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Full Length Article

Validation of Functioning Resistant Genes against Malaysian Biotype of Brown Planthopper in Rice Variety, Rathu Heenati

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Abstract

Rathu Heenati is a donor parent commonly used in the development of resistant rice varieties against brown planthopper (BPH), *Nilaparvata lugens*, which is a devastating insect pest of rice. The variety is reported to have a broad-spectrum resistance against BPH, through the action of multiple genes and quantitative trait loci (QTL) including *Bph3*, *Qbph3*, *Qbph4* and *Qbph10*. The present study was carried out to validate the effectiveness of those genes against Malaysian BPH population. The study was conducted using an F_2 segregating population which was obtained from a cross between Rathu Heenati and a Malaysian commercial variety, MR219. Plant damage score was used to estimate the degree of plant tolerance, while the amount of honeydew excretion was used to measure the level of antibiosis. The gene presence in an individual plant was determined based on the segregation pattern of the flanking microsatellite markers of the respective reported genes. Results confirmed the role of *Bph3* and the three other *QTLs* in conferring resistance against the Malaysian biotype of BPH. Marker assisted breeding can facilitate the monitoring of the introgressed genes in the plants of a breeding population. © 2022 Friends Science Publishers

Keywords: *Oryza sativa* L.; Brown plant hopper (BPH); Resistance gene; Quantitative trait locus; Microsatellite markers

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops that feed more than three billion people in the world (Khush 2005; Yudo *et al.* 2018). It is estimated that the growing demand of the world population for rice consumption in 2030 would reach 873 million tons (Purevdorj and Kubo 2000; Yudo *et al.* 2018). This target however is being jeopardised by the loss of rice production in the field. Biotic factors contribute to a 52% reduction of global rice yield (Yarasi *et al.* 2008) where almost 21% resulted from infestations by various species of insect pests. One of the major insects that cause a huge problem to rice is Brown Planthopper (BPH), *Nilaparvata lugens* (Stål). Many countries have reported that BPH greatly damaged the plant and significantly reduced the rice yield (Huang *et al.* 1997; Sogawa *et al.* 2003; Sun *et al.* 2005; Shabanimofrad *et al.* 2017). In Asia, the annual economic loss caused by BPH is estimated to be over $300 million (Min *et al.* 2014; Yuexiong *et al.* 2019), with several devastating outbreaks previously reported in China, Vietnam, Philippines, Indonesia, Thailand, Japan, Korea, India, Bangladesh, and Malaysia (Heong 2009). BPH has long been identified as one of the most economically important insect pests of rice in Malaysia.

The utilization of resistant varieties is regarded as the most effective method in minimizing yield losses. Several BPH resistant varieties have been bred and released to farmers (Jairin *et al.* 2017). Currently, more than 30 BPH resistant genes have been reportedly present in several rice varieties including in wild type species (Fujita *et al.* 2013; Sarao and Bentur 2016). To date, a Sri Lankan rice variety Rathu Heenati has shown high resistance and is resistant to all four BPH biotypes worldwide (Jairin *et al.* 2007a; Li *et al.* 2017), including the BPH population in Malaysia (Ito *et al.* 1994). The resistance of a variety to BPH is measured phenotypically through the mechanisms of antibiosis, plant tolerance, and antixenosis

(Bhanu *et al.* 2014; Hu *et al.* 2016). These mechanisms were used in the breeding and screening processes for the development of BPH resistant varieties.

Another breeding approach is currently available to breeders, i.e. the application of molecular markers in plant breeding, whereby selection is performed based on the presence or absence of genotypic markers instead of relying only on the observation of phenotypic expression. The selection of resistant plants using marker-assisted selection (MAS) is based on the linkage markers that are located nearby or on the resistant genes themselves. The introgression of desirable genes from one plant to another or from the parent plant to their progenies can be monitored through the presence of those markers (Mekonnen *et al.* 2017).

The *Bph3* gene in Rathu Heenati was mapped on the short arm of chromosome 6 and is flanked by related markers, RM589 and RM588 (Jairin *et al.* 2007a). Previous studies have also shown that Rathu Heenati's resistance to BPH was also related to the presence of other genes such as the *Bph17* and other minor quantitative trait loci, QTLs located on various chromosomes (Sun *et al.* 2005; Jairin *et al*. 2007a; Kumari *et al*. 2010; Hu *et al.* 2016). A QTL named *Qbph3* is located on chromosome 3, positioned between markers RM313 and RM7. Whereas the second QTL, *Qbph4* was found on the short arm of chromosome 4 between markers RM8213 and RM5953 with a map distance of 3.6 cM and 3.2 cM. On the other hand, the *Qbph10* was flanked by markers RM484 and RM496 on chromosome 10 (Sun *et al.* 2005).

This study aimed to validate the reported gene/QTLs that may have an association with the resistance of Rathu Heenati to the Malaysian biotype of BPH. While all the four genes/QTLs and their respective markers were known, the relative resistance expression of each of these resistant factors or multiple combinations of them is yet to be determined. Previous attempts using Rathu Heenati as donor parent failed to produce lines with resistance scores comparable to Rathu Heenati. It is then hypothesized that all the four resistant factors are responsible for the high resistance of Rathu Heenati. Assuring the presence of all the four genes in the breeding lines may ensure the development of highly resistant varieties. Marker-assisted selection could facilitate the monitoring and introgression of these gene/QTLs in the plants.

Materials and Methods

Plant materials and insect population

A total of 167 F_2 progenies were derived from the cross of Rathu Heenati and MR219. Rathu Heenati (MRGB07637) is a traditional Sri Lankan rice variety and was used as the BPH resistant donor, whereas MR219 (MRGB11633) is a commercial high-yielding Malaysian variety that shows susceptibility to the current field population of BPH. Rice variety Taichung Native One (TN1) (MRGB01760) was used as the susceptible check variety. The BPH population used in the study was originally collected from rice fields surrounding the Malaysian Agricultural Research and Development Institute (MARDI) station in Seberang Perai, Malaysia. These insects were subsequently reared and maintained on seedlings of MR219 under greenhouse conditions. It was previously reported that MR219 harbored the *Bph1* gene (Habibuddin 2012) and hence, the insect population used in this study was expected to represent the Biotype-2 of BPH.

Honeydew test

Honeydew test is a test for the antibiosis mechanism of resistance. A piece of filter paper (Whatman No. 1) was dipped into a solution containing 0.02% of bromocresol green in ethanol (Pathak and Heinrichs 1982; Horgan *et al*. 2016). The experiment was conducted in a plant growth room at 22 ± 3ºC with 60 ± 10% humidity and artificial photoperiodic lighting of 16h: 8h (light: dark). The dried bromocresol green-treated filter paper was then placed at the base of individual 40-day-old plant samples inside a feeding chamber. The filter paper was placed 2 cm above the soil surface to protect the filter paper from excess humidity of the soil. Gravid brachypterous BPH females of similar age were starved for 2 h 30 min and 5 brachypterous BPH were subsequently released into the feeding chamber and left to feed on a single tiller for 24 h. Honeydew droplets dropped onto the bromocresol green-treated filter paper. The area of each blue spot on the bromocresol green-treated filter papers was measured using a square (mm) grid.

Plant damage score test

Plant damage score is a measure of plant damage tolerance upon infestation. The 5 brachypterous BPH and their nymphs were left to continue feeding on their respective test seedlings for 7–14 days or until yellowing or death of the MR219 test seedlings were first observed. Each test plant was scored individually according to the criteria established by the 5th Standard Evaluation System (SES) (IRRI 2013) based on the damage (scale from 1 to 9) of individual plants as a result of the BPH feeding (Table 1).

Extraction and quantification of DNA samples

Genomic DNA was extracted from fresh, young leaves of *Oryza sativa* using the Taco™ Plant DNA Extraction Kit (GeneReach Biotechnology Corp, Taiwan) according to the manufacturer's protocol. The purified DNA was stored at -20ºC. The concentration and quality of the extracted DNA were determined using NanoQuant™ spectrophotometer (TECAN Infinite 200 PRO, USA).

Evaluation of candidate markers

Sets of flanking microsatellite markers linked to the respective BPH resistant genes were evaluated for their polymorphisms on the parental varieties. The selection of these markers was based on the previous study by Sun *et al.* (2005) who suggested the involvement of three different QTL regions in Rathu Heenati, namely *Qbph3*, *Qbph4/Bph17* and *Qbph10* and a major BPH resistant gene (*Bph3*). To enhance the density of markers used in this study, 56 microsatellite markers were further mined from the GRAMENE database (http://www.gramene.org/) based on their relative position surrounding the above mentioned gene and QTLs (Fig. 1). The best polymorphic, shortest distance to the respective targeted gene or QTLs were then selected for subsequent used in the study.

Polymorphism of linked markers on F_2 plants

Only 8 polymorphic markers nearest to the target gene or QTLs were selected based on their preliminary polymorphism results. The selected polymorphic markers for the respective gene or QTLs were as follows: a) RM7 and RM1256 for *Qbph3*, b) RM8213 and RM5473 for *Qbph4/Bph17*, c) RM8072 and RM588 for *Bph3*, and d) RM5352 and RM5471 for *Qbph10*. Polymorphisms of the respective markers on individual F_2 plants were assessed. The results of this test were subsequently merged with the corresponding data on plant resistance assessments.

The expected presence of resistance loci on F_2 plants

The use of two flanking markers in the MAS breeding program could produce as high as 99 percent selection efficiency as compared to using a single marker (Kelly and Miklas 1998; Collard and MacKill 2008). The expected presence of a resistant gene locus in the individual F_2 plants was then based on the segregation pattern of their flanking markers, A and B, on the left and right-hand sides of the gene, respectively. For example, 'AA' represented the homozygous genotype of marker A, 'Aa' is heterozygous, and 'aa' denotes the absence of marker A in a plant, and similarly with the marker B. Hence, the plants with their representative flanking markers genotypes as 'A_B_' are considered to have a gene for resistance at a 99% probability. Likewise, plants with either combination of 'A_bb' or 'aaB_' genotypes may also indicate the presence of the resistant gene at 95% probability, depending on the proximity of the gene to the marker alleles A or B of Rathu Heenati. On the other hand, plants with the genotype of 'aabb' of MR219 were classified as individuals without the gene for resistance.

Statistical analyses

Data analysis was performed using Microsoft Excel. The Pearson's Chi-square (χ^2) test was performed to evaluate the expected segregation ratio of the F_2 population in the Mendelian segregations. The χ^2 value was estimated based on the procedure outlined by Panse and Sukhatme (2000) as shown below:

$$\chi^2 = \sum_{i=1}^{k} \frac{(Oi-Ei)^2}{Ei}$$

With the degree of freedom (df) = k-1, where O = Observed frequency of the class, E = Expected frequency of the respective class, $\sum$ = Summation of all classes. The non-significant χ^2 values justified the agreement between the observed and expected ratio, therefore the null hypothesis is accepted as true.

The presence of a respective marker 'A' in the individual F_2 plants was genotyped as either 'AA', 'Aa' or homozygous 'aa'. The χ^2 test was also used to test their expected 1:2:1 segregation ratio for a dominant factor. A co-segregation ratio of 9:7 was also tested for the co-segregation of the two-ends flanking markers A and B. The association between plants having respective resistant genes and their phenotypic resistance parameters was evaluated using association analysis (SAS version 9.3) where Cramer's V coefficient was used to measure the association strength. The relationship between genes and susceptible parameter was evaluated using correlation analysis (SAS version 9.1).

Results

Honeydew test and plant damage scores of the parents

The mean honeydew excretion (measured as area in mm^2) obtained from the release of 5 gravids BPH on three rice varieties is presented in Table 2. The honeydew excretions appeared as a blue-rimmed spot. As expected, the susceptible control variety (TN1) displayed the highest (211.43 ± 22.93 mm^2) mean score of honeydew spots. Likewise, the mean score of honeydew spots of MR219 was 185.71 ± 16.74 mm^2, which was slightly lower than that of TN1. In contrast, the honeydew droplet area of Rathu Heenati was only 45.71 ± 6.17. Additionally, a low coefficient of variation (CV) values was observed on TN1 (28.70%) and MR219 (23.85%), while Rathu Heenati showed a high CV value (40.93%). A similar pattern was also shown for plant damage measurement. The mean score for susceptible varieties, TN1 and MR219, was 8.71 ± 0.29 and 7.86 ± 0.60, respectively, while the lowest (1.57 ± 0.37) score was observed for the resistant donor parent, Rathu Heenati. The CV value was also recorded higher for Rathu Heenati as compared to MR219 and TN1. At the end of the study duration, almost all of the MR219 and TN1 plants were wilted and died due to heavy feedings by the BPH. These results confirmed the resistance of Rathu Heenati to BPH which was used as the donor parent and the susceptibility of MR219 as the recipient parent.

Table 1: Modified damage rating score of the test plants following of a continuous BPH feeding (IRRI, 1998)

Scale	Description	Reaction
1	No damage on the leaves	Highly resistant
3	Very slight damage on the leaves	Resistant
5	One to 2 leaves were yellowing	Moderately resistant
7	More than half of the leaves shrank	Susceptible
9	The plant died	Highly susceptible

Table 2: Overall mean scores (± standard errors, SE) for BPH resistance and coefficient of variation (CV) of parental and control rice varieties based on the honeydew and plant damage tests

Variety	N	Resistance score (Mean ± SE)				Reaction
		Honeydew (mm^2)	% CV	Plant damage score	% CV	
Rathu Heenathi	7	45.71±6.17	40.93	1.57±0.37	62.10	R
MR219	7	185.71±16.74	23.85	7.86±0.60	20.03	S
TN1 (Control)	7	211.43±22.93	28.70	8.71± .29	8.67	HS

R, S, and HS denote resistant, susceptible, and highly susceptible plants, respectively

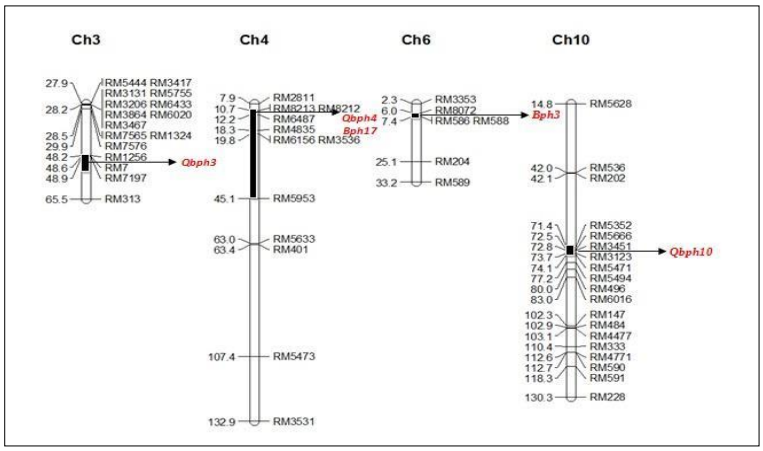

Fig. 1: Chromosomal location of BPH resistant genes and QTLs in the F_2 population. The marker names are listed on the right-hand side of the chromosome with the distance (in cM) displayed on the left-hand side (Mapchart 2.30)

Phenotypic segregation of F_2 plants

The frequency distribution of the 167 F_2 plants against the amount of honeydew excretion displayed a continuous normal distribution, although it was slightly skewed towards the resistant parent with a mean score of 85.21 mm^2 (Fig. 2), which may indicate that this antibiosis mode of resistance might be controlled by more than one gene. The majority of the progenies (119 plants) showed score values of ≤100mm^2, while Rathu Heenati and MR219 had score values of 42.0 mm^2 and 181.0 mm^2, respectively. However, the frequency distribution of F_2 plants for plant damage scores is in bimodal distribution (Fig. 3). A total of 36 plants (including Rathu Heenati) displayed a score of 1. These plants maintained their green-colored leaves even after 9 days of infestation. Additionally, 45 F_2 plants had a score of 3, and 23 plants were wilted and die and had a score of 9 (including MR219). Plants with scores of 1, 3, and 5 were pooled and classified as resistant, while plants with the scores of 7 and 9 were classified as susceptible. In total, 130 resistant plants and 37 susceptible plants were observed (Table 3). The χ^2 test showed that the phenotypic scoring of plant damage score was consistent with the expected Mendelian segregation ratio of 3:1, an indication of the role of a dominant gene effect. A similar result was also reported by Hu *et al.* (2018) who also conducted studies on Rathu Heenati. The results also showed that BPH faced difficulties to carry on with durable feeding or ingestion on the majority of the F_2 progenies, suggesting that these plants had been successfully introgressed with the resistant genes originated from Rathu Heenati.

Segregation and Co-segregation of the flanking markers

The segregation of individual markers in the F_2 population was analyzed. Except for marker RM8072, results from the χ^2 analysis show that the segregation of all other markers followed the 1:2:1 segregation ratio which suggested that each of these flanking markers was independently inherited as a dominant factor (Table 4). The calculated χ^2 value for marker RM8072 was high (135.18), resulting in deviation from the expected ratio at $P<0.001$, which could be due to

Table 3: Segregation of the F_2 plants of Rathu Heenati x MR219 for resistance to BPH infestation based on their plant damage scores

Phenotypic expression of corresponding F_2 plants	Expected F_2 genotype	Observed number of F_2 individuals
5 ≤ Resistant	AA, Aa	130
Susceptible ≥ 7	aa	37

N=167
Calculated χ^2 value for 3:1 ratio is 0.720 ($\chi^2_{0.05, 1}$ = 3.841)
The resistant plants are expected to possess homozygous AA or heterozygous Aa genomes inherited from Rathu Heenati, while the susceptible plants are carrying the 'aa' genome of MR219

Table 4: Segregation of marker alleles of eight microsatellite markers in the F_2 progenies derived from the cross of Rathu Heenati and MR219

Markers	Chr	Gene/QTL	Ratio (1:2:1)			χ^2
			AA	Aa	aa	
RM7	3	*Qbph3*	44	69	45	2.54
RM1256			40	66	48	3.96
RM8213	4	*Qbph4*	47	85	34	2.14
RM5473			33	76	49	3.44
RM8072	6	*Bph3*	98	16	48	135.18
RM588			26	94	37	7.66
RM5352	10	*QBph10*	33	59	55	12.36
RM5471			33	72	44	1.78

'AA' and 'aa' represent the genotypes of Rathu Heenati and MR219, respectively. 'Aa' is the heterozygous genotype.
Tabulated $\chi^2_{0.05, 0.01, 0.001,}$ df_2 = 5.991, 9.210 and 13.82, respectively

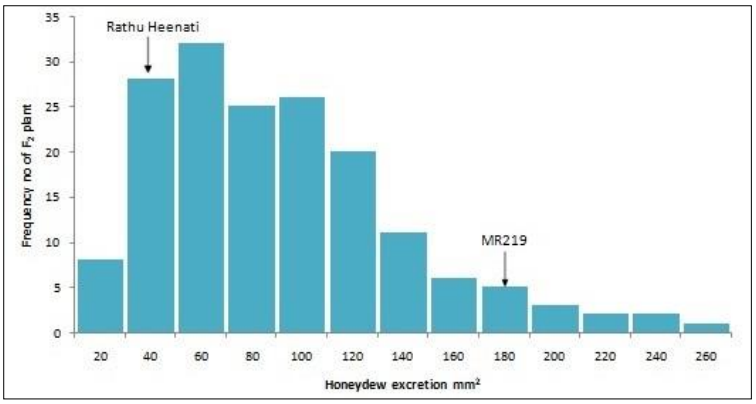

Fig. 2: Frequency distribution of the amount of honeydew excreted by BPH feeding on the F_2 population of Rathu Heenati/MR219, N=169

segregation distortion. This distortion was due to the high frequency number of AA (98) and the lesser number of the heterozygous Aa (16) genotypes. The cause of this segregation distortion could be genetic and environmental factors. This segregation distortion phenomenon was commonly found during population mapping due to biological selection or sampling, and this phenomenon usually interferes with the creation of genetic maps (Xu *et al*. 1997; Soundararajan *et al*. 2004).

In this study, two flanking markers were assumed to be closely linked if they were inherited in an allelic or co-segregated manner, of which the flanked gene is positioned between them. The plants with genotypes 'A_B_' are highly predicted to harbor resistant genes originated from Rathu Henati, while the plants which co-segregated with the genotype 'aabb' may indicate the absence of the resistant gene as the alleles were inherited from MR219. Our results showed that the calculated χ^2 value for the co-segregation of markers RM8213 and RM5473 is 0.31 (Table 5), a value lower than that of the tabulated value at P>0.05, thus suggesting that the two flanking markers are closely linked, co-segregated and hence the possibility for the presence of the gene *Qbph4* between them is very high. The calculated χ^2 values for the co-segregation of *Qbph3* and *Qbph10* markers, respectively were also low, albeit at a lower confidence level of P>0.01, and may still support the acceptance of the 9:7 ratio. On the other hand, the χ^2 value for the marker set RM588/RM8072 of *Bph3* was relatively high (P>001), due to distortion resulting from a higher number of 'A_B_' relative to other genotypes. The co-existing of both the flanking markers in a plant indicated that they inherited resistant genes from Rathu Heenati at the particular loci.

Association between the gene presence and phenotypic expression of the plants

An association analysis was conducted to estimate the

Table 5: Co-segregation of the flanking markers of the four putative BPH resistant genes in the F_2 progenies of Rathu Heenati and MR219 cross

Flanking Markers	RM7/ RM1256	RM8213/ RM5473	RM588/ RM8072	RM5471/ RM5352
(Gene)	***(Qbph3)***	***(Qbph4)***	***(Bph3)***	***(Qbph10)***
Segregation ratio	9: 7	9: 7	9: 7	9: 7
Observed	101: 53	81: 69	108: 49	64: 71
χ^2	5.45	0.31	10.03	4.28

Tabulated $\chi^2_{0.05, 0.01, 0.001, df1}$ = 3.841, 6.635 and 10.828, respectively

H_0= The segregation ratio of the flanking markers is in the ratio of 9:7 for the A_B_: other allele combinations, respectively

Table 6: The association of putative gene or QTLs and the phenotypic expression of the F2 plants

	Plant damage score (Tolerance)		Honeydew excretion (Antibiosis)	
Gene combination	χ^2	Cramer's V coefficient	χ^2	Cramer's V coefficient
Bph3	16.1910*	0.4172	29.6626*	0.5588
Qbph3	12.260*	0.3442	19.7283*	0.4420
Qbph4	13.2328*	0.3946	32.6712*	0.5632
Qbph10	12.9218*	0.3994	20.7747*	0.5064

*Significant at P=0.05

Cramer's V coefficient: V=0.25-0.75 (moderately strong)

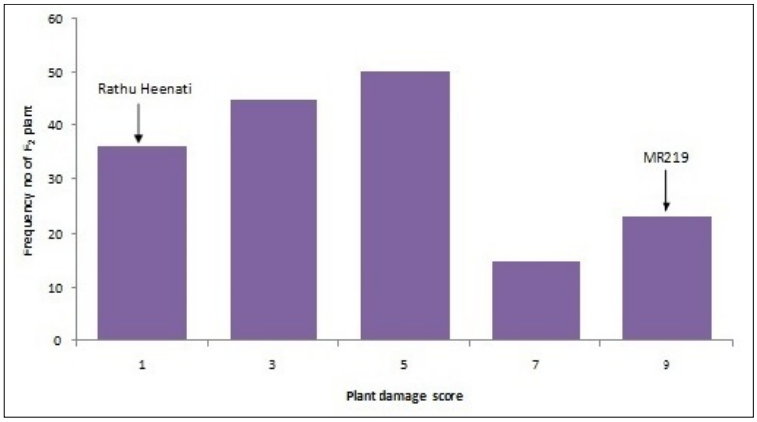

Fig. 3: Frequency distribution of F_2 plants of a cross between Rathu Heenati and MR219 when based on the plant damage scores following the infestation by BPH

The scores of Rathu Heenati and MR219 as control varieties were 1 and 9, respectively, N=169

association between the presence of *Bph3* gene or the other three respective QTLs in a plant and their phenotypic expression. All the χ^2 values are significant at P<0.05 (Table 6). These results indicated that the resistance expression of a plant is associated with the respective gene or QTL harbored by them. Plants harboring any of those resistant genes showed degrees of resistance expressed in the form of plant damage score and levels of antibiosis, as compared to those plants without any of the resistant gene or QTL. The strength of association between genes presence and the expression of the phenotypes is measured by Cramer's V coefficient, which is moderate.

Rathu Heenati as the donor parent is harboring *Bph3* gene and three other QTLs. The variety showed the lowest plant damage score of 1.80 as compared to the susceptible (without any gene) MR219 with the score of 8.00, indicating the high level of BPH resistance in Rathu Heenati (Table 7). A similar pattern of resistance expression is also demonstrated in the form of honeydew excretion, where the area of honeydew droplets in Rathu Heenati is 42 mm, significantly lower than that of MR219 (181.0 mm). The F_2 plants in groups with different gene combinations showed variable degrees of resistance, mostly moderate. The F_2 plants in the group ABCD which are having all the four genes showed a low plant damage score (2.27) and a small honeydew droplet area (46.36mm), which are not significantly different from that of Rathu Heenati, demonstrated an equivalent level of resistance to that of Rathu Heenati. A correlation analysis was also conducted to estimate the relationship between plant damage score resulted from the BPH feeding and the amount of honeydew excreted by those feeding BPH. A high correlation coefficient value was observed between the two parameters (r=0.83009***), which was as expected, which supported the assumption that excessive feeding, draining of the water, and nutrients from the plants in the form of honeydew, lead to their wilting and death of the plants.

Table 7: Plant damage score and honeydew excretion on plants with different number of BPH resistant gene and QTLs

Gene/QTL combination	No. of gene	Plant damage score		Honeydew excretion (mm^2)	
MR219 (Susceptible parent)	0	8.0	ab	181.0	a
No gene (H)	0	7.7	a	143.3	ab
Bph3 (A)	1	4.4	cdef	77.9	bcdef
Qbph3 (B)	1	4.6	bcde	88.2	bcde
Qbph4 (C)	1	1.0	f	30.0	ef
Qbph10 (D)	1	3.7	cdef	66.7	cdef
Bph3/Qbph3 (AB)	2	4.3	cdef	76.5	bcdef
Bph3/Qbph4 (AC)	2	7.0	abc	110	bcd
Bph3/Qbph10 (AD)	2	4.3	cdef	76.7	bcdef
Qbph3/Qbph4 (BC)	2	5.3	abcde	113.3	bc
Qbph3/Qbph10 (BD)	2	1.0	f	10.0	f
Qbph4/Qbph10 (CD)	2	3.8	cdef	69.0	cdef
Bph3/Qbph3/Qbph4 (ABC)	3	5.4	abcde	94.5	bcde
Bph3/Qbph3/Qbph10 (ABD)	3	5.8	abcd	107.5	bcd
Bph3/Qbph4/Qbph10 (ACD)	3	2.8	def	51.1	cdef
Qbph3/Qbph4/Qbph10 (BCD)	3	5.0	abcde	107.1	bcd
Bph3/Qbph3/Qbph4/Qbph10 (ABCD)	4	2.3	def	46.4	cdef
Rathu Heenati (Resistant parent)	4	1.8	ef	42.0	def

Means within a column with a similar letter are not significantly different by DMRT

Discussion

Identification of suitable resistant candidate genes is one of the most important steps in an effective resistant breeding program. Therefore, there is a need to validate all reported genes of interest towards the local BPH population to make sure their effective function, and that the breeding activity could be carried out effectively at a reasonable cost. In this study, a Sri Langkan rice variety, Rathu Heenati, has been selected to be used as the donor of resistant genes and QTLs for the development of local rice varieties resistant against BPH, *Nilaparvata lugens*. The level of BPH resistance in Rathu Heenati was measured and compared to the susceptible check variety TN1 and the recurrent parent MR219. The measurement was based on the assessment of the quantity of honeydew excreted area by BPH sucking on the plant (as an antibiosis assessment) and the plant damage score following BPH infestation (an assessment for plant tolerance). From the antibiosis perspective, the BPH feeding on Rathu Heenati excreted the lowest amount of honeydew excretion as compared to those feeding on MR219 or TN1. The low amount of honeydew excretion on Rathu Heenati could be due to the difficulties in phloem ingestion by the BPH, thus contributing to the variety's resistance. The higher coefficient of variation (CV) value recorded in Rathu Heenati as compared to those on MR219 and TN1 showed clear evidence of the inconsistency of BPH feeding ability on Rathu Heenati as the proportion of honeydew droplets of BPH is hugely influenced by its host varieties (Ghaffar *et al.* 2011).

Because of the inability of BPH to consistently feed on the resistant rice plant, the feeding data as measured by the level of antibiosis has also contributed to plant tolerance measurement data where Rathu Heenati also showed the lowest plant damage score. This result was in line with the observation of Akanksha *et al.* (2019) who showed that the amount of honeydew excretion and plant damage was highly correlated and significantly affected by host varieties. It was previously demonstrated that BPH primarily fed on the phloem of the susceptible varieties, ingesting high-value nutrients such as amino acids to support its growth and reproduction. During the early phase of stylet penetration in Rathu Heenati, undesirable constituent elements in the variety may disturb the BPH and prevent effective phloem ingestion (Ghaffar *et al.* 2011). This mechanism thus enables resistant plants to survive BPH attacks. The inability to consistently feeding on the host plant has caused less injury to the plant's tissues and hence the potential plant dehydration and loss of nutrients was prevented. This describes the reason behind the lower plant damage scores recorded in Rathu Heenati as compared to the scores on the susceptible control variety TN1 and MR219. This result confirmed the earlier reports by Habibuddin (1989) and Jairin *et al.* (2007) claiming that Rathu Heenati showed high and broad-spectrum resistance characteristics to BPH populations in Thailand and Malaysia, respectively. The variety is thus a very good candidate donor parent for used in the local BPH resistant breeding program.

However, the strong resistance of Rathu Heenati to BPH of variable biotypes, in multiple localities and countries is attributed to its harboring of at least the *Bph3* gene and the three mentioned QTLs, the *Qbph3, Qbph4* and *Qbph10*. Many attempts in the previous BPH resistance breeding program which utilized Rathu Heenati as the donor parent produced progenies or new varieties with moderate levels of resistance. Breeders have difficulty in developing new cultivars having the equivalent levels of BPH resistance to that of Rathu Heenati. This could be due to the failure to transfer or ensuring the maintenance of all the relevant genes or QTLs in the newly developed varieties. Our results in Table 7 showed that different F_2 plants may harbor different combinations of BPH resistant genes or QTLs in the plants, resulting in the variable degree of resistance to BPH. To ensure the subsequent progenies or generations of the crosses involving Rathu Heenati to continuously harbor the

desired gene or QTL is a difficult task when resistance assessment is solely based on phenotypic expression.

Application of marker assisted selection (MAS) in resistant breeding programs may offer an opportunity to overcome this limitation. Linked or functional markers could be used to determine the presence or absence of the genes in the plants. Through MAS, the introgression of genes would be monitored among breeding lines from generation to generation until the new varieties are ready to be selected for released and commercialized. This study showed that the introgression of the four genes and QTLs could be ascertained, and newly improved, resistant Malaysian major varieties could be developed, having a resistance level to BPH which is equivalent to that of Rathu Heenathi.

Conclusion

This study confirmed that the resistance of Rathu Heenati to BPH is controlled by *Bph3* gene and three other QTLs, namely the *Qbph3*, *Qbph4* and *Qbph10*. The introgression of these four resistant factors in the plants and breeding lines could be monitored through the detection of their flanking microsatellite markers. Application of marker-assisted selection (MAS) could thus enhance resistant breeding programs and the development of resistant or improved popular major cultivars is much easier to achieve.

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Author Contributions

MBAG, RMY and NAS planned the experiments, MBAG, MMS and SAR interpreted the results, MBAG, HH and RMY made the write up and MF statistically analyzed the data and made illustrations.

Conflicts of Interest

The authors declare no conflicts of interest

Data Availability

All data were presented in this paper and additional information can be obtain from corresponding author

Ethics Approval

Not applicable in this paper

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