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Marker-assisted selection for rice brown planthopper (*Nilaparvata lugens*) resistance using linked SSR markers

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Abstract: Developing cultivars resistant against different biotypes of brown planthopper (BPH) through marker-assisted selection (MAS) is an effective management strategy to control this pest. In this study, 28 polymorphic simple sequence repeat (SSR) markers were analyzed in 108 F_3 progenies derived from the cross of Rathu Heenati and MR276 rice cultivars to investigate the association with BPH resistance against biotypes 2 and 3. For statistical analysis, parents and their offspring were grouped into 2 phenotypic classes based on their levels of BPH resistance. Chi-square analysis demonstrated a good fit to a ratio of 3:1 for the segregation of resistance and susceptibility. Association of SSR markers with phenotypic traits in F_3 progenies were identified. Six SSR markers (RM401, RM5953, RM217, RM210, RM242, and RM1103) were significantly associated with BPH resistance to biotypes 2 and 3 of BPH. Out of these 6 markers, RM401, RM5953, and RM217 accounted for about 17% of total phenotypic variation and RM210, RM242, and RM1103 accounted for 20% of total phenotypic variation against biotypes 2 and 3, respectively. Therefore, the above 6 markers were confirmed for MAS in rice breeding programs to develop BPH-resistant rice cultivars.

Key words: *Bph* resistance gene, F_3 progenies, microsatellites, rice

1. Introduction

Rice (*Oryza sativa* L.) is a primary source of nutrition for over half of the world's population (Nagadhara et al., 2003; Latif et al., 2011; Ashkani et al., 2012). Brown planthopper (BPH) is one of the most destructive rice pests, causing considerable losses to this important crop worldwide (Jena and Mackill, 2008; Krishnaiah and Varma, 2011). It has been reported in all major rice-producing areas in Malaysia (Hashim, 1989).

Since the early 2000s, after major outbreaks of the BPH, much attention has paid to developing effective and economical controls against this insect pest, particularly in Southeast Asian countries (Wang et al., 2010). However, there is no high-yielding BPH-resistant variety available in Malaysia to date. Knowledge about the biotypes of BPH, the function of the insect-resistance gene, and the molecular mechanism of host-plant resistance to BPH is important for controlling this insect pest (Zhang, 2007; Du et al., 2009). Therefore, characterizing the BPH-resistant genes in order to develop an increase in long-term stabile resistance is highly desirable. It may provide security for a long period of time in various agroclimatic conditions. In this regard, molecular markers are a powerful technique that may provide realistic information about resistant cultivars through creation of high-resolution genetic maps among the important crop traits (Sandhu et al., 2003; Moose and Mumm, 2008; Alsaleh et al., 2013). Marker-assisted selection (MAS) has become an important tool in plant breeding in recent years and is now used to detect valuable traits in individuals or populations, such as insect resistance (Cock et al., 2009; Furbank and Tester, 2011; Miah et al., 2013; Balta et al., 2014). During the last decade, the genetics of BPH resistance have been extensively studied and more than 28 loci related to BPH resistance have been reported in numerous cultivated and wild species (Brar et al., 2009). Thus, the molecular markers linked to BPH resistance are advantageous to screen for in the progenies and to use in molecular MAS,

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map-based selection, and gene pyramiding for cloning (Ali and Chowdhury, 2014). Simple sequence repeats (SSRs) are extensively used in population genetic analyses and MAS (Zhou et al., 2003; Pashley et al., 2006; Steele et al., 2006; Ellis and Burke, 2007). SSR makers are widely distributed in the rice genome and could be used to scan and identify the markers linked to BPH-resistant genes and quantitative trait loci (QTLs) on rice chromosomes. This offers a wide scope for improving the efficiency of conventional plant breeding (Yang et al., 2002; Kim and Sohn, 2005; Jairin et al., 2007).

Previously reported SSR markers (RM401, RM217, RM545, RM204, and RM242) linked to *Bph3*, *bph4*, *Bph13(t)*, *bph19(t)*, and *Qbph-9* were tightly linked to BPH-resistant genes (Chen et al., 2006; Jairin et al., 2007; Liu et al., 2009). Therefore, these markers can be used in MAS for the identification of resistant genes. In this study, 28 linked SSR markers for BPH resistance were used. The objective of the present investigation was to determine the inheritance patterns of BPH resistance and to identify suitable SSR markers linked to the resistance against BPH biotypes 2 and 3 in F_3 families derived from the crossing of Rathu Heenati (a resistant cultivar) and MR276 (a susceptible rice cultivar) for MAS.

2. Materials and methods

2.1. Plant materials and insects

An F_3 population consisted of 108 families from the cross between MR276 and Rathu Heenati. Both varieties were indica type. MR276 is a Malaysian high-yielding variety but susceptible to BPH, whereas Rathu Heenati is a traditional Sri Lankan rice cultivar resistant to BPH (Lakshminarayana and Khush, 1977). The BPH populations used for infestation were biotypes 2 and 3, which were provided by the MARDI Research Station at Pulau Pinang, Malaysia, and reared on TN1 (a BPH-susceptible variety) under a net house (25 ± 5 °C, relative humidity = 80%) at the Rice Research Centre, Universiti Putra, Malaysia.

2.2. Evaluation of BPH resistance

The levels of BPH resistance among parents, their F_3 progenies, and TN1 (the susceptible control) were bioassayed by a standard seed-box screening test with some modifications as described by Heinrichs et al. (1985). The seeds were presoaked and sown in rows in plastic trays of 110 × 80 × 10 cm along with the susceptible control TN1. After 7 days, the seedlings were infested with first- to third-instar nymphs of BPH biotypes 2 and 3 at the rate of approximately 5 to 6 nymphs per seedling. When more than 90% of TN1 plants died, the plants of F_3 lines were examined and each individual plant was given a score of 0–9 (0, 1, 3, 5, 7, and 9), according to the criteria listed in Table 1, which were based on those of the International Rice Research Institute (IRRI, 1998).

For inheritance analysis of a single dominant gene model, the plants with scores of 0, 1, 3, or 5 were considered resistant, while plants rated with scores of 7 or 9 were considered susceptible. The 2-gene model was also analyzed by classification of resistance to BPH 2 and 3 using 4 classes: resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S). According to Mendel's principle of segregation, the phenotypic segregation for a 2-gene model is 9:3:3:1 for R:MR:MS:S, respectively. In the present study, scores of 0 to 1 were considered as R, 3 as MR, 5 as MS, and 7 to 9 as S (IRRI, 1998).

2.3. DNA extraction

Isolation of genomic DNA was done from fresh leaf tissues of all F_3 progenies and parents using the modified hexadecyltrimethylammonium bromide protocol described by Doyle and Doyle (1990). DNA was quantified by using a NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). For requantification, DNA was run by 1% agarose gel in 1X TBE buffer (Trizma base (FW = 121.1) with EDTA and boric acid; pH was adjusted to 8.0 with NaOH) at 80 V for 30 min and then observed under a UV transillumination lamp by staining with 1 µL of Midori Green DNA stain. DNA was diluted with TE buffer to a

Table 1. The scoring criteria for brown planthopper resistance (IRRI, 1988).

Scale	Description	Reaction
0	No damage	Highly resistant
1	Very slight damage	Resistant
3	One to 2 leaves were yellowing or slight stunting	Moderately resistant
5	More than half the leaves shrank	Moderately susceptible
7	More than half of the plant wilting or dead, the remaining plant still alive	Susceptible
9	The plant died	Highly susceptible

concentration of 20 ng/µL and kept in a refrigerator at –20 °C for further polymerase chain reaction (PCR) analysis.

2.4. PCR amplification for microsatellite analysis

About 110 microsatellites primers with known chromosomal positions distributed on rice chromosomes were selected from the Gramene database (www.gramene.org) related to the BPH resistance gene (Renganayaki et al., 2002; Sun et al., 2005; Chen et al., 2006; Jena et al., 2006). Parental varieties were screened for SSR polymorphisms associated with the BPH resistance gene. A total of 28 polymorphic SSR markers linked to BPH resistance were used for analysis in the F_3 generation (Table 2). For PCR amplification, final concentrations of the components were 7.5 µL of Master Mix (2X) (contains DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 0.5 µM of forward and reverse primers, and 20 ng of genomic DNA. PCR amplification was carried out on a Bio-Rad T100 Thermal Cycler using a touch-down PCR program as follows: 94 °C for 5 min; followed by 10 cycles of 94 °C for 15 s, 62 °C for 15 s (decreasing 0.5 °C per cycle), and 72 °C for 15 s; and 30 cycles of 94 °C for 15 s, 52 °C for 15 s, and 72 °C for 15 s; and a final extension at 72 °C for 10 min followed by rapid cooling to 4 °C prior to analysis. Amplified PCR products were resolved in a 3.0 % MetaPhor Agarose gel with 1 µL of Midori Green DNA stain to detect amplicons in 1X TBE buffer (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, and pH 8.0). The gel was run at a constant voltage of 90 V for 120 min and

Table 2. List of the SSR markers.

SSR markers	Primer sequences (5'-3')		Chr.	Repeat motif	Expected product size (bp)
	F: Forward primer	R: Reverse primer			
RM545	CAATGGCAGAGACCCAAAAG	CTGGCATGTAACGACAGTGG	3	(GA)30	226
RM312	GTATGCATATTTGATAAGAG	AAGTCACCGAGTTTACCTTC	1	(ATTT)4(GT)9	97
RM401	TCGAAGCCATCCACCAACGAAG	TCCGTACGCCGACGAGGTCGAG	2	(GA)15	156
RM154	ACCCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC	2	(GA)21	183
RM573	CCAGCCTTTGCTCCAAGTAC	TCTTCTTCCCTGGACCACAC	2	(GA)11	201
RM36	CAACTATGCACCATTGTCGC	GTACTCCACAAGACCGTACC	3	(GA)23	192
RM514	AGATTGATCTCCCATTCCCC	CACGAGCATATTACTAGTGG	3	(AC)12	259
RM222	GGCTTACTGGCTTCGATTTG	CGTCTCCTTTGGTTAGTGCC	3	(CT)15	266
RM5953	AAACTTTCTGTGATGGTATC	ATCCTTGTCTAGAATTGACA	4	(CAC)6	129
RM3872	GGAAGAAAGGATCTATATCA	TACGATTTGTTTAAGTTCAA	3	(GA)36	181
RM136	GAGAGCTCAGCTGCTGCCTCTAGC	GAGGAGCGCCACGGTGTACGCC	6	(AGG)7	101
RM3827	GGACGGATTGTAGGTAGGAC	CCTTTCTTCAATCTGCATTC	6	(GA)21	160
RM435	ATTACGTGCATGTCTGGCTG	CGTACCTGACCATGCATCTG	6	(ATG)7	166
RM11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG	7	(GA)17	140
RM25	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC	8	(GA)18	146
RM42	ATCCTACCGCTGACCATGAG	TTTGGTCTACGTGGCGTACA	8	(AG)6-(AG)2T(GA)5	166
RM544	TGTGAGCCTGAGCAATAACG	GAAGCGTGTGATATCGCATG	8	(TC)9	248
RM210	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG	8	(CT)23	140
RM547	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG	8	(ATT)19	235
RM242	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG	9	(CT)26	225
RM217	ATCGCAGCAATGCCTCGT	GGGTGTGAACAAAGACAC	6	(CT)20	133
RM120	CACACAAGCCCTGTCTCACGACC	CGCTGCGTCATGAGTATGTA	11	(GA)9TAG(ATC)4	173
RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	11	(AAG)8(AG)13	157
RM229	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT	11	(TC)11(CT)5C3(CT)5	116
RM12	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	12	(GA)21	184
RM1103	CAGCTGCTGCTACTACACCG	CTACTCCACGTCCATGCATG	12	(AG)12	216
RM512	CTGCCTTTCTTACCCCCTTC	AACCCCTCGCTGGATTCTAG	12	(TTTA)5	214
RM6947	ATTAAACGTCCACTGCTGGC	GCTAGGTTAGTGGTGCAGGG	12	(TTC)8	155

photographed on a UV transilluminator (ChemiDoc XRS gel documentation system, Bio-Rad).

2.5. Marker genotyping

The 108 F_3 progenies were genotyped with linked 28 SSR markers of BPH resistance. These marker alleles were detected on 3% MetaPhor agarose gels. The allele size of an individual was compared with the allele size of the parents. For each individual gel, a DNA ladder marker was used to create a standard band and confirm the allele sizes observed in the parental and progeny survey. The individuals that were the same size as the resistant parent alleles were labeled "A", those with a banding pattern similar to the susceptible parent alleles were labeled "B", and the heterozygous plants were labeled "H".

2.6. Statistical analysis

The goodness-of-fit to 3:1 and 9:3:3:1 ratios between observed and expected distributions were tested using chi-square analysis. To analyze the single-gene model, the observed segregation data were tested by chi-square analysis against the expected Mendelian ratio (1:2:1). To establish phenotype–marker association as suggested by Soller and Beckmann (1983), the general linear model procedure in SAS (SAS, 2003) was used to identify the mean of the groups formed based on the segregation pattern of each SSR marker locus for each trait. A threshold significance level ($P < 0.01$ and $P < 0.05$) indicated segregation of marker locus with genotypic classes or with phenotype.

3. Results

3.1. Phenotypic data analysis

In total, 108 F_3 progenies, including the 2 parents, were screened for BPH resistance based on the standard evaluation system of BPH damage to rice (Table 1).

Among the 108 evaluated F_3 progenies, 83 progenies were resistant to BPH 2 and 82 progenies were resistant to BPH 3 with scores of 0, 1, 3, and 5, while 25 progenies were susceptible to BPH 2 and 26 progenies were susceptible to BPH 3 with rated scores of 7 or 9. It is evident from our results that the expected number of resistant and susceptible plants in the segregation ratio for a single dominant gene model fit well and was not significantly different from the number of observed resistant and susceptible seedlings at the $P \leq 0.05$ significance level (Table 3). Phenotypic segregation patterns of the F_3 populations infested with biotype 2 and biotype 3 of BPH in the 2-gene model are shown in Table 4.

3.2. Trait frequency distribution

The frequency distribution of resistance scores of the 108 F_3 progenies infested with biotypes BPH 2 and 3 are shown in Figure 1. The resistance score in biotype BPH 2 showed 3 apparent peaks around 1.5, 2.5, and 6.0 in the distribution curve, while biotype BPH 3 showed a continuous distribution ranging from 0.0 to 9.0, with 3 apparent peaks around 2.0, 3.0, and 5.0 in the distribution curve.

3.3. Molecular marker assays

Twenty-eight SSR markers were analyzed for the alleles existing in F_3 plants. All of these markers exhibited visible polymorphic bands between Rathu Heenati and MR276. Segregation ratios for used markers are shown in Table 5. Segregation analysis revealed that Rathu Heenati carried resistant genes associated with the SSR markers RM545, RM401, RM22, RM5953, RM210, RM242, RM217, RM224, and RM1103. As indicated in Table 5, these markers showed a good fit to the expected marker segregation ratio (1:2:1) in a Mendelian fashion ($df = 1.0$, $P \leq 0.05$). For MAS, parental bands that were amplified as controls along with the F_3 individuals were used in screening. Plants that

Table 3. Observed and expected segregation ratios of resistant and susceptible plants in the F_3 population for single-gene models (3:1) obtained from the cross between rice cultivars MR276 and Rathu Heenati infested with BPH biotypes 2 and 3.

BPH biotype	Population	Bioassay		χ^2 (3:1)	P-value
		Reaction	No. of lines observed		
Biotype 2	F_3	R	83	0.11	0.74
		S	25		
		Total	108		
Biotype 3	F_3	R	82	0.01	0.92
		S	26		
		Total	108		

According to a model on a single dominant gene; S: susceptible, R: resistant. $df = 1.0$; $\chi^2(0.05, 1) = 3.84$.

Table 4. Chi-square test for 2 independent genes (9:3:3:1) for BPH resistance in an F_3 population from the cross between rice cultivars MR276 and Rathu Heenati infested with biotype 2 and biotype 3 of BPH.

BPH biotype	Gene models	Total no. of F_3 seedlings	No. of lines observed				Expected ratio	χ^2	P-value
			R	MR	MS	S			
Biotype 2	Independent genes	108	38	39	22	9	9:3:3:1	26.78*	<0.0001
Biotype 3	Independent genes	108	28	42	26	12	9:3:3:1	46.73*	<0.0001

χ^2 0.05, 3 = 7.81, df = 3 at P ≤ 0.05; R: resistant; MR: moderately resistant; MS: moderately susceptible, S: susceptible.

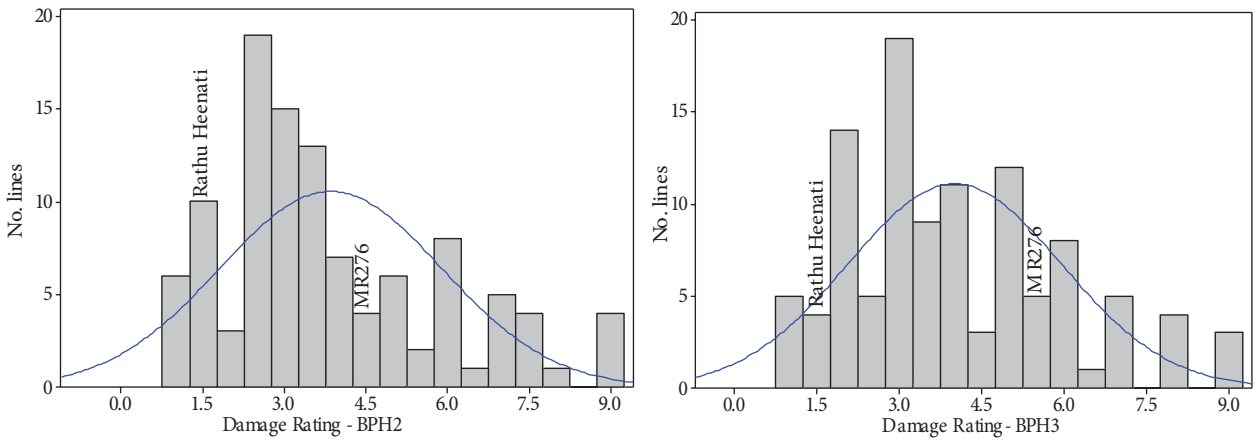

Figure 1. Distribution of damage rating (biotypes BPH 2 and BPH 3) in the F_3 families.

showed the resistant gene alleles of linked markers, namely RM401, RM5953, RM217, RM210, RM242, and RM1103, were retained in the selection program as resistant plants; the others were discarded. In Figure 2, the progenies having a resistant parent allele (205 bp) of marker RM1103 represent BPH resistance. These markers correlated to resistance effects of about 17% and 20% for phenotypic variation to biotypes 2 and 3 (Table 6).

4. Discussion

Since 1980, more than 20 BPH resistance genes affecting rice cultivars have been genetically mapped; only 2 of them (*bph14* and *bph18*) have been cloned and characterized (Jena and Kim, 2010; Zhou et al., 2013). Validation and practical application of those discoveries in the form of MAS in rice breeding programs shows that the target genes can be identified more efficiently in segregating populations at any plant growth stage with the use of tightly linked DNA markers (Collard and Mackill, 2008). Identification of suitable SSR markers associated with BPH resistance is essential for MAS application for development of resistant variety.

In this study, F_3 progenies segregating for BPH resistance were derived from 2 crosses that involved indica rice cultivars Rathu Heenati and MR276. The plants resistant to BPH biotypes 2 and 3 from the F_3 population showed a 3:1 ratio for a single dominant gene model and were linked to SSR markers RM401, RM5953, and RM217 for BPH 2 and RM210, RM242, and RM1103 for BPH 3, respectively. These markers were inherited with a single dominant gene and have shown good and high association with BPH resistance. Gomathi (2002) and Kumari et al. (2010) identified 4 SSR markers, namely RM168, RM186, RM3180, and RM2453, associated with BPH resistance using the F_3 and F_7 population of IR50/Rathu Heenati.

These findings showed that the observed segregation ratios in F_3 were likely to behave as a single dominant gene. Our results are in agreement with the findings of earlier researchers (Latif et al., 2008; Ashkani et al., 2011; Latif et al., 2013). The segregation ratio (3:1) suggested a single specific resistant gene segregating against BPH in biotypes BPH 2 and BPH 3 in the F_3 progenies. The highly resistant cultivar Rathu Heenati could be the result of the complement of BPH-resistant genes. Thus, it could be obvious from the previous studies that resistance to insect pests, including BPH, is governed either by a single gene or moderate and polygenic resistance, depending on the genotypes or cultivars (Latif et al., 2008; Ashkani et al., 2011; Latif et al., 2013).

Table 5. Markers analyzed in the F_3 progenies derived from the cross between rice cultivars MR276 and Rathu Heenati.

Marker	Chr.	Ratio (A:H:B)	Chi-square	P-value
RM545	Chr03	34:43:31	4.65	0.0979
RM312	Chr01	36:36:36	12**	0.0025
RM401	Chr04	32:44:32	3.7	0.1569
RM154	Chr02	35:39:34	8.35*	0.0154
RM573	Chr02	37:36:35	12.07**	0.0024
RM36	Chr03	38:28:42	25.33***	0
RM514	Chr03	35:37:36	10.72**	0.0047
RM222	Chr10	29:48:31	1.41	0.4947
RM5953	Chr04	35:42:31	5.63	0.0599
RM3872	Chr03	38:31:39	19.61***	0.0001
RM136	Chr06	42:36:30	14.67***	0.0007
RM3827	Chr06	40:30:38	21.41***	0
RM435	Chr06	33:36:39	12.67**	0.0018
RM11	Chr07	36:38:34	9.56**	0.0084
RM25	Chr08	40:35:33	14.28***	0.0008
RM42	Chr08	38:40:30	8.44*	0.0147
RM544	Chr08	33:38:37	9.78**	0.0075
RM210	Chr08	34:42:32	5.41	0.067
RM547	Chr08	39:34:35	15.11***	0.0005
RM242	Chr09	35:44:29	4.37	0.1125
RM217	Chr10	33:46:29	2.67	0.2636
RM120	Chr11	35:40:33	7.33*	0.0256
RM224	Chr11	34:44:30	4	0.1353
RM229	Chr11	41:43:24	9.83**	0.0073
RM12	Chr12	35:38:35	9.48**	0.0087
RM1103	Chr12	31:47:30	1.83	0.3998
RM512	Chr12	35:32:41	18.59***	0.0001
RM6947	Chr12	37:37:34	10.87**	0.0044

df = 1.0; χ^2 (0.05, 1) = 3.84; χ^2 (0.01, 1) = 6.63.

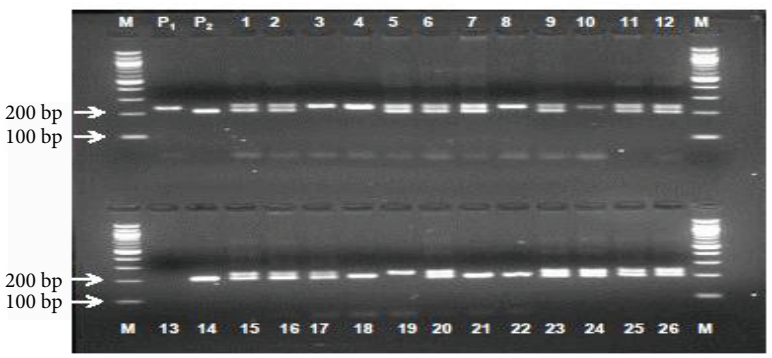

Figure 2. PCR products for genotyping, with marker RM1103 linked to *Bph* resistance genes in F_3 population of rice derived from MR276 (P1) × Rathu Heenati (P2). Running on 3% MetaPhor agarose gel stained with Midori Green. Only 26 samples plus the 2 parents for this marker are shown (M = 100-bp ladder).

Table 6. Molecular markers statistics associated with brown planthopper resistance to biotypes 2 and 3 in the F_3 progenies.

Traits	Marker	Chr.	SMR (R^2)	SMR (F)	Percentage[a]	P[b]
	RM401	Chr04	0.055	3.031	5	0.04828
Damage rating for Biotype 2	RM5953	Chr04	0.066	3.718	7	0.02485
	RM217	Chr06	0.054	3.013	5	0.04913
	RM210	Chr08	0.055	3.078	6	0.04611
Damage rating for Biotype 3	RM242	Chr09	0.058	3.222	6	0.0401
	RM1103	Chr12	0.076	4.295	8	0.01431

SMR: single marker regression; [a]proportion of the total phenotypic variance accounted for the markers; [b]the probability of an association.

Analysis of our selected SSRs markers in F_3 segregating populations indicated that these markers correlated to significant resistance, about 17% and 20% for phenotypic variation, to biotypes BPH 2 and BPH 3, respectively. These markers had high selection accuracy for resistant plant sources and could be used in MAS for the resistant gene. Therefore, the genetic studies on this variety have provided very useful information for local rice breeders in developing BPH-resistant rice cultivars. The characterization of SSR markers will not only help identify markers close to the genes of importance in breeding programs, but will also help evaluate germplasm and breeding materials. This information will help rice breeders to improve BPH resistance in rice by MAS.

In conclusion, chi-square analysis showed a good fit to a ratio of 3:1 for the segregation of resistance and susceptibility for biotypes 2 and 3 of BPH. SSR markers RM545, RM401, RM22, RM5953, RM210, RM242, RM217, RM224 and RM1103 were significantly associated with BPH resistance to biotypes 2 and 3 of BPH in rice ($P \leq 0.01$). These markers showed high selection accuracy for resistant plant sources with confirmation of resistance effect of about 17%–20% for phenotypic variation and can be used in MAS for the resistant gene. The resistance gene markers reported here provide rice breeders and geneticists with a valuable tool for MAS of the insect resistance gene.

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