IDENTIFICATION OF POLYMORPHISM ON SIMPLE SEQUENCE REPEATS MARKERS ASSOCIATED WITH BROWN PLANTHOPPER RESISTANCE GENES IN TWENTY RICE GENOTYPES AND THEIR GENETIC RELATIONSHIP

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ABSTRACT

Brown planthopper is one of the most destructive insect pest of rice in Indonesia and other Asian countries. Pyramiding some brown planthopper resistance genes is a valuable approach to create more durable resistance against the pest. The objective of this study was to identify polymorphisms of Brown Planthopper Resistance genes (Bph) on 20 genotypes of rice, and to obtain genetic relationship among genotypes tested. The experiment was conducted from June to September 2012 at Green House and Laboratory of Plant Analysis and Biotechnology, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor. Twenty genotypes were analyzed, and two of them were used as check varieties. Simple Sequence Repeat (SSR) markers were applied to detect *Bph3*, *Bph4, Qbph3,* and *Qbph4* genes. Polymorphic levels were analyzed by calculating PIC (*Polymorphic Information Content*). The grouping of rice genotypes were done based on principal components analysis (PCA) of SSR data, and the genetic relationship based on the presence of Bph genes was estimated using UPGMA (*Unweighted Pair Group With Arithmetic mean*). Results showed that RM313, RM8072, RM8213, RM5953, RM586, and RM589 markers were polymorphic. Rice genotypes PTB 33, Diah Suci, Cibogo, Cisantana, Digul, Ciherang, Inpari 13, Inpari 10, and Memberamo had *Bph3*, *Qbph3, Bph4*, and *Qbph4.* Meanwhile *Bph3, Qbph3*, and *Bph4* were supposed to be belonged by IR 64, Aek Sibundong, Batang Gadis, IR 66, and Mekongga. Kalimas and Tukat Penatu had *Bph3, Qbph3*, dan *Qbph4*. IR 74 had *Bph3* and *Qbph3*, and Fatmawati had *Bph3* and *Bph4.* UPGMA clustering resulted in two main clusters, in which the first cluster consisted of 2 subclusters. PTB-33 was closely related with Memberamo, Tukat Penatu, Digul, Diah Suci, and Kalimas. The SSR markers used in this study were proven to be valuable in molecular detection of *Bph* genes and in estimating genetic relationsips of rice genotypes. PTB-33 was a good donor of resistance genes, as well as Memberamo, Tukat Penatu, Digul, Diah Suci, and Kalimas which were identified as promising donors in rice breeding resistance to brown planthopper.

Key words : *Bph* gene, Brown Planthopper, Genetic relationship, SSR markers.

INTRODUCTION

Indonesia and the other Asian countries are currently facing problems in securing supplies of rice. One reason of the decline in rice production is the brown planthoppers (BPH; *Nilaparvata lugens* Stal.) pests. BPH is a global threat because not only attacking rice crops in Indonesia, but also attacking rice crops in China, Thailand, Vietnam, India, Bangladesh, Malaysia, Philippines, Japan, and Korea (Baehaki, 2007). Interesting phenomenon in the interaction between the rice varieties to brown planthopper is the appearance of biotype, the

strains of brown planthoppers that have adapted physiologically or genetically to certain varieties. In Indonesia, a lot of BPH biotypes were found, such as biotype 2, 3, 4 and also a possible mix among them. To prevent the spread of brown planthoppers, control action has been carried out in many ways since 1970. One of the most effective, economical, and environmental friendly approach is by using resistant rice varieties. This approach was chosen due to the fact that resistance to brown planthoppers is genetically controlled, which generally based on the resilience of the major genes (Kisimoto, 1981; Mochida, 1978). To identify brown planthopper resistance genes in rice varieties, molecular markers (DNA-based markers) is a promising way to perform such effort. Simple Sequence Repeats is frequently applied for assessing genetic diversity, QTL analysis and other molecular tasks. SSR markers are PCR-based DNA markers in a form of DNA sequence with a long repetition of a nucleotide bases (Foulet *et al*, 2005). For SSR markers have been known to be associated with the BPH resistance genes: RM589, RM586, RM19291 and RM8072 with Bph3 (Jairin *et al.*, 2007a), RM313 and RM7 with Qbph3 (Sun *et al.*, 2005), RM589 and RM586 with Bph4 (Jairin *et al.*, 2007a), RM5953 and RM8213 with Qbph4 (Sun *et al.*, 2005).

Identification of *Bph* resistance genes in rice resistant varieties by using SSR markers is valuable tool in order to detect the composition of BPH resistance genes and also to make an attempt on pyramiding some resistance genes into one genotype. Gene pyramiding combines more than two resistance genes into a single genotype achieved through cross-breeding (Sharma *et al.*, 2003), in order to obtain rice varieties with horizontal resistant composted by multiple BPH biotypes which is expected to be more durable. Meanwhile, genetic relationship of rice genotypes based on the presence of their Bph genes should be evaluated for selecting promising parent for hybridization. In this research, testing for polymorphism of SSR markers was evaluated. Level of polymorphism was estimated by counting the PIC (Polymorphic Information Content) value, which was done to determine the informative level of SSR markers used, in which polymorphism occurs due to differences in the number of repetitions of DNA sequences (Liu, 1998). This study was aimed to identify Bph resistance genes on twenty rice genotypes, to assess genetic relationship among genotypes, and to identify better markers for detecting Bph genes especially for Indonesian rice varieties.

MATERIALS AND METHODS
Plant Materials

Twenty rice genotypes derived from Indonesian Rice Research Institute (BB Padi Sukamandi) were used: PTB33, Diah Suci, IR74, IR64, Aek Sibundong, Batang Gadis, Cibogo, Cisantana, Digul, Ciherang, Fatmawati, Inpari13, Inpari10, IR66, Kalimas, Mekongga, Memberamo, and Tukat Penatu. IR42 and Taichung 65 were used as check varieties (susceptible). The seeds were germinated on petridish and then sown in trays. Each genotype consisted of 10 plants in pots that were planted in separate pot. Young green leaf from 45-days old plant was taken for DNA analysis.

DNA preparation and SSR analysis

Molecular markers as listed in Table 1 were applied. These SSR markers were se-

lected based on literature studies and further confirmed by initial laboratory experiment.

Genomic DNA was isolated from young leaves using CTAB method (Doyle and Doyle, 1987). Concentration of DNA was estimated using spectrophotometer (Rayleigh UV-9200) at wavelength of 260nm and 280nm. Total volume of PCR mixture was 25 μL, consisted of 9,5 μL *Nuclease-free* water, 12,5 μL Go *Taq®* Green Master Mix, 1 μL Forward Primer, 1 μL Reverse Primer, and 1 μL Template (DNA) ~ 20ng.

Amplification reaction was performed on a thermal cycler (Eppendorf), and programmed as follows: one cycle of 94°C for 5 min for initial denaturation, 36 cycles of 94°C for 1 min for denaturation, 55°C for 1 min for annealing, and 72°C for 1 min for elongation, followed by 72°C for 7 min for final elongation. PCR product was run on agarose gel (3%) electrophoresis for 45 minutes at 100 volt. Agarose gel was stained with ethidium bromide solution for 20-30 minutes then subsequently visualized in chemidoc gel system.

Table 1. SSR markers used

No	SSR Markers	Sequence		PCR product size*	Bph target	Literature
		Forward	Reverse			
1	RM7	ttcgccatgaagtctctcg	cctcccatcatttcgttgtt	180bp	*Qbph3*	Sun *et al.*, 2005
2	RM586	acctcgcgttattaggtacccatcat	gagatacgccaacgagataccaggtt	271bp	*Bph3* and *Bph4*	Jairin *et al.*, 2007
3	RM589	ggtcggtggcttaac	ccaaccagacactg	186bp	*Bph3* and *Bph4*	Jairin *et al.*, 2007
4	RM5953	aaactttctgtgatggtatc	atccttgtctagaattgaca	129bp	*Qbph4*	Sun *et al.*, 2005
5	RM8072	gatcactcaggtcatccattc	aatcagagaggctaaagacaataat	146bp	*Bph3*	Jairin et al., 2007
6	RM8213	agcccagtgatacaaagatg	gcgaggagataccaagaaag	177bp	*Qbph4*	Sun et al., 2005
7	RM19291	cacttgcacgtgtcctctgtacg	gtgtttcagttcaccttgcatcg	146bp	*Bph3*	Jairin et al., 2007
8	RM313	tgctacaagtgttcttcaggac	gctcaccttttgtgttccac	111bp	*Qbph3*	Sun et al., 2005

Note: * Derived from Gramene (2008).

Data analysis

The PIC value e" 0.5, which was calculated according to Botstein *et al.* (1980): Molecular data in terms of PCR bands derived from image visualization system using Geldoc under UV transilluminator (captured by Genesnap software program) was interpreted. The images were then analyzed using a GeneTool software (Syngene, UK) for ensuring the differences in size of each band. Polymorphic level was assessed by calculating the PIC, which provides an estimate level of informativeness of SSR markers. SSR markers were considered to be informative if

$$PIC = 1 - \left(\sum pi^2\right)^2$$

with PIC = Polymorphic Information Content, i = 1, 2, 3, ... n, pi^2 is the i-th allele frequency.

Principal component analysis (PCA), which is a multivariate technique, was peformed to analyze several variables, to identify the principal directions in which the data varies and to display the similarity of the pattern of observations and variables as points on a map. To determine genetic relationship among genotypes, clustering with the UPGMA (Unweighted

Pair Group With Arithmetic mean) was carried out with NTSYS program (Rohlf, 1998) using XLStat 2013.

RESULTS AND DISCUSSION

Molecular screening by means of confirmation of SSR markers in their association with brown planthoppers (Bph) resistance genes in 20 genotypes is an essential effort for selecting promising parental for crossing and developing new genetic combination with some *Bph* genes in one genotype in order to create a horizontal resistance which is believed to be more durable. Molecular detection by using RM8072 as an example for detecting *Bph3* gene wass presented in Figure 1. Results showed that PTB 33 (*Bph3* with 146bp PCR product size referred to Gramene, 2008) had the same band with Inpari 13 (#12), IR 64 (4), Ciherang (#10), IR 42 (#19), IR 74 (#3), Aek Sibundong (#5), Batang Gadis (#6), Cibogo (#7), Cisantana (#8), Digul (#9), Fatmawati (#11), IR 66 (#14), Mekongga (#16) and Memberamo (#17). Diah Suci (#2) and Inpari 10 (#13) had a slightly different size with PTB 33, meanwhile Taichung 65 (#20), Kalimas (#15) and Tukat Penatu (#18) clearly had different band with PTB 33. Genotypes with the same banding pattern were suspected to have same *Bph* genes. According to the genome sequence of Nipponbare, *Bph3* locus was finally localized approximately in a 190 kb interval flanked by marker RM19291 and RM8072, which contained twenty-two putative genes. This resistance gene has been identified and mapped on the short arm of chromosome 6 (Jairin *et al.,* 2007b). It is very much believed that RM8072 is SSR marker that associated with *Bph3* gene.

Table 2 showed compilation of the number of SSR markers detected in 20 genotypes. DNA banding pattern of each primer used in this study was presented. Form this table it was supposed that genotypes could be classified into three types of SSR markers they belong i.e.: (i) genotype with 0-2 SSR markers i.e.: IR 42 and Taicung genotypes; (ii) genotype with 3-5 SSR markers i.e.: IR 74, IR 64, Aek Sibundong, Batang Gadis, Cisantana, Inpari 10, IR 66, Kalimas, Menkongga, and Tukat Penatu genotypes; (iii) genotype with 6-8 SSR markers i.e.: PTB 33, Diah Suci, Cibogo, Digul, Ciherang, Inpari 13, and Memberamo. These data were consistent with the results of BPH resistance tests performed by Indonesian Rice Research Institute. Cultivar Inpari 13 proved to be resistant against BPH biotypes 1, 2, and 3, which was better than IR64 and Ciherang that only resistant to BPH biotypes 1 and 2.
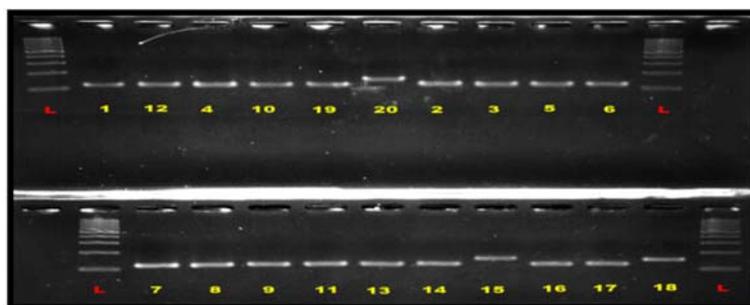

Figure 1. Visualisation of Agarose Gel Electrophoresis Using RM 8072 to Detect *Bph3* Gene (*Ladder* (L); PTB 33 (#1); Diah Suci (#2); IR 74 (#3); IR 64 (#4); Aek Sibundong (#5); Batang Gadis (#6); Cibogo (#7); Cisantana (#8); Digul (#9); Ciherang (#10); Fatmawati (#11); Inpari 13 (#12); Inpari 10 (#13); IR 66 (#14); Kalimas (#15); Mekongga (#16); Memberamo (#17); Tukat Penatu (#18); IR 42 (#19); Taichung 65 (#20)

Results showed that Kalimas and Tukat Penatu had *Qbph3, Bph3*, and *Qbph4* genes. According to the description of rice varieties published by Indonesian Rice Research Institute (2009), it was mentioned that Tukat Penatu was resistant to BPH biotype 3 and Kalimas was moderately resistant to BPH biotype 3. Fatmawati was supposed to have *Bph4* and *Bph3* which wass moderately resistant to BPH biotype 2 and biotype 3. Cultivar IR 74 was found to have *Bph3* and *Qbph3* genes, and Indonesia Rice Research Institute reported that this cultivar was resistant to BPH biotype 1 and biotype 2.

Table 2. Number of SSR markers detected in 20 rice genotypes

Genotypes	RM 7 (Qbph3)	RM 313 (Qbph3)	RM 8072 (Bph3)	RM 19291 (Bph3)	RM 5953 (Qbph4)	RM 8213 (Qbph4)	RM 586 (Bph3 dan Bph4)	RM 589 (Bph3 dan Bph4)	Number of positive detection
PTB 33 (#1)	+	+	+	+	+	+	+	+	8
Diah Suci (#2)	-	+	.	+	+	+	+	+	6
IR 74 (#3)	+	+	+	+	-	-	-	-	4
IR 64 (#4)	+	-	+	+	-	-	+	-	4
Aek Sibundong (#5)	+	-	+	+	-	-	+	-	4
Batang Gadis (#6)	+	-	+	+	-	-	+	-	4
Cibogo (#7)	+	+	+	+	-	+	+	-	6
Cisantana (#8)	+	-	+	+	-	+	+	-	5
Digul (#9)	+	-	+	+	+	-	+	+	6
Ciherang (#10)	+	+	+	+	+	+	+	-	7
Fatmawati (#11)	-	-	+	+	-	-	+	-	3
Inpari 13 (#12)	+	+	+	+	+	+	+	-	7
Inpari 10 (#13)	+	-	-	+	-	+	+	-	4
IR 66 (#14)	+	+	+	-	-	-	-	+	4
Kalimas (#15)	+	+	-	+	+	+	-	-	5
Mekongga (#16)	+	+	+	+	-	-	+	-	5
Memberamo (#17)	-	+	+	+	+	-	+	+	6
Tukat Penatu (#18)	+	+	-	+	+	-	-	-	4
IR 42 (#19)	-	-	+	+	-	-	-	-	2
Taichung 65 (#20)	-	-	-	-	-	-	-	-	0

Note: (+) = Supposed to have resistance gene, (-) = Supposed do not have resistance gene

Based on Table 2, Memberamo and Diah Suci had *Qbph3*, *Qbph4, Bph3*, and *Bph4* genes. These varieties were resistant to brown planthopper biotypes 1, 2, and were moderately resistant to biotype 3. Meanwhile, although Inpari 10 had *Qbph3, Qbph4, Bph3,* and *Bph4*, these cultivars were identified as slightly resistant to BPH biotype 1 and 2 (Suprihatno

et al., 2010). This might be due to resistance to BPH is not governed by a single gene but controlled by many genes (Nugaliyadde *et al*, 2000; Jairin *et al*, 2005; Chen *et al*, 2006; Murata, 1998).

In general, genotypes PTB 33, Diah Suci, Cibogo, Cisantana, Digul, Ciherang, Inpari 13, Kalimas, Mekongga, and Memberamo were well serve as parent because positively detected as having resistance genes using many markers (Table 2). The more *Bph* genes they have the more resistant they were expected.

PIC (Polymorphic Information Content)

The polymorphic information content of 8 SSR markers used in this study was presented in Table 3. Based on Table 3, RM313, RM8072, RM5953, RM8213, RM586, and RM589 were considered as informative markers, while RM7 and RM19291 were classified as uninformative markers. This criterion refers to Zhang *et al.* (2011) who stated that if the PIC values e" 0.5, then the markers are classified to be informative. The greater the PIC value corresponds to the better primer to be molecular markers (Anderson *et al.*, 1993).

It was found that sometimes PCR product of informative markers was not polymorphic, which may hinder the analysis. This might be related to the purity of genomic DNA used for amplification. In such case, DNA extraction, PCR reaction and electrophoresis were repeated again in assuring a trusted data. Optimization was also performed for various conditions such as concentration of DNA polymerase, annealing temperature, concentration of DNA samples and the length of the PCR process. The success of DNA amplification was also influenced by the purity and integrity of the DNA samples (Bardakci, 2001) but in some genomes, the purity of the DNA samples did not affect amplification (Caetano-Anollés, 2004). DNA concentration which is too high may increase the contaminants that interferes amplification reaction (Chen, 2000).

Table 3. Polymorphic Information Content (PIC) of SSR markers

Markers	PCR product Size	Number of band	Number of Allele	PIC
RM7 (*Qbph3*)	180bp	19	3	0,42
RM313 (*Qbph3*)	111bp	20	5	0,73
RM19291(*Bph3*)	146bp	20	4	0,35
RM8072 (*Bph3*)	146bp	20	7	0,66
RM5953 (*Qbph4*)	129bp	20	3	0,54
RM8213 (*Qbph4*)	177bp	29	3	0,63
RM586 (*Bph3 & Bph4*)	271bp	20	5	0,76
RM589 (*Bph3 & Bph4*)	186bp	20	5	0,77

Principal Component Analysis

Based on result of principal component analysis (PCA) presented in Figure 2, there were three main groups identified. Group one consisted of PTB-33, Tukat Penatu, Memberamo, Diah Suci, Kalimas, IR-74 and IR-66 genotypes. Taichung-65 was separated as group two, and the other genotypes clustered together in group three. Genotypes clustered in group one was thought to have *Bph* resistance gene close to PTB-33, whereas Taichung-65 (susceptible genotype) did not have *Bph* resistance gene as has been reported in previous studies, and located on the opposite site with quadrant of PTB-33 on the PCA plot.
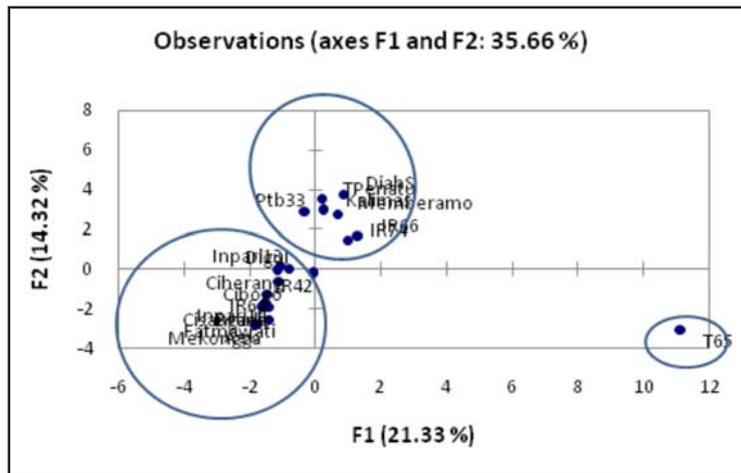
Figure 2. PCA of 20 Rice Genotypes

Genetic Relationship

Results of cluster analysis on 20 rice genotypes using eight SSR markers was shown in Figure 3. This grouping of genotypes was resulted based on molecular data using UPGMA Jaccard's coefficient using XLSTAT 2013 program to determine a genetic relationship based on the presence of *Bph* genes. Two main clusters were recognized, with the first cluster consistsed of 2 sub clusters. The first sub cluster was formed by PTB 33, Memberamo, Tukat Penatu, Digul, Diah Suci, Kalimas, while IR 64, Cisantana, Inpari 10, Aek Sibundong, Mekongga, Batang Gadis, Fatmawati, Cibogo, Ciherang, Inpari 13 formed the second sub cluster. The first and second sub cluster joined by coefficient value of 3.25. The second cluster consisted of IR 74, IR 66 and IR 42 and joined with the first cluster by coefficient value of 3.49.
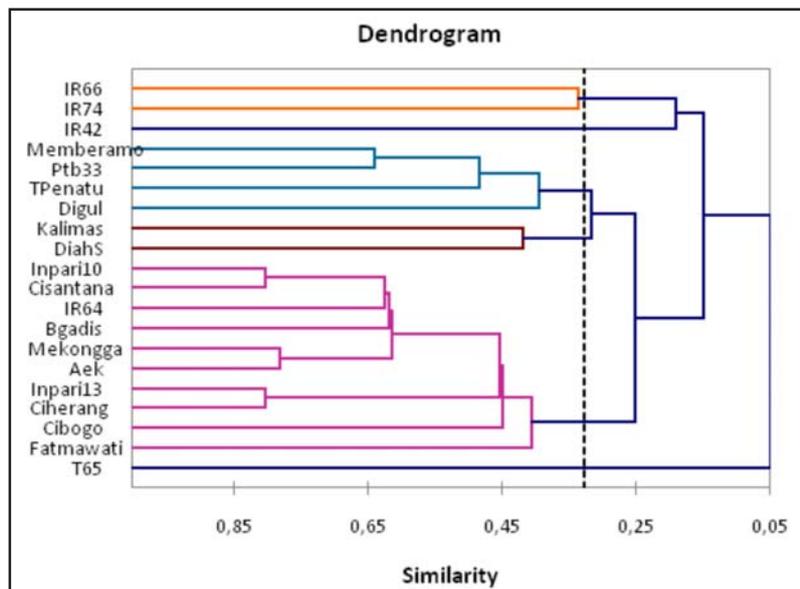

Figure 3. Clustering Analysis of 20 Rice Genotypes based on 8 SSR Markers

Figure 3 revealed that the smaller coefficient value between genotypes indicated that they were closely related in terms of resistance gene to BPH. PTB 33 was known to be resistant to BPH along with Memberamo, Tukat Penatu, Digul, Diah Suci, and Kalimas.

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Based on the results of SSR, the PTB 33, Memberamo, Digul, and Diah Suci was supposed to have *Qbph3 gene, Bph3, Qbph4,* and *Bph4*, as well as Tukat Penatu and Kalimas which have *Qbph3, Bph3*, and *Qbph4*. These genotypes were thought to have similar genes that resistant to BPH.

CONCLUSION

1. The informative SSR markers were RM586, RM589, RM313, RM8072, RM5953 and RM 8213 while the uninformative markers were RM7 and RM19291.
2. The SSR markers used in this study were capable of detecting the presence of *Bph* genes.
3. Genetic relationship based on the presence of *Bph* genes showed that PTB 33 was closely related to Memberamo, Tukat Penatu, Digul, Diah Suci, and Kalimas.

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