreme resistant and 5 individual

Table 19: Similarity matrix among parents, resistant and susceptible bulks obtained using the allelic diversity at 15 SSR loci

	HD 29	Bulk R	Bulk S	WH 542
HD 29	1.0000			
Bulk R	0.9701	1.0000		
Bulk S	0.9253	0.9552	1.0000	
WH 542	0.7910	0.8208	0.8656	1.0000

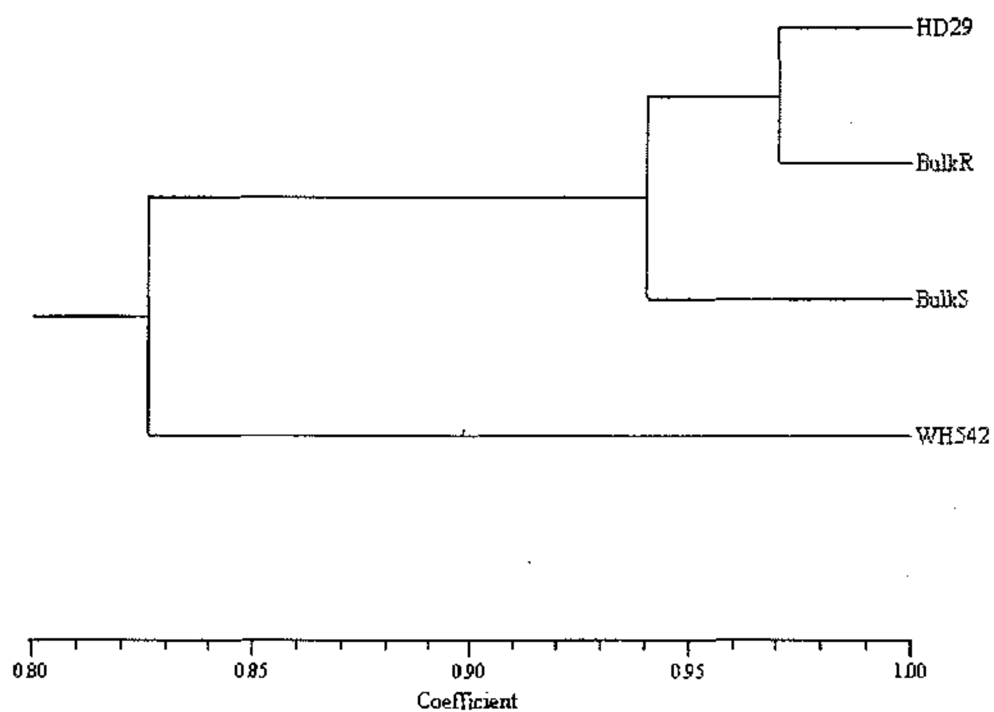

Fig.13: Dendrogram of parents and both the bulks based on SSR diversity data at 15 loci

RILs belonging to extreme susceptible bulk was carried out with these three primers. In case of Xgwm 334-6A, out of the five RILs belonging to resistant bulk, four showed profile similar to that of the resistant parent while all the five RILs belonging to susceptible bulk gave profile similar to that of susceptible parent. Using Xgwm 337-1D primer pair, the results revealed that four out of five RILs from resistant and all five RILs from susceptible bulk showed amplification profiles characteristic of the resistant and susceptible parents, respectively. Similarly, in case of Xgwm 637-4A, four out five RILs belonging to each of the two bulks showed amplification profiles characteristic of the corresponding parents (Figs. 14-16). This suggested an association of these three markers Xgwm 334-6A, Xgwm 337-1D and Xgwm 637-4A) with karnal bunt resistance.

4.4 RAPD analysis

4.4.1 Primers not showing DNA amplification

Ninety two primers of arbitrary nucleotide sequence were used to amplify DNA segments from the genomic DNA of both the parents (HD 29 and WH 542). Fifteen primers *viz.,* OPM-08, OPM-09, OPY-19, OPZ-19, OPAA-16, OPAB-13, OPQ-07, OPAF-10, OPA-06, OPE-01, OPE-02, OPH-01, OPI-01, OPI-08 and OPI-17 did not amplify genomic DNA of any of the parent.

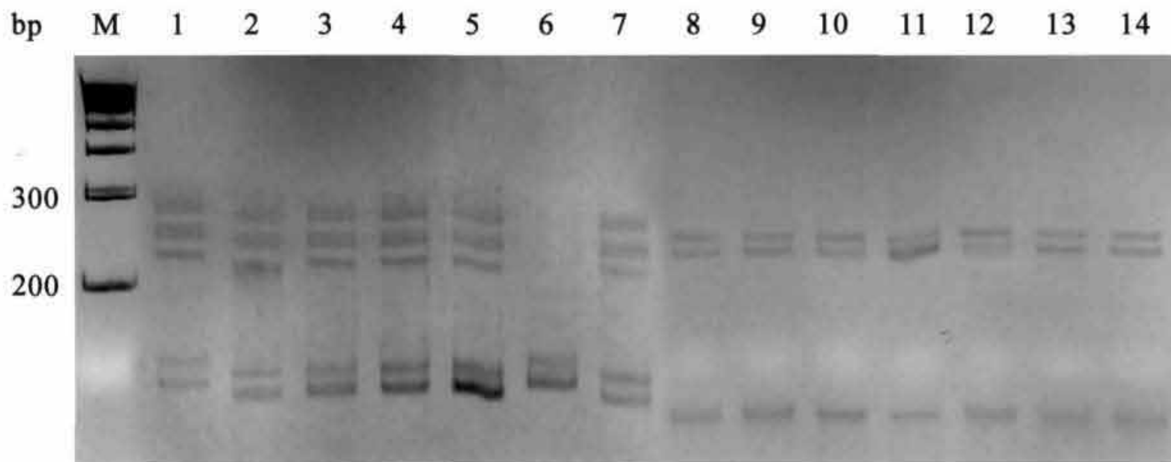

Fig. 14 : Selective genotyping of RILs (representing extreme groups)with gwm 334-6A primer. Lane M : 100-bp ladder marker;1,8: parents HD29 & WH542; 2,9: resistant & susceptible bulks; 3-7: RILs resistant to karnal bunt; 10-14: RILs susceptible to karnal bunt.

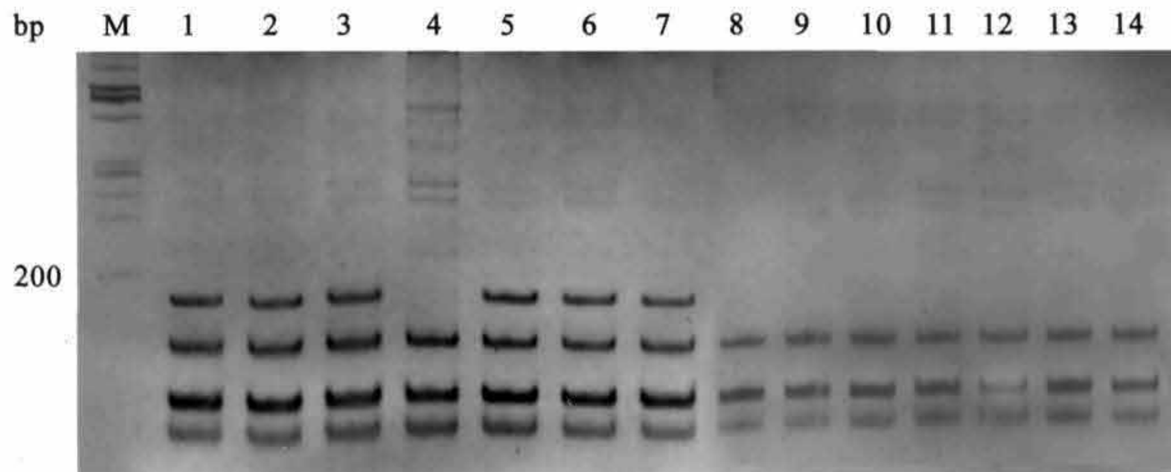

Fig. 15 : Selective genotyping of RILs (representing extreme groups)with gwm 337-1D primer. Lane M : 100-bp ladder marker;1,8: parents HD29 & WH542; 2,9: resistant & susceptible bulks; 3-7: RILs resistant to karnal bunt; 10-14: RILs susceptible to karnal bunt.

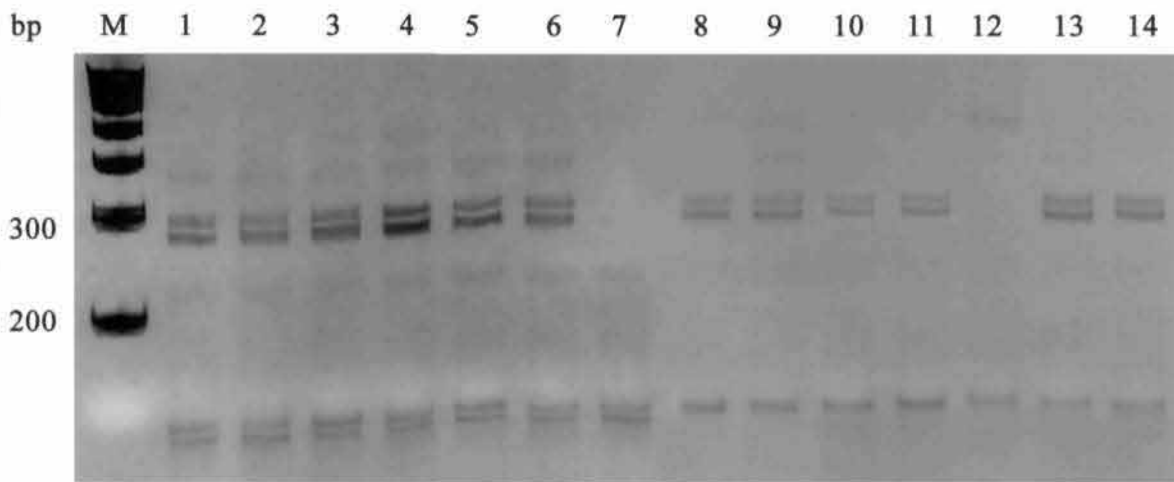

Fig. 16 : Selective genotyping of RILs (representing extreme groups)with gwm 637-4A primer. Lane M : 100-bp ladder marker;1,8: parents HD29 & WH542; 2,9: resistant & susceptible bulks; 3-7: RILs resistant to karnal bunt; 10-14: RILs susceptible to karnal bunt.

4.4.2 Primers amplifying DNA of either parent

Twelve primers, OPA-09, OPB-09, OPB-14, OPB-16, OPD-09, OPG-12, OPH-15, OPI-05, OPO-09, OPO-18, OPQ-01 and OPS-20 amplify either of the parents. So these were not included in the present study.

4.4.3 RAPD markers based polymorphism among the parents

Sixty five primers amplified DNA of both the parents, HD 29 and WH 542. Gels displaying allelic polymorphism among the parents for some of the RAPD markers have been shown in Figs 17-20. A total of 165 alleles were detected at 65 RAPD loci. Data recorded for different alleles for each primer as present (scored as 1) or absent (scored as 0), of amplified products is presented in Table 20. Out of 65 primers used in the study, both the parents showed polymorphism at 25 RAPD loci and 41 primers yielded monomorphic amplified products. The number of alleles per locus ranged from 1 (OPN-01, OPN-08, OPN-17, OPN-19, OPM-01, OPM-18, OPM-19, OPAD-06, OPAF-04, OPAH-03, OPAR-05 and OPAS-10) to 6 (OPAS-06) with an average of 2.5 alleles per locus (Table 21.).

The data matrix was entered in the NTSYS-PC programme and analysed using Simqual to generate Jaccard's similarity coefficient. The genetic similarity matrix was produced for the RAPD data are presented in Table 22. The similarity coefficient between the two parental genotype was 0.836 at 65 RAPD loci.

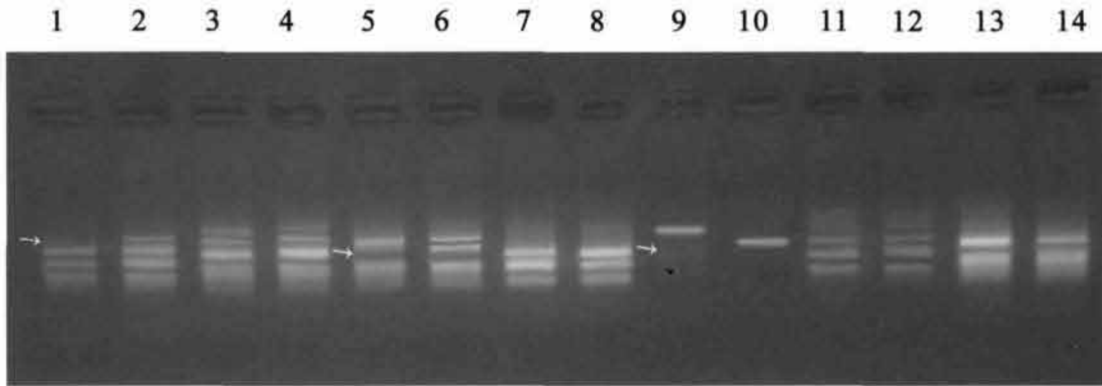

Fig. 17 : A representative sample of RAPD polymorphism between parents HD 29 (Lanes 1,3,5,7,9,11,13) and WH542 (Lanes 2,4,6,8,10,12,14) using seven primers: OPD-01 (Lanes 1,2),OPD-02 (Lanes 3,4),OPD-03 (Lanes 5,6),OPD-05 (Lanes 7,8), OPD-06(Lanes 9,10),OPD-07 (Lanes 11,12) and OPD-08 (Lanes 13,14).

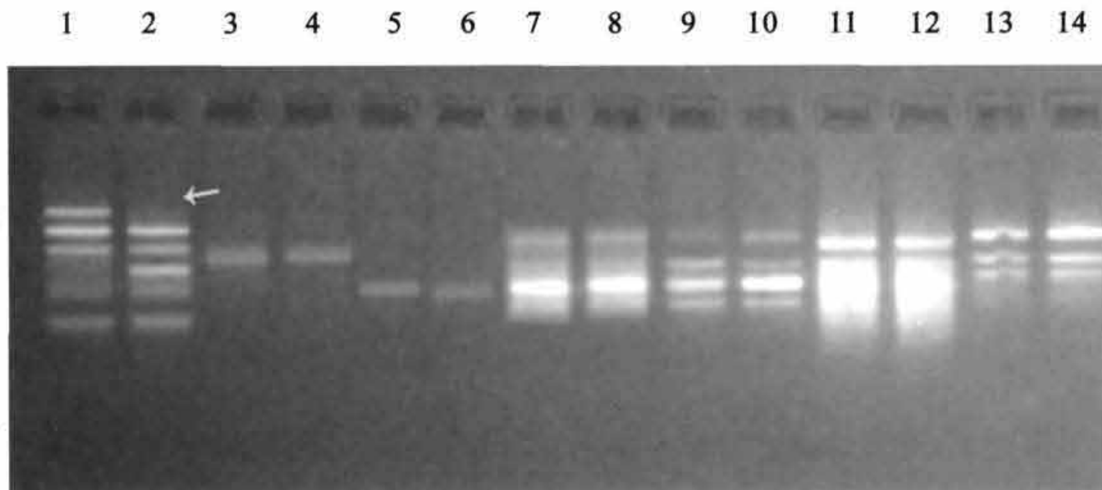

Fig. 18 : A representative sample of RAPD polymorphism between parents HD 29 (Lanes 1,3,5,7,9,11,13) and WH542 (Lanes 2,4,6,8,10,12,14) using seven primers: OPAS-06 (Lanes 1,2),OPAS-10 (Lanes 3,4),OPAR-05 (Lanes 5,6),OPAQ-07 (Lanes 7,8),OPAP-07 (Lanes 9,10),OPAO-03 (Lanes 11,12) and OPAK-03 (Lanes 13,14).

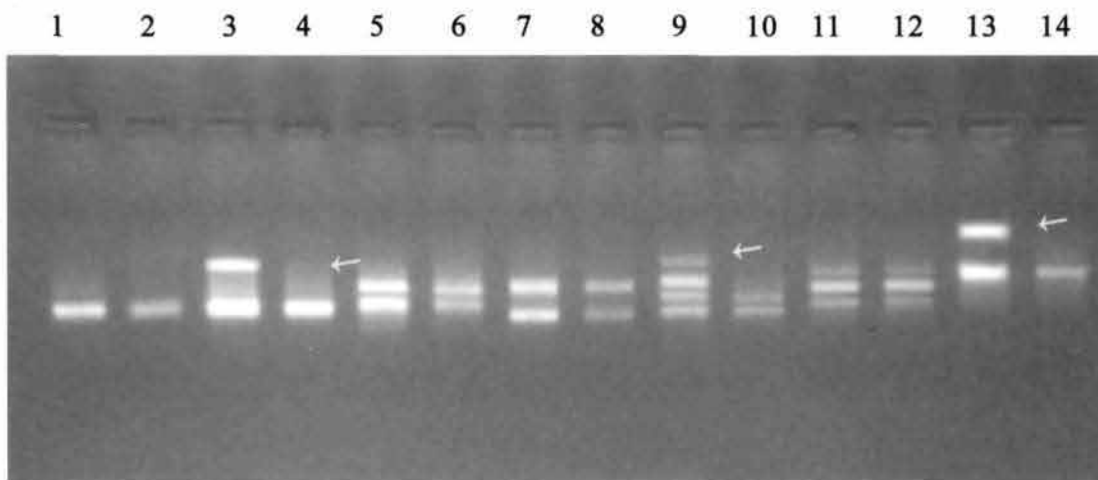

Fig. 19 : A representative sample of RAPD polymorphism between parents HD 29 (Lanes 1,3,5,7,9,11,13) and WH542 (Lanes 2,4,6,8,10,12,14) using seven primers: OPN-08 (Lanes 1,2),OPN-09 (Lanes 3,4),OPN-10 (Lanes 5,6),OPN-11 (Lanes 7,8), OPN-12 (Lanes 9,10),OPN-13 (Lanes 11,12) and OPN-14 (Lanes 13,14).

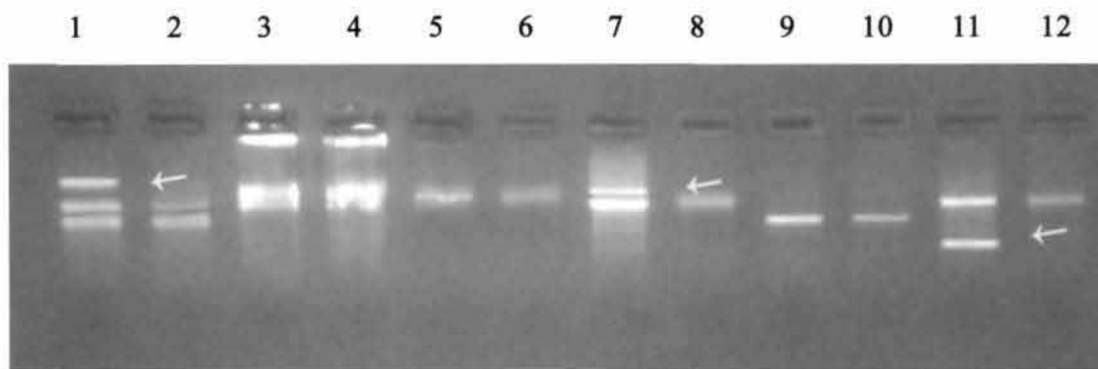

Fig. 20 : A representative sample of RAPD polymorphism between parents HD 29 (Lanes 1,3,5,7,9,11) and WH542 (Lanes 2,4,6,8,10,12) using six primers: OPN-15 (Lanes 1,2),OPN-16 (Lanes 3,4),OPN-17 (Lanes 5,6),OPN-18 (Lanes 7,8),OPN-19 (Lanes 9,10) and OPN-20 (Lanes 11,12).

Table 20: DNA amplification profile as obtained on parents with 65 RAPD primers

Marker	HD 29	WH542	Marker	HD 29	WH542
OPD-01 A1	0	1	OPN-10 A1	1	1
A2	1	1	A2	1	1
A3	1	1	OPN-11 A1	1	1
A4	1	1	A2	1	1
OPD-02 A1	1	1	OPN-12 A1	1	0
A2	1	1	A2	1	0
A3	1	1	A3	1	1
A4	1	1	A4	1	1
OPD-03 A1	1	1	OPN-13 A1	1	1
A2	0	1	A2	1	1
A3	1	1	A3	1	1
OPD-05 A1	1	1	OPN-14 A1	1	0
A2	1	1	A2	1	1
A3	1	1	OPN-15 A1	1	0
OPD-06 A1	1	0	A2	1	1
A2	0	1	A3	1	1
OPD-07 A1	1	1	OPN-16 A1	1	1
A2	1	1	A2	1	1
A3	1	1	OPN-17 A1	1	1
OPD-08A1	1	1	OPN-18 A1	1	0
A2	1	1	A2	1	1
OPN-01 A1	1	1	OPN-19	1	1
OPN-02 A1	1	1	OPN-20 A1	1	1
A2	1	1	A2	1	0
OPN-03 A1	1	0	OPM-01 A1	1	1
A2	1	1	OPM-02 A1	1	1
A3	1	1	A2	1	1
OPN-04 A1	0	1	A3	1	1
A2	1	1	A4	1	1
A3	1	1	OPM-03 A1	1	1
OPN-05 A1	1	0	A2	1	1
A2	1	1	A3	1	1
A3	1	1	OPM-04 A1	1	0
OPN-06 A1	1	1	A2	1	1
A2	1	1	OPM-05 A1	1	1
A3	1	1	A2	1	1
OPN-07 A1	1	1	A3	1	1
A2	1	1	A4	1	1
A3	1	1	OPM-06 A1	1	0
OPN-08 A1	1	1	A2	1	1
OPN-09 A1	1	0	A3	1	1
A2	1	1	OPM-07 A1	1	1

Contd..

Marker	HD 29	WH542	Marker	HD 29	WH542
A2	1	1	A4	1	1
OPM-10 A1	1	1	OPAH-03 A1	1	1
A2	1	1	OPAH-10 A1	1	1
OPM-11 A1	1	0	A2	1	1
A2	1	1	A3	1	1
OPM-12 A1	1	1	OPAI-08 A1	1	0
A2	1	1	A2	1	1
A3	1	1	A3	1	1
OPM-13 A1	1	1	A4	1	1
A2	1	1	A5	1	1
OPM-14 A1	1	1	OPAJ-09 A1	1	1
A2	1	1	A2	1	1
OPM-15 A1	1	0	OPAK-03 A1	1	1
A2	1	1	A2	1	1
A3	1	1	A3	1	1
A4	1	1	OPAL-06 A1	1	1
OPM-16 A1	1	1	A2	1	1
A2	1	1	OPAM-07 A1	1	1
A3	1	1	A2	1	1
A4	1	1	A3	1	1
OPM-17 A1	1	1	OPAN-10 A1	0	1
A2	1	0	A2	1	1
OPM-18 A1	1	1	A3	1	0
OPM-19 A1	1	1	A4	1	1
OPM-20 A1	1	0	OPAO-03 A1	1	1
A2	1	1	A2	1	1
A3	1	1	OPAP-07 A1	1	1
OPAC-07 A1	1	1	A2	1	1
A2	1	1	A3	1	1
A3	1	1	A4	1	1
A4	1	1	OPAQ-07 A1	1	1
OPAD-06 A1	1	1	A2	1	1
OPAE-10 A1	0	1	OPAR-05 A1	1	1
A2	1	1	OPAS-06 A1	1	0
A3	1	1	A2	1	1
OPAF-04 A1	1	1	A3	1	1
OPAG-04 A1	1	1	A4	1	1
A2	1	0	A5	1	1
OPAG-10 A1	1	1	A6	1	1
A2	1	1	OPAS-10 A1	1	1
A3	1	1			

A – represents allele number
0 - represents absence of band
1 - represents presence of band

Table 21: Allelic diversity among parents HD29 and WH 542 as assessed by 65 RAPD markers

Number of primersused	65
Number of alleles	165
Range of alleles	1-6
Average number of alleles	2.5
Number of polymorphic markers	25
Number of monomorphic markers	40

Table 22: Similarity matrix among parents HD29 and WH 542 as obtained using the allelic diversity at 65 RAPD loci

	HD 29	**WH 542**
HD 29	1.000	
WH 542	0.836	1.000

4.4.4 Molecular analysis of HD 29 X WH 542 segregating RILs using RAPD markers

A total of 25 RAPD markers, which were found to be polymorphic on both the parents were tested on both the resistant and susceptible bulks. With 23 of the 25 RAPD primers, no apparent association between the markers and the karnal bunt resistance was observed. Two primers (OPN – 04 and OPM - 20) exhibited amplification profiles characteristics of extreme resistant and susceptible parents in the corresponding bulks following bulk segregant analysis. Gels displaying allelic polymorphism among the parents, resistant and susceptible bulks for two RAPD markers have been shown in Fig. 21.

4.4.5 RAPD markers associated with karnal bunt resistance

The expected association of two markers *viz.* OPN-04 & OPM-20 with resistance to karnal bunt was suggested using selective genotyping of individual RILs belonging to the two bulks. Using the OPN-04 primer, 4 out of 5 RILs belonging to the resistant bulk showed the characteristic amplification profile of the resistant parent and all 5 RILs showed the characteristic amplification profile of the susceptible parent. With the OPM-20 primer, it was observed that 4 out of 5 RILs from each of the two bulks showed amplification profiles characteristic of the corresponding parents (Figs. 22 & 23). This suggested that OPN-04 and OPM-20 arc associated with karnal bunt resistance.

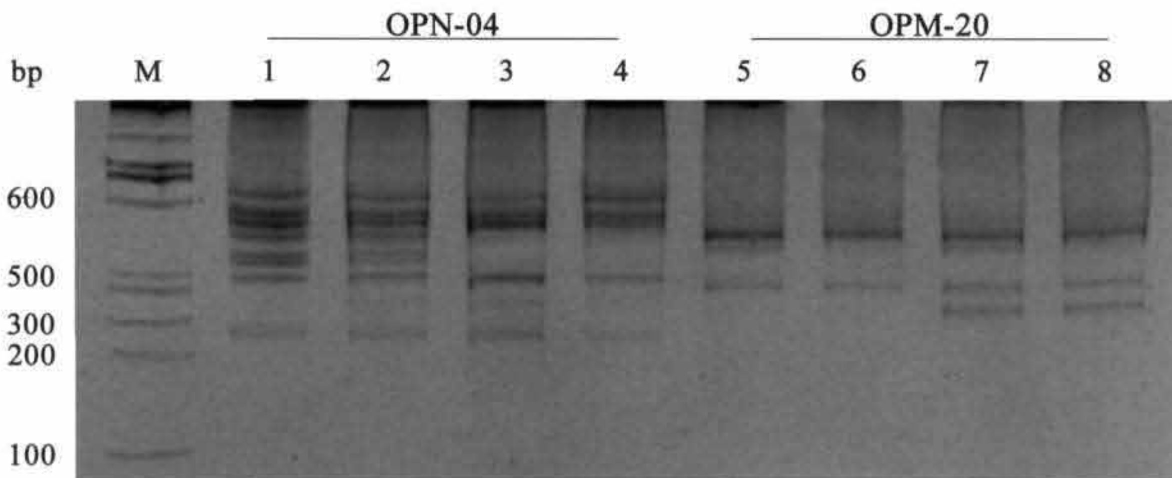

Fig. 21 : Bulked segregant analysis of RILs (representing extreme groups) with OPN-04 and OPM-20 primers. Lane M: 100-bp ladder marker; 3,7: parent HD29; 2,6: parent WH542; 4,8: bulk segregants for resistant and 1,5: bulk segregants for susceptible to karnal bunt.

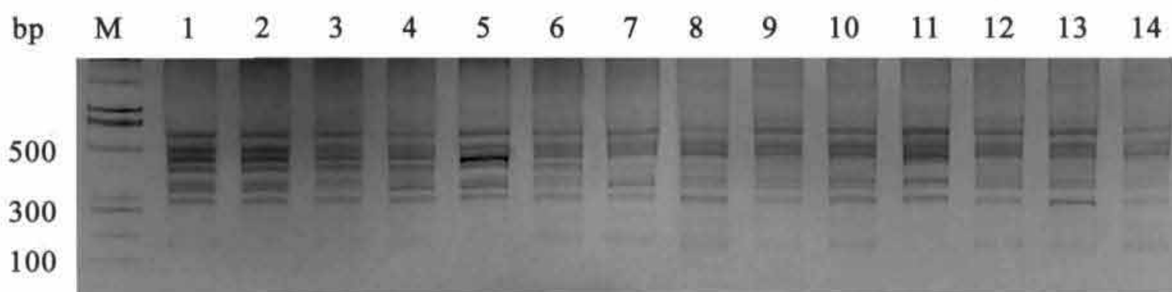

Fig. 22 : Selective genotyping of RILs (representing extreme groups) with OPN-04 primer. Lane M: 100-bp ladder marker; 1,8: parents WH542 & HD29; 2,9: susceptible & resistant bulks; 3-7: RILs susceptible to karnal bunt; 10-14: RILs resistant to karnal bunt.

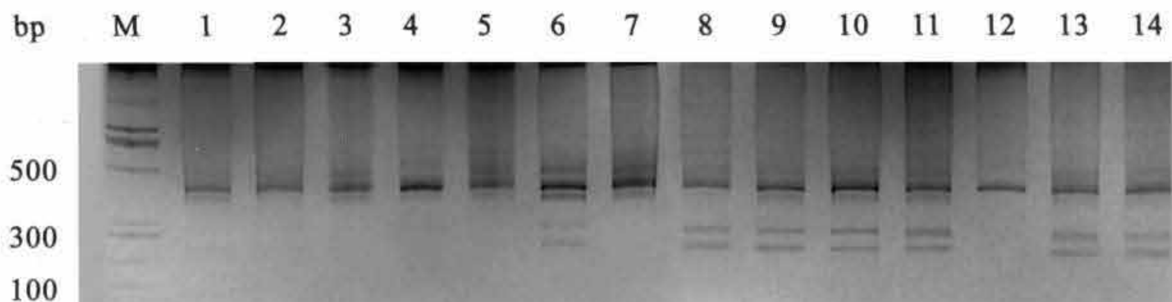

Fig. 23 : Selective genotyping of RILs (representing extreme groups) with OPM-20 primer. Lane M: 100-bp ladder marker; 1,8: parents WH542 & HD29; 2,9: susceptible & resistant bulks; 3-7: RILs susceptible to karnal bunt; 10-14: RILs resistant to karnal bunt.

5

DISCUSSION

Wheat among cereals being elite crop gets infected with number of diseases. Karnal bunt (*Neovossia indica*) is one of them. The pathogen causes infection on the floral parts, entering into the glumes, rachis and ovary. Although yield losses are low, international quarantine policies against the disease may restrict the free flow of the global wheat trade. Conventional approaches for controlling karnal bunt include cultural practices such as crop rotation, sowing of disease free seeds, and adjustment of the time of irrigation to minimize disease infection. The most effective and economical method of disease management is through host plant resistance. The wheat cultivars with sufficient levels of resistance to karnal bunt have been observed among Indian, Chinese and Brazilian wheats. Developing karnal bunt resistant wheat cultivars with conventional methods is time consuming and labour intensive. An alternative to direct selection for karnal bunt resistance is the indirect selection for DNA markers linked to genes conferring karnal bunt resistance. Many of the linked markers identified in various studies are restriction fragment length polymorphisms (RFLPs) that are

co-dominant and reproducible but are of limited use in wheat breeding programme. On an average, only 15-20 per cent of the RFLPs are polymorphic between lines of *T. aestivum*. Microsatellite belongs to an important class of molecular markers, since they are abundant in all animal and plant genomes and are hypervariable in nature, easier to use and more polymorphic than RFLP markers. Therefore, present investigation was undertaken to evaluate the recombinant inbred lines for karnal bunt resistance and to study the DNA polymorphism among RILs populations using microsatellite markers.

5.1 Assessment of recombinant inbred lines for karnal bunt resistance

Green house studies were conducted on parents viz., HD 29 and WH 542 and their recombinant inbred lines (RILs) generated through single seed descent method. Percentage of seeds infected with karnal bunt was calculated. Analysis of variance indicated that there were significant variations among the RILs. Segregation patterns of recombinant inbred lines indicated that the resistance of HD29 is conferred by a single dominant gene. Singh *et al.* (1995a) while determining the genetic basis of resistance in fourteen Mexican genotypes also reported that resistance in Cettia, Irena, Turacio, Oparta, Picus and Yaco was conditioned by a single dominant gene. The distribution of karnal bunt disease severity on the RILs was skewed towards the resistant parent in both the years, thereby suggesting the segregation of multiple genes with dominant or complementary gene action in wheat line HD 29. The inheritance of karnal bunt

resistance have also been reported to be controlled by 2 recessive genes (Singh *et al.*, 1993a; Fuentes-Davila *et al.*, 1995). Identification of RILs with lower and higher disease incidence than HD 29 and WH 542 respectively, suggested that WH 542 probably has minor genes for karnal bunt resistance. Singh *et al.* (1996) reported that resistance was controlled by few major gene (s) along with some minor genes.

5.2 SSR marker based polymorphism among parents

In conventional plant breeding, during selection of desirable plants from the segregating population, the plant breeder is faced with the following problems: (i) a large segregating population needs to be screened for a desirable trait e.g., disease resistance, etc,; (ii) one has to wait for advanced generations (F_5, F_6) to exercise selection for quantitative traits, where the selection in early generation is not effective; (iii) it becomes very difficult, if not possible to screen a segregating population for a desired trait when the trait is influenced by environment; (iv) contrasting forms are often not distinguishable at the seedling stage, making it necessary to grow population up to adult stage; (v) it is difficult to undertake pyramiding of resistance genes, since selection of additional genes in presence of an existing resistance gene would be difficult. In view of these difficulties, the plant breeder would like to exercise indirect marker aided selection at the seedling stage in early generations, if possible. Availability of tightly linked molecular markers for a trait will facilitate such an indirect selection and help plant breeding

by saving time and expense; for this purpose microsatellites have become the markers of choice.

The polymorphism between both the parental genotypes, obtained by microsatellite marker amplification, is a strong and authentic tool for the assessment of genetic diversity and polymorphism (Lang *et al.* 2001a, b). The data of polymorphism obtained between karnal bunt resistant and susceptible parent was used for undertaking genes/ QTL mapping studies. A high level of polymorphism was observed among the parental genotypes using 46 SSR primers. Of these 15 (32 per cent) 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 more informative than RFLPs (Plashchke *et al.*, 1995; Roder *et al.*, 1995; Ma *et al.*, 1996; Bryan *et al.*, 1997; Singh *et al.*, 2003) in bread wheat. If a large number of SSR markers are developed for wheat, the observed rate of intraspecific allelic variation should be sufficient to construct genetic maps of adequate density to allow the detection of QTLs / genes with a large effect.

A total of 179 alleles were detected at 46 SSR loci with an average of 3.9 alleles per locus (ranges from 1-8 alleles per locus). Since microsatellite primers are locus specific, only one specific locus is expected to be amplified by each primer and it was unexpected that one primer amplified more than one locus. In two earlier studies also, more than one locus per microsatellite primer pair was

detected and mapped in bread wheat (Roder *et al*, 1998a; Stephenson *et al*, 1998; Vasu *et al*, 2000). Prasad *et al.* (2000) also reported relatively higher alleles per locus. Struss and Plieske (1998) reported 8.6 alleles per locus in barley. 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. The detection of multiple alleles per locus is probably due to heterogeneity of the accessions rather than genetic heterozygosity. The size of PCR products amplified ranged from 105 to 290 bp. Variation in the size of SSR alleles results from a change in the number of repeats. In our study, relative to other SSRs, more alleles were detected at $(GA)_n$ loci followed by $(GT)_n$. In barley also, more alleles were detected for $(GA)_n$ than for $(GT)_n$ repeats (Struss and Plieske , 1998).

5.3 Identification of SSR marker for karnal bunt resistance

In this study, a total of 15 polymorphic SSR markers were tested on two bulked DNAs, one obtained by pooling DNA from 10 karnal bunt resistant RILs and the other similarly obtained from 10 karnal bunt susceptible RILs. Two parental wheat genotypes, namely HD 29 and WH 542 had a similarity coefficient 0.86, which indicates that two parents are genetically not much divergent. The genetic diversity between the two parental lines was evident from the results of UPGMA based tree cluster. Ahmed (2002) reported that genetic similarities ranged from 30.1 ('Era' and 'Klasic') to 90.1 ('Neepawa and 'Thatcher') between genotypes. The low genetic diversity in our study might be due to the reason that

only the genome regions conferring karnal bunt resistance were tagged, since the primers generate polymorphism between resistant and susceptible parents. The results of genetic diversity based on the dendrogram derived using genetic similarity found that resistant parent, resistant bulk and susceptible bulk were present in the same group.

5.4 Specific SSR markers probably linked to karnal bunt resistance

Problem arises in finding useful genes / QTLs for a particular trait when numerous genes / QTLs are associated with the expression of that trait. The smaller the contribution of individual gene, the more difficult they are to detect. It is important for breeding crops that all the major QTLs associated with a trait should be detected. Several new types of markers are now available for gene/ QTL mapping. SSR markers are certainly the best for most purposes (McCouch *et al*, 2001). These markers are highly polymorphic, abundant and position of SSR markers are mapped on linkage map. 15 SSR markers distributed over all the chromosomes of wheat genome were selected for the further studies. Earlier studies have reported the presence of genes/ QTLs on many chromosomes. Vasu et al. (2000) tagged microsatellite markers for karnal bunt resistance gene (s); microsatellite loci gwm382, gwm369, gwm637, gwm156 and gwm617 mapped on 2AS, 3AS, 4AL, 5AL, and 6AL chromosomes, respectively were found to be associated with karnal bunt resistance. Singh *et al.* (2003) linked SSR marker *gwm538*, for marker-assisted selection against karnal bunt resistance in

wheat. In the present study, alleles amplified by 3 of the 15 SSR markers (Xgwm 334, Xgwm 337 and Xgwm 637) located on chromosomes 6AS, 1DS and 4AL were present in resistant parent, resistant bulk and individual RILs indicating their association with karnal bunt resistance. Several other researchers have associated molecular markers to agronomic traits in wheat. Liu *et al.* (2001) reported that SSR markers WMS67 and WMS213 mapped on 5BL in wheat genome were linked to salt tolerance gene(s). Buerstmayer *et al.* (2002) identified the QTL 1B associated with high molecular weight glutenin locus. Khletskina *et al.* (2002) mapped three red coleoptile colour genes in wheat on chromosomes 7AS, 7BS and 7DS, respectively. Our results, together with the above reports, suggest that several genes / QTLs for karnal bunt resistance reside 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 karnal bunt in wheat. Because RFLP markers in a polyploid crop like wheat are relatively unsuitable for marker assisted breeding, additional PCR based markers should be developed to make marker-assisted selection for efficient karnal bunt resistance.

5.5 Molecular analysis using RAPD markers

One of the advantages of RAPDs method is that arbitrarily designed random primers for PCR provide a broadened scope of potential templated DNA. The entire plant genome is targeted for primer annealing, facilitating development

of a higher density map. Polymorphism can be successfully scored and used for studying gene tagging, genetic variation and diversity. The RAPD protocol was thus optimized with respect to PCR reaction components and amplification conditions. Such optimizations of RAPD analysis has been done by Devos and Gale (1992).

Ninety two decanucleotide primers of random sequences having 60 per cent or higher (G+C) content were used for priming amplification of DNA for RAPD analysis. Of the 92 primers used 15 did not amplify DNA of any of the wheat genotypes. These primers perhaps did not find any complementary binding sequences on the genomic DNA of any of the wheat genotypes. Other workers have also reported such non-amplifying primers. Tao *et al.* (1993) used 30 primers for studying polymorphism in sorghum and one primer did not show amplification. Zhenshen *et al.* (1996) used 150 primers for studying polymorphism in Chinese common wild and cultivated rice, 41 of which did not amplify any genotype. Twelve primers amplified, either of the parents. Similarly, Kresovich *et al.* (1995) also observed no amplification in one or two *Brassica* lines. In the present investigation, a total of 165 alleles were detected by 65 primers in two genotypes with an average of 2.5 alleles per locus (ranging from 1-6 alleles per locus). About 38 per cent of these were polymorphic. Rafalski *et al.* (1991) postulated that in RAPD reaction, the composition of amplified products was determined by a competition between potential priming site in the template

rather than by the total number of priming sites available. It is for this reason, variation in amplification and polymorphism is observed for different primers.

The two parents had a similarity coefficient of 0.83. The level of RAPD polymorphism within wheat itself has been reported to be low (Devos and Gale, 1992). Joshi and Nguyen (1993) in a study of common bread wheat for 109 RAPD markers found that similarity among cultivars ranges from 0.60 to 0.90. Sun *et al.* (2003) also reported genetic similarity ranging from 0.64 to 0.98, while studying different level of Fusarium resistance in wheat using RAPD.

A total of 25 polymorphic RAPD markers were tested on two bulked (resistant and susceptible) DNAs. With 23 of the 25 primers, no apparent association between the markers and karnal bunt resistance was observed. The remaining two primers, OPN-04 and OPM-20 were found to be associated with the karnal bunt resistance as they exhibited amplification profile characteristic of resistant and susceptible parents in the corresponding bulks followed by bulk segregant analysis and selective genotyping of individual RILs. Demeke *et* al. (1996) identified a DNA marker for Bt-10 common bunt resistance gene in wheat. Lili *et al.* (1996) reported that one RAPD marker, specific to chromosome arm 6VS, OPH17-1900 could be used as a molecular marker for the detection of gene *Pm21* in breeding material. Hu *et al.* (1997) detected 3 RAPD markers closely linked to a gene for resistance to *B. graminis*, causes powdery mildew in wheat. Robert *et* al. (1999) reported one RAPD marker OP-Y15 (580)

was closely linked to the yellow rust resistance gene (*Yr17*). Cherkuri *et al.* (2003) tagged leaf rust resistance gene *Lr19* with a RAPD marker S73 (728). Sun *et al.* (2003) detected three RAPD markers; H191000, F2500 and B12400 associated with FHB-resistant genotypes. He and Huges (2003) reported two markers $UBC548_{590}$ and $UBC274_{988}$, flanked the resistance gene with a map distance of 9.1 and 18.2 cM, respectively, for resistance to common bunt *Tilletia tritici* in spelt wheat. Two RAPD markers have been detected in the present investigation associated with karnal bunt resistance. These can be useful for marker assisted breeding and can also be used as candidate markers for further gene mapping and cloning.

6

SUMMARY AND CONCLUSIONS

The present investigations were carried out on two genotypes namely, HD 29 and WH 542, and their recombinant inbred lines. The investigations were conducted to evaluate the recombinant inbred lines for karnal bunt resistance and to study DNA polymorphism among RILs populations using molecular markers. The two parents and their RILs were sown in green house for *in vivo* studies. DNA was isolated from both the parents and RILs and further subjected to polymorphic studies. The major findings of the study are given below:

1. The range of coefficient of infection (CI) on the RILs was 0– 81.25 and 0–80.80 in years 2001 and 2002, respectively. Analysis of variance for coefficient of infection and per cent infected grains showed significant variations among all the recombinant inbred lines.

2. There was a wide variation among the 104 RILs for karnal bunt resistance. The distribution of karnal bunt disease on the RILs was towards the resistant parent type in both the years.

3. The Chi-square data suggested goodness fit ($\chi^2 = 0.05$) indicating a single dominant gene was responsible for karnal bunt resistance.

4. A good quality of high molecular weight DNA was obtained from the leaves of two wheat genotypes and their RILs. The SSR and RAPD conditions were optimized.

5. Microsatellite markers based fingerprint database were generated using 46 SSR primers for both the parents differing in karnal bunt resistance. A total of 179 alleles were detected at 46 SSR loci. Large number of alleles showed the superiority of microsatellites in detecting DNA polymorphism.

6. Out of 46 primers used in the study, both the parents showed polymorphism at 15 SSR loci and 31 primers yielded monomorphic amplified products.

7. The number of alleles per locus ranged from 1(Xgwm 60, Xgwm 102, Xgwm 160, Xgwm 282 and Xgwm 299) to 8 (Xgwm 205 and Xgwm 383) with an average of 3.9 alleles per locus.

8. The overall size of PCR products amplified ranged from 105 to 290 bp. The molecular size difference between the smallest and the largest allele at a SSR locus varied from 0 (Xgwm 60, Xgwm 102, Xgwm 160, Xgwm 282 and Xgwm 299) to 150 bp (Xgwm 637).

9. SSR loci with di-nucleotide motifs were found to be more polymorphic as compared to those with tri or tetra-nucleotide motif. Loci with GA repeat motif were found to be more polymorphic compared to loci with GT, CT and CA motifs.

10. The similarity matrix showed a close genetic relationship between both the parents, HD 29 and WH 542.

11. The cluster analysis led to distribution of both the bulk and parental genotypes into two groups. Group 1 was further divided into two subgroups. Subgroup-I had HD 29 and resistant bulk. Subgroup-II had susceptible bulk and the two subgroups merged at similarity coefficient of 0.94. Group 2 had alone WH 542, which merged with group 1 at similarity coefficient of 0.82.

12. Among all the primers used with the two bulk DNAs, three primer pairs (Xgwm 334-6A, Xgwm 337-1D and Xgwm 637-4A) were identified to be associated with karnal bunt resistance.

13. A total of 92 random sequence commercially available decanucleotide primers of 60 per cent or more of G+C contents were used for the amplification of DNA for RAPD analysis. Of these 15 primers did not amplify DNA of any of the parents, while 12 primers amplified either of the parents.

14. Sixty five primers amplified both the parental genotypes. These primers produced a total of 165 alleles. Out of 65, 25 primers were polymorphic while 40 primers were monomorphic.

15. Total number of DNA fragments amplified by a specific primer varied from minimum of one (OPAS-10, OPAR-05, OPAH-03, OPAD-06, OPAF-04, OPN-01, OPN-08, OPN-17, OPN-19, OPM-01, OPM-18 and OPM-19) to six (OPAS-06) with an average of 2.5 alleles per locus.

16. The genetic similarity coefficient between the two parental genotypes was 0.836 at 65 RAPD loci.

17. Two primers (OPN-04 and OPM-20) exhibited amplification profiles characteristics of extreme resistant and susceptible parents in the corresponding bulks following bulk segregant analysis and selective genotyping of RILs. This suggested that OPN-04 and OPM-20 were associated with karnal bunt resistance in wheat.

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