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Exploring the Resistance of Pigmented Rice to Brown Planthopper (*Nilaparvata lugens* Stål) by Phenotypic and Genotypic Analyses

(Mengkaji Kerintangan Beras Berpigmen terhadap Wereng Coklat (*Nilaparvata lugens* Stål) melalui Analisis Fenotip dan Genotip)

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ABSTRACT

Developing new pigmented rice varieties that meet consumers' preferences requires addressing resistance to brown planthopper (BPH), a highly destructive insect pest of rice. To effectively manage this pest, it is essential to explore BPH resistance genes from rice germplasms and incorporate them into elite varieties. The objective of this study was to identify the source of resistance among local and improved pigmented rice varieties in Indonesia against field BPH. Furthermore, the study aimed to characterize their genetic diversity using molecular markers associated with *Bph* genes and functional markers for these genes. Eighty-eight local varieties from the Indonesian Agricultural Gene Bank, collected across 18 provinces between 1971 and 2019, and four improved varieties, were evaluated using the standard seed box screening test method. Among these entries, 6 local accessions and 1 improved variety exhibited moderate resistance. Genetic analysis of these entries and three randomly selected susceptible entries using 12 SSR and InDel markers associated with *Bph* gene loci showed that the moderately resistant entries had higher allele numbers (11 to 13) compared to the susceptible entries (4 to 7). These markers could distinguish the resistant group from the susceptible ones in cluster analysis. Genotyping using functional markers further showed that the moderately resistant entries had 2 to 4 combinations of *Bph* genes. In contrast, the susceptible entries contained neither *Bph* genes nor only one gene. Local accessions with a 2 to 4 *Bph* gene combination can be donors of BPH resistance in improving pigmented rice varieties.

Keywords: *Bph* gene; brown planthopper resistance; local rice accession; screening

ABSTRAK

Membangunkan varieti padi berpigmen baharu yang memenuhi keutamaan pengguna memerlukan usaha menangani rintangan terhadap wereng coklat (BPH), serangga perosak pemusnah beras. Untuk menguruskan perosak ini dengan berkesan, adalah penting untuk mengkaji gen rintangan BPH daripada germplasma padi dan memasukkannya ke dalam varieti elit. Objektif kajian ini adalah untuk mengenal pasti punca rintangan dalam kalangan varieti padi tempatan dan padi berpigmen dipertingkat di Indonesia berbanding BPH ladang. Tambahan pula, penyelidikan ini bertujuan untuk mencirikan kepelbagaian genetik mereka menggunakan penanda molekul yang dikaitkan dengan gen *Bph* dan penanda berfungsi untuk gen ini. Lapan puluh lapan varieti tempatan daripada Bank Gene Pertanian Indonesia yang dikumpulkan daripada 18 wilayah antara 1971 dan 2019 dan empat varieti yang dipertingkat dinilai menggunakan kaedah ujian saringan kotak benih piawai. Antara entri ini, 6 aksesi tempatan dan 1 varieti dipertingkat menunjukkan rintangan yang sederhana. Analisis genetik bagi entri ini dan tiga entri rentan yang dipilih secara rawak menggunakan 12 penanda SSR dan InDel yang dikaitkan dengan lokus gen *Bph* menunjukkan bahawa entri tahan sederhana mempunyai nombor alel yang lebih tinggi (11 hingga 13) berbanding dengan entri rentan (4 hingga 7). Penanda ini boleh membezakan kumpulan rintangan daripada kumpulan rentan dalam analisis kelompok. Genotip menggunakan penanda berfungsi selanjutnya menunjukkan bahawa entri tahan sederhana mempunyai 2 hingga 4 gabungan gen *Bph*. Sebaliknya, entri yang rentan tidak mengandungi gen *Bph* atau hanya satu gen. Aksesi tempatan dengan gabungan gen 2 hingga 4 *Bph* boleh menjadi penderma rintangan BPH dalam meningkatkan varieti beras berpigmen.

Kata kunci: Aksesi beras tempatan; gen *Bph*; ketahanan terhadap wereng coklat; penyaringan

Introduction

Pigmented or coloured rice is a type of rice that is characterized by red, brown, black, and dark purple colour in their aleurone layer due to the presence of flavonoids anthocyanin and proanthocyanidin (Tiozon Jr., Sartagoda & Sreenivasulu 2023). It has been known to have many nutritional and health benefits, such as antioxidant, anti-inflammatory, anticancer, antidiabetic, antiatherosclerosis, and antiallergic activities due to its high content of flavones, tannin, phenolics, sterols, tocols, γ-oryzanols, amino acids, micronutrients, such as iron and zinc (Tiozon Jr., Sartagoda & Sreenivasulu 2023). This type of rice has long been consumed in East and South Asian countries such as China, Korea, Japan, and India (Deng et al. 2013).

Various pigmented rice, especially red rice, is widely distributed in Indonesia and shows high genetic diversity, as shown by molecular markers (Risliawati et al. 2021). In recent years, the consumption of pigmented rice has been gaining popularity in Indonesia due to a preference for a healthier lifestyle and an increase in people's income (Abdullah 2017; Risliawati et al. 2021). However, pigmented rice is less cultivated by farmers because it is late maturing, easily logged at maturity, is vulnerable to main pests and diseases, and has low grain yield and low eating quality (Abdullah 2017; Afza 2017). From 1985 to 2019, 14 improved pigmented rice varieties were released in Indonesia, but only one red rice variety (IR24 Gabusan) is favoured by consumers and profitable for farmers (Abdullah 2017). Breeding of pigmented rice varieties that can meet the preferences of consumers and farmers needs to be intensified, but their development must pay attention to resistance to important biotic stresses (Abdullah 2017).

As with white rice, pigmented rice is also prone to attack by brown planthopper, *Nilaparvata lugens* Stål (BPH), a highly destructive insect pest in East, South and Southeast Asian rice growing regions (Dyck & Thomas 1979). The first BPH outbreak in Indonesia occurred in 1968 when a 52,000-ha rice growing area was affected. The attacked area increased to 283,000 ha in 1976, then decreased to 120,000 ha and 42,247 ha in 1977 and 1978, respectively, but increased sharply in 1985, 1986, 2010, 2011, and 2017 to 80,000 ha; 200,000 ha; 137,768 ha; 218,060 ha; and 407,000 ha, respectively (Ariefiansyah 2018; Baehaki & Mejaya 2014). The cause of the BPH population explosion is the emergence of virulence variations known as biotypes and the adoption of high-yielding rice cultivation techniques in the 1960s (Dyck & Thomas 1979). This new cultivation technique relies on high nitrogen fertiliser inputs and insect pest control with insecticides that destroy natural enemies (Dyck & Thomas 1979).

BPH biotype 1 appeared in Indonesia in 1971 after adopting the variety IR5 and IR8 introduced by the International Rice Research Institute (IRRI) in 1967 (Baehaki 2012). Massive screenings of rice germplasm were conducted at IRRI to identify resistance donors. Through classical genetic studies, BPH resistance genes were identified and used to improve the resistance of rice varieties (Brar et al. 2009). IR26 was the first high-yielding variety to contain BPH resistance genes *Bph1* (now renamed *Bph18*) from Mudgo (Brar et al. 2009; Yang et al. 2023). This variety was widely planted in the Philippines, Indonesia, and Vietnam from 1973 to 1974 and successfully controlled BPH populations. Still, another outbreak occurred in 1977 after the emergence of a new virulent variant of BPH called biotype 2 (Brar et al. 2009). After that, IR36 and IR42 with the *bph2* gene (now renamed *Bph26*) were released in 1980 and widely adopted in those countries (Brar et al. 2009; Yang et al. 2023). This gene successfully saved rice production in Indonesia until the emergence of a virulent variant of BPH to IR42 in North Sumatera Province and to IR36 in several regions in 1982 (Baehaki 2012; Brar et al. 2009). IR72 with the *Bph3* gene was released in 1988 and widely grown in Asia (Brar et al. 2009).

Since the first identification of the *Bph1* gene in 1967, more than 40 *Bph* genes and 22 quantitative trait loci (QTLs) have been identified in both *Oryza indica* and *japonica* subspecies and four wild relatives, *O. australiensis*, *O. eichingeri*, *O. latifolia*, and *O. offiinalis* (Haliru et al. 2020). These genes mainly concentrate on chromosomes 1, 3, 4, 6, and 12 (Haliru et al. 2020). Recent molecular genetic studies have shown that certain *Bph* genes, despite having different numbers, are in fact identical genes or multiple alleles of the same gene (Yang et al. 2023; Zhao et al. 2016). The closely linked markers for *Bph* genes/QTLs have been used in marker-assisted breeding to monitor the gene transfer in the progeny of hybridization (Kamal et al. 2023) and to screen rice germplasms (Harini et al. 2013; Jena et al. 2015; Muduli et al. 2023; Ramkumar et al. 2016). Seventeen *Bph* genes have been cloned and characterized at the molecular level (Yang et al. 2023). Specific markers for genes located on chromosomes 3, 4, and 12 have been designed and successfully detected the presence of *Bph* genes in rice germplasms from different parts of the world (Ramkumar et al. 2016; Yang et al. 2023).

Growing resistant varieties is a low-cost and environmentally friendly method of controlling BPH compared to applying insecticides. Still, appropriate strategies are required to avoid triggering the adaptation of BPH populations. Recently, there has been an increase in the virulence of BPH field populations from several regions in Indonesia. Virulence tests showed that most IRRI's differential varieties were susceptible, while Swarnalata and PTB33 were still effective in most BPH populations (Chaerani et al. 2016; Horgan et al. 2015). Therefore, there is an urgent need to search for new sources of resistance from diverse rice germplasms, including pigmented rice, to widen the genetic base of durable resistance varieties. So far, a limited number of pigmented rice accessions has been evaluated for resistance to BPH, and the results showed that most responded resistant to BPH population (Busniah, Kasim & Winarto 2020; Dwipa et al. 2018). Using markers linked to *Bph* genes and functional markers for the genes can increase the precision of donor selection and accelerate the resistance breeding programme (Muduli et al. 2023; Yang et al. 2023).

This study aimed to evaluate the resistance of local accessions and improved varieties of pigmented rice in Indonesia and to analyse their genetic diversity using molecular markers linked to *Bph* resistance genes and functional markers for the genes.

Materials and Methods

Plant material

The pigmented rice materials used in this study comprised 88 local varieties from the Agricultural Gene Bank of the Indonesian Ministry of Agriculture collected across 18 provinces between the years 1971 and 2019. Additionally, the study included four improved varieties, i.e., Inpari24 Gabusan, Pamelen, Pamera, and Jelitheng. Most of the entries had a red bran layer (Table 1). Six differential rice varieties i.e., TN1 (no known *Bph* genes), Mudgo (*Bph18* [=*Bph1*]), ASD7 (*Bph26* [=*bph2*]), Rathu Heenathi (*Bph3*+*Bph17*), Swarnalatha (*Bph6*), and PTB33 (*Bph26* [=*bph2*]+*Bph3*+*Bph17*-*ptb*+*Bph32*) were included in the tests. TN1 and PTB33 were used in the screening test as the susceptible and resistant check, respectively.

TABLE 1. Origin and bran layer colour of Indonesian pigmented rice used in the resistance screening to brown planthopper (*Nilaparvata lugens* Stål)

Origin	Bran layer colour						Total
	Red	Black	Dark purple// black	Dark brown	Light brown	Purple spotting	
Aceh	11	0	1	0	1	0	13
North Sumatera	5	0	0	0	0	0	5
West Sumatera	4	0	0	0	0	0	4
Riau	2	0	0	0	0	0	2
Jambi	1	0	0	0	0	0	1
Banten	1	0	0	0	0	0	1
West Java	10	0	7	0	0	0	17
Central Java	3	0	3	0	0	0	6
Special Region of Yogyakarta	0	0	2	0	0	0	3
East Java	3	0	1	0	0	0	4
West Kalimantan	2	0	1	0	0	0	3
Central Kalimantan	3	0	0	0	1	0	4
South Kalimantan	4	0	0	0	0	0	4
East Kalimantan	3	0	0	0	1	1	5
West Sulawesi	3	0	0	0	0	0	3
South Sulawesi	1	0	0	0	0	0	1
North Sulawesi	1	0	0	1	0	0	2
East Nusa Tenggara	5	0	1	0	0	3	10
Improved varieties	3	0	1	0	0	0	4
						Total	92

MASS REARING OF BPH

A virulent BPH colony collected from a rice field in Klaten, Central Java, in 2011 (Chaerani et al. 2016) was maintained continuously on the Ciherang variety in insect-proof cages under a glasshouse environment. To obtain nymphs of uniform age, gravid females and a few males were released in oviposition cages containing pre-cleaned 40 to 45 days old (d.o.). rice plants in pots intended for egg-laying and feeding plants. After three days, all insects were removed from the oviposition cage. The depleted food plants were replaced every two days or as needed for the hatched nymphs. The 2nd and 3rd stages of the nymphs were used in the resistance screening.

EVALUATION OF BPH RESISTANCE

Resistance phenotyping was performed in a greenhouse using the standard seedbox screening test (SSST) method described by Li et al. (2019) with a slight modification. Seeds were pregerminated in Petri dishes for three days. Thirty seeds at pigeon state from each genotype were sown in a row of 35 cm long in a plastic box (L 60 cm × W 45 cm × H 7 cm). Each genotype was randomly assigned in each box and replicated three times. Each box was planted with 16 to 18 entries along with three rows of TN1 placed in the border-left, centre and border-right, and two rows of PTB33 on the left and right side in each box. Pregerminated seeds of one of the other four differential varieties i.e., Mudgo (*Bph18* [=*Bph1*]), ASD7 (*Bph26* [=*bph2*]), Ratu Heenathi (*Bph15* [=*Bph3*])+*Bph17*), PTB33 (*Bph26*[*bph2*]+*Bph15*[*Bph3*]+*Bph17-ptb*+*Bph32*), and Swarnalata (*Bph6*) were also planted in three replicates. Ten days after sowing, each entry was thinned to 20 seedlings/row. The seed boxes were transferred to a pool of water made of tarpaulin. Each box was covered with a lid that could be opened at the top, glass walls at the front, back, and top, and insect-proof wire mesh walls on the sides. Through the top wall, the 2nd to 3rd instar BPH nymphs were infested on seedlings at the 2 or 3-leaf stage (or 14 days after sowing of pregerminated seeds) using the tapping method. The number of infested nymphs was visually checked to reach 4-6 per seedling. Seedling damages were scored on an individual plant basis when ≥90% of TN1 seedlings died using the method following Sun et al. (2005): 0=no visible damage, 1=one leaf showed yellowing, 3=one to two leaves showed yellowing, or one leaf shrank, 5=one to two leaves shrank, or one leaf shrivelled, 7=three to four leaves shrank, or two to four leaves shrivelled, the plant was still alive, and 9=plant died. The most frequent score from a total of 60 seedlings acrooss three replicates was used to determine the resistance level of a particular entry (Effendi & Munawar 2013). The 0, 1, 3, 5, 7, and 9 damage scores were categorised as immune, highly resistant, resistant, moderately resistant, susceptible, and highly susceptible, respectively (Jena et al. 2015).

GENETIC ANALYSIS

Total genomic DNA was extracted from young leaves of resistant entries, three selected susceptible entries, and six differential varieties using the Genomic DNA Mini Kit (Geneaid). DNA concentration was quantified using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific). A total of 29 molecular markers flanking the *Bph* loci and markers co-segregated with *Bph* genes on chromosomes 1, 2, 3, 4, 6, 8, 11, and 12 (Table 2) were tested for polymorphisms on TN1, a DNA pool of three resistant entries and three susceptible entries. Recently published nine functional markers for *Bph* genes (Yang et al. 2023) were also included in the test. Polymerase chain reactions (PCRs) were performed in 15 µL reaction volumes containing 20 ng of template DNA, each 0.5 µM of forward and reverse primers, 7.5 µl MyTaq HS mix (Bioline) and ultrapure water. PCRs were carried out on BiometraT1 thermocycler using the amplification profile described in Yunus et al. (2023): 3 min pre-denaturation at 94 °C followed by 35 cycles of 15 s denaturation at 94 °C, 15 s primer annealing at 50 °C or 55 °C and 20 s primer extension at 72 °C, and then terminated with 5-min final extension at 72 °C. PCR products from amplification with SSR and InDel primers were separated by electrophoresis on 6% non-denaturing polyacrylamide gels at 90V for 100 min or 4% agarose gels at 150V for 2.5 h. In comparison, those functional markers were run on 1.5% agarose gels at 100V for 45 min. DNA size marker of 100 bp ladder (Vivantis) was run along with the PCR products. DNA bands were stained with GelRed (Biotium) according to the protocol set by the manufacturer and were visualized on the Uvitec gel imaging system.

STATISTICAL ANALYSIS

DNA bands' presence (1) and absence (0) for polymorphic SSR and InDel markers of resistant and selected susceptible entries were entered into a binary data matrix. This data matrix was used to calculate the number of alleles per locus, genetic diversity, heterozygosity, and polymorphism information content (PIC) using PowerMarker ver. 3.25 software (Liu & Muse 2005). This software was also used to generate a similarity matrix among genotypes based on the proportion of shared alleles, which was subsequently used to construct a phylogenetic tree using the unweighted pair group method with arithmetic means (UPGMA).

TABLE 2. List of molecular markers used in polymorphism test across selected resistant and susceptible genotypes of pigmented rice to a field population of brown planthopper (*Nilaparvata lugens* Stål)

Resistance gene	Chr[a]	Marker name	Marker type and association with *Bph* gene	Brown planthopper population	Reference
Bph33(t)	1	RM488	SSR (flanking)	N.A.	Naik et al. (2018)
		RM11522	SSR (flanking)		
Bph37	1	YM35	InDel (flanking)	N.A.	Yang et al. (2019)
Bph13(t)	2L	RM240	SSR (flanking)	N.A.	Liu et al. (2001)
		RM250	SSR (flanking)		
bph19(t)	3S	RM3134	SSR (flanking)	Biotype 2	Chen et al. (2006)
		RM6308	SSR (flanking)		
Bph31	3L	PA26	InDel (flanking)	Biotype 4	Prahalada et al. (2017)
		RM2334	SSR (flanking)		
Bph35	4	PSM20	InDel (flanking)	N.A.	Zhang et al. (2020)
		RM3471	SSR (flanking)		
Bph20(t)	4S	MS10	InDel (flanking)	N.A.	Rahman et al. (2009)
		RM5953	SSR (flanking)		
Bph30	4S	RM16294	SSR (tightly linked)	Biotype 1 and 2	Wang et al. (2018)
Bph36	4S	S13	InDel (flanking)	Biotype 1 and 2	Li et al. (2019)
		X48	InDel (flanking)		
Bph6	4L	RM5742	SSR (flanking)	Biotype 4	Qiu et al. (2010)
		RM6997	SSR (flanking)		
Bph34	4L	RM16994	SSR (flanking)	Biotype 4	Kumar et al. (2018)
Bph3	6S	RM588	SSR (flanking)	Biotype 1, 2, 3, and 4	Jairin et al. (2007)
		RM589	SSR (flanking)		
Bph25	6S	S00310	InDel (flanking)	N.A.	Myint et al. (2012)
Bph23(t)	8	RM2655	SSR (flanking)	N.A.	Hou et al. (2011)
		RM3572	SSR (flanking)		
Bph43	11	16-22	InDel (flanking)	N.A.	Kim et al. (2022)
		16-30	InDel (flanking)		
Bph18	12L	18-7	InDel (flanking)	N.A.	Hu et al. (2013)
		RM3331	SSR (flanking)		
Bph26 (=*bph2*)	12L	RM5479	SSR (flanking)	N.A.	Myint et al. (2012)
Bph1/9	12L	B1279D	Functional marker	Field	Yang et al. (2023)
Bph15 (=*Bph3*)	12L	B3D	Functional marker	Field	Yang et al. (2023)
Bph6	12L	B6D	Functional marker	Field	Yang et al. (2023)
Bph7	12L	B7D	Functional marker	Field	Yang et al. (2023)
Bph9	12L	B9D	Functional marker	Field	Yang et al. (2023)
Bph14	12L	B14D	Functional marker	Field	Yang et al. (2023)
Bph18 (=*Bph1*)	12L	B18D	Functional marker	Field	Yang et al. (2023)
Bph26 (=*bph2*)	12L	B26D	Functional marker	Field	Yang et al. (2023)
Bph32	12L	B32D	Functional marker	Field	Yang et al. (2023)

[a]Chr: chromosome

Results and Discussion

Resistance evaluation

Compared to white rice, resistance evaluation of pigmented rice to BPH is scarcely conducted in Indonesia (Busniah, Kasim & Winarto 2020; Dwipa et al. 2018). Considering the rapid evolution of BPH virulence in Indonesia (Baehaki 2012; Chaerani et al. 2016), new resistance donors must continue to be sought using virulent BPH populations. This study found that only 7 out of 92 (8%) entries showed moderate resistance or damage score 5 (Table 3). These entries comprise six local accessions from five provinces and one improved variety (Pamera). The moderately resistant local accessions consisted of four red rice, i.e., Merah 30475 (C. Java), Merah 30756 (East Java); Slegreng Merah 30403 (Aceh), and Slegreng Merah 30477 (North Sumatera) and two black rice accessions, i.e., Badigal (West Java) and Padi Hitam Karang Anyar (Central Java) (Table 4). The moderately resistant improved variety Pamera is a red rice type which was reported resistant to BPH biotypes 1, 2, and 3 (https://agrikan.id/4-varietas-unggul-beras-merah-untuk-kesehatan/); thus it had a broad resistance spectrum to BPH.

TABLE 3. Resistance response of local and improved varieties of Indonesian pigmented rice to a field population of brown planthopper (*Nilaparvata lugens* Stål) based on standard seed box screening test method

Province/origin	Number of genotypes[a]						Total
	0	1	3	5	7	9	
Aceh	0	0	0	1	0	12	13
Riau	0	0	0	0	0	2	2
North Sumatera	0	0	0	1	0	4	5
West Sumatera	0	0	0	0	0	3	4
Jambi	0	0	0	0	0	1	1
Banten	0	0	0	0	0	1	1
W. Java	0	0	0	1	0	17	17
Central Java	0	0	0	2	0	4	6
Special Region of Yogyakarta	0	0	0	0	0	3	3
East Java	0	0	0	1	0	3	4
W. Kalimantan	0	0	0	0	0	3	3
South Kalimantan	0	0	0	0	0	4	4
C. Kalimantan	0	0	0	0	0	3	4
E. Kalimantan	0	0	0	0	0	5	5
N. Sulawesi	0	0	0	0	0	1	2
South Sulawesi	0	0	0	0	0	1	1
W. Sulawesi	0	0	0	0	0	3	3
East Nusa Tenggara	0	0	0	0	0	10	10
Improved variety	0	0	0	1	0	3	4
Total	0	0	0	7	0	83	92

[a]0: immune; 1: highly resistant; 3: resistant; 5: moderately resistant; 7: susceptible; and 9: highly susceptible

The result of resistance screening can be affected by the genotype studied, phenotyping test method, and BPH virulence. We used a variation of the SSST method, i.e., 4-6 nymphs of a highly virulent BPH population were infested on 3-leaf seedlings (or 14 days after sowing of pregerminated seed) to avoid early seedling death. On the other hand, the two previous studies, which screened 9 to 17 brown rice varieties of West Sumatera origin, used a different method where one BPH insect (the stage was unknown) of biotype 3 was infested on 30 d.o. plants individually planted in pots (Busniah, Kasim & Winarto 2020; Dwipa et al. 2018). Under lenient screening conditions compared to ours, they obtained a higher number of resistant genotypes (41-60%).

Classically, plant resistance mechanisms are categorized into: 1) antibiosis, a trait that adversely affects the biology of an insect following the ingestion of host tissue; 2) antixenosis (non-preference), a trait that limits a plant from serving as a host to an insect; and 3) tolerance, properties that enable plant to yield more biomass than a susceptible plant despite high infestation by insect (Ling & Weilin 2016; Prahalada et al. 2017; Smith 1999). Plants with an antibiosis resistance mechanism would impose heavy selection pressure on insects and favour the rapid evolution of BPH virulence (Prahalada et al. 2017). On the contrary, antixenosis confers more durable resistance to BPH attack (Yang et al. 2023). Both antibiosis and antixenosis resistance mechanisms can reduce crop damage from insect infestation. Still, the impact on yield depends on the plant's tolerance, i.e., the ability to compensate for resource losses due to insect feeding (Horgan et al. 2021).

The SSST is a rapid method to assess the BPH resistance of hundreds of lines in one experiment and has been used in many rice breeding programs (Horgan et al. 2021; Ling & Weilin 2016). However, since this method is qualitative and detects only antixenosis resistance mechanisms, many entries rated susceptible just because they are not attractive to the insect for settling are eliminated (Horgan et al. 2021). Antibiosis and tolerance resistance mechanisms can be detected in older plants (ca. 30 days). At this stage, the fitness of insects (growth, survival, and reproduction) and plant response (biomass and yield) are measured (Heinrichs, Medrano & Rapusas 1985; Horgan et al. 2021). Further tests on the seven moderately resistant genotypes will be needed to determine if they also have these two resistance mechanisms. The susceptible entries can be retested using a modified SSST (MSSST) that uses fewer nymphs on older plants (20 d.o.) (Horgan et al. 2021). With fewer nymphs and older seedlings, plant damage occurs later, caused by the offspring of the infested nymphs. In this way, genotypes that express resistance at a later stage of the plant or 'field resistance', can be identified (Horgan et al. 2021). When planted in the field, genotypes with all three resistance mechanisms are expected not to impose strong selection pressure against BPH and thus do not lead to rapid evolution of BPH virulence.

GENOTYPIC VARIATION

With the increasing number of closely linked and tightly bound markers to the identified *Bph* genes, the development of rice resistance to BPH can be accelerated via a marker-assisted selection process. We tested 29 markers flanking *Bph* gene loci and tightly linked markers to the gene to characterize the genetic performance of seven moderately resistant and three randomly selected susceptible entries. Fourteen markers linked to 12 BPH resistance genes (*Bph18* [=*Bph1*], *Bph15* [=*Bph3*], *Bph6*, *Bph20(t)*, *Bph23(t)*, *Bph26* (=*bph2*), *Bph31*, *Bph33(t)*, *Bph34*, *Bph35*, *Bph36*, and *Bph37*) distributed on five chromosomes showed polymorphism (Table 4). They were used to analyse nine moderately resistant genotypes and three randomly selected susceptible genotypes. A total of 50 alleles was produced among these genotypes, with the average number of alleles per locus being 3, ranging from 2 to 5 (Table 4). The PIC values of the markers ranged from 0.09 (RM16994) to 0.73 (PA26), with an average of 0.42. Five markers (PA26, RM6997, RM589, RM3471, and MS10) with PIC values of >0.50 were informative for describing the genetic diversity of the studied genotypes.

The number of alleles detected in each genotype ranged from 4 to 13 (Table 5). The range of allele number is concordant to the resistance level, i.e., moderately resistant genotypes had 11 to 13 alleles, while the susceptible ones had 4 to 7 alleles. Cluster analysis using 14 polymorphic markers divided the 10 genotypes into two major clusters, coinciding with the resistance phenotypes where the susceptible genotypes are separated from the moderately resistant genotypes (Figure 1). The cluster of moderately resistant genotypes was further divided into two subclusters. A similar finding was also reported by Harini et al. (2013). In their study, 30 SSR markers closely linked to *Bph* genes were able to distinguish the resistance among 28 elite rice varieties. However, with a small number of genotypes to analyse, it is possible to obtain groupings of genotypic variation that correspond to resistance phenotypes. Jena et al. (2015) and Muduli et al. (2023), who analysed 58 rice genotypes with 22 *Bph* gene-linked markers and 24 rice genotypes with 12 *Bph* gene-linked markers, respectively, obtained

partial clustering of genotypes based on phenotype. One cluster contained only genotypes with resistant phenotypes, while the other clusters consisted of a mixture of resistant, moderately resistant, and susceptible genotypes (Jena et al. 2015; Muduli et al. 2023). The reliability of our marker set in predicting resistance phenotypes needs to be tested on more rice genotypes with varying levels of resistance before being used to differentiate the resistance of rice germplasm in the future.

TABLE 4. Polymorphic SSR and InDel markers used in the genetic analysis of pigmented rice resistant and susceptible to a field population of brown planthopper (*Nilaparvata lugens* Stål)

Marker	Resistance gene	Number of alleles	Range of allele size (bp)	PIC
PA26[a]	*Bph31*	5	0-210	0.73
RM6997	*Bph6*	5	0-175	0.70
RM589	*Bph15* (=*Bph3*)	4	0-160	0.61
RM3471	*Bph35*	4	0-125	0.60
MS10	*Bph20(t)*	3	0-140	0.55
RM3331	*Bph18* (=*Bph1*)	3	0-155	0.49
RM5479	*Bph26* (=*bph2*)	4	0-150	0.48
RM588	*Bph15* (=*Bph3*)	3	0-95	0.47
RM488	*Bph33(t)*	3	0-100	0.47
YM35[a]	*Bph37*	3	150-170	0.35
RM3572	*Bph23(t)*	2	170-177	0.33
X48[a]	*Bph36*	2	0-240	0.33
PSM20[a]	*Bph35*	2	180-210	0.16
RM16994	*Bph34*	2	160-170	0.09
	Mean	3		0.45

[a]InDel marker

TABLE 5. Genetic diversity of pigmented rice with varying levels of resistance to a field population of brown planthopper (*Nilaparvata lugens* Stål) across 14 polymorphic SSR and InDel markers linked to *Bph* genes

Genotype	Origin	Bran layer colour	Resistance phenotype	Number of alleles
Merah 30475	Central Java	Red	MR	11
Slegreng Merah 30477	North Sumatera	Red	MR	13
Pamera	Improved variety	Red	MR	11
Merah 30756	East Java	Red	MR	12
Badigal	West Java	Dark purple/black	MR	11
Padi Hitam Karang Anyar	C. Java	Dark purple/black	MR	12
Slegreng Merah 30477	Aceh	Red	MR	13
Si Topas	W. Sumatera	Red	HS	5
Karamanting	C. Kalimantan	Red	HS	4
Iden	N. Sulawesi	Red	HS	7
			Mean	13

[a]HS: highly susceptible (damage score 9); MR: moderately resistant (damage score 5) based on bulk seedling test using the standard seed box screening test method

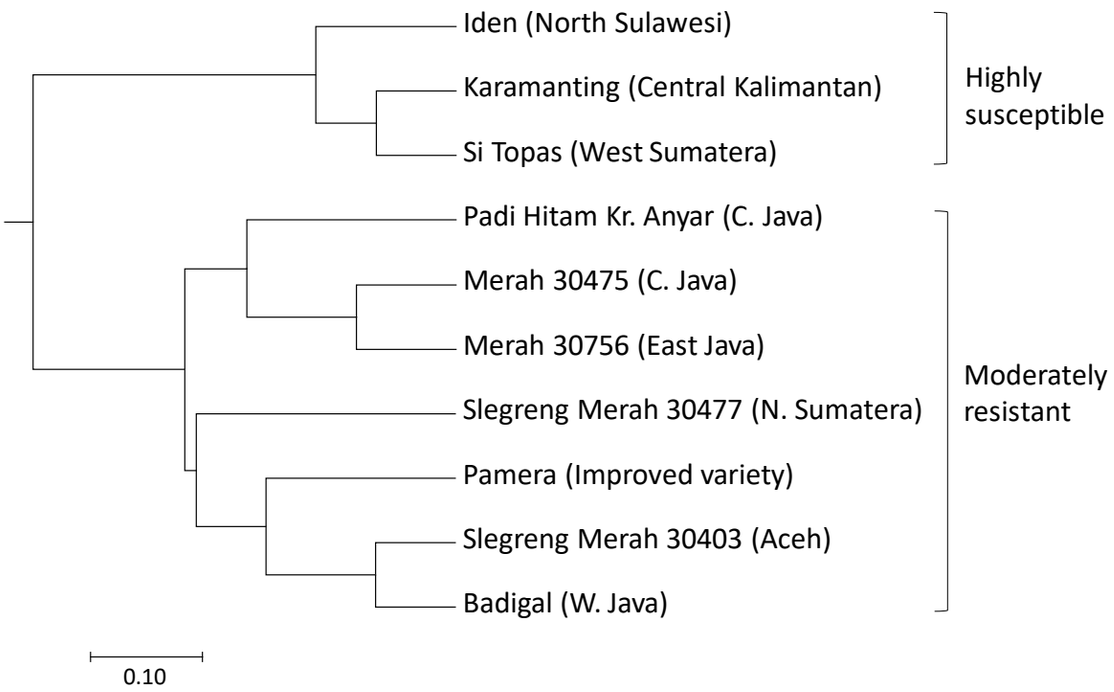

FIGURE 1. Dendrogram of pigmented rice with differential resistance to a field population of brown planthopper (*Nilaparvata lugens* Stål) based on 14 polymorphic SSR and InDel markers linked to *Bph* genes

DISTRIBUTION OF *Bph* GENES

Further analysis of the 10 genotypes with nine functional markers developed by Yang et al. (2023) indicated that six primers (B18D, B26D, B3D, B6D, B32D, and B1279D) amplified the expected allele sizes (Figure 2). These markers were specific for the gene *Bph18* (=*Bph1*), and *Bph26* (=*bph2*), *Bph15* (=*Bph3*), *Bph6*, *Bph32*, and *Bph1/9*, respectively. The remaining primers amplified unexpected fragment size or multiple fragments; thus, the data was excluded.

The number of *Bph* genes present in each genotype ranged from one (Iden and Padi Hitam Karang Anyar) to four (Merah 30475, Pamera, and PTB33) (Table 6). The locus *Bph1*/*9* was predominant, followed by *Bph18* and *Bph26*. Yang et al. (2023) also reported that these genes were the most frequently detected in a collection of 560 traditional rice accessions with resistance to at least one BPH biotype. The *Bph1/9* gene was amplified by marker B1279D which also amplified fragments from accession carrying *Bph18* (=*Bph1*) and *Bph26* (=*bph2*), *Bph7*, or *Bph9* (Yang et al. 2023). Accordingly, our study showed that genotypes carrying *Bph1/9* gene had either *Bph18* (=*Bph1*) or *Bph26* (=*bph2*) gene (Table 6).

However, the results of genotypic analysis on differential varieties indicated that some functional markers were not as specific as they should have been. Besides being detected to contain the *Bph18* (=*Bph1*) gene, Mudgo was also detected to contain the *Bph32* gene. Meanwhile, Ratu Heenathi was not detected to contain the *Bph15* (=*Bph3*) gene as it should be. Research by Yang et al. (2023) also showed similar findings, e.g., Mudgo was detected to contain the *Bph26* (=*bph2*) gene instead of the *Bph18* (=*Bph1*) gene.

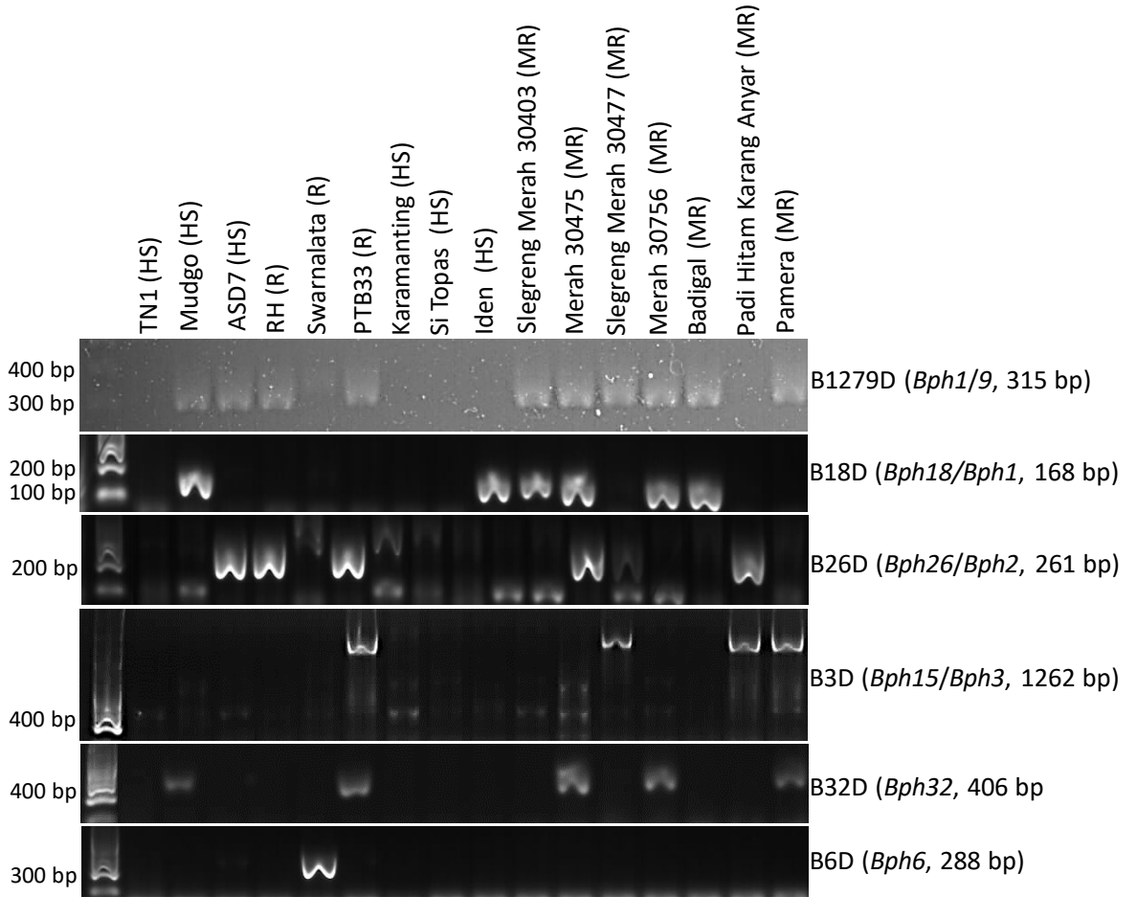

FIGURE 2. PCR amplification of the resistance gene *Bph18* (=*Bph1*), and *Bph26 (=bph2*), *Bph15* (=*Bph3*), *Bph32*, *Bph1/9* detected by the functional marker B18D, B26D, B3D, B32D, and B1279D, respectively, on pigmented rice with varying level of resistance to a field population of brown planthopper (*Nilaparvata lugens* Stål). R: resistant (damage score 3), MR: moderately resistant (damage score 5), and HS: highly susceptible (damage score 9)

Two susceptible accessions (Karamanting and Si Topas) were not detected to contain the *Bph* gene, while the other susceptible accessions had the *Bph18* (=*Bph1*) gene. This result is consistent with the fact that this gene is no longer effective against the BPH population in Indonesia (Chaerani et al. 2016). Yang et al. (2023) reported that genotypes carried any of *Bph18* (=*Bph1*), *Bph26* (=*bph2*), or *Bph9* gene were also susceptible to virulent BPH population of Bangladesh type in China. Combining the *Bph18* (=*Bph1*) gene with at least one other *Bph* gene increases the resistance to BPH. For example, the moderately resistant Slegreng Merah 30403 and Badigal contained the *Bph1/9* gene besides *Bph18* (=*Bph1*) (Table 6).

The observed resistance phenotype was evaluated through the SSST method, which does not differentiate between antixenosis and antibiosis mechanisms (Horgan et al. 2021). Therefore, the number and combination of *Bph* genes do not seem to affect the resistance level in moderately resistant genotypes detected under the SSST method. For example, all moderately resistant genotypes except for Padi Hitam Karang Anyar had 2 to 4 *Bph* genes. This black rice variety contained only *Bph15* (=*Bph3*), effective against several BPH populations in Indonesia (Chaerani et al. 2016). The alleles derived from 12 polymorphic SSR and InDel markers in this genotype may contribute to the resistance level, or other *Bph* gene(s) could not be detected with the functional markers tested. To differentiate the resistance phenotypes, all moderately resistant genotypes must be further tested using methods that detect antibiosis and tolerance mechanisms.

TABLE 6. Distribution of *Bph* genes in resistant and susceptible rice to a field population of brown planthopper as detected by functional markers

Genotype and *Bph* gene	Origin/type	Bran layer colour	Resistance phenotype[a]	*Bph* gene and marker name						Number of genes
				Bph18 (=*Bph1*) (B18D)	*Bph26* (=*bph2*) (B26D)	*Bph15* (=*Bph3*) (B3D)	*Bph32* (B32D)	*Bph6* (B6D)	*Bph1/9* (B1279D)	
Karamanting	Central Kalimantan	Red	HS	0	0	0	0	0	0	0
Si Topas	West Sumatera	Red	HS	0	0	0	0	0	0	0
Iden	North Sulawesi	Red	HS	1	0	0	0	0	0	1
Slegreng Merah 30403	Aceh	Red	MR	1	0	0	0	0	1	2
Merah 30475	C. Java	Red	MR	1	0	0	1	0	1	3
Slegreng Merah 30477	N. Sumatera	Red	MR	0	1	1	0	0	1	3
Merah 30756	East Java	Red	MR	1	1	0	1	0	1	4
Badigal	W. Java	Dark purple/ black	MR	1	0	0	0	0	1	2
Padi Hitam Karang Anyar	C. Java	Dark purple/ black	MR	0	0	1	0	0	0	1
Pamera	Improved variety	Red	MR	0	1	1	1	0	1	4
TN1	Differential variety	White	HS	0	0	0	0	0	0	0
Mudgo (*Bph18*[=*Bph1*])	Differential variety	White	HS	1	0	0	1	0	1	3
ASD7 (*Bph26*[=*bph2*])	Differential variety	White	HS	0	1	0	0	0	1	2
Ratu Heenathi (*Bph15*[*Bph3*]+*Bph17*)	Differential variety	White	R	0	1	0	0	0	1	2
PTB33 (*Bph26*[*bph2*]+*Bph15*[*Bph3*]+*Bph17-ptb*+*Bph32*)	Differential variety	White	R	0	1	1	1	0	1	4
Swarnalata (*Bph6*)	Differential variety	White	R	0	0	0	0	1	0	1
			Total	6	6	4	5	1	10	

[a]R: resistant (damage score 3); MR: moderately resistant (damage score 3); HS: highly susceptible (damage score 9)

Pyramiding significant resistance genes is one method to increase the level and broaden the spectrum of rice resistance to make the resistance more durable (Ramkumar et al. 2016). If the target genes are already present in a single source, transferring them to elite varieties is easier than if the target genes are scattered in different donors (Ramkumar et al. 2016). All the moderately resistant genotypes can be a good source of BPH resistance in the gene pyramiding program because they contain 2 to 4 *Bph* genes. The improved variety Pamera already had four *Bph* genes and broad-spectrum resistance to BPH, so it does not need further improvement unless a new virulent variant of BPH that can defeat this gene combination emerges.

Conclusion

Screening of 88 local accessions and four improved varieties of pigmented rice against a field population of BPH using the SSST method obtained six accessions (Badigal, Merah 30475, Merah 30756, Padi Hitam Karang Anyar, Slegreng Merah 30403, and Slegreng Merah 30477) and one improved variety (Pamera) with moderately resistant response. Genetic analysis of moderately resistant and three randomly selected susceptible entries using 12 SSR and InDel markers linked to the *Bph* gene showed that the resistant entries had more alleles (11 to 13) than the susceptible entries (4 to 7). Cluster analysis clearly separated the moderately resistant genotypes from the susceptible ones. Using six published markers specific for *Bph* genes located on chromosome 12 showed that the moderately resistant genotypes had more diverse numbers and combinations of *Bph* genes. In contrast, the susceptible genotypes contained no *Bph* genes or had only one *Bph* gene.

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