Plant Omics Journal POJ

POJ 8(3):212-219 (2015) ISSN:1836-3644

Analysis of SSR markers linked with brown planthopper resistance genes (*Bph*) using high-resolution melting (HRM) in rice

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Abstract

Developing rice cultivars with host-plant resistance is widely considered the best strategy for the long-term control of the brown planthopper (BPH). The use of molecular markers in many aspects of rice (*Oryza sativa* L.) studies, such as the genetic analysis of insect and disease resistance, is increasing. In the present study, 110 simple sequence repeat (SSR) markers that are associated with *Bph* resistance genes were selected from the Gramene database and used to develop SSR marker-based strategies for the reliable selection of BPH-resistant genotypes. Fifty-seven of the best polymorphic markers were used to identify the segregation ratio in 176 individual F_2 rice progeny from a MR276 (susceptible) × Rathu Heenati (resistant) interspecific cross. Thirty-five SSR markers, including RM544, RM547, and RM8213, showed a good fit to the expected segregation ratio (1:2:1) for the single gene model (d.f. = 1.0, $p \leq 0.05$) in chi-square (χ^2) analyses. The remaining markers did not fit the expected Mendelian segregation ratios. The genetic information generated in this research will be useful in rice breeding programmes to provide varieties with durable resistance to BPH. Additionally, this research showed that high-resolution melting analysis (HRM) is powerful and applicable for accurately and quickly genotyping many samples.

Keywords: Rice (*Oryza sativa* L.); Brown planthopper (BPH); Resistance genes; Simple sequence repeat markers; F_2 population.
Abbreviations: BPH_Brown planthopper, SSR_Simple sequence repeat, QTL_Quantitative trait loci, HRM_High-resolution melting analysis

Introduction

Rice (*Oryza sativa* L.), which is one of the most important crops in the world, is a staple food for more than half of the world population (Fitzgerald et al. 2009; Song et al. 2007). Biotic factors and abiotic stresses limit the production in rice growing areas in many tropical regions (Giri and Laxmi 2000). The brown planthopper (BPH) *Nilaparvata lugens* is the most destructive insect pest in Asia (Jena et al. 2006). This monophagous pest is a major threat to rice production by sap-sucking and by acting as a vector of rice stripe virus, rice grassy stunt virus and ragged stunt virus, which can cause even more serious yield reduction (Li et al. 2010; Ram et al. 2010; Zhang et al. 2010). Several strategies that are being deployed in breeding to transfer resistance genes into rice varieties have been proposed for combating insect pests (Huang et al. 2001). Planting resistant rice varieties is the preferred breeding strategy for BPH management (Bottrell and Schoenly 2012; Jena et al. 2006). Polygenic (i.e., through several genes that each have smaller effects) and moderate resistance to insect pests may be a useful approach (Huang et al. 2001). However, for more effective protection, the pyramiding of multiple resistance genes of different origins is clearly an advantageous strategy for increasing the durability of resistance because the insect would not likely be able to overcome resistance from multiple genes simultaneously. Additionally, closely linked molecular markers should be useful for transferring resistance genes to develop cultivars that carry multiple resistance genes. Selecting appropriate parental genotypes, followed by selecting the types of DNA markers, is of prime importance for constructing a linkage map and for performing QTL analysis (Javed et al. 2013). Microsatellites, which are also called simple sequence repeats (SSRs), are a popular type of co-dominant molecular marker and are widely used in rice genetic analyses, genome mapping and marker-assisted breeding. Because of their widespread distribution in the genome, SSRs have become a valuable source of genetic markers (Ashkani et al. 2011;

Table 1. Fifty-seven polymorphic microsatellite markers that were used for segregation analysis in an F_2 population of rice.

SSR markers	Primer sequences (5'-3')		Chromosome	Repeated Motif
	F: Forward primer	R: Reverse primer		
RM5	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG	1	(GA)14
RM312	GTATGCATATTTGATAAGAG	AAGTCACCGAGTTTACCTTC	1	(ATTT)4(GT)9
RM319	ATCAAGGTACCTAGACCACCAC	TCCTGGTGCAGCTATGTCTG	1	(GT)10
RM431	TCCTGCGAACTGAAGAGTTG	AGAGCAAAACCCTGGTTCAC	1	(AG)16
RM110	TCGAAGCCATCCACCAACGAAG	TCCGTACGCCGACGAGGTCGAG	2	(GA)15
RM154	ACCCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC	2	(GA)21
RM6	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC	2	(AG)16
RM208	TCTGCAAGCCTTGTCTGATG	TAAGTCGATCATTGTGTGGACC	2	(CT)17
RM555	TTGGATCAGCCAAAGGAGAC	CAGCATTGTGGCATGGATAC	2	(AG)11
RM573	CCAGCCTTTGCTCCAAGTAC	TCTTCTTCCCTGGACCACAC	2	(GA)11
RM22	GGTTTGGGAGCCCATAATCT	CTGGGCTTCTTTCACTCGTC	3	(GA)22
RM36	CAACTATGCACCATTGTCGC	GTACTCCACAAGACCGTACC	3	(GA)23
RM218	TGGTCAAACCAAGGTCCTTC	GACATACATTCTACCCCCGG	3	(TC)24ACT5(GT)11
RM514	AGATTGATCTCCCATTCCCC	CACGAGCATATTACTAGTGG	3	(AC)12
RM517	GGCTTACTGGCTTCGATTTG	CGTCTCCTTTGGTTAGTGCC	3	(CT)15
RM545	CAATGGCAGAGACCCAAAAG	CTGGCATGTAACGACAGTGG	3	(GA)30
RM3872	GGAAGAAAGGATCTATATCA	TACGATTTGTTTAAGTTCAA	3	(GA)36
RM261	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC	4	C9(CT)8
RM5953	AAACTTTCTGTGATGGTATC	ATCCTTGTCTAGAATTGACA	4	(CAC)6
RM401	TGGAACAGATAGGGTGTAAGGG	CCGTTCACAACACTATACAAGC	4	(CT)15
RM8213	AGCCCAGTGATACAAAGATG	GCGAGGAGATACCAAGAAAG	4	(TC)10
RM348	CCGCTACTAATAGCAGAGAG	GGAGCTTTGTTCTTGCGAAC	4	(CAG)7
RM13	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	5	(GA)6-(GA)16
RM122	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC	5	(GA)7A(GA)2A(GA)11
RM163	ATCCATGTGCGCCTTTATGAGGA	CGCTACCTCCTTCACTTACTAGT	5	(GGAGA)4(GA)11C(GA)20
RM413	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC	5	(AG)11
RM3	ACACTGTAGCGGCCACTG	CCTCCACTGCTCCACATCTT	6	(GA)2GG(GA)25
RM136	GAGAGCTCAGCTGCTGCCTCTAGC	GAGGAGCGCCACGGTGTACGCC	6	(AGG)7
RM217	ATCGCAGCAATGCCTCGT	GGGTGTGAACAAAGACAC	6	(CT)20
RM510	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	6	(GA)15
RM3827	GGACGGATTGTAGGTAGGAC	CCTTTCTTCAATCTGCATTC	6	(GA)21
RM435	ATTACGTGCATGTCTGGCTG	CGTACCTGACCATGCATCTG	6	(ATG)7
RM11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG	7	(GA)17
RM455	AACAACCCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC	7	(TTCT)5
RM25	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC	8	(GA)18
RM42	ATCCTACCGCTGACCATGAG	TTTGGTCTACGTGGCGTACA	8	(AG)6-(AG)2T(GA)5
RM210	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG	8	(CT)23
RM515	TAGGACGACCAAAGGGTGAG	TGGCCTGCTCTCTCTCTCTC	8	(GA)11
RM544	TGTGAGCCTGAGCAATAACG	GAAGCGTGTGATATCGCATG	8	(TC)9
RM547	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG	8	(ATT)19
RM3572	AGTGCTGTCTGGTTTTTGGC	CCCCTCCCTTTCTTTCTTTG	8	(GA)12
RM205	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	9	(CT)25
RM242	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG	9	(CT)26
RM160	AGCTAGCAGCTATAGCTTAGCTGGAGATCG	TCTCATCGCCATGCGAGGCCTC	9	(GAA)23
RM222	CTTAAATGGGCCACATGCG	CAAAGCTTCCGGCCAAAAG	10	(CT)18
RM228	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC	10	(CA)6(GA)36
RM496	GACATGCGAACAACGACATC	GCTGCGGCGCTGTTATAC	10	(TC)14
RM120	CACACAAGCCCTGTCTCACGACC	CGCTGCGTCATGAGTATGTA	11	(GA)9TAG(ATC)4
RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	11	(AAG)8(AG)13
RM229	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT	11	(TC)11(CT)5C3(CT)5
RM6894	AATCTCCACTGCAGCGATTC	CGAATGGTCAAACGTAGGTG	11	(TTA)11
RM12	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	12	(GA)21
RM1103	CAGCTGCTGCTACTACACCG	CTACTCCACGTCCATGCATG	12	(AG)12
RM7376	TCACCGTCACCTCTTAAGTC	GGTGGTTGTGTTCTGTTTGG	12	(GAAA)6
RM179	CCCCATTAGTCCACTCCACCACC	CCAATCAGCCTCATGCCTCCCC	12	(TG)7
RM512	CTGCCTTTCTTACCCCCTTC	AACCCCTCGCTGGATTCTAG	12	(TTTA)5
RM6947	ATTAAACGTCCACTGCTGGC	GCTAGGTTAGTGGTGCAGGG	12	(TTC)8

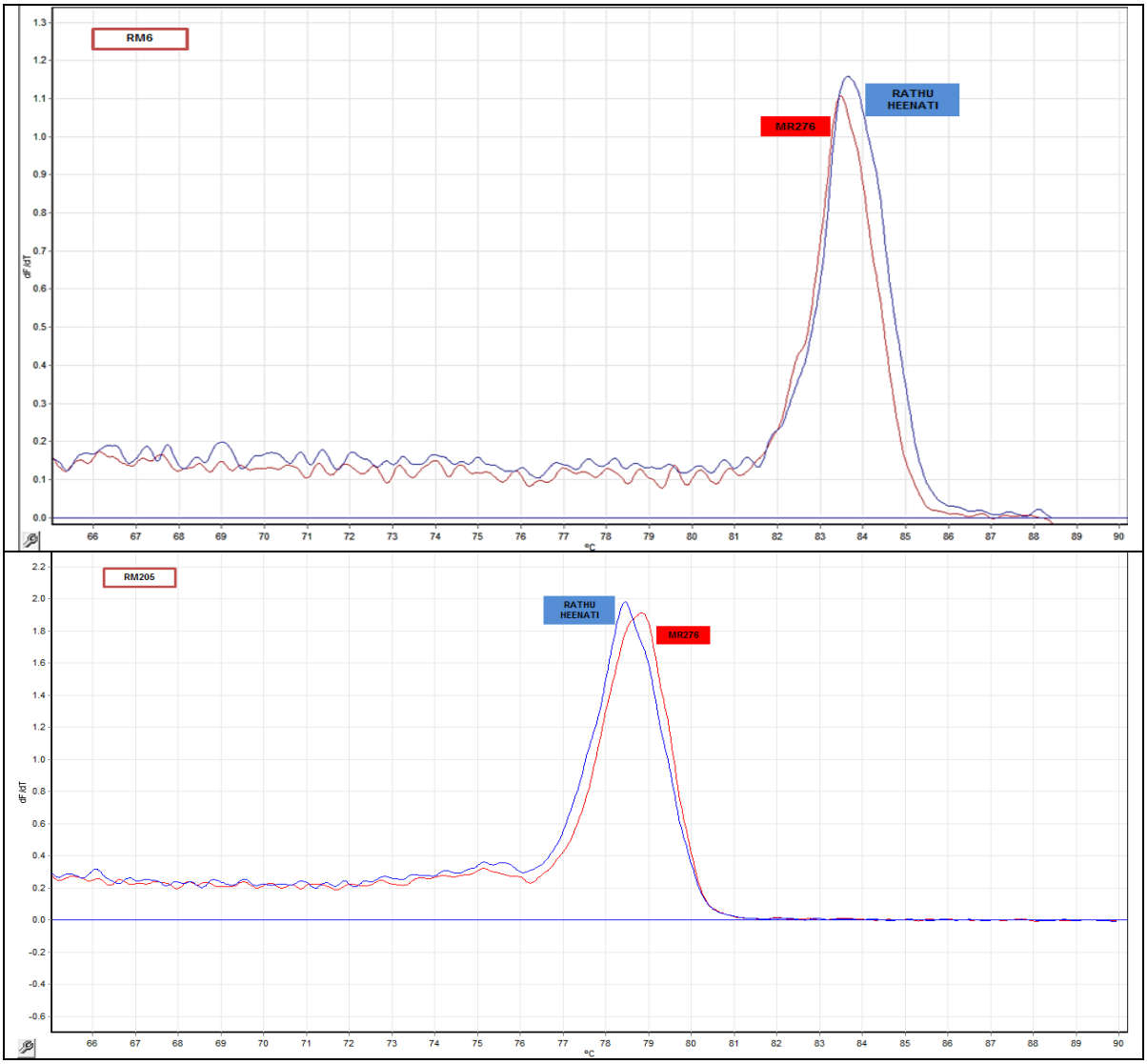

Fig 1. Screening to identify the polymorphic microsatellite loci between parental varieties MR276 (susceptible, red line) and Rathu Heenati (resistant, blue line) for the SSR markers RM6 and RM205 using HRM analysis.

Ashkani et al. 2014; McCouch et al. 2001; Sun et al. 2011). Many researchers have investigated the genetics of brown planthopper resistance, and several SSR markers that are closely linked to *Bph* resistance genes have been identified (Renganayaki et al. 2002). Recently, high-resolution melting (HRM) has been introduced as a homogenous, closed-tube, post-PCR method for rapidly and efficiently detecting mutations, polymorphisms and variations in DNA fragments and has been identified as a powerful method that can be used for barcoding and genotyping (SNP and SSR markers) (Ganopoulos et al. 2011a, b; Golding et al. 2010; Xanthopoulou et al. 2014). To date, more than 28 brown planthopper resistance genes have been identified (Chen et al. 2006; Harini et al. 2010; Jairin et al. 2007; Jena et al. 2006; Kawaguchi et al. 2001; Rahman et al. 2009; Sun et al. 2005; Yang et al. 2002; Yang et al. 2004). Resistance to BPH has not yet been identified in Malaysian rice, and the development of resistant rice is generally considered the best strategy for long-term insect control.

The objective of this study was to use HRM to analyse SSR markers linked to *Bph* resistance genes in an F_2 population derived from an interspecific cross between the resistant variety Rathu Heenati and the susceptible rice cultivar MR276 to develop SSR marker-based strategies for the reliable selection of BPH-resistant genotypes.

Results

Marker polymorphism between parents

One hundred and ten SSR primers were used to screen for polymorphisms between parental varieties. The melting curve patterns, which were amplified with 110 SSR primers for the parents, MR276 and Rathu Heenati, were sampled for visual scoring. The melting points (T_m) of the amplification products for the parental DNA varied from 73 and 74.3°C (RM312) to 84.2 and 84.6°C (RM517) for MR276 and Rathu Heenati, respectively. Of the 110 SSR markers used to screen for polymorphisms between parental varieties, approximately 51% (57 primers) showed clear polymorphisms between the

Table 2. Marker analyses in an F_2 population derived from the cross between MR276 (susceptible) and Rathu Heenati (resistant) rice varieties.

Marker Name	Chromosome	Size (P1)	Size (H)	Size (P2)	x^2	Pr> x^2	HetBand
RM5	1	50	86	40	1.23	0.541	Codominant
RM312	1	45	84	47	0.41	0.815	Codominant
RM319	1	55	69	52	8.31*	0.016	Codominant
RM431	1	42	75	59	7.13*	0.028	Codominant
RM110	2	50	77	49	2.76	0.251	Codominant
RM154	2	41	91	44	0.31	0.858	Codominant
RM6	2	59	71	46	8.49*	0.014	Codominant
RM208	2	54	64	58	13.27**	0.001	Codominant
RM555	2	30	103	43	7.03*	0.030	Codominant
RM573	2	50	78	48	2.32	0.314	Codominant
RM22	3	52	96	28	8.00*	0.018	Codominant
RM36	3	40	98	38	2.32	0.314	Codominant
RM218	3	40	90	46	0.50	0.779	Codominant
RM514	3	53	78	45	3.00	0.223	Codominant
RM517	3	49	93	34	3.13	0.210	Codominant
RM545	3	50	85	41	1.13	0.570	Codominant
RM3872	3	47	84	45	0.41	0.815	Codominant
RM261	4	47	71	58	7.94*	0.019	Codominant
RM5953	4	40	94	42	0.86	0.649	Codominant
RM401	4	53	82	41	2.45	0.293	Codominant
RM8213	4	45	86	45	0.09	0.956	Codominant
RM348	4	58	65	53	12.31**	0.002	Codominant
RM13	5	50	70	56	7.77*	0.021	Codominant
RM122	5	54	69	53	8.22*	0.016	Codominant
RM163	5	59	66	51	11.73**	0.003	Codominant
RM413	5	57	72	47	6.95*	0.031	Codominant
RM3	6	59	74	43	7.36*	0.025	Codominant
RM136	6	39	91	46	0.76	0.683	Codominant
RM217	6	48	76	52	3.45	0.178	Codominant
RM510	6	49	99	28	7.76*	0.021	Codominant
RM3827	6	38	96	42	1.64	0.441	Codominant
RM435	6	40	89	47	0.58	0.748	Codominant
RM11	7	47	87	42	0.31	0.858	Codominant
RM455	7	53	66	57	11.18**	0.004	Codominant
RM25	8	47	86	43	0.27	0.873	Codominant
RM42	8	45	78	53	3.00	0.223	Codominant
RM210	8	45	82	49	1.00	0.607	Codominant
RM515	8	58	70	48	8.5*	0.014	Codominant
RM544	8	43	89	44	0.03	0.983	Codominant
RM547	8	43	88	45	0.05	0.978	Codominant
RM3572	8	47	70	59	9*	0.011	Codominant
RM205	9	29	100	47	6.95*	0.031	Codominant
RM242	9	52	81	43	2.03	0.362	Codominant
RM160	9	60	68	48	10.73**	0.005	Codominant
RM222	10	47	80	49	1.50	0.472	Codominant
RM228	10	45	69	62	11.49**	0.003	Codominant
RM496	10	41	73	62	10.13**	0.006	Codominant
RM120	11	49	82	45	1.00	0.607	Codominant
RM224	11	48	95	33	3.67	0.160	Codominant
RM229	11	52	83	41	1.94	0.379	Codominant
RM6894	11	59	82	35	7.36*	0.025	Codominant
RM12	12	47	85	44	0.31	0.858	Codominant
RM1103	12	45	75	56	5.22	0.074	Codominant
RM7376	12	41	78	57	5.18	0.075	Codominant
RM179	12	43	85	48	0.49	0.783	Codominant
RM512	12	46	85	45	0.22	0.898	Codominant
RM6947	12	39	95	42	1.22	0.545	Codominant

χ^2 test: Statistical testing for the segregation distortion of markers; Pr > χ^2: Corresponding probability for the χ^2 test statistics, which is equal to probability when x is greater than χ^2; * χ^2 values: 5.99 at p 0.05 and 9.21 at $p \leq 0.01$; HetBand: For populations with heterozygosity, e.g., F_2 and F_3, this indicator represents whether the marker is codominant, dominant, or recessive.

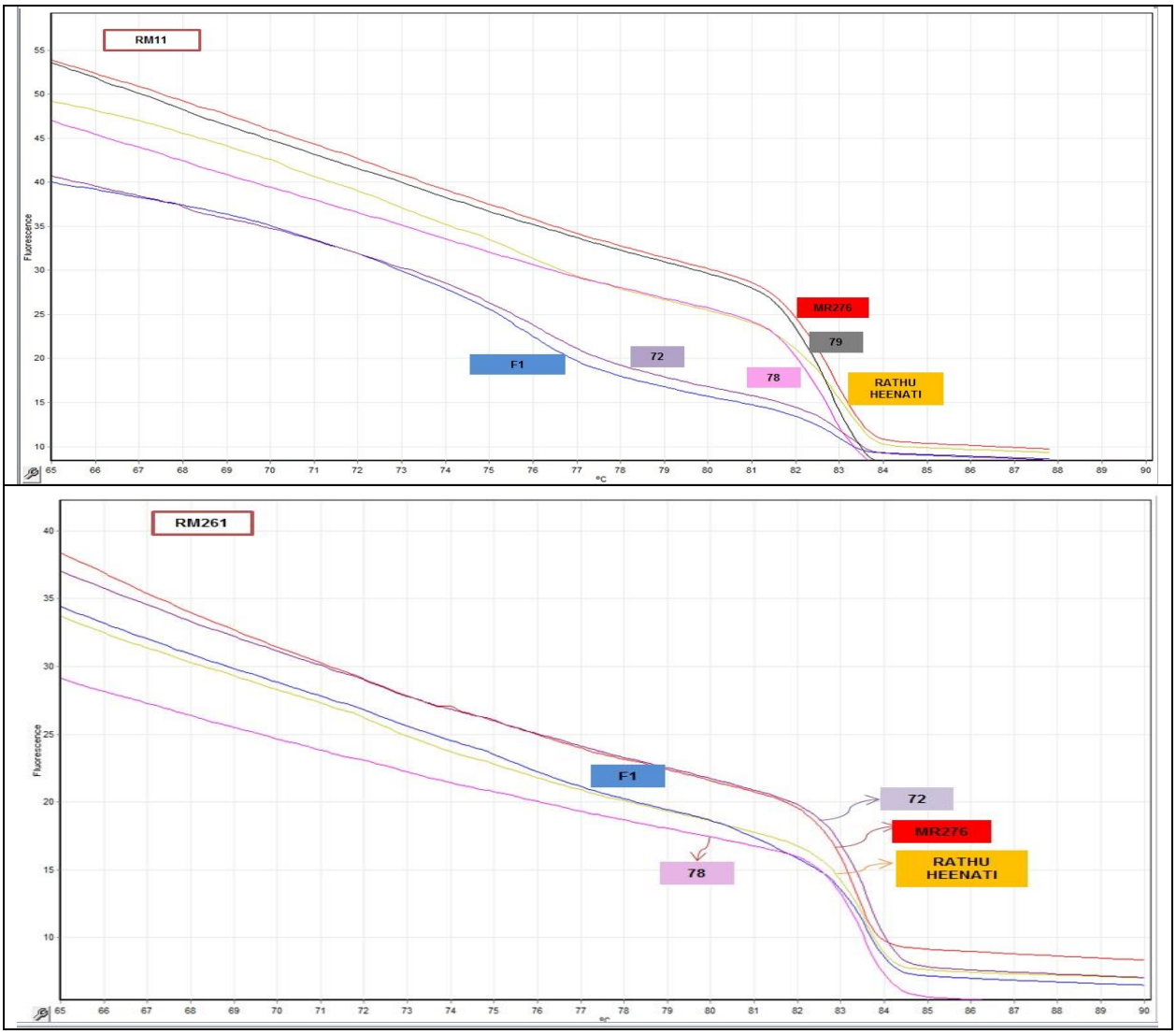

Fig 2. HRM curve analysis showing the segregation patterns of the F2 population that was derived from a cross between MR276 and Rathu Heenati at microsatellite loci RM261, RM11 and RM6 on chromosomes 4, 7 and 2, respectively. Rotor-Gene ScreenClust HRM software automatically classified the genotypes of the 176 F_2 individual plants based on the curve patterns of the parental and F_1 varieties.

susceptible and resistant parents. The melting curve patterns of the microsatellite primer pairs for two polymorphic markers linked with *Bph* genes, namely, RM6 and RM205, are shown in Fig. 1.

Marker segregation data analysis

Of the 110 SSR loci surveyed, the 57 best polymorphic markers with high-quality melting curve patterns (Table 1) were evaluated for 176 F_2 progeny derived from MR276 × Rathu Heenati. The patterns of all the polymorphic markers varied in the segregating F_2 population. These 57 SSR markers were unambiguous and easily scorable based on the distinct HRM peak patterns. The F_2, parent, and F_1 patterns of three polymorphic SSR markers, RM261, RM6, and RM11, are shown in Fig. 2. All of the markers were co-dominant with either MR276 or Rathu Heenati alleles (Table 2). The number of polymorphic SSR loci surveyed ranged from 2 (chromosome 7) to 7 (chromosomes 3 and 8) (Table 1). The survey of 176 F_2 progeny was performed using the primer pairs of 57 polymorphic SSR loci that were distributed across the 12 rice chromosomes (Table 2). The chi-square test was used to examine the deviation of SSR markers at specific loci from the normal segregation pattern. The segregation patterns of the SSR markers RM261, RM11 and RM6 are shown in Fig. 2. In total, 35 SSR markers showed good fit to the expected segregation ratio (1:2:1) for a single-gene model (d.f. = 1.0, $p \leq 0.05$). Twenty-two SSR markers deviated from the expected segregation pattern. Of these 22 SSR markers, 12 markers were skewed in favour of MR276 alleles, whereas 10 markers were skewed in favour of Rathu Heenati alleles. The segregation distortion detected in the present study was 61.4%. Fifteen SSR markers exhibited significant deviation from the normal distribution pattern, and seven (RM208, RM348, RM163, RM455, RM160, RM228 and RM496) displayed highly significant deviation from the normal distribution pattern (Table 2).

Discussion

Since the early 1990s, the usage of molecular marker technology in commercial breeding programmes has greatly increased the efficiency of these programmes and accelerated the transfer of useful traits into agronomically desirable

varieties and hybrids (Guimarães 2007). Complex traits and polygenic inheritance that were previously more difficult to elucidate using conventional breeding methods can now be easily followed using molecular markers (Schrodi et al. 2014). The screening and identification of polymorphic markers for parental polymorphisms can form the basis for plant genome analysis and are suitable for generating mapping populations, tagging resistance genes and subsequently aiding marker-assisted breeding programmes (Ilango and Sarla 2010; Meenakshisundaram et al. 2011). Polymorphic SSR markers can be used in analysing genetics, constructing genetic maps and fine mapping rice brown planthopper (*Nilaparvata lugens* Stål)-resistance genes (Chen et al. 2006; Huang et al. 2013; Renganayaki et al. 2002; Wu et al. 2014). Segregation distortion using molecular markers has been observed across different mapping populations. Sun et al. (2011) and Ashkani et al. (2011) attempted to map segregation distortion loci using SSR markers. These types of markers have the potential to improve the speed and effectiveness of germplasm development and to identify desirable genotypes for the purposes of gene and QTL mapping. Several studies have demonstrated that distorted marker segregation is common in rice and that segregation in the F_2 population is typically expected to follow the Mendelian ratio pattern in the offspring (Dettori et al. 2001; Hanley et al. 2002; Kubisiak et al. 1995; Liebhard et al. 2002). Ashkani et al. (2011) investigated the segregation distortion of 23 SSR markers across the F_2 rice population derived from a cross between Pongsu Seribu 2 and Mahsuri rice cultivar. These authors reported 47.8% segregation distortion for these markers across the studied populations. Wu et al. (2010) reported 23.9% and 27.1% segregation distortion in two F_2 populations that were derived from japonica and indica crosses. In the present study, 35 polymorphic markers clearly showed a good fit to the expected segregation ratio for the single-gene model. These results revealed that a significant segregation distortion (61.4%) was recorded in the indica mapping population, but a low segregation distortion (47.8%) was reported in indica rice (Ashkani et al. 2011). Overall, our result was in agreement with previous marker segregation analyses, which indicated that one or two dominant genes that are present in the cultivars can confer complete resistance against BPH (Jairin et al. 2009; Su et al. 2006). The analysis of our selected SSR markers in the F_2 population indicated that these markers were useful tools for population genetics studies. This finding has potential uses in genetic analyses for the marker-assisted selection and confirmation of *Bph* resistance genes to develop rice cultivars with durable resistance in Malaysian rice breeding programmes.

Materials and methods

Plant materials

An F_2 population of 176 individuals that was developed from a cross between the Malaysian rice variety MR276 (susceptible parent: female) and Rathu Heenati (resistant parent: male) (Renganayaki et al. 2002) was genotyped using selected SSR markers that are associated with *Bph* resistance genes. Rathu Heenati (a traditional Sri Lankan rice cultivar) is a resistant variety that shows broad-spectrum resistance to all four biotypes (Biotypes 1, 2, 3, and 4) of BPH. MR276 (ER3722 × Y1279) was one of the Malaysian high-yielding varieties obtained from an advanced yield trial; however, this variety is susceptible to BPH.

Sample collection and DNA extraction

Leaf tissues were harvested from 4-week-old individual F_2 and parental seedlings that were grown in a greenhouse. Then, the tissues were stored at -80°C until DNA isolation was performed. Total genomic DNA was extracted from freshly frozen leaf material using the CTAB method as described by Doyle and Doyle (1990) with some modifications. Leaf tissue was ground for 150 s in a TissueLyser II (Qiagen Inc., Valencia, CA), which was equipped with a 3 mm diameter tungsten carbide bead. Then, 800 µl extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA (ethylenediaminetetraacetate) (pH 8.0), 1.4 mM NaCl, 2% (w/v) CTAB, and 2% (w/v) PVP (polyvinylpyrrolidone)) and 3 µl β-mercaptoethanol were added to 0.1 g ground leaf tissue. Then, the mixture was incubated at 65°C for 60 min and centrifuged at 13,000 rpm for 5 min. Next, 600 µl chloroform:isoamyl alcohol (24:1 (v/v)) was added to each incubated sample, and the sample was centrifuged at 13,000 rpm for 5 min. Isolated DNA was precipitated from the aqueous phase by adding an equal volume of 4°C isopropanol and incubating at -20°C overnight. Then, after centrifugation at 13,000 rpm for 10 min, the precipitated DNA was washed by adding 400 µl ice-cold 70% (v/v) ethanol. The air-dried pellet was re-suspended in 50 µl TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)), treated with 1 µl RNase and incubated at 37°C for 2 h to remove RNA from the isolated genomic DNA.

SSR amplification

In total, 110 SSR primer pairs that are related to *Bph* resistance genes (*Bph*-Genes) and that have been mapped by Jena et al. 2006, Sun et al. 2005, Chen et al. 2006, Renganayaki et al. 2002, Yang et al. 2002 and Sun et al. 2007 were selected from the Gramene database (www.gramene.org) and used to amplify SSR markers for analysis in individual F_2 plants.

Polymerase chain reaction (PCR) and HRM analysiss

SSR amplification and HRM analysis were performed using a Rotor-Gene Q (Qiagen, Hilden, Germany) with a Type-it HRM PCR Kit (Qiagen, http://www.qiagen.com). A touchdown PCR protocol was used to amplify the SSRs. The reaction mixture consisted of 20 ng genomic DNA, 10 µM of each primer, and 2× HRM PCR Master Mix, which included HotStarTaq Plus DNA Polymerase, Type-it HRM PCR Buffer, Q-Solution, and dNTPs, in a total volume of 10 µl. A negative control containing all reagents minus DNA was included in each run. The HRM reaction procedure and melting analysis were 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; then 25 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. The melting curves were obtained once amplification was completed by ramping the temperature from 65 to 90°C, with 0.1°C per step and 10 fluorescent acquisitions per degree Celsius. Rotor-Gene ScreenClust HRM software version 1.10.1.2 was used to classify (autocall) the genotypes of individual lines.

Genotyping for marker segregation

Marker segregation analysis using high-resolution melting (HRM) curve analysis was performed on 176 individual F_2 plants. Two samples from the maternal, paternal and F_1 lines

were included in every PCR run. F_2 individuals were genotyped using 57 polymorphic SSR markers. The HRM curve for each F_2 plant was compared with the paternal, maternal and F_1 curves, and the genotype of each F_2 was determined based on the similarity of the curves. This analysis was performed using Rotor-Gene ScreenClust HRM software. The plants that showed a curve pattern similar to the resistant parent alleles were scored as "R", the plants with a curve pattern similar to the susceptible parent alleles were scored as "r", and those with a curve pattern similar to the F_1 curve were scored as "Rr".

Statistical analysis

Segregation data were analysed using the chi-square test (α = 0.01, with n-1 degrees of freedom). The goodness of fit of the observed value (O) to the expected values (E) for the F_2 population was calculated using R software version 2.7.1 and QTL IciMapping version 4.0 software. The critical value for the chi-square is 3.84 for the single-gene model.

Conclusions

In total, 57 polymorphic markers were used to identify the segregation ratios in an F_2 population of rice that was derived from a cross of MR276 (susceptible) × Rathu Heenati (resistant) cultivars. The chi-square analyses of 35 SSR markers showed the expected segregation ratio of 1:2:1 and simple Mendelian inheritance. The segregation distortion that was detected in the present study using SSR markers was significantly high (61.4%). This study found that HRM analysis should be applied to cereal genomics research and to similar analyses of any species. These results will be useful for rice breeding, leading to the deployment of the genes and QTLs that confer BPH resistance in rice fields.

Acknowledgements

This study was supported by the Fundamental Research Grant Scheme FRGS/1/11/STWN/UPM/02/24 Vot No. 5524145, Ministry of Higher Education, Malaysia.

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