Screening of IR50 x Rathu Heenati F_7 RILs and identification of SSR markers linked to Brown Planthopper (*Nilaparvata lugens Stål*) resistance in Rice (*Oryza sativa* L.)

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ABSTRACT

Screening of IR50 x Rathu Heenati F_7 RILs and identification of SSR markers linked to Brown Planthopper (*Nilaparvata lugens* Stål) resistance in Rice (*Oryza sativa* L.)

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A total of 268 F_7 RILs derived between a Brown Planthopper (BPH) susceptible IR50 and moderately resistant Rathu Heenati were phenotyped for their level of resistance against BPH by the standard seedbox screening test (SSST) in the greenhouse. The parents namely IR50 and Rathu Heenati had the mean score of 5 and 3 respectively. Among the F_7 RILs, the leaf damage score ranged from 2.0 to 9.0. Out of the 268 F_7 RILs screened, 34 lines were found to be resistant with a damage score between 1 and 3.9, 46 lines were found to show moderate resistance reaction with a damage score between 4 and 4.9, 151 lines were found to be moderately susceptible with a damage score between 5 and 8.9 and 37 lines were scored as susceptible with a damage score 9. In the present study, a total of 53 SSR primers mapped on the chromosome 3 were used to screen the polymorphism between the parents IR50 and Rathu Heenati, out of which eleven were found to be polymorphic between IR50 and Rathu Heenati. The eleven primers that have shown polymorphism between the IR50 and Rathu Heenati parents were genotyped in a set of 5 resistant RILs and 5 susceptible RILs along with the parents for co-segregation analysis. Among the eleven primers, two primers namely RM3180 (18.22 Mb) and RM2453 (20.19 Mb) showed complete co-segregation with resistance.

LIST OF ABBREVIATION

AFLP	Amplified Fragment Length Polymorphism
APS	Ammonium per sulphate
ASAL	*Allium sativum leaf agglutinin*
BAC	Bacterial Artificial Chromosome
BPH	Brown Planthoppper
BSA	Bulked Segregant Analysis
CTAB	Cetyl Trimethyl Ammonium Bromide
DAS	Days After Sowing
DNA	Deoxyribo Nucleic acid
EDTA	Ethylene Diamine Tetraacetic Acid
EST	Expressed Sequence Tag
GI	Galvanized iron
GLH	Green leafhopper
GNA	*Galanthus nivalis agglutinin*
HPR	Host Plant Resistance
IPM	Integrated pest management
IRM	International Rice Microsatellite Initiative
IRRI	International Rice Research Institute
ISSR	Inter Simple Sequence Repeat
LOD	Logarithm of Odd
MAS	Marker Assisted Selection
Mb	Megabase
NILs	Near Isogenic Lines
OP	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism

RILs	Recombinant Inbred Lines
SNP	Single Nucleotide Polymorphism
SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeats
SSST	Standard Seedbox Screening Test
STR	Short Tandem Repeat
STS	Sequence Tagged Site
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
WBPH	Whitebacked Planthopper

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INTRODUCTION

Globally, more than 3 billion people from Asia and other countries depend on rice (*Oryza sativa*, *L*.) as their staple food, and by 2025 about 60% more rice must be produced to meet the needs of the growing population (Khush, 1997). Rice productivity is adversely impacted by numerous biotic and abiotic factors. Diseases and insect pests are the major biotic agents causing significant yield losses. An approximate 52% of the global production of rice is lost annually owing to the damage caused by biotic factors, of which ~21% is attributed to the attack of insect pests (Yarasi *et al*.,2008). Productivity losses resulting from herbivorous insects have been estimated between 10 – 20% for major crops grown worldwide (Ferry *et al*., 2004). Rice is infested by more than 100 species of insects. About 20 of them are considered as serious pests as they cause significant damage to rice crop. Among them, brown plant hopper (BPH), *Nilaparvata lugens* Stål, is one of the most destructive insect pests causing significant yield loss in rice cultivars every year (Khush *et al.,* 1997; Sogawa *et al*., 2003). In addition to causing physiological damage to rice plant, BPH also causes indirect damage by acting as a vector for rice grassy stunt virus and ragged stunt virus (Heinrichs, 1979). The details on BPH outbreak in India were reviewed by Gunathilagaraj *et al*., (1997).

The primary methods of control are chemical insecticides and host plant resistance (HPR) as part of an integrated pest management (IPM) strategy. The cost of chemical control is often exorbitant, destroys the natural balance of BPH predators that helps to keep the BPH populations in check and can ultimately cause development of new insecticide resistant strains. Host - plant resistance is the most effective way of controlling BPH, and thus, insect resistance breeding has priority in rice improvement programs.

DNA marker - based technology is being increasingly used to overcome difficulties of plant breeding based on phenotypic characters like insect resistance. Molecular breeding approaches facilitate the early and efficient selection for resistance genes. It is most appropriate for inter-sub specific and intra-specific transfer of insect resistance that has been difficult to improve using conventional methods. It also paves the way for selecting the target gene based on DNA marker with a predictable rate of accuracy.

The availability of molecular linkage maps in rice (McCouch *et al.,* 1988; Causse *et al.,* 1994; Kurata *et al.,* 1994; Huang *et al.,* 1997; Chen *et al.,* 1997; Harushima *et al.,* 1998; Temnykh *et al.,* 2000) has facilitated the identification and easy manipulation of major genes and polygenes (Quantitative Trait Loci or QTL), conferring resistance to insects. Molecular marker technique has

opened the possibility for marker assisted selection and breeding using gene-tags, to evolve durably resistant cultivars in shorter span of time with greater accuracy.

Hence, in HPR programmes, screening diverse germplasm and identification of tightly linked markers is more important, as pest populations continue to change their virulence pattern and new genes for resistance must be constantly identified (Panda and Khush, 1995). Map-based cloning represents one possible approach to isolate BPH resistance genes and elucidating the BPH resistance mechanism in rice. Until now, many genes have been assigned to, or mapped on, rice chromosomes 3, 4, 6, and 12, using RFLP and microsatellite markers (Hirabayashi *et al.*,1999; Murata *et al.*, 2000; Kawaguchi *et al.*, 2001). These linkage maps, however, are not fine enough for map-based cloning. To achieve map-based cloning, construction of a high-resolution linkage map with DNA markers is required.

Researchers have succeeded in identifying 21 major genes associated with resistance to BPH and locating them on the genetic map of rice. It has long been proposed that moderate and polygenic resistance to insect pests, including BPH, should provide more durable resistance than single major genes (Heinrichs, 1985). Alam and Cohen (1998) and Soundararajan *et al*. (2004) mapped several QTL associated with resistance to BPH in rice. The number of resistance QTL in rice germplasm is expected to be very large and the quantitative resistance to BPH in rice can be further enhanced by pyramiding genes/QTL with different origin by MAS. A major QTL for the BPH resistance was reported in the chromosome 3 by Renganayaki *et al*., 2002; Buna et *al*., 2001; Tan *et al*., 2004; Huang *et al*., 2001; Sun *et al*., 2005; Chen *et al.,* 2006; Ramalingam *et al*., 2003.

In the present study, attempts were made to locate the genomic region associated with BPH resistance in rice chromosome 3 by involving F_7 families of IR50/Rathu Heenati cross. The objectives of the present study are as follows:

- Phenotypic screening of F_7 families of IR50/Rathu Heenati for the inheritance of BPH resistance.
- Identification of polymorphic SSR markers between IR50 and Rathu Heenati parents in rice chromosome 3.
- Selective genotyping of the F_7 families using identified polymorphic SSR markers and identification of SSR markers associated with BPH resistance in rice chromosome 3.

REVIEW OF LITERATURE

Rice, the world's most important cereal crop, is the primary source of food and calories for about half of the human population (Liu *et al*., 2008). Taxonomically, rice is classified in the family *Poaceae* and subfamily *Oryzoideae*. Due to the importance of rice as a major food crop, its origin and diversity of has attracted greater interest. The genus *Oryza*, to which cultivated rice belongs, probably originated at least 130 million years ago and spread as a wild grass in Gondwana land which eventually broke up and drifted apart to Asia, Africa and Australia (Chang, 1976). Today's species of *Oryza* is distributed in all of these continents except Antarctica. There are 21 wild species and two cultivated species of *Oryza*. The cultivated species has about 11,500 years of domestication in the river valleys of South and Southeast Asia and China (Normile, 1997). The Asian cultivated rice, *Oryza sativa*, is grown worldwide and the African rice, *Oryza glaberrima* is grown on a limited scale in West Africa. *Oryza sativ*a has different ecotypes *viz*., *indica*, *japonica* and *javanica* which represent specialized gene pools that make it possible to cultivate rice under diverse conditions including both tropical and temperate climates, varying altitudes and irrigated and rainfed environments. The genetic diversity of *indica* sub species is thought to be more than that of *japonica* subspecies (Zhang *et al*., 1992).

In India, during past decades increasing demand for rice has been met mainly through yield-enhancing measures of the "Green Revolution" in the 1970s, which introduced improved rice varieties and improved production technologies. Green revolution technologies made the insect pests as the major biotic constraints in rice production. In the 1970s, BPH became a threat to rice intensification programs in Indonesia, Thailand, India, Solomon Islands and the Philippines. IRRI organized the first BPH international conference in 1977 which brought together scientists from all rice producing countries to tackle the problem. Activities triggered by this conference that followed, including IPM, reducing unnecessary insecticide use and breeding resistant varieties that contributed to improved management of the pest that kept it under control for the next 20 years. To cope with the increasing demand for rice a key element is the development and implementation of effective rice insect pest management strategies. The thinking has led to the development of the strategy and philosophy of integrated pest management (IPM) (Huffaker and Smith, 1980). Host plant resistance (HPR) is the basic component of IPM on which several other methods of pest suppression can be superimposed with a high degree of complementarity (Chelliah, 1985). HPR may be due to inherent genetic capacity and ecology of the host. The potential of HPR as an

insect control method was not fully appreciated until the 1960s, as the over dependence on chemical pesticides forced entomologists to explore alternative strategies for pest control (Panda and Khush, 1995). During the last 40 years, interest in HPR has been rejuvenated as a result of accumulation of adequate knowledge on the important insect pests of crop species and understanding phenomena such as impact of damage by these pests, genetics of resistance, mechanisms of resistance, factors affecting breeding and genetic engineering of insect resistant crops.

However, in the last 5 years, planthopper problems have intensified in several countries, like China and Vietnam. The Second International Conference on Rice Planthoppers held at IRRI, Philippines during 23–25 June 2008 and sponsored by FAO, Government of Japan, Thailand and a few asian countries along with the private sector to develop sustainable solutions to the planthopper problems to tackle rice price increases and climate change. Classical host plant resistance had been IRRI's main approach to BPH management but this may not suffice, as the pest population structures constantly evolve. Several BPH resistance genes *(bph 1, 2, 3, 10, 18, 25* etc) and QTLs have been identified by breeders. Planthopper outbreaks are signs of ecosystem deterioration and in order to implement sustainable management strategies, there is need to adjust the management and policy facilitation facet to favor ecological management techniques. Ecological research showed that BPH is a secondary pest induced by ecological perturbations. To build sustainable systems that will keep BPH in low densities, a broader perspective incorporating landscape ecology, ecological engineering and population ecology to manage "system resistance" (which includes plant resistance) will be needed. To achieve this over a large scale will require developing communication strategies with multi stakeholder participation. Such approaches will also be needed to build system resilience in order to enhance adaptation strategies and local capacities to combat climate change.

Breeding for insect resistance has only been a focus of rice development programmes since the early 1960s. All the major rice producing countries in South and Southeast Asia have breeding programmes for resistance to major insect pests and diseases. Rice cultivars resistant to the BPH were first identified at International Rice Research Institute (IRRI) in 1963. Since 1963 about 50,000 accessions have been tested and more than 400 resistant accessions have been identified (Brar and Khush, 1997).

2. 1. Brown planthopper (BPH)

The brown planthopper, *Nilaparvata lugens* Stål (Homoptera: Delphacidae), is a destructive and widespread insect pest throughout the rice growing areas in Asia. The BPH feeds specifically in rice, using stylet like mouthparts to penetrate the plant tissues and sucks assimilates from the phloem. Feeding by a large number of BPH may result in drying of the leaves and wilting of the tillers resulting in a condition called 'hopper burn'. BPH causes severe damage on rice plants either directly by feeding on phloem sap or indirectly by acting as vectors for tungro, grassy stunt and rugged stunt viral diseases (Heinrichs, 1979). The most severe outbreak of BPH in India occurred in Kerala at the end of 1973 and early in 1974 (Nalinakumari and Mammen, 1975). Widespread occurrence of BPH and the associated grassy stunt virus disease have been primarily due to the indiscriminate use of pesticides and selective elimination of bio-control agents (Nagarajan, 1994). Susceptible rice cultivars often suffer severe yield loss up to 60% from its attacks (Panda and Khush, 1995; Xu *et al*., 2002). Initially, BPH populations were thought to belong to the same general biotype. However, four BPH biotypes have been reported so far. Biotypes 1 and 2 are widely distributed in Southeast Asia, biotype 3 is a laboratory biotype produced in Philippines and biotype 4 occurs in the Indian subcontinent (Khush and Brar, 1991; Huang *et al*., 2001). Based on the reaction pattern of different rice varieties to different biotypes the proportion of resistance genes in these varieties varies.

2. 2. Genetics of BPH resistance in rice

The genetics of BPH resistance is extensively studied and 21 major genes conferring resistance to BPH have been reported until now in *indica* cultivar and four wild relatives. *O. australiensis, O. eichingeri*, *O .latifolia* and *O. officinalis* (Myint *et al.,*2009). The genes conferring resistance to South and Southeast Asian biotypes are mostly dominant in nature. The genes in Mudgo, ASD 7, Rathu Heenati and Babawee were designated as *Bph1, bph2, Bph3* and *bph4* respectively (Martinez and Khush, 1974; Lakshminarayana and Khush, 1977). Further, genetic analysis for BPH resistance revealed the presence of new genes *viz., bph5* in ARC 10550 (Khush *et al*., 1985), *Bph6* in Swarnalatha and *bph7* in T12 (Kabir and Khush, 1988), *bph8* in Chin Saba (Nemoto *et al*., 1989) and *Bph9* in Kaharamana, Balamwee and Pokkali (Ikeda, 1985). Another gene *Bph10* was identified in an introgression line of *O. australiensis* (Jena and Khush, 1990).

The recessive genes, *bph2* and *bph4* are linked to the dominant genes *Bph1* and *Bph3* respectively, but are independent of each other (Kawaguchi *et al*., 2001). Two recessive genes, *bph11*

and *bph12*, confers resistance to the BPH biotype of Japan. The resistance genes *Bph1, bph2, Bph9* and *Bph10* are located on chromosome 12; *Bph13, Bph15 and bph12* on chromosome 4; *Bph3* and *bph4* on chromosome 6; *Bph6* on chromosome 11; and *bph11* and *Bph13*, *Bph14* and *Bph19* on chromosome 3, (Ishii *et al*., 1994; Hirabayashi *et al*, 1999; Jena *et al*., 2003; Renganayaki *et al*., 2002; Sharma *et al*., 2003). Recently, Jena *et al*. (2006) identified a new BPH resistance gene *Bph18(t)* and mapped it on chromosome 12.

Several QTL for BPH resistance have also been identified and major QTL conferring resistance to BPH biotypes 1 and 2 have been reported (Alam and Cohen 1998; Xu *et al*., 2002; Soundararajan *et al*., 2004). However, two dominant genes, *Bph14* and *Bph15* previously named as *Qbp1* and *Qbp2*, conferring strong resistance to the BPH biotype of China have been mapped on the long arm of chromosome 3 and the short arm of chromosome 4, respectively (Ren *et al*., 2004; Yang *et al*., 2004).

Like the gene-for-gene system in disease resistance, there seems to exist a similar system between BPH and the resistance genes. For more effective protection, however, pyramiding resistance genes from multiple sources, especially from wild relatives would be beneficial. It has been reported that several wild *Oryza* species, *e.g.*,*O. latifolia*, *O. minuta*, *O. nivara*, *O. officinalis* and *O. punctata* possessing resistance to various biotypes of BPH (Wu *et al*., 1986).

2. 3. Biochemicals associated with resistance to BPH in rice

Resistance to insects enables a plant to avoid or inhibit host selection, inhibit oviposition and feeding, reduce insect survival and tolerate or recover from injury from insect populations that would cause greater damage to other plants of the same species under similar environmental conditions. Apart from the scorable and measurable parameters so far made available in the study of host plant resistance to BPH in rice, many of the biochemical components contributing towards plant's resistance to BPH and insect behavioural responses are not well elucidated.

The attempts to find out the causative agents responsible for orientation of BPH and stimulation of probing and sucking resulted in the identification of several biochemical compounds influencing the insect behaviour. Obata *et al*. (1981) established that the combination of volatile compounds *viz.,* methyl palmitate, methyl linolenate and ethyl linolenate play a definite role in the BPH attraction and persistence on the rice plant. Kuwatsuka (1962) detected a set of flavonoids peculiar to rice *viz*., tricin-5-glucoside, glucotricin, orizatin and homoinetin acting as probing stimulants for BPH. Salicylic acid was found to be another probing stimulant and its effect markedly

enhanced in combination with sucrose (Sekido and Sogawa, 1976). The other category of stimulants found to be associated with sucking by BPH includes sucrose (Koyama, 1981) and amino acids such as aspartic acid, glutamic acid, alanine, asparagine and valine (Sogawa, 1971). The chemicals *viz*., soluble salicylic acid (Yoshihara *et al*., 1980), oxalic acid, maleic acid, itaconic acid and benzoic acid were found to be strong sucking inhibitors. Among the above chemicals influencing feeding behaviour of BPH, salicylic acid was found to act as probing stimulant (Sekido and Sogawa, 1976) and sucking inhibitor (Yoshihara *et al*., 1980).

Over the last two decades considerable progress has been made in the development of technologies and tools to describe the expression of genes and the protein complement and rapid determination of metabolites important in both primary and secondary metabolism (Roessner *et al*., 2002). Biochemical phenotyping of plants by determining the steady state concentrates of a broad spectrum of metabolites will expand the horizon of host plant resistant research.

2. 4. Molecular markers

With the advent of molecular-marker technology, scanning the whole genome of crops for quantitative trait loci (QTLs) controlling traits of interest is now possible. Rice is considered the model plant for mapping genes of importance among cereals due to its small genome size of 430 Mb (Yano and Sasaki, 1997). It is a true diploid (2n=24) with twelve chromosome pairs and 12 linkage groups with 5.8 x 10^5 kb/haploid genome (Bennet and Smith, 1976). Though it has a relatively small genome, the DNA of rice shows high polymorphism. The DNA content per map unit in rice is two to three times greater than that of *Arabidopsis thaliana*, the model dicot for genome analysis. QTLs conferring resistance for BPH in several crops have been identified (Zhang *et al*., 2001), thereby leading the way to marker assisted breeding. Selection for desirable alleles at molecular markers closely linked to specific QTL can be done (Shen *et al*., 2001). They have several advantages over traditional phenotypic markers because selection is indirect and does not depend on phenotyping every time. They are also not environmentally regulated and are detected in all stages of plant growth (Mohan *et al*., 1997).

2. 4. 1. Types of Molecular Markers

PCR based DNA markers are preferred for molecular breeding because of their simplicity and low cost. They have been used to evaluate genetic diversity in different crop species (Cooke, 1995) and for QTL mapping. The important ones are Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al*., 1980), Random Amplified Polymorphic DNA (RAPD)

(Williams *et al*.,1990), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995), Single Sequence Repeats / Short Tandem Repeats (SSR/STR) (Hearne *et al*., 1992), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz *et al*., 1994), Single Nucleotide Polymorphism (SNP) (Jordan and Humphries, 1994), Oligonucleotide Polymorphism (OP) (Beckmann, 1988), Microsatellite / Simple Sequence Length Polymorphism (SSLP) (Saghai-Maroof *et al*., 1994) and Sequence Tagged Sites (STS) (Fukuoka *et al*., 1994).

Development of molecular markers for use as probes for genomic DNA has provided the geneticists, physiologists, agronomists and breeders with valuable new tools to identify traits of importance in improving crop resistance to biotic stresses (Chopra and Sinha, 1998). Some of these techniques are robust and reliable e.g., RFLP and AFLP, while some are quick, e.g., RAPD and some others are quick and reliable e.g., microsatellites. The limitations in the use of RFLP and AFLP markers are time consuming (Kochert, 1994), complicated methodology and requirement of large amount of DNA (Vos *et al*., 1995). PCR based markers such as microsatellites and RAPD have been of great use in genetic diversity analysis, but microsatellite markers need prior sequence information. RAPD markers offer many advantages such as higher frequency of polymorphism, rapidity, technical simplicity, use of fluorescence, requirement of only a few nanograms of DNA, no requirement of prior information of the DNA sequence and feasibility of automation (Subudhi and Huang, 1999).

2.4. 2. Microsatellites or Simple Sequence Repeats

Microsatellites, also termed Simple Sequence Repeats (SSRs), are tandemly arranged repeats of short DNA motifs, 1- 6 bp in length. They frequently exhibit variation in the number of repeats at a locus (Temnykh *et al*., 2001). Microsatellites are among the most variable types of DNA sequence in plant and animal genomes (Wang *et al*., 1994). These are also known as Single Sequence Length Polymorphism (SSLP) (McCouch *et al*., 1997). The International Rice Microsatellite Initiative (IRMI) was formed to increase the density and utility of the SSR map in rice. IRMI is comprised of an international group of researchers from both public and private sector institutions that worked collaboratively to augment the number of experimentally validated SSR markers (McCouch *et al.,* 2002).

Since SSR markers are highly polymorphic, abundant and easy to use, they have become the marker of choice for genetic mapping and population studies (Goldstein and Schlotterer, 1999). The unique sequences bordering the SSR motifs provide templates for specific primers to amplify

the SSR alleles via the polymerase chain reaction (Weber and May, 1989). High level of allelic diversity, technical efficiency and multiplex potential of microsatellites make them preferable for many forms of high throughput mapping, genetic analysis and marker assisted plant improvement strategies (Coburn *et al*., 2002; McCouch *et al*., 1997).

Hence, microsatellite markers are considered to be the most informative molecular genetic markers (Tautz, 1989) for DNA fingerprinting and varietal identification (Ramakrishna *et al*., 1994; Udupa *et al*., 1999), genome mapping (Chen *et al*., 1997; McCouch *et al*., 1997), gene tagging (Blair and McCouch, 1997) and studies on population dynamics (Yang *et al*., 1994). Being highly reproducible molecular tools for genotyping, SSRs are very useful in any genotype based genetic analysis (Ribaut and Betran, 1999).

2. 4. 2. 1. Rice microsatellite markers

The reported frequency of specific SSR motifs varies significantly among different organisms (Lagercrantz *et al*., 1993). The most abundant microsatellite motif reported in plants is $(AT)_n$, while $(AC)_n$ is most abundant in human genome (McCouch *et al*., 1997). Akagi *et al*. (1996) first noted that AT- rich microsatellites tended to show more length variation and suggested that these would make best SSR markers for rice. $(GATA)_n$ is the most frequent tetranucleotide motif while the dinucleotide $(AC)_n$ is the second highest frequency in the rice genome (Panaud *et al.,* 1995; McCouch *et al*., 1997). The trinucleotide motif $(CGG)_n$ has been reported to be very abundant in rice and interspersed throughout the genome (Zhao and Kochert,1992). In a rice genome of 450 Mb (McCouch *et al.,* 1997), based on hybridization assay using clone libraries, earlier work predicted about 5,500 to 10,000 microsatellite loci in rice (Wu and Tanksley, 1993; Panaud *et al*., 1996).

Temnykh *et al*. (2001) examined 47,430 kb of a BAC end sequence (~0.11 genome equivalent) and predicted that rice genome contained approximately 11,000 class I ($\geq$20 nt) and an additional 22,000 class II (12-20 nt) microsatellites. Estimates of total microsatellite frequencies in these sequences were three times those based on BAC sequences suggesting a total of about 1,00,000 SSR motifs in the rice genome (McCouch *et al*., 2001). Microsatellites may be obtained by screening sequences in databases or by screening libraries of clones. A pre-sequencing screening step was used to eliminate clones where the microsatellite repeat was too near to one of the primers and to determine which end should be sequenced with priority (Panaud *et al.,* 1996). Microsatellites are abundant and well distributed throughout the rice genome and genetic maps

were developed using microsatellite markers (Wu and Tanksley, 1993; Akagi *et al*., 1996; Panaud *et al*., 1996; Chen *et al*., 1997; Temnykh *et al*., 2001).

2. 4. 2. 2. Application of microsatellite markers

SSR markers have been used as the powerful genetic markers in plants (Morgante and Olivieri, 1993; Powell *et al*., 1996). Because of their high levels of polymorphism in number of repeats, they have been widely used as markers in studies of kinship, population structure and genetic mapping. Microsatellite markers generate enough allelic diversity to differentiate cultivars within a subspecies or ecotype (Yang *et al.,* 1994), making it possible to analyze germplasm commonly used in breeding program.DNA fingerprinting and diversity study in rice by SSR markers has been used to visualize genetic relationships among the elite breeding lines (Chakravarthi and Naravaneni, 2006). They are also stable enough to reliably trace the flow of monogenic or QTL of interest in rice pedigree (Panaud *et al.,* 1996). Gupta *et al*. (1996) reviewed and discussed the use of microsatellites in areas such as selection and diagnosis of segregating population, cultivar identification, germplasm characterization, estimation of genetic relatedness, genome selection during gene introgression (in a backcrossing program), genome mapping, gene tagging, etc.

Yang *et al*. (1994) used SSR markers to demonstrate the higher levels of allelic diversity in a collection of landraces. Olufowote *et al*. (1997) demonstrated that a selected set of highly informative SSR markers could be used to differentiate varieties and these markers are especially useful in identifying allele frequencies in complex mixtures of pure lines that are characteristic of many traditional (landrace) varieties. Microsatellites have been proved to be useful in evaluating diversity in narrowly defined gene pool in which other kinds of molecular markers such as AFLP, RFLP and RAPD are unable to detect polymorphism (Powell *et al*., 1996). Examples in rice include *O. glaberrima* accessions from West Africa (Semon *et al*., 2001) and *O. rufipogon* Grif. (Zhou *et al*., 2003). Microsatellites have been used for the assessment of genetic diversity in wild barley, *Hordeum spontaneum* (Beak *et al.,* 2003). Thomson *et al*. (2007) has reported the use of microsatellite markers in the genetic diversity analysis of traditional and improved Indonesian rice germplasm.

SSRs are economically employed in hybrid rice breeding programs. These markers have also been used to define heterotic groups in rice (Xiao *et al*., 1996), to study the genetics of heterosis (Hua *et al*., 2000), transgressive variation (Xiao *et al*., 1998), hybrid fertility (Zhang *et al*., 1997), to transfer traits via marker-assisted selection (He *et al*., 2000), to define introgressions in wide hybridization programs (Brar *et al*., 2000), to construct ordered sets of substitution lines

(Lorieux *et al*., 2000) and for the study of microsynteny in the chloroplast genomes of *Oryza* and eight other Graminae species (Ishii and McCouch, 2000).

Microsatellite markers have become the molecular markers of choice for a wide range of applications in genome mapping (Chen *et al.,* 1997; McCouch *et al*., 1997; Ramsay *et al.,* 1999), linkage mapping in many crop plants (Cho *et al.,* 1998; Rae *et al.,* 2000; Flandez-Galvez *et al.,* 2003) and to identify genes and QTLs in both intra and interspecific mapping populations (McCouch *et al*., 1997; Xiao *et al*., 1998 and Yu *et al*., 2000). It is predicted that the availability of an increasing number of SSR markers, well distributed in the rice genome, will provide an increasingly useful resource for many applications in genetics and breeding.

2. 5. DNA markers identified for BPH resistance genes

In rice, molecular linkage maps have been constructed using RFLP and randomly amplified polymorphic DNA (RAPD) markers (Huang *et al*, 1997). Of the three DNA markers tagged to different BPH resistance genes, one RFLP marker has been tagged to a BPH resistance gene derived from *O. australiensis* (Ishii *et al*., 1994) and two RFLP markers were tagged to BPH resistance genes derived from cultivated rice germplasm (Mei *et al*., 1996). *Bph1* is the first major resistance gene identified at IRRI, Philippines (Athwal *et al*., 1971). The *Bph1* locus was mapped on the rice chromosome 12; the closest RFLP marker XNpb248 was 10.7cM from the *Bph1* locus (Hirabayashi and Ogawa, 1995). *bph2,* a BPH resistance gene in 'Norin-PL4', was mapped at 3.5cM from the closest RFLP marker, G2140 on the long arm of chromosome 12 (Murata *et al*., 1998). Murai *et al.* (2001) identified eight AFLP markers linked to the BPH resistance gene *bph2*, of which one marker (KAM4) showed complete co-segregation with *bph2* and converted KAM4 into PCR-based sequence- tagged- site (STS) marker. Kim and Sohn (2005) through bulked segregant RAPD analysis, developed an STS marker, designated as *BpE18-3*, linked (3.9cM) to the BPH resistance gene, *Bph1*.

PCR based RAPD markers have been used for tagging agronomic traits in several crops as a less labour-intensive alternative to using RFLP markers. Jena *et al.* (2003) identified a RAPD marker $OPA16_{938}$ linked to the BPH resistance gene on chromosome 11 at a distance of 0.52cM. SSR markers are widely distributed in the rice genome (McCouch *et al*., 1997) and can be easily and economically analysed by polymerase chain reaction. SSR markers have the advantages of both rapidity and simplicity of RAPD and the stability of RFLP and can be the markers of first choice for genetic mapping in rice. Yang *et al.* (2002) identified a closest SSR marker, RM261 to the BPH

resistance gene *Bph12(t)* at 1.8cM map distance and three polymorphic RFLP markers C820, R288 and C946 linked to the gene and thus confirmed its location on the short arm of chromosome 4.

Biotype-4 resistance gene *Bph13(t)*, derived from *Oryza officinalis* were mapped on the chromosome 3 by the RAPD analysis. The RAPD marker *AJ09b* was mapped 1.3cM from the resistant gene. The most closely linked RAPD marker, *AJ09b* was converted to a co-domimant linked sequence tagged sites (STS) marker. The closely linked *AJ09b-STS* marker co-segregated with RG100 on chromosome 3, when mapped by the 96 DH lines by Temnykh *et al*. (2000). RG100 and *AJ09b-STS* were flanked by RZ892 and RG191.Using the 252 RI lines from Lemont X Teqing population, *AJ09b-STS* mapped to chromosome 3 flanked by RG100 and RM7 (Renganayaki *et al*., 2002).

The resistance gene locus *bph19(t)* was finely mapped to a region of about 1.0 cM on the short arm of chromosome 3, flanked by markers RM6308, RM3134 and RM1022 (Chen *et al*., 2006).The resistant gene *Bph9* in kaharamana was located between SSR markers RM463 and RM5341 on chromosome 12 with linkage distances of cM and 9.7cM respectively (Chao *et al*.,2006). Through linkage analysis, *Bph17* was located between two SSR marker RM8213 and RM5953 on the short arm of chromosome 4 with map distances of 3.6 cM and 3.2 cM, respectively (Sun *et al.,* 2005). Physical mapping of *Bph3* was performed using a BC_3F_3 population derived from a cross between Rathu Heenati and KDML105. *Bph3* locus was localized approximately in a 190 kb interval flanked by markers RM19291 and RM8072 (Jairin *et al.,* 2007).

Fine mapping of the *Bph1* has been done on chromosome 12 in 273 F_8 recombinant inbred lines (RILs) derived from a cross between Cheongcheongbyeo, an *indica* type variety harboring *Bph1* from Mudgo, and Hwayeongbyeo, a BPH susceptible *japonica* variety (Cha *et al*., 2008). The two major genes conferring resistance to BPH, *bph20(t*) and *Bph21(t),* derived from an indian rice variety ADR52 were recently mapped on rice chromosomes 6 and 12, respectively (Myint *et al.,* 2009).

2. 6. QTL mapping for BPH resistance in rice

Recent advances in DNA marker technology and molecular biology have greatly facilitated studies to understand the genetic basis of complex phenotypes. Genes contributing to quantitative trait variation, or quantitative trait loci (QTL), related to a wide range of complex phenotypes, have been mapped in rice. Several QTL for BPH resistance have also been identified and major QTL conferring resistance to BPH biotypes 1 and 2 have been reported (Alam and Cohen 1998; Xu *et al*., 2002; Soundararajan *et al*., 2004).

Alam and Cohen (1998) reported two QTL for BPH resistance of which one QTL was located on the short arm of chromosome 3 (between RG191 and RZ678) and the other was located on the long arm of chromosome 4 (between RG163 and RG620). Huang *et al.* (2001) detected two QTL for BPH resistance, *Qbp1* with a LOD score of 12.89 was located in the 14.3cM length interval between R1925 and R2443 on the long arm of chromosome 3 and *Qbp2* with a LOD score of 7.69 was located in the 0.4cM interval between C820 and R288 on the short arm of chromosome 4 (Geethanjali, 2001) detected four QTL on chromosome 3, 7, 8 and 10 which conferred resistance to WBPH in the DH lines derived from the cross IR64/Azucena. Kadirvel *et al*. (2003) used 262 RILs derived from a cross of Basmati370/ASD16 and identified two QTL associated with whitebacked planthopper (WBPH) resistance on chromosome 3 and 7.

The nine QTL for BPH resistant were located to chromosomes 3, 4, 6, 11 and 12 by resistance gene analogs (RGAs) and putative defence response (DR) as a marker. The QTL on chromosome 3 were identified between the RG191 and RZ678 in the double haploid population of IR64 and Azucena (Ramalingam *et al*., 2003). Sun *et al., (*2005) identified the QTL between RM3131 and RM7 with a LOD score of 2.32 and phenotypic variance of 6.5% in the F_2 population of Rathu Heenati/02428.

Two dominant genes, *Bph14* and *Bph15* previously named as *Qbp1* and *Qbp2*, conferring strong resistance to the BPH biotype of China have been mapped on the long arm of chromosome 3 and the short arm of chromosome 4, respectively (Ren *et al*., 2004; Yang *et al*., 2004). Ren *et al.* (2004) performed QTL analysis for BPH resistance trait involving a RIL population derived from a cross between B5 and Minghui 63 using a linkage map based on RFLP, SSR and EST markers. A total of 4 QTL was identified and mapped on chromosome 2, 3, 4 and 9. Soundararajan *et al.* (2004) identified six QTL associated with BPH resistance in the double haploid (DH) population derived from the cross IR64/Azucena and mapped them on chromosome 1, 2, 6 and 7. Su *et al.* (2005) identified one major QTL, *Qbph11* for BPH resistance involving a set of 81 recombinant inbred lines (RILs) of Kinmaze/DV85, with a LOD score of 10.1 between X202 and C1172 on chromosome 11.

2. 7. Marker assisted selection

The molecular markers are especially advantageous to tag agronomic traits such as resistance to insects, pathogens and nematodes, tolerance to abiotic stress, quality parameters and quantitative traits. Molecular marker studies using Near Isogenic Lines (NIL) (Martin *et al*., 1994), Bulked Segregant Analysis (BSA) (Michaelmore *et al*., 1991) or Recombinant Inbred Lines (RILs) (Mohan *et al*., 1994) have accelerated mapping many genes in different plant species.

Molecular marker-assisted selection, often simply referred to as marker-assisted selection (MAS) involves selection of plants carrying genomic regions that are involved in the expression of traits of interest through molecular markers. Availability of tightly linked genetic markers for resistance genes will help in identifying plants carrying these genes and simultaneously without subjecting them to the pathogen or insects attack in early generations.

With MAS, it is now possible for the breeder to conduct many rounds of selection in a year without depending on the natural occurrence of the pest. In general, the success of a marker-based breeding system depends on (i) their inherent repeatability (Weeden *et al.,*1992) (ii) a genetic map with an adequate number of uniformly-spaced polymorphic markers to accurately locate desired QTLs or major gene(s); (iii) close linkage (<10cM) between the QTL or a major gene of interest and adjacent markers (Timmerman *et al.,*1994; Kennard *et al.,*1994); (iv) adequate recombination between the markers and rest of the genome; and (v) an ability to analyse a larger number of plants in a cost- effective manner.

The success of MAS depends on location of the markers with respect to genes of interest. Three kinds of relationships between the markers and respective genes could be distinguished; (i) the molecular marker is located within the gene of interest, which is the most favourable situation for MAS and in this case, it could be ideally referred to as gene-assisted selection. While this kind of relationship is the most preferred one, it is also difficult to find this kind of markers. (ii) the marker is in linkage disequilibrium (LD) with the gene of interest throughout the population. LD is the tendency of certain combination of alleles to be inherited together. Population- wide LD can be found when markers and genes of interest are physically close to each other. Selection using these markers can be called as LD-MAS. (iii) the marker is in linkage equilibrium (LE) with the gene of interest throughout the population, which is the most difficult and challenging situation for applying MAS.

MAS is gaining considerable importance as it would improve the efficiency of plant breeding through precise transfer of genomic regions of interest (foreground selection) and accelerating the recovery of the recurrent parent genome (background selection). MAS has been more widely employed for simply inherited traits than for polygenic traits, although there are a few success stories in improving quantitative traits through MAS. Since a variety of molecular markers have become available in recent years, efforts are being made to identify the most efficient and cost effective markers that can be used by plant breeders (Mohan *et al*., 1997 and Gupta *et al*., 1999).

Attempts have been made by Jena *et al*. (2006) to incorporate new brown planthopper (BPH) resistance gene *Bph18(t)* into modern rice cultivars. An STS marker 7312.T4A was generated and was validated using 433 BC_2F_2 individuals. 94 resistant BC_2F_2 individuals completely co-segregated with the resistance specific marker allele (1,078 bp) in either homozygous or heterozygous state. The F_2 segregation showed a 1:2:1 segregation ratio indicating the presence of a major dominant gene conferring resistance to BPH. The gene pyramided *japonica* line has been constructed in which two BPH resistant genes *Bph1* and *bph2* on the long arm of chromosome 12 independently derived from two *indica* resistance lines were combined through the recombinant selection (Sharma *et al.,* 2004).

Genetic enhancement of rice through conventional methods is often constrained by narrow gene pools besides strong barriers to crossability. Transgenic technology can be adopted as an alternative approach for evolvement of insect resistant varieties by introducing exotic resistance genes into leading rice cultivars. Lectin (*asal)* gene from *Allium sativum* was isolated, cloned and characterized and it was expressed in elite indica rice cultivars using Agrobacterium-mediated genetic transformation method. The stable transgenic lines, expressing *ASAL,* showed explicit resistance against major sap-sucking insects, viz., BPH, GLH and WBPH (Yarasi *et al.*, 2008).

MATERIALS AND METHODS

The present study was undertaken with a view of i) understanding phenotypic variation for BPH resistance in F_7 RILs of IR50/Rathu Heenati under green house conditions; ii) surveying of SSR markers in IR50 and Rathu Heenati and selective genotyping of the resistant and susceptible F_7 RILs of IR50/Rathu Heenati and iii) identification of markers associated with BPH resistance. All the phenotypic experiments were conducted at greenhouse of Department of Agricultural Entomology Unit and Paddy Breeding Station (PBS), Tamil Nadu Agricultural University (TNAU), Coimbatore. All the genotyping experiments were conducted at Department of Plant Molecular Biology and Biotechnology, Centre for Plant Molecular Biology, TNAU, Coimbatore during 2007-2009. The materials used and methods adopted in this study are described below.

3.1. Understanding phenotypic variation for BPH resistance

3.1.1. Plant Materials and insects

A population of 268 F_7 RILs of rice were generated and maintained by DPMB&B, CPMB, TNAU and were used in the present study . The F_7 RIL population was derived from a cross between IR50, an elite BPH susceptible *indica* cultivar and Rathu Heenati, a known BPH resistant *indica* cultivar. The biotype 4 of insects (Plate 1) was collected from the rice fields at wetland and Paddy Breeding Station, Tamil Nadu Agricultural University (TNAU), Coimbatore.

3.1.2. Status of RILs population and method used for development

The RIL population was developed by making crosses between IR50 x Rathu Heenati .The F_4 population was phenotyped during summer 2005.(Jenifer 2006). By the single seed descent method, the population was forwarded to F_7 generation. In the present study, the F_7 generation seeds were used for the phenotyping for reaction to the BPH.

3.2. Methods

3.2.1. Evaluation of insect resistance

3.2.1.1. Mass rearing of the BPH

Mass culturing of the BPH is essential for continuous screening of rice accessions under greenhouse. BPH undergoes 5 instars to reach the adult stage. Male insects are dark brown and winged. Female insects are wingless and light brown to dark brown. The BPH was mass reared on the susceptible rice variety Taichung Native 1 (TN1) by following the method of Heinrichs *et al*. (1985). Initially, BPH population was collected from the rice fields at wet land and PBS, TNAU, Coimbatore.

Plate 1. Brown Planthopper

Plate 2. Mass rearing of Brown Planthopper

The gravid females were collected with an aspirator and left on pre-cleaned 35 days old potted plants of TN1 which was placed in oviposition cages (45 x 45 x 60cm) having wooden frames, glass top, door and wire mesh sidewalls. Twenty females along with five males were released per plant. The ovipositing insects were removed three days later and plants with eggs were taken out of cages and placed in separate cages for the nymphs to emerge. The emerged nymphs were then transferred to 15 days old TN1 seedlings raised in the germination trays. The seedling trays were changed as and when necessary. Using this technique, a continuous pure culture of the BPH was maintained during the period of study (Plate 2).

3.2.1.2. Standard Seedbox Screening Test

Evaluation test for BPH resistance were conducted in the three seasons namely winter, spring and rainy. The main objective of mass screening was to rapidly identify resistant and susceptible lines. Two hundred and sixty eight F_7 individuals along with the parents, Rathu Heenati, IR50, TN1 (susceptible check) and PTB33 (resistant check) were screened in the green house.

The pre-germinated seeds of test lines were sown 3 cm apart in 30 cm rows in 50 x 40 x 10 cm plastic trays. Each line was planted in two replications across the width of the seedling box in such a way so as to have at least 15 plants per row. One row each of the susceptible check TN1 and the resistant check, PTB33 were sown at random in all the seedling trays. The seedlings were infested with first to third instar nymphs of BPH at the rate of approximately 5 to 8 nymphs per seedling. After infestation, the seedling trays were covered with wire mesh wooden cages (45 x 45 x 60 cm). The test plants were observed daily for damage by BPH. Damage rating of the test lines was done on individual plant basis when 90 per cent of the plants in the susceptible check (TN1) row were killed. The test lines were graded using 1-9 scale (Heinrichs *et al*., 1985).

The extent of damage on each plant was examined by visual scoring and evaluated according to the criteria of Standard Evaluation system for rice (IRRI, 1988), which were

graded as given below:

Grade	Damage	Categories
1	Very slight damage	R
3	Partial yellowing of the first and second leaf	MR
5	Pronounced yellowing of half of the plant	MS
7	Wilting of more than half of the plant	S
9	Whole plant dead	HS

3.3 Molecular marker analysis

3.3.1. Isolation of genomic DNA

DNA was extracted from fresh leaf tissue for all the F_7 individuals and their parents using the modified CTAB protocol as described by Ausubel *et al*., (1994). The quality of DNA was checked by agarose gel electrophoresis and quantified by Nanodrop Spectrophotometer (Nanodrop Spectrophotometer ND-1000).

3.3.1.1. Requirements

a) Leaf samples (leaf samples were collected from 30 days old seedlings and stored at –80° C till use.

b) Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction buffer (100 ml):

1. CTAB 2% (w/v)
2. Tris HCl (pH 8.0) 100 mM
3. Sodium chloride 1.4 M
4. EDTA 20 mM

Note: Tris, sodium chloride and EDTA were autoclaved and 2% CTAB was added after autoclaving and preheated before using the buffer.

a) Tris EDTA (TE) Buffer

Tris HCl (pH 8.0) 10 mM

EDTA (pH 8.0) 1 mM

This was dissolved and made up to 100 ml, autoclaved and stored at 4°C.

b) Ice-cold Isopropanol

c) Chloroform: Isoamylalcohol 24:1 (v/v)

d) Sodium acetate (3.0 M, pH 5.2) (pH adjusted using glacial acetic acid)

e) Ethanol (70% and 100%)

f) RNase A - 10 mg/ml

(RNase A was dissolved in TE buffer and boiled for 15 minutes at 100°C to destroy DNase and stored at -20°C.)

3.3.1.2. Protocol

- About 200 mg of leaf samples were cut into small bits with the help of sterile scissors and transferred to sterile mortar.
- The leaf tissues were ground in liquid nitrogen and extracted with 600 µl of CTAB buffer and incubated for 30 minutes at 65°C in water bath with occasional mixing.
- The tubes were removed from the water bath and equal volume of chloroform: Isoamylalcohol mixture (24:1 v/v) was added and mixed by inversion for 15 minutes.
- It was centrifuged at 10,000 rpm for 20 minutes at room temperature.
- The clear aqueous phase was transferred to a new sterile eppendorf tube.
- Equal volume of ice cold isopropanol was added and mixed gently by inversion and then kept in the freezer until DNA was precipitated out.
- Using blunt end tips, the precipitated DNA was spooled out into an eppendorf tube.
- The spooled DNA was air dried after removing the supernatant by brief spin.
- 100 µl of TE was added to dissolve the DNA and then 10 µl of RNase was added and incubated at 37°C for 35 minutes.
- 500 µl of Chloroform: Isoamylalcohol mixture was added and centrifuged for 10 minutes.
- Aqueous phase was transferred to another eppendorf without disturbing the inner phase.
- 2.5 volume of absolute alcohol and 1/10 volume of sodium acetate were added and kept for overnight incubation.
- Then it was centrifuged and the supernatant was discarded. To this 500 µl each of 70% and 100% ethanol was used subsequently to wash the DNA by centrifugation.
- The alcohol was discarded and DNA was completely air-dried.
- Then the DNA pellet was dissolved in 100 µl of TE and stored at -30°C.

3.3.2. Assessing the quality of DNA by agarose gel electrophoresis

3.3.2.1. Chemicals used

a) Loading Dye

Glycerol 50% (v/v)

Bromophenol blue 0.5% (w/v)

b) 10X TAE (Tris Acetate EDTA buffer)

Tris Base	48.4 g
Acetic acid	11.42 ml
0.5MEDTA	20 ml

Dissolved in 800 ml of sterile water and made up to 1000 ml.

3.3.2.2. Protocol

- The Pyrex gel casting plate open ends were sealed with cello tape and the comb was placed properly in casting plate kept on a perfectly horizontal platform.
- 0.8 % (0.8 g/100 ml) agarose was added to 1X TAE, boiled until the agarose dissolved completely and then allowed to cool. Ethidium bromide (DNA intercalating agent) was added when temperature reached 55-60^0 C as a staining agent.
- Then it was poured into the gel mould and allowed to solidify.
- The comb and the cello tape were removed carefully after solidification of the agarose.
- The casted gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1X TAE to a depth of about 1cm.

3.3.2.3. Loading the DNA samples

- 2 μl of DNA sample dissolved in TE was pipetted onto a parafilm and mixed well with 4 μl of 6X loading dye by pipetting up and down several times.
- The gel was run at 8 V/cm for 1 hour
- Post staining was done by keeping the gel in Ethidium bromide (DNA intercalating agent) staining agent and bands were visualized and documented using a gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., USA).

3.3.3. Quantification of DNA

DNA was quantified by using Nanodrop. 1 µl of genomic DNA was loaded for quantification. 1µl of TE buffer was used as blank. The absorbance for all samples was measured at 260 nm as double stranded DNA has maximal absorbance at 260 nm. If the quantified DNA in Nanodrop shows 'x' ng/µl, then dilution is done 'y' times (where, 'y' = 'x'/50). Based on the quantification data; DNA dilutions were made in 1X TE buffer to a final concentration of 50ng/µl and stored in -20°C for further use.

3.3.4. SSR genotyping of parents and F_7 RILs

Microsatellite (SSR) markers showing polymorphism between the parents IR50 and Rathu Heenati were used for screening the F_7 RILs. SSR genotyping includes the following steps:

- PCR amplification of genomic DNA was done using forward and reverse microsatellite primers
- Resolution of polymorphism through agarose gel electrophoresis and denaturing Polyacrylamide gel electrophoresis. (PAGE)
- Staining and developing the gel
- Analysis of banding pattern

3.3.4.1. PCR amplification

The cocktail for PCR amplification was prepared as follows:

A) Reaction mixture (15 µl)

Stock	1X	Final concentration
a) DNA 50 ng/µl	2.00 µl	66.7ng
b) dNTPs (2.5 mM)	0.50 µl	75.0mM
c) Forward primer (10 µM)	1.00 µl	1.5µM
d) Reverse primer (10 µM)	1.00 µl	1.5µM
e) Assay buffer (10 X)	1.50 µl	1 X
f) *Taq* DNA polymerase (3 units/µl)	0.20 µl	0.04 units
g) Sterile distilled H_20	8.80 µl	
Total	**15µl**	

(dNTPs, assay buffer and *Taq* DNA polymerase used were obtained from Bangalore

Genei Ltd., India and primers used were obtained from Research Genetics Inc., USA.)

B) The reaction mixture was given a momentary spin for through mixing of the cocktail components. Then 0.20 ml PCR tubes were loaded in a thermal cycler.

C) The reaction in thermal cycler (PTC-100™, MJ Research Inc, Massachusetts, USA and BIO-RAD, DNA Engine®, Peltier Thermal Cycler) was programmed as follows:

a) Profile 1:	95˚C for 5 minutes	Initial denaturation
b) Profile 2:	94˚C for 1 minute	Denaturation
c) Profile 3:	56-61˚C for 1 minute	Annealing
d) Profile 4:	72˚C for 1 minute	Extension
e) Profile 5:	72˚C for 5 minutes	Final extension
f) Profile 6:	4˚C for hold	Hold the samples

Profiles 2, 3 and 4 were programmed to run for 36 cycles.

After PCR amplification, the products were resolved by agarose gel electrophoresis and banding pattern was scored after EtBr staining.

3.3.5. Parental polymorphism

A total of 53 SSR Primers were selected covering whole region of chromosome 3 for identifying polymorphism between the parents namely IR50 and Rathu Heenati using agarose gel electrophoresis and denaturing polyacrylamide gel electrophoresis (PAGE).

3.4. Phenotypic screening of F_7 RILs of IR50/Rathu Heenati

A total of 268 F_7 RILs were screened to assess the level of resistance to BPH based on the standard seedbox screening test (SSST) in the greenhouse. The 268 RILs were screened for the BPH reaction in different time period. Every time the resistant (PTB33) and susceptbile (TN1) check variety were used for maintaining the level of uniformity in data points. The time-period of the phenotyping screening was as follows:

RILs	Greenhouse Experiment Time
1 - 45, 134 - 178	October – January
46 - 90, 179 - 223	February – March
91 - 133, 224 - 268	April - July

3.5. Selective genotyping of F_7 RILs of IR50/Rathu Heenati

Based on the phenotyping results obtained from green house screening experiment the susceptible (Suscetible score 9) and resistance (Resistance score 3) F_7 RILs of IR50/Rathu Heenati were selected for SSR genotyping. The susceptible plants F_7 RILs include the 11, 12, 41, 43 and 211 RILs and the resistant F_7 RILs include the 2, 28, 49, 135, 265 (Table 1). Eleven polymorphic primers were screened in the five selected RILs of BPH resistant and five susceptible along with parents for co-segregation using denaturing polyacrylamide gel electrophoresis (PAGE). The list of polymorphic primers used for segregation analysis is given in the Appendix 1.

3.6. Electrophoretic analysis of SSR products using denaturing polyacrylamide gels (PAGE)

After PCR amplification, the PCR products were separated by and 6% denaturing polyacrylamide.

3.6.1. Denaturing Polyacrylamide gel electrophoresis (PAGE) and silver staining materials

a) 40% Acrylamide stock (19:1)

Acrylamide	38 g
Bis- acrylamide	2 g

Dissolved in 50 ml MilliQ water and volume made up to 100 ml and stored in a brown bottle.

b) 6% Polyacrylamide denaturing stock solution

Urea	210 g
40% Acrylamide	75 ml
MilliQ water	150 ml

Stirred with low heat until urea dissolved, filtered through 0.22 μm cellulose acetate filter paper and 50 ml of 10X TBE buffer was added and then final volume was made to 500 ml with MilliQ water and stored at 4˚C.

c) 10x Tris borate EDTA (TBE) buffer

Trizma base	107.8 g
Boric acid	55.03 g
EDTA disodium salt	8.19 g

Dissolved in 800 ml MilliQ water, filtered through 0.22μm filter paper and made up to 1000 ml and stored at 4˚C.

d) 10% Ammonium per sulfate

Table 1. List of ten recombinant inbred lines of IR50 and Rathu Heenati selected for selective genotyping and their damage scores based on standard seedbox screening test.

S.No.	BPH susceptible RILs	Damage Score	BPH resistant RILs	Damage Score
1	11	9	2	3
2	12	9	28	3
3	41	9	49	3
4	43	9	135	3
5	211	9	265	3
6	IR50	5	Rathu Heenati	3

Ammonium per sulfate	0.1 g
Sterile water	1.0 ml

e) Bind silane (For 500 ml silanizing solution)

Ethanol	497.5 ml
Glacial acetic acid	2.5 ml
Bind silane	0.75 µl

f) Repel silane RainX (Glass repellent)

g) Fixer 10% acetic acid

2 litre was prepared by adding 200 ml acetic acid and 1800 ml sterile water and kept in a brown bottle.

h) Staining solution

Silver nitrate	2 g
Formaldehyde	3 ml
MilliQ water	2 l

Keep this solution in a dark brown bottle. Solution could be used twice.

i) Developing solution

Sodium carbonate	60 g
MilliQ water	2 l

Pre-chilled at 4˚C and 400 µL of sodium thiosulphate and 3 ml formaldehyde were added immediately before use.

j) Manual sequencing loading dye

Formamide	10 ml
Bromophenol blue	10 mg
Xylene cyanol FF	10 mg
0.5M EDTA	200 µl

3.6.2. Preparation of plates for denaturing PAGE gel casting

- The large and small glass plates were soaked in 2% Sodium hydroxide solution overnight and then the plates were cleaned using scrubber in tap water and again in distilled water.
- Both the plates were again wiped with 70% alcohol.
- 3 ml of repellent on small plate and bind silane on large plate was applied and spread uniformly with Kim wipes.

- Vaseline was applied to both the sides of the spacers.
- The spacers were placed with rubber adapter on either sides of the large plate and the small plate was placed on top of the large plate in such a way that it was seated uniformly on the edges and sides.
- Then the plates were clamped and the edges were sealed with cello tape.

3.6.3. Gel matrix preparation and gel casting

- 70 ml of a 5% polyacrylamide denaturation solution was taken in a conical flask and 500 µl of 0.1 % APS solution and 50 µl of N', N', N', N'-tetramethylethylenediamine (TEMED) were added and mixed well.
- The plates were kept in slanting position in such a way that the gel matrix flows freely into the plates and air bubble comes out freely.
- The solution was poured between the plates with the help of 10 ml syringe. After the matrix spreads uniformly throughout the plate, the comb was placed and the plates were clamped on the top.
- The plates were left for 1 hour for polymerization.

3.6.4. Sample loading and gel running (electrophoresis)

- After polymerization, comb was removed and the gel setup was mounted on an electrophoresis apparatus (Sequi-Gen® GT, BIO-RAD sequencing cell, USA).
- After flushing the wells with running buffer (0.5X TBE), the gel was pre-run for 45 minutes.
- 3 µl of loading dye were added to 15 µl of DNA taken for sample preparation and from this sample 2 µl were used for loading in the well. The sample was denatured at 95°C for 5 minutes and snap cooled on ice.
- After flushing the wells again, the denatured DNA samples were loaded on to the gel.
- The electrophoresis was resumed and allowed to proceed at 100 watts (constant) till bromophenol blue reached the bottom of the gel.
- Finally plates were dismantled from the electrophoresis apparatus.

3.6.5. Staining of gel

After careful removal of the small plate with gel from the assembly, the gel with plate was stained with washing treatments of various solutions. Silver staining was done in following steps:

3.6.6. Fixing

- Gel was soaked in fixer for 15 minutes or till the dye disappears with mild shaking.
- Then washed twice in double distilled water for 5 minutes.

3.6.7. Staining

- Gel was soaked in staining solution for 15 minutes with mild shaking followed by brief wash in double distilled water for 10 seconds.

3.6.8. Developing

- Gel was soaked in developer for 3-5 minutes or till bands appeared.
- Again gel was soaked in fixer for 5 minutes to stop the reaction and followed by washing with double distilled water for 2 minutes.
- Then the gel was dried and scanned using a UMAX scanner.

EXPERIMENTAL RESULT

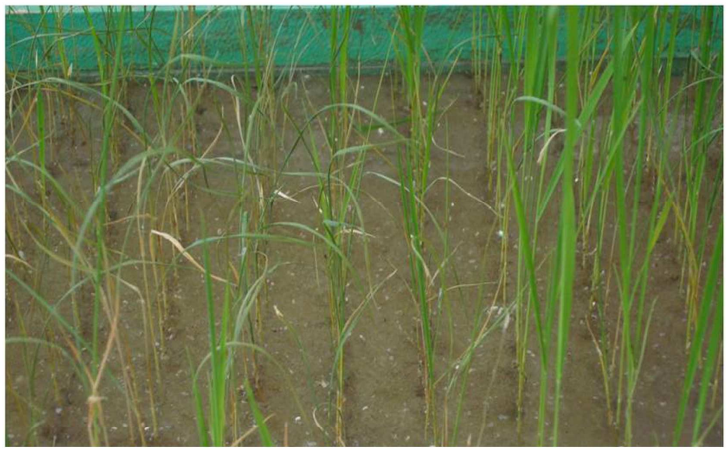
The present study was undertaken with a view of i) understanding phenotypic variation for BPH resistance in F_7 RILs of IR50/Rathu Heenati under green house conditions; ii) surveying of markers in IR50 and Rathu Heenati and selective genotyping of the resistant and susceptible F_7 RILs of IR50/Rathu Heenati and iii) identification of markers associated with BPH resistance. The results obtained are presented as below:

4.1. Phenotyping the IR50 x Rathu Heenati F_7 RIL population for BPH resistance

The BPH resistant parent Rathu Heenati, and susceptible parent IR50, have been used for the development of RIL mapping populations. A total of 268 F_7 RILs were screened to assess the level of resistance to BPH based on the standard seedbox screening test (SSST) in the greenhouse. The 268 F_7 RILs were screened for the BPH reaction in different time period due to want of insect populations and space for the screening. Every time the resistant and susceptible check variety were used for maintaining the level of uniformity in data points.

As BPH biotype 4 is prevalent in India, the biotype 4 were collected from the rice field at wetland and Paddy Breeding Station, TNAU, Coimbatore and used for the screening under greenhouse condition, the level of resistance to BPH was assessed based on the severity of the symptoms caused by the insects at the day on which 90 percent of the plants in susceptible check (TN1) row were killed by the insects. The severity of symptom on TN1, PTB33, IR50, Rathu Heenati and F_7 RILs are shown in the Plate 3. The parents namely IR50 and Rathu Heenati had the mean score of 5 and 3, respectively. The leaf damage score ranged from 2.0 to 9.0 in the F_7 RILs (Figure 1). The mean damage score recorded was 6.3 with a standard deviation of 1.9. A maximum of 59 plants were found to possess the damage score between 6-6.9. Out of the 268 F_7 RILs screened, 34 lines were found to be resistant with a damage score between 1 and 3.9, 46 lines were moderately resistant with a damage score between 4 and 4.9, 92 lines were moderately susceptible with a damage score between 5 and 8.9 and 37 lines were scored as susceptible with a damage score 9(Table 2).

Chi-square test was attempted to find out the phenotypic variation based on the leaf damage score by standard seedbox screening test for BPH resistance in F_7 RILs of IR50/Rathu Heenati. The calculated value of χ^2 was 43.52 against the tabulated value of χ^2 at 5 % (3.841) for



TN1 RILs IR50 RILs PTB33 Rathu Heenati

Plate 3.Phenotypic screening of F_7 RILs of IR50/Rathu Heenati

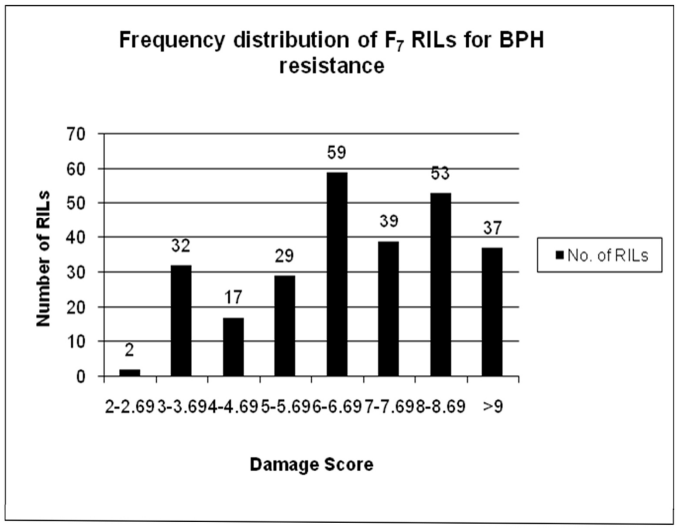


Figure 1. Frequency distribution of F_7 RILs of IR50/Rathu Heenati showing different levels of resistance to BPH based on standard seedbox screening test

Table 2. Number of F_7 families showing different levels of resistance to BPH based on the average damage score recorded in standard seedbox screening test.

Damage score ± SE	**Level of resistance**	**Number of F_7 families showing respective scale of damage**
1.0 – 3.9	Resistant	34
4.0 – 4.9	Moderately resistant	46
5.0-8.9	Moderately Susceptible	151
9	Susceptible	37

the expected 1: 1 segregation. The frequency of susceptible individuals was found to be more, which showed segregation distortion at 5% level (Table 3).

4.2. Parental Polymorphism Survey using SSR Primers

Total 53 SSR primers mapped throughout the chromosome 3 were selected and used to amplify the parents namely IR50 and Rathu Heenati to find out polymorphism between them. Out of which, only 11 primers RM2346, RM3180, RM6283, RM3117, RM1002, RM2453, RM3646, RM60, RM16, RM520 and RM227 were found to be polymorphic between the parents (Plate 4). The polymorphic primers between IR50 and Rathu Heenati are listed in Appendix 1. These polymorphic primers are positioned on the chromosome 3 in Figure 2.

4.3. Selective genotyping of the resistant and susceptible F_7 RILs

Based on the damage score of the standard seedbox screening test, the susceptible (score 9) and resistance (score 3) F_7 RILs of IR50/Rathu Heenati were selected for SSR genotyping. The susceptible F_7 RILs consists of 11,12,41,43 and 211. The resistant F_7 RILs consists of RILS 2, 28, 49, 135 and 265 (Table 1). Identified 11 polymorphic primers were screened in the five selected RILs of BPH resistant and five susceptible RILs along with parents for co-segregation analysis using Urea denaturing polyacrylamide gel electrophoresis (PAGE).

4.4. Identification of SSR markers linked to BPH resistance

The eleven primers that have shown polymorphism between the IR50 and Rathu Heenati parents were tested against the 5 resistant RILs and 5 susceptible RILs for co-segregation analysis. These 10 RILs were selected based on the damage score of standard seedbox screening test. Out of the 11 polymorphic primers, only two primers showed complete co-segregation among the selected susceptible and resistant individual RI lines. The segregation pattern of different primers on RILs is given as follows:

4.5. Co-segregation Analysis

4.5.1. Primer RM3180

BPH susceptible RILs have shown susceptible parent alleles at 150 bp. All the 5 resistant RILs were co-segregated with the resistant parent by having the allele at 135 bp (Plate 5).

4.5.2. Primer RM2453

BPH susceptible RILs having alleles 135 bp are similar to susceptible parent. All the 5 resistant RILs co-segregated with the resistant parent by having allele at 125 bp (Plate 6).

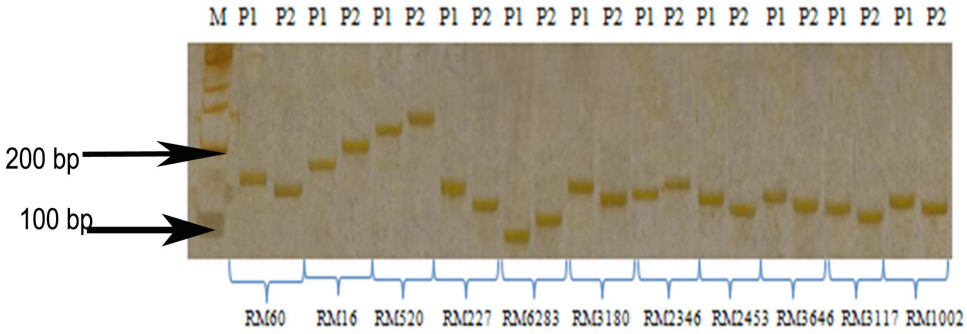


Plate 4. Parental Polymorphism Survey in IR50/Rathu Heenati

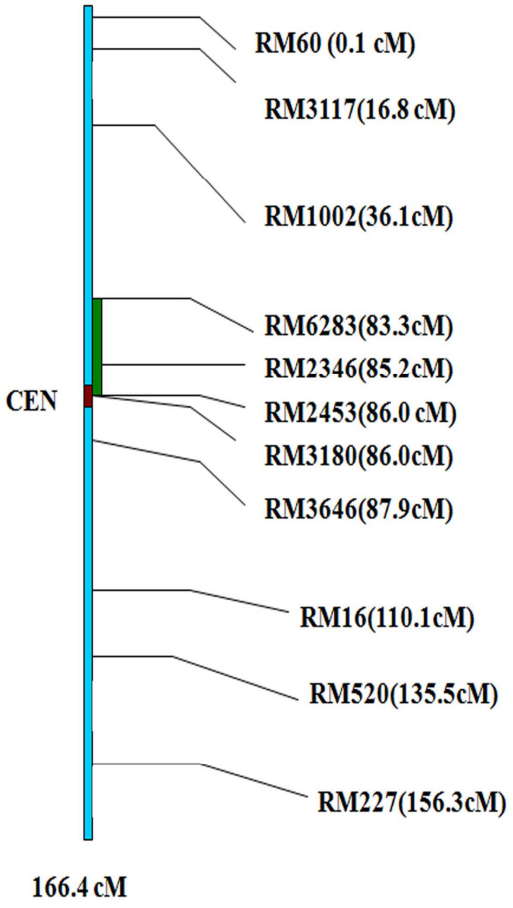


Figure 2. Polymorphic primers identified on chromosome 3 in IR50/ Rathu Heenati

Table 3. Chi-square test for BPH resistance and susceptible reaction in F_7 RILs of IR50/Rathu Heenati

	Observed value	**Expected value**	**χ^2 calculated**	**χ^2 table at 5%**
Resistant RILs	80	134	21.76	3.841
Susceptible RILs	188	134	21.76	
			43.52	

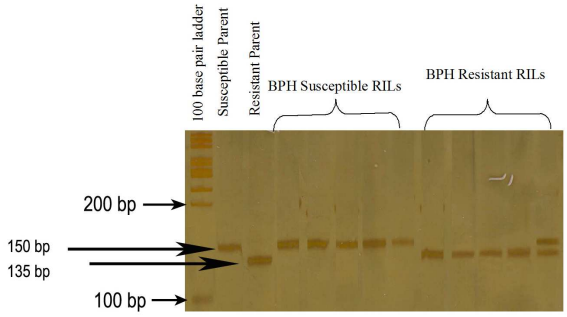


Plate 5. Selective genotyping of the susceptible and resistant F_7 RILs of IR50/Rathu Heenati by RM3180

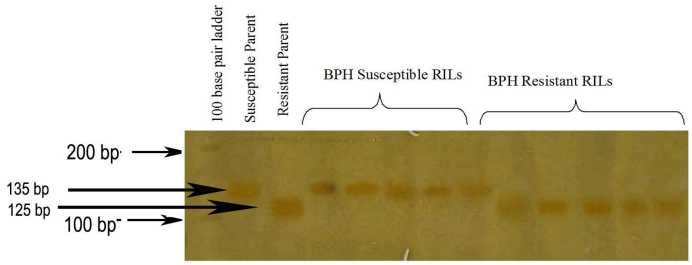


Plate 6. Selective genotyping of the susceptible and resistant F_7 RILs of IR50/Rathu Heenati by RM2453

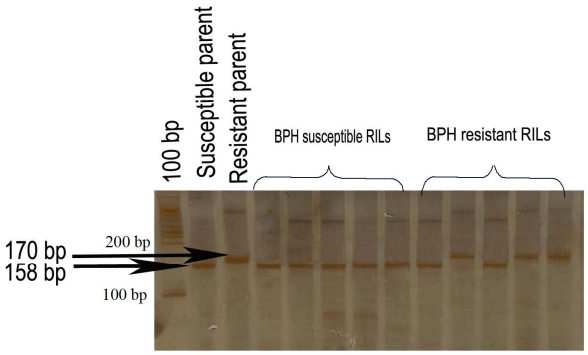


Plate 7. Selective genotyping of the susceptible and resistant F_7 RILs of IR50/Rathu Heenati by RM2346

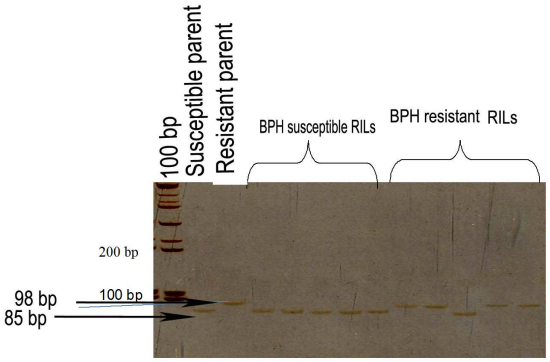


Plate 8. Selective genotyping of the susceptible and resistant F_7 RILs of IR50/Rathu Heenati by RM6283

4.5.3. Primer RM2346

The susceptible RILs having alleles at 168 bp are similar to susceptible parent. However, among the resistant lines, RILs#2 and RILs#49 produced the allele (168 bp) similar to susceptible parent, while all other lines having susceptible parent allele at 170 bp (Plate 7).

4.5.4. Primer RM6283

BPH susceptible RILs having alleles 85 bp are similar to susceptible parent. The alleles of all the resistant RILs, except RIL#49, produced the allele at 98 bp similar to resistant parent (Plate 8).

4.5.5. Primer RM1002

Three BPH susceptible RI lines have shown alleles at 155 bp similar to susceptible parent and remaining two susceptible RI lines produced an allele at 145 bp similar to resistant parent. One BPH resistant RI lines produced alleles at 145 bp similar to resistant parent and 2 resistance RI lines produced an allele (155 bp) similar to susceptible parent and 2 resistance RI lines produced both resistant and susceptible allele.

4.5.6. Primer RM3646

Among the 5 BPH susceptible RILs, 4 RILs produced an allele at 142 bp similar to susceptible parent and the RIL#78 have shown allele at 137 bp similar to the resistant parent. Out of 5 BPH resistant RILs, 3 RILs produced alleles at 137 bp similar to resistant parent and two RILs produced an allele at 142 bp similar to susceptible parent.

4.5.7. Primer RM3117

Four BPH susceptible RILs have shown alleles at 125 bp similar to susceptible parent and one RIL produced an allele at 110 bp similar to resistant parent. Out of five BPH resistant RIL_S two RIL_S produced alleles at 110 bp similar to resistant parent and and 3 RILs produced an allele at 125 bp similar to susceptible parent.

The primers RM60, RM16 RM227 and RM520 tested against the five BPH resistance and five BPH susceptible RILs. All the susceptible RILs produced susceptible parent allele and and all resistance RILs produced resistance parent allele.

DISCUSSION

Rice is infected by more than 100 species of insects. About 20 of them are considered as serious pests as they cause significant damage to the rice crop. Unfortunately by the mid 1980s, insecticides were shown to cause resurgence in green revolution-induced pests like brown planthopper (BPH), whitebacked planthopper (WBPH) and green leafhopper (GLH) (IRRI, 1984). Among the pest management strategies, host plant resistance (HPR) served as a viable alternative to chemical control methods (Khush and Brar, 1991). Much effort has been directed towards understanding the genetics of HPR to insects in crop plants. Based on vertical resistance, several genes conferring resistance to BPH, WBPH and GLH were identified (Heinrichs *et al*., 1985). There have been many notable successes in conventional breeding for improved plant resistance to insects. However, difficulties were encountered in incorporating these genes to elite varieties, since selection procedures for identifying resistant phenotypes were tedious and highly complex.

Breeding for resistance requires the development of suitable and reliable screening techniques, identification of heritable resistance and a strategy to transfer the trait of interest. Major and minor genes for a particular trait are determined in relation to their effects and the ability to detect them. The ability of detecting genes rests on the nature of screening method used on the phenotype of the trait. In other words, screening methods vary according to the phenotype under study. In understanding the genetic architecture of resistance to insect pests in a crop, the magnitude or level of resistance can be qualitatively determined by analysis of standard scoring systems or quantitatively by insect establishment.

In this study, Biotype 4 of insects was used for the screening of F_7 RILs. This biotype generally occurs in the Indian subcontinent (Khush *et al*., 1991). The different biotypes have been identified based on their abilities to feed and infest rice varieties with different resistant gene. More than 10 resistant genes have been identified according to their reactions to different BPH biotypes and their locations on chromosome (Yang *et al*., 2002). Rathu Heenati shows the resistance against all of the BPH biotype. Three genes *bph5, Bph6 and bph7* are resistant to biotype 4 but susceptible to biotypes 1, 2 and 3.

In order to map the BPH resistance gene, the development of mapping population and construction of linkage map is the first step in the marker assisted breeding approach. The genetic map construction followed by QTL mapping requires a suitable mapping population derived from divergent parents with markers and trait polymorphism. In the present study, two divergent parents

namely IR50 (*indica*), an elite BPH susceptible rice cultivar and Rathu Heenati (*indica*) , a BPH resistant rice cultivar were crossed and advanced to produce F_7 population by single seed descent method. Most of the earlier genetic map construction in rice was carried out by involving inter-subspecific (*indica/japonica*) crosses. The populations involving one *indica* parent and another *japonica* parent always shared higher level of marker polymorphism when compared to *japonica/japonica* or *indica/indica* parents.

Study of genetics of resistance to BPH was established by adopting the standard seed box screening test. It is a rapid method for screening large numbers of rice germplasm for qualitative resistance (Heinrichs *et al*., 1985). The screening of 268 F_7 RILs of IR50 and Rathu Heenati against BPH resulted in the identification susceptible and resistance RILs. The level of resistance showed a continuous variation for the trait of interest with a larger proportion of RILs falling towards susceptibility class. The leaf damage score ranged from 2.0 to 9.0. The mean of damage score recorded was 6.3 with a standard deviation of 1.9. A maximum of 59 plants were found to possess the damage score between 6 - 6.9. Out of the 268 F_7 RILs screened, 34 lines were found to be resistant with a damage score between 1 and 3.9, 46 lines were moderately resistant with a damage score between 4 and 4.9, 92 lines were moderately susceptible with a damage score between 5 and 8.9 and 37 lines were scored as susceptible with a damage score 9.

The leaf damage score of resistance and susceptible class were subjected to Chi-square test to assess the genetics of BPH resistance. The frequency of susceptible individuals was found to be more which resulted in segregation distortion at 5% level. Segregation distortion has been common in mapping population due to sampling or biological selection and it usually impede with the process of genetic map construction. It may be caused by genetic, physiological and environmental factors (Xu *et al*., 1997). Wang *et al*. (1994) developed a genetic map using a set of RILs from two distantly related rice varieties CO39 and Moroberekan was the first report wherein segregation distortion was significant for most of the marker loci surveyed. Subsequently, Xu *et al*. (1997) analysed the segregation distortion in six different mapping populations involving marker data established and the range of segregation distortion was 6.8 – 31.8 per cent.

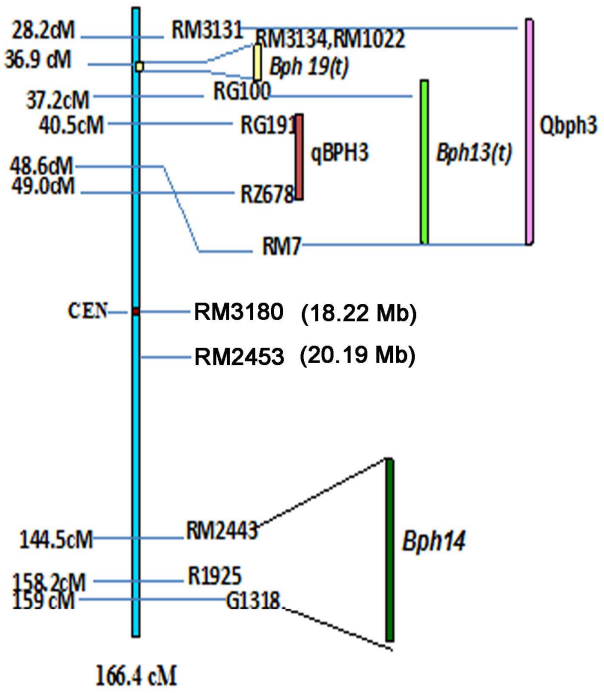
Several genetic maps using SSR markers have been constructed in rice (Temnykh *et al*., 2001 and McCouch *et al*., 2002). Mapping with high resolution requires a high density of genetic markers covering the entire genome having normal segregation pattern. Many workers (Renganayaki *et al*., 2002; Buna *et al.,* 2001; Tan *et al*., 2004; Huang *et al*., 2001; Sun *et al*., 2005;

Chen et al., 2006; Ramalingam *et al*., 2003) have reported major QTL for BPH resistance in chromosome 3. Further SSR marker survey and association mapping work resulted in identification of SSR markers linked to BPH resistance in chromosome 3 in the region of 83.3 - 86.0 cM (Jennifer, 2006). Hence an attempt was made to explore the chromosome 3 for a possible major gene to BPH resistance. A total of 11 polymorphic markers were identified out of 53 marker loci surveyed, between IR50 and Rathu Heenati covering linkage group of 3. The survey of two parents revealed an average polymorphism of 20.75 per cent for the SSR marker loci. This low level of polymorphism could be due to the involvement of both the rice varieties from *indica* group.

Based on the damage score of the standard seedbox screening test, 5 BPH resistant and 5 BPH susceptible recombinant inbred lines (RILs) were selected. The eleven primers that have shown polymorphism between the IR50 and Rathu Heenati parents were tested against the 10 (5 resistant lines and 5 susceptible lines) individual RILs along with parents for co-segregation. Out of the 11 polymorphic primers, two primers namely RM3180 (18.22 Mb) and RM2453 (20.19 Mb) showed complete co-segregation with resistance and other nine primers showed recombination among the selected susceptible and resistant individual RI lines in selected markers region. Earlier, Jennifer (2006) reported that RM3180 (18.22Mb) is linked with BPH resistance which is located near to the RM3180 (18.22Mb) and RM2453 (20.19 Mb) reported in the present study.

The selected resistant RILs include 2, 28, 49,135 and 265. The phenotype of the selected resistant RILs possessing towards the resistant parent Rathu Heenati and the two primer RM3180 and RM2453 segregation also shows the allele similar to the resistant parent Rathu Heenati. It ensures that these markers are linked with the region for BPH resistance in the selected RILs. These RILs can be used for the testing in the field in multilocation trial and it can be used for the development of the resistant varieties.

According to Lakshminarayana and Khush (1977), the Sri Lankan cultivar Rathu Heenati has a dominant gene for resistance, and designated it as *Bph3*. Gomathi (2002) identified two SSR markers namely RM168 and RM186 associated with BPH resistance using the F_3 population of IR50/Rathu Heenati. Biotype-4 resistance gene *Bph13(t)*, derived from *Oryza officinalis* was mapped on the chromosome 3 by the RAPD analysis. Sun *et al.,* (2005) identified the QTL between RM3131 and RM7 with a LOD score of 2.32 and phenotypic variance of 6.5% in the F_2



Chromosome 3

Bph 19(t)- Chen *et al.,* 2006
qBPH3 - Ramalingam *et al.,* 2003
Bph13(t) - Renganayaki *et al.,* 2002
Qbph3 - Sun *et al.,* 2005
Bph14 - Huang *et al.,* 2001; Buna *et al.,* 2001; Tan *et al.,* 2004

Figure 3. SSR markers flanking the QTL for BPH resistance gene on chromosome 3 and location of the markers identified for BPH resistance in F_7 RILs of IR50 and Rathu Heenati

population of Rathu Heenati / 02428. The QTL reported are mainly present between the region 36.9 cM to 48.6 cM and 144.5 to 159 cM on chromosome, which is shown in the figure 3.

In the present study, genome wide QTL analysis could not be made due to the non-availability of a molecular marker based linkage map of IR50/Rathu Heenati. This was due to the low level of polymorphism observed between parents (20.75). Higher order of segregation distortion has a negative impact on genetic map construction by unwanted pseudo linkages between marker loci (Wang *et al*., 1994). Establishing linkage maps showing segregation distortion may not be ideal considering the utility of genetic maps for QTL mapping. In the present study, the linkage map for chromosome 3 could not be established based on the segregation distortion observed across the SSR markers surveyed and moreover forced linkage map construction using segregation data may lead to dubious linkages between marker loci.

Fine mapping of the *Bph1* has been done on chromosome 12 in 273 F_8 recombinant inbred lines (RILs) derived from a cross between Cheongcheongbyeo, an indica type variety harbouring *Bph1* from Mudgo, and Hwayeongbyeo,a BPH susceptible *japonica* variety. (Cha *et al*., 2008). There is possibility of the fine mapping of BPH resistant gene of the reported region on the chromosome 3 with more markers. Other *in silico* approaches are expected to give closer markers to the gene of interest facilitating marker assisted breeding programme Map-based cloning represents the most promising approach for isolating the BPH resistance genes. The detailed genetic and physical maps of the locus can facilitate marker-assisted gene pyramiding and cloning of the gene present on chromosome 3.

SUMMARY

The present study was undertaken with a view of understanding phenotypic variation for BPH resistance in F_7 RILs of IR50 / Rathu Heenati under green house conditions and surveying of the markers in IR50 and Rathu Heenati and selective genotyping of the resistant and susceptible F_7 RILs of IR50 / Rathu Heenati and to identify the markers associated with BPH resistance.

1. A total of 268 F_7 RILs derived between a Brown Planthopper (BPH) susceptible IR50 and moderately resistant Rathu Heenati were phenotyped for surveying their level of resistance against BPH by the standard seedbox screening test (SSST) in the greenhouse.
2. The parents namely IR50 and Rathu Heenati had the mean score of 5 and 3 respectively.

3. Among the F_7 RILs, the leaf damage score ranged from 2.0 to 9.0. Out of the 268 F_7 RILs screened, 34 lines were found to be resistant with a damage score between 1 and 3.9, 46 lines were found to show moderate resistance reaction with a damage score between 4 and 4.9, 151 lines were found to be moderately susceptible with a damage score between 5 and 8.9 and 37 lines were scored as susceptible with a damage score 9.
4. A total of 53 SSR primers mapped on the chromosome 3 were used to screen the polymorphism between the parents IR50 and Rathu Heenati, out of which eleven were found to be polymorphic between IR50 and Rathu Heenati.
5. The eleven primers that have shown polymorphism between the IR50 and Rathu Heenati parents were genotyped in a set of 5 resistant RILs and 5 susceptible RILs along with the parents for co-segregation analysis.
6. Among the eleven primers, two primers namely RM3180 (18.22 Mb) and RM2453 (20.19 Mb) showed complete co-segregation with resistance.

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APPENDIX 1. – Polymorphic RM primers between IR50 and Rathu Heenati

Primer name	Sequence	Number of bases
RM3180	F-5'GGGTCGGATAGCCACACAC3' R-5'GAGGTAATCTCGCGGAGTTG3'	19 20
RM2346	F-5'TTCAGGGATGTGAAATATAC3' R-5'ATCGTGCTTTTATTGAAATA3'	20 20
RM6283	F-5'TGGAGACTGAGCTGATGCC3' R-5'TCAGGTGGTCGGTTCCTTAC3'	19 20
RM2453	F-5'TAGGTGTTCAGGAGTAAAGA3' R-5'AAACCAGTATTGCTTACAAG3'	20 20
RM3117	F-5'GCCATCTCTCTCTCTCTCTCTC3' R-5'CCTTAGCTCATCAAGCGAGG3'	22 20
RM3646	F-5'ACTAGAGCACCCTCGCTGAG3' R-5'CTCAGCCACCCCATCAAC3'	20 18
RM1002	F-5'GAACCAGACAAGCAAAACGG3' R-5'AGCATGGGGATTTAGGAACC3'	20 20
RM60	F-5'AGTCCCATGTTCCACTTCCG3' R-5'ATGCCTACTGCCTGTACTAC3'	20 20
RM16	F-5'CGCTAGGGCAGCATCTAAA3' R-5'AACACAGCAGGTACGCGC3'	19 18
RM227	F-5'ACCTTTCGTCATAAAGACGAG3' R-5'GATTGGAGAGAAAAGAAGCC3'	21 20
RM520	F -5'AGGAGCAAGAAAAGTTCCCC3' R-5'GCCAATGTGTGACGCAATAG3'	20 20