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# SSR Mapping of Brown Planthopper Resistance Gene *Bph*9 in Kaharamana, an *Indica* Rice (*Oryza sativa* L.)

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**Abstract:** The brown planthopper (BPH) is one of the most serious insects pests of rice, and the host resistance has been recognized as one of the most economic and effective measures for BPH management. In this study, we conducted a molecular-based genetic analysis of *Bph*9 in Kaharamana, a Sri Lanka rice variety resistant to BPH insects of East and Southeast Asia. An $F_2$ segregating population composed of 180 plants was constructed from the cross between Kaharamana and 02428, and each $F_2$ plant was self-crossed to obtain $F_{2:3}$ family. The bulked seedling test method was used to evaluate the resistance of $F_{2:3}$ families, and the genotype of each $F_2$ plant was inferred from the phenotype of corresponding $F_{2:3}$ family. Linkage analysis indicated that the resistant gene *Bph*9 in Kaharamana was located between SSR markers RM463 and RM5341 on chromosome 12 with linkage distances of 6.8 cM and 9.7 cM, respectively. The time- and money-saving SSR markers would be helpful in the application of *Bph*9 in breeding program via marker-assisted selection.

**Key words:** SSR mapping; *Nilaparvata lugens* Stål; *Bph*9; rice (*Oryza sativa* L.)

The brown planthopper (BPH), *Nilaparvata lugens* Stål, is one of the most serious insect pests of rice (*Oryza sativa* L.) in Asian rice growing area. BPH causes direct damage by sucking plant sap, and transmitting several viral diseases such as rice grassy stunt [1] and rugged stunt [2].

Using of insecticides to control BPH insects is not only costly in terms of labor and money, but also causes environment damage. In addition, resurgence, a phenomenon of pest population increase after application of insecticides [3], has also been reported. To solve the problem, naturally evolved resistance systems would provide a promising and readily acceptable means of control.

Utilization of host resistance has been recognized as one of the most economic and effective measures for BPH management. Many donors of BPH resistance have been identified and genetics of resistance have been investigated. So far, 13 BPH resistance genes have been reported, of which *Bph*1, *Bph*2 and *Bph*10 (t) were mapped on chromosome 12 [4-6]. Four additional resistance genes *Bph*4, *Bph*11(t), *Bph*12 (t) and *Bph*13(t) were assigned to chromosome 6, 3, 4 and 2 [7-9]. Analysis of quantitative trait loci (QTL) contributing to BPH resistance in IR64, Kasalath, Teqing and wild rice *Oryza officinalis* has also been carried out [10-13].

Three Sri Lanka varieties, Kaharamana, Balamawee and Pokkali, were found to be resistant to BPH insects of biotype1, biotype2 and biotype3 but not biotype4 from Bangladesh, and the resistance of these three varieties was controlled by a dominant gene which was allelic to each other but was different from

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*Bph*1 and *Bph*3 [14]. Despite of the versatility of *Bph*9, the use of the gene in breeding program has not yet been reported. Nemoto *et al.*[15] carried out allelism tests between Kaharamana, Balamawee and Pokkali, and proved that a dominant gene, which was designated as *Bph*9, governed the resistance. RFLP and RAPD analysis of the resistant gene has been carried out in Pokkali, and this gene has been mapped between OPRO4 and S2545 on the long arm of chromosome 12 with a map distance of 8.8 cM from OPRO4 and 12.5 cM from S2545 [16]. But the molecular analysis of resistant gene in Kaharamana and Balamawee has not yet been reported.

To facilitate a marker-assisted selection in breeding BPH resistant variety, we carried out SSR tagging of *Bph*9 in Kaharamana. In the study reported here, we constructed an $F_2$ segregating population and conducted a molecular-based genetic analysis of *Bph*9 in Kaharamana. The objective was to determine the chromosomal location of *Bph*9 in Kaharamana and find SSR markers closely linked to *Bph*9 that might be useful for cloning this gene and for improving BPH resistance in rice breeding programs.

## 1 Materials and Methods

### 1.1 Plant materials and insects

The genetic materials were an $F_2$ population of 180 plants and $F_1$ plants from a cross between Kaharamana and 02428. The *japonica* 02428 is a BPH susceptible cultivated rice variety with wide compatibility. Each $F_2$ plant was selfed to obtain $F_{2:3}$ family. Rathu Heenati, carrying *Bph*3 [17] and Taichung Native 1 (TN1) with no resistance genes were used as resistant and susceptible controls, respectively.

The BPH population used for infestation was biotype 1, which was first collected from rice fields at Hangzhou, China, and then maintained on TN1 in the greenhouse for ten generations. In this experiment, insects were maintained on TN1 under natural condition in the greenhouse of Nanjing Agriculture University, Nanjing, China.

### 1.2 Evaluation of BPH resistance

A modified bulked seedling test was conducted to phenotype the reaction to BPH infestation on parents, $F_1$ plants and 180 $F_{2:3}$ families. To ensure all seedlings at the same growth stage for all seedlings for insect infestation, seeds were first germinated in petri dishes. After which about 70 germinated seeds of each entry were sown in two plastic pots of 10 cm-diameter with a hole in the button. Generally, 28 pots including one pot of parents and control varieties were placed in a 68 cm × 42 cm × 16 cm plastic seed-box. About 2cm deep water was kept in the seed-box until resistance evaluation was completed.

At the second-leaf stage, 15 d after the seeds were sown; the seedlings were infested with 2nd to 3rd-instar BPH nymphs at a density of six insects per seedling. When all seedlings of TN1 died, the plants of $F_{2:3}$ families were examined and each seedling was given a score of 0, 1, 3, 5, 7 or 9 according to Su *et al.* [11], which were based on IRRI [18] and Athwal *et al* [19]. The genotype of each $F_2$ plant was then determined by assaying the phenotype of corresponding $F_{2:3}$ families, which was inferred based on the weighted average of the seedlings tested.

### 1.3 DNA preparation and SSR assay

DNA samples were extracted from young leaves of each entry using the method described by Dellaporta *et al*[20]. The extracted DNA samples were then dissolved in TE buffer (10 mmol/L Tris-base, 0.1 mmol/L EDTA) and tested for quality and quantity using a MBA 2000 UV/VIS Spectrometer (Perkin Elemer Co.). The samples were then diluted into 20 ng/μL with autoclaved double distilled water (dd $H_2O$) for further analysis.

SSR analysis was performed following the procedure of Chen *et al.* [21] with minor modifications. The original sources and motifs for all the SSR markers used in this study could be found in the gramene database (http://www.gramene.org/) and McCouch *et al.* [22](or http://www.dna_res.kazusa.or.jp/9/6/05/spl_table1/table.pdf). Amplification reactions were carried out in 10 μL containing 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl,

1.5 mmol/L $MgCl_2$, 50 μmol/L each of dNTPs, 0.2 μmol/L each primers, 0.5 U *Taq* polymerase (TaKaRa, Dalian) and 20 ng of DNA template. Reactions were performed using a PTC-200 thermal cycler (MJ Research Inc.) programmed as 94℃ for 4 min, followed by 35 cycles of 1 min at 94℃, 1 min at 55℃ 1.5 min at 72℃ with a final extension of 7 min at 72℃. Amplification productions were separated in 8% non-denaturing PAGE, and observed by silver staining method based on Sanguinetti CJ [23]. The bands were then scored on a light box with fluorescent lamps.

### 1.4 Linkage analysis

Co-segregation of SSR markers with BPH resistance was analyzed using MAPMAKER/3.0 software to determine the linkage relationships between Lander *et al* [24]. The Kosambi function was transformed into centimorgan (cM).

## 2 Results

### 2.1 BPH resistance evaluation

In bulk seedlings test, the resistance score of Kaharamana and 02428 were 1.63 and 7.07, respectively, which indicated that Kaharamana was resistant to biotype-1 of BPH while 02428 was susceptible. The $F_1$ plants with resistance score of 0.65 were highly resistant to BPH, which indicated Kaharamana was governed by a dominant gene. It seemed that Kaharamana was less resistant than Rathu Heenati whose resistance score was 0.4 (Table 1).

The resistance scores of the 180 $F_{2:3}$ families infested with BPH of biotype-1 showed a continuous distribution, ranging from a low of 0.47 to a high of 9.00, with three apparent peaks around 2, 5 and 8 in the distribution curve ( Fig.1). Genotype of each $F_2$ plant was inferred from the corresponding $F_{2:3}$ family. All the 180 $F_{2:3}$ families were classified into three categories on the resistance scores as resistance, segregating or susceptibility and the corresponding $F_2$ plant was genotyped as *RR* (homozygous resistance), *Rr* (Segregating heterozygous) and *rr* (homozygous susceptibility). The segregation of $F_2$ population showed a good fit to the expected ratio of 1:2:1 ($\chi^2$= 1.51, $\chi^2_{0.05,\ 2}$= 5.99 ) (Table 2).

**Table 1 The scores of BPH resistance in parents and control varieties**

| Varieties[a] | Number of seedlings tested | Resistance score (0-9) |
|---|---|---|
| Kaharamana | 60 | 1.63 |
| 02428 | 60 | 7.07 |
| $F_1$ | 26 | 0.65 |
| Rathu Heenati | 60 | 0.4 |
| TN1 | 65 | 9.0 |

[a] Rathu Heenati and TN1 were used as resistance and susceptible controls for BPH biotype-1, respectively.

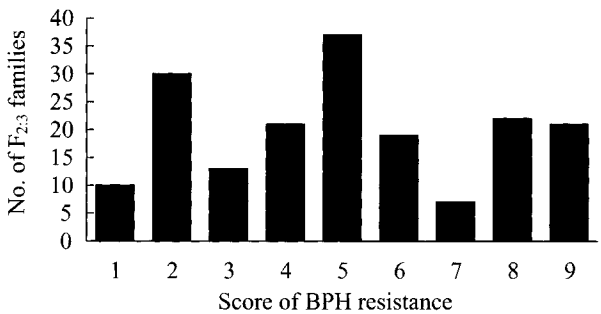


**Fig. 1 Distribution of BPH resistance scores of the 81 RILs**

The scores of Kaharamana, 02428 and $F_1$ were 1.63,7.07 and 0.65, respectively.

**Table 2 Segregation of BPH resistance in $F_2$ population derived from the cross Kaharamana/02428**

| $F_2$ genotype | Number of $F_2$ individuals[a)] | Phenotype of corresponding $F_{2:3}$ family[b)] |
|---|---|---|
| *RR* | 38 | RS<2 |
| *Rr* | 96 | 2≤RS<7 |
| *rr* | 46 | RS≥7 |

[a)] $\chi^2$ value for 1RR: 2Rr: 1rr is 1.51 ($\chi^2_{0.05,\ 2}$=5.99 );

[b)] Number in this column was the range of resistance score weighted average; RS: Resistance score.

### 2.2 Mapping of *Bph*9 by SSR assay

According to the previous report on the chromosome position of *Bph*9 in Pokkali [16], a total of 35 SSR primer pairs on chromosome 12 were firstly

used to survey polymorphisms between Kaharamana and 02428 in this study, of which 12 (34%) SSR primer pairs showed to be polymorphic. An SSR marker RM463 on the long arm showed significant cosegregation with BPH resistance (Table 3, Fig.2A). The other SR markers showing polymorphism around this region were further used to analyze segregation in the mapping population and a linkage map was constructed using SSR markers on the long arm of chromosome 12. Finally, the resistant gene *Bph*9 in Kaharamana was located between RM463 and RM5341 with distances of 6.8 cM and 9.7 cM, respectively (Fig.2B).

**Table 3 Distribution of resistance score by genotype of RM463 in $F_2$ population derived from the cross Kaharamana/02428**

| Marker | Marker genotype [a)] | Number of $F_2$ plants in three genotype according to the phenotype of corresponding $F_{2:3}$ family | | |
|---|---|---|---|---|
| | | *RR* | *Rr* | *rr* |
| RM463 | 1/1 | 29 | 4 | 2 |
| | 1/2 | 5 | 80 | 1 |
| | 2/2 | 1 | 6 | 41 |

[a)] 1/1 denotes marker genotype of Kaharamana; 2/2 denotes marker genotype of 02428; 1/2 denotes marker genotype of $F_1$ cross of Kaharamana/02428.

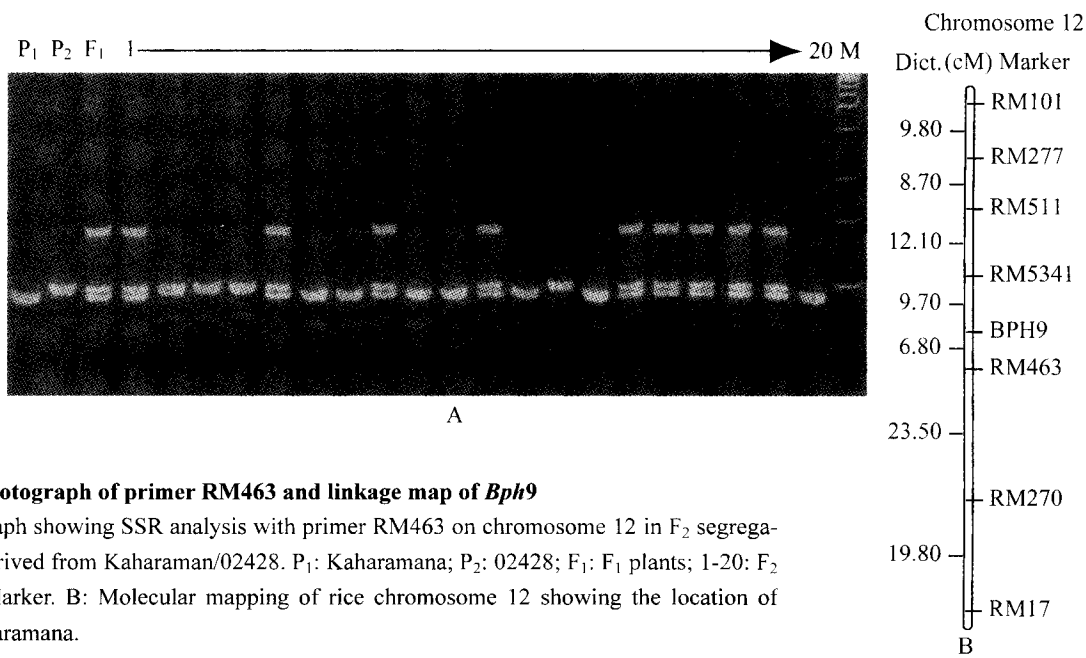


**Fig. 2 PAGE photograph of primer RM463 and linkage map of *Bph*9**

A: PAGE photograph showing SSR analysis with primer RM463 on chromosome 12 in $F_2$ segregation population derived from Kaharaman/02428. $P_1$: Kaharamana; $P_2$: 02428; $F_1$: $F_1$ plants; 1-20: $F_2$ individuals; M: Marker. B: Molecular mapping of rice chromosome 12 showing the location of *Bph*9 gene in Kaharamana.

## 3 Discussion

The threat of BPH to rice has resulted in a constant search for genes showing resistance and an accelerated effort to develop resistant varieties [25, 26]. However, the difficulty of breeding varieties for resistance was greatly exacerbated by the ability of BPH to develop new biotypes to "breakdown" resistant varieties rapidly and become virulent on novel plant genotype. An outbreak of BPH prompted IRRI to initiate a breeding program for the development of resistant varieties of rice. Resistant varieties with *Bph*1 and *Bph*2 were released sequentially in 1973 and 1976, but the varieties became ineffective within a few years; the dominant *Bph*3 gene was released in 1982, which has been currently broken down in many countries [27]. Therefore, more effective genes for BPH resistance must be constantly identified and incorporated into BPH resistance breeding program.

The resistant gene *Bph*9 in Kaharamana was

mapped between RM55341 and RM463 on chromosome 12 (Fig.2B). Murata *et al.* [16] reported that *Bph*9 in Pokkali was located between G2140 and S2545 (Fig.3d). On the newly published genetic linkage map that integrated RFLP and STS markers with SSR markers, the SSR marker RM5341 was located between G2140 and S2545 and only 0.6 cM to G2140 (Fig.3c). The other SSR marker RM463 was 1.0 cM to RM309 on the IR64/Azucena map (Fig.3a), while RM309 was 3.0 cM to S2545 on the integrated map (Fig.3c). Hence we confirmed that *Bph*9 in Kaharamana and Pokkali were located in the same region of chromosome 12. We found that the map distance between RM511 and RM463 in this study was 12.9 cM greater than the corresponding distance on the IR64/Azucena (Fig.3, a and b). This might be ascribed to the use of different mapping populations and the hybrid sterility in the progenies of *indica/japonica* cross. The same phenomenon was also reported by Murata *et al* [16].

To slow down the evolution of BPH population, several approaches have been proposed over the years, such as rotating different cultivars, planting multiple resistant lines within the same field, and combing different resistance genes within the same cultivars[28-31]. Because of the ineffectiveness of *Bph*1, *Bph*2 and *Bph*3, the resistant gene *Bph*9 should provide an alternation for carrying out strategies of rotating different cultivars or pyramiding different resistant genes in the same cultivar. The molecular markers linked to *Bph*9, especially the

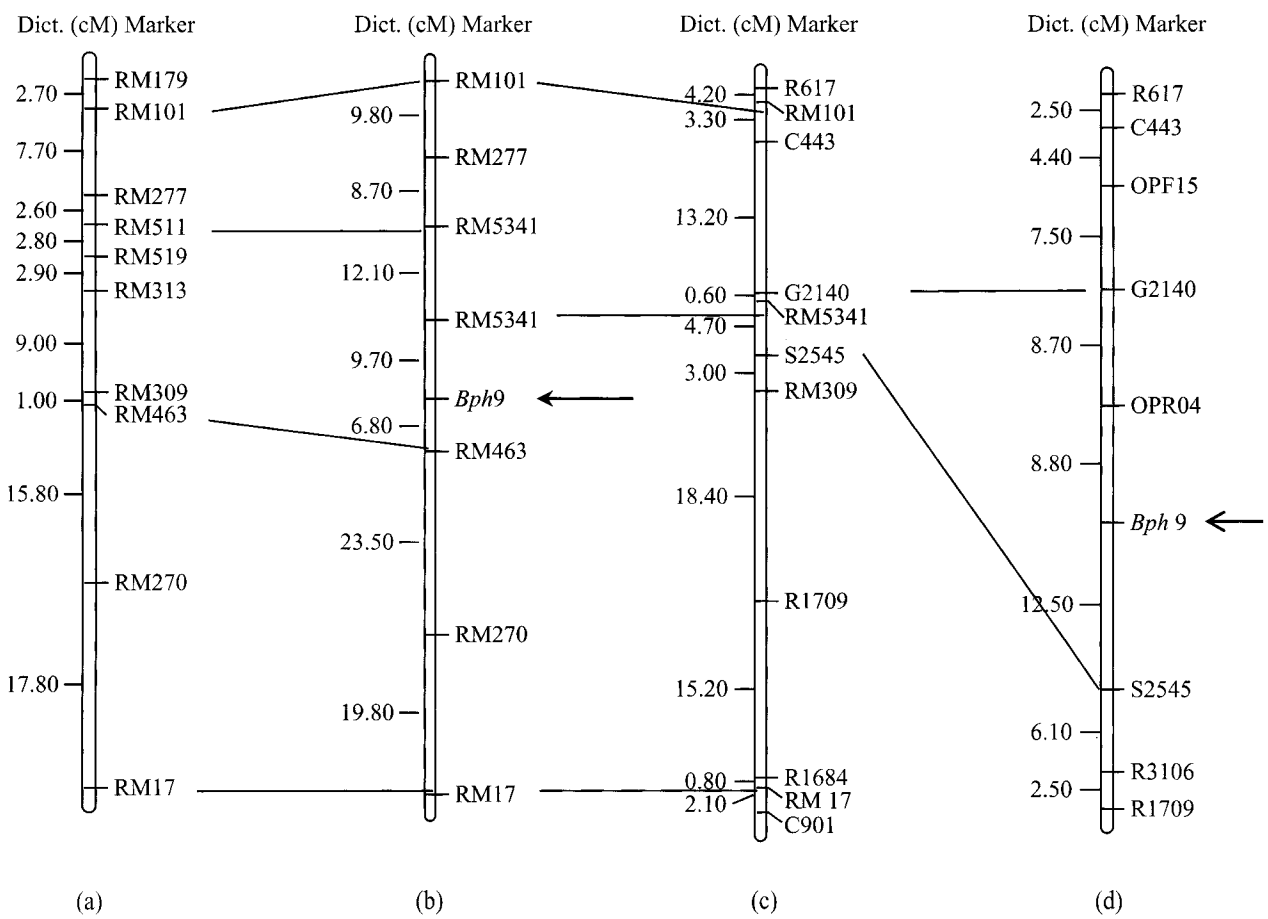


**Fig. 3 The map position of *Bph*9 on the long arm of chromosome 12**

(a): The IR64/Azucena map constructed in reference to gramene database (http://www.gramene.org/). (b): The Kaharamana/02428 map constructed in the present study; (c): The map constructed in reference to the database in http://www.dna_res.kazusa.or.jp/9/6/05/spl_figure1/fig1.pdf; (d): The Norin-PL9/Pokkali map constructed by Murata *et al.* (2002); " ← ", Chromosome location of *Bph*9 in Kaharamana in this study; " ← ", Chromosome location of *Bph*9 in Pokkali by Murata *et al.* (2002).

time- and money-saving SSR markers found in this study would inevitably promote the use of this resistant gene in marker-assisted selection program for breeding new BPH resistance cultivars.

Although *Bph*9 in Kaharamana and Pokkali were located in the same region of chromosome 12, no evidence has yet been obtained that they might share the same genomic sequence and encode the same protein. Cloning of the gene in Kaharamana, Pokkali and Balamawee would eventually lead to the elucidation of the difference.

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# 利用 SSR 定位籼稻品种 Kaharamana 中抗褐飞虱基因 *Bph9*

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**摘 要**：褐飞虱是危害水稻生产最重要的害虫之一，利用寄主抗性被认为是防治褐飞虱最经济而有效的方法。斯里兰卡水稻品种 Kaharamana 对东亚和东南亚的褐飞虱种群均表现抗虫性，利用分子遗传学的方法对其携带的 *Bph9* 基因进行了 SSR 定位。所用的遗传群体为来源于 Kaharamana 和 02428 的含有 180 个单株的 $F_2$ 分离群体，每个 $F_2$ 单株套袋自交获得 $F_{2:3}$ 家系。利用苗期集团鉴定对 $F_{2:3}$ 家系进行抗褐飞虱鉴定，以推测相应 $F_2$ 单株的基因型。连锁分析表明，*Bph9* 位于第 12 染色体上的两个 SSR 标记 RM463 和 RM5341 之间，分别与之相距 6.8 cM 和 9.7 cM。该标记有助于将 *Bph9* 用于分子标记辅助选择育种研究。

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