Molecular screening of blast resistance genes in selected Southeast Asia rice varieties

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The blast disease due to *Magnoparthae oryzae* results in yield losses in rice production. Cultivation of blast resistant rice varieties has been proven to be the best approach to address this problem. This study identified the rice varieties that harbor *Pi5*, *Piz-t*, *Pik*, and *Pib* genes. A total of 93 rice varieties that derived from Malaysia, Indonesia, Laos, and Philippines were screened using PCR-based DNA marker targeting *Pi-d2*, *Pi5*, *Piz-t*, *Pik*, and *Pib* genes. Out of the 93 rice varieties, 75 were found positive for *Pi-d2* gene, 21 for *Pi5* gene, 33 for *Piz-t* gene, 81 for *Pik* gene, and 85 for *Pib* gene. The varieties that originated from Indonesia harbored the highest number of *Pi-d2*, *Pi5*, *Pik*, and *Pib* genes, when compared to those derived from other countries. The varieties from Malaysia seemed to harbor the highest number of *Piz-t* gene. A total of eight varieties appeared to be positive for all four genes. These eight varieties displayed the potential to be implemented in breeding program as blast resistant genes donor for durable resistance against blast disease. This present study offers useful information to enhance blast resistance in future rice breeding programs. The benefits of using gene specific DNA marker in characterizing germplasm revealed that the gene-tracking process in programs that involve resistance breeding can be tailored using molecular tools.

Keywords: Blast resistant rice variety; *Magnoparthae oryzae*; PCR-based marker.

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Introduction

Rice, scientifically known as *Oryza sativa* L., is an important crop across the globe as it is a source of food for most of the global population. The escalating demand for rice is on par with the increase in population, particularly across Latin American, Asian, and African regions. In 2010, the population in Malaysia was 28.6 million, and this figure has been projected to increase by 10 million (35.0%) to 38.6 million in 2040. Malaysia's rice industry production increased from 2.19 to 2.74 metric tons from 2006 to 2015 with slight increase of paddy field area from 676,034 hectares in 2006 to 681,559 hectares in 2015. A steady increment of the average annual yield from 3.24 tons/hectare to 4.02 tons/hectare was recorded for 2006 to 2015 [1]. Despite the reported increment of average annual yield and rice production, the rice production in Malaysia demands a significant increment in order to feed the exponential human population growth in Malaysia.

Rice yield has been continuously threatened by diseases, blights, and pests. A major disease that

has been affecting rice yield is the rice blast disease due to *Magnaporthe oryzae*, a non-obligate filamentous ascomycete. The common way to overcome this blast disease is by using fungicide. Nonetheless, excessive use of fungicide is harmful to the environmental and evolves the disease strain. Thus, cultivation of the resistant rice variety appears to be the best approach to address this issue. The conventional pathogenicity test that applies differential races to detect resistance genes in rice germplasm lacks in efficiency, demands close environmental control, and consumes much time. The emergence of tightly linked DNA markers has addressed such issues, due to its advantage in identifying and incorporating resistive gene for breeding program [2].

In light of resistance genes to *M. oryzae*, about 350 QTLs and above 86 dominant R genes have been determined as resistant to rice blast, with 23 characterized molecularly. These R genes, nevertheless, address only certain pathogenic race. This calls for identification of new alleles or R genes for pyramiding multiple resistant genes for durable resistant varieties [3]. The R genes for *M. oryzae* have been determined in cultivars, landraces, and wild rice via differential physiological races of *M. oryzae* [4]. As for this study, 93 rice varieties from four Southeast Asia countries, namely Malaysia, Indonesia, Philippines, and Laos, were characterized using molecular marker controlling blast resistant gene. Characterizations of genetic resources have widely adopted the molecular genetic markers as there are unexplored distinct alleles that demand screening to identify their potential use. Little has been reported on the genetic basis of blast resistance amidst the identified alleles. This present study is aimed at expanding the body of knowledge in this subject matter by determining the resistance genes to *M. oryzae* from selected Southeast Asia rice varieties using molecular markers.

Materials and Methods

Plant materials and DNA isolation

Ninety-three rice varieties composed of modern and conventional rice varieties originated from Malaysia, Indonesia, Philippines, and Laos, were selected for screening of blast resistant genes. Seed of the respective varieties was germinated for 20 days prior to DNA extraction. Fresh leaves were collected, cut into pieces, and placed into 96-well DNA extraction plate containing stainless steel beads (2.3 mm diameter) and immediately stored at -80^{o}C for 24 hours. Each variety has three biological replicates of sample. The frozen tissue was ground using Tissue Lyser (Qiagen, Hilden, Germany) immediately after adding Extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH8), 0.05% β-mercaptoethanol, and 20 mM EDTA). The total genomic DNA was extracted using high-throughput DNA extraction protocol [5]. Integrity of the extracted DNA was determined on 0.8% agarose gels, whereas DNA concentration was measured using Fluoraskan Ascent (Thermo Fisher Scientific, Carlsbad, California, USA).

Markers specific for rice blast genes

Screening of germplasm accession was performed to determine the existence of four major blast resistances in genes, *Pi5* (Forward: TCCTCCTCTTCGGACACCTC, Reverse: CGGACGAG CGATAGTGATCC) [6], *Piz-t* (Forward: TTGCTGAGC CATTGTTAAACA, Reverse: ATCTCTTCATATATATG AAGGCCAC), *Pib* (Forward: GCCACATCAATGGCTA CAACGTT, Reverse: CCAGAATTTACAGGCTCTGG), and *Pik* (Forward: GACTCGGTCGACCAATTCGCC, Reverse: ATCAGGCCAGGCCAGATTTG) [2]. The PCR-based markers had been selected to determine blast resistance genes amidst the chosen rice varieties. All markers were synthesized from Integrated DNA technologies (IDT) (Science Park Rd, Singapore).

DNA marker analysis

Allele-specific PCR-marker assays the genotype by determining the existence of PCR amplification product. Reaction mixture of PCR had been prepared to 10 µL final volume, which consisted of 1x buffer (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA), 10 µM each

of forward and reverse primers, 2 µM of dNTP (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA), 0.1 µL of bovine serum albumin (BSA) as PCR enhancer, and 1 U of Taq polymerase (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA). Amplification had been performed via GeneAmp® PCR System 9700 (Applied Biosystems, United States) with initial denaturation for 2 min at 94°C, followed by 35 cycles for 30 s at 94°C, 45 s for annealing (47.5°C for *Pi5* and *Piz-t*, 58.3°C for *Pik*, and 63.0°C for *Pib*), and 45 s at 72°C, as well as 7 min at 72°C as the final extension. After the amplification, the PCR product was resolved on 2.0% agarose gel. The DNA marker analysis was performed twice in order to evaluate the consistency of the result before the PCR bands were scored. These fragments that were amplified had been scored as (0) for absence and (1) for presence of amplicon associated with each gene DNA fragment.

Results and discussion

One destructive disease that destroys rice yield refers to rice blast disease. In order to fight this disease, resistance gene deployment has been proven to be an environment-friendly and effective method. Nevertheless, the genome evolution of *M. oryzae*, including the presence of diverse strains derived from other regions, is indeed a challenge amongst rice breeders. The highly varied genetic in *M. oryzae* permits fungus to spread and infect the prior resistant genotypes. Hence, identifying alternative resistant donors or accessions is vital to ensure successful breeding programs to meet agricultural demands.

The analysis of *Pi5* gene showed that this gene is the least gene harboring by several studied varieties with only 21 varieties out of 93 harboring this gene. Only three varieties originated from Malaysia displayed the presence of this gene. Previously, *Pi5* gene was mapped on chromosome 9 encode proteins that carry a leucine-rich repeat (LRR) motif, an N terminal coiled-coil (CC) motif, and a nucleotide-binding (NB) domain (the three features of R genes), which were linked with broad-spectrum resistance to a range of blast isolates [7]. Screening of *Piz-t* revealed 33 varieties with positive result for this gene with most varieties originated from Malaysia, wherein a total of 20 out of 24 varieties exhibited the presence of this gene. Similar to *Pi5*, *Piz-t* was position at rice chromosome 6 that conferred broad-spectrum resistance to *M. oryzae*, which is also part of the gene cluster of nucleotide binding site-leucine rich repeat (NBS–LRR). The *Piz-t* gene was assumed to derive from Indica cultivars [8]. *Pik* screening analysis revealed 81 varieties harboring this gene, respectively. The *Pik* gene found on rice chromosome 11 was encoded for the resistance protein of NBS-LRR [9]. The direct interaction between Avr-Pik effector protein (generated by pathogen) and *Pik* resistance protein led to a response of defense by the host. *Pik* and Avr-Pik genes displayed a range of alleles [10]. The analysis of *Pib* gene exhibited that most of the varieties (85 out of 93 varieties) harbored the *Pib* gene. The *Pib* gene was previously mapped on chromosome 2 and part of NBS-LRR class of plant disease resistance genes. *Pib* was reported to confer high resistance to most Japanese blast races [11]. The segregation of the genes based on country is summarized and visualized in Figure 1 and Table 1, respectively. The analysis revealed a total of five varieties as positive across all the five genes. These five varieties displayed potential to be employed in breeding program as blast resistant genes donor for durable resistant against blast disease. Eight varieties showed the presence of resistant alleles for all four resistant genes meanwhile three varieties namely Minanok, MR103, and MR106, did not harbor any resistant allele for all four genes. The segregation of the genes based on individual varieties were summarized in Table 2.

Adaptation of pathogens, as well as susceptibility to other stresses, has become continuous threats to the present rice varieties. Domestication and artificial selection at breeding program has narrowed crops diversity and variability [2]. Low genetic variation tends to limit the options of

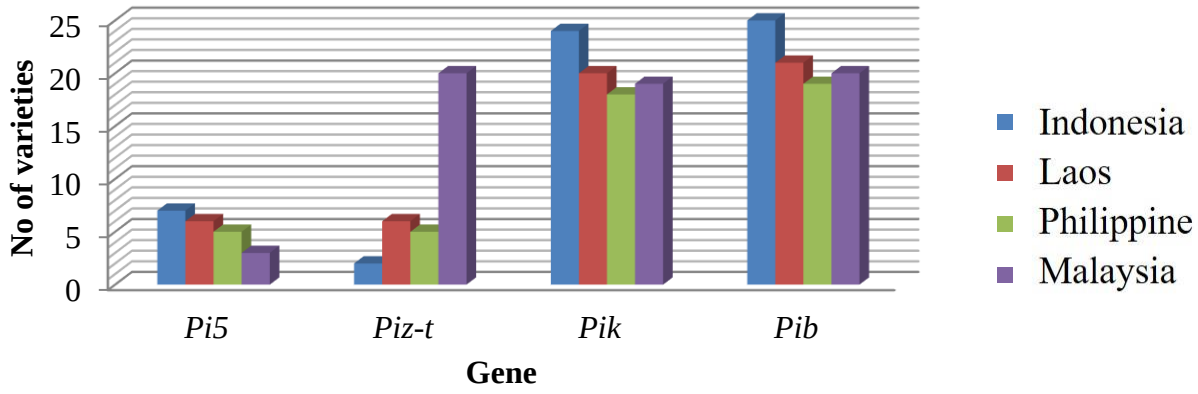

Figure 1. Segregation of the gene based on the origin of the varieties.

Table 1. Segregation of gene based on origin of the varieties.

Gene	Indonesia	Laos	Philippine	Malaysia	Total
Pi5	7	6	5	3	21
Piz-t	2	6	5	20	33
Pik	24	20	18	19	81
Pib	25	21	19	20	85

developing new varieties against unpredictable and destructive biotic, as well as abiotic stresses, encountered within the agricultural domain. The molecular marker, nonetheless, has great potential to screen genetic resources for blast resistant gene. The varieties of identified regulative genes may be applied for future breeding program as a donor variety for blast resistant. This study introduces five varieties that can potentially harbor five resistant genes to serve as donor for stacking gene breeding. Implementation of multiple resistant genes with overlapping pattern of resistance to enhance resistance against rice blast has been proven as an effective method [12]. Brunner *et al*. [13] asserted that the combination of multiple alleles of resistant gene can reduce the pressure of selecting certain pathogen(s).

Conclusion

The benefit of using gene-specific DNA marker for germplasm characterization showcases that gene tracking in resistance breeding programs should be performed precisely by using molecular tools. The sequence analysis of segments, which was positive for marker, verifies the variation in alleles, as well as the importance of the evolutionary aspect in the studied gene pool. This study contributes by identifying potential blast resistant gene in rice. Information gathered after characterizing the accessions of GenBank via modern molecular tools should be incorporated into the databases. Apposite cataloguing of information related to phenotypic and genome enables flora biologists and breeders to apply the present genotypic diversity found in GenBank in assessing the biological importance of phenotypic and molecular diversifications.

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Table 2. Segregation of the four studied genes based on individual varieties.

Variety ID	Sample name	Origin	Pi5	Piz-t	Pik	Pib	Total
IT01	Melati	Indonesia	*0*	*0*	*1*	*1*	*2*
IT02	Gondil	Indonesia	*1*	*1*	*1*	*1*	*4*
IT03	Sebedol	Indonesia	*1*	*0*	*1*	*1*	*3*
IT04	Deli	Indonesia	*0*	*0*	*1*	*1*	*2*
IT05	Iden	Indonesia	*0*	*0*	*1*	*1*	*2*
IT06	Padi Pulut Ayang	Indonesia	*0*	*0*	*1*	*1*	*2*
IT07	Wajo Kuning	Indonesia	*0*	*0*	*0*	*1*	*1*
IT08	Jerneng Kuning	Indonesia	*1*	*1*	*1*	*1*	*4*
IT09	Widas	Indonesia	*0*	*0*	*1*	*1*	*2*
IT10	Mama laka	Indonesia	*0*	*0*	*1*	*1*	*2*
IT11	Kemala Water	Indonesia	*0*	*0*	*1*	*1*	*2*
IT12	Ramos	Indonesia	*0*	*0*	*1*	*1*	*2*
IT13	Lokal Buntu Sangala	Indonesia	*0*	*0*	*1*	*1*	*2*
IT14	Batanghari	Indonesia	*0*	*0*	*1*	*1*	*2*
IT15	Empat	Indonesia	*1*	*0*	*1*	*1*	*3*
IT16	Si Gumpal Kandang	Indonesia	*0*	*0*	*1*	*1*	*2*
IT17	Balacung	Indonesia	*0*	*0*	*1*	*1*	*2*
IT18	Gading	Indonesia	*0*	*0*	*1*	*1*	*2*
IT19	Katimbung	Indonesia	*1*	*0*	*1*	*1*	*3*
IT20	Ketan Maronto	Indonesia	*0*	*0*	*1*	*1*	*2*
IM01	IR82480	Indonesia	*1*	*0*	*1*	*1*	*3*
IM02	IR85627	Indonesia	*0*	*0*	*1*	*1*	*2*
IM03	Inpari 40	Indonesia	*0*	*0*	*1*	*1*	*2*
IM04	Inpari Blas	Indonesia	*0*	*0*	*1*	*1*	*2*
IM05	Inpari HDB	Indonesia	*1*	*0*	*1*	*1*	*3*
LT01	Ea Loup 284	Laos	*1*	*1*	*1*	*1*	*4*
LT02	Ea Loup 313	Laos	*0*	*1*	*1*	*1*	*3*
LT03	Peud Nam	Laos	*1*	*0*	*1*	*1*	*3*
LT04	Hom Thong 2125	Laos	*0*	*1*	*1*	*1*	*3*
LT05	Ea non 2213	Laos	*0*	*0*	*1*	*1*	*2*
LT06	Phae deng	Laos	*0*	*0*	*1*	*1*	*2*
LT07	Ea non 5643	Laos	*0*	*0*	*1*	*1*	*2*
LT08	Hom Thong 5718	Laos	*1*	*1*	*1*	*1*	*4*
LT09	Chao Deng	Laos	*1*	*0*	*1*	*1*	*3*
LT10	Chao loy	Laos	*0*	*0*	*1*	*1*	*2*
LT11	Phon ngan	Laos	*0*	*0*	*1*	*1*	*2*
LT12	Kay noi lai	Laos	*0*	*0*	*1*	*0*	*1*
LT13	Chao mali	Laos	*0*	*0*	*0*	*1*	*1*
LT14	Beua nam	Laos	*0*	*0*	*0*	*1*	*1*
LT15	Nam yen	Laos	*0*	*1*	*1*	*1*	*3*
LT16	Hom mali gnay	Laos	*0*	*0*	*1*	*1*	*2*
LT17	Mak Yom	Laos	*0*	*0*	*1*	*1*	*2*
LM01	TDK1	Laos	*0*	*0*	*1*	*1*	*2*
LM02	TDK8	Laos	*0*	*0*	*1*	*1*	*3*
LM03	TDK11	Laos	*1*	*0*	*1*	*1*	*3*
LM04	VTE450-2	Laos	*1*	*0*	*1*	*1*	*3*
LM05	XBF-2	Laos	*0*	*1*	*1*	*1*	*3*
PT01	Alaminos	Philippines	*0*	*1*	*1*	*1*	*3*
PT02	Azucena	Philippines	*0*	*0*	*1*	*0*	*1*
PT03	Bacao	Philippines	*0*	*0*	*1*	*0*	*1*

PT04	Balibud	Philippines	*1*	*1*	*1*	*1*	*4*
PT05	Campena	Philippines	*1*	*0*	*1*	*1*	*3*
PT06	Ca-ong (white)	Philippines	*1*	*0*	*1*	*1*	*3*
PT07	Dicula	Philippines	*0*	*1*	*1*	*1*	*3*
PT08	Dingras	Philippines	*0*	*0*	*1*	*1*	*2*
PT09	Inaporaonon	Philippines	*0*	*0*	*0*	*1*	*1*
PT10	Kalagnon	Philippines	*0*	*0*	*1*	*1*	*2*
PT11	Katsiyam Tabao	Philippines	*0*	*0*	*1*	*1*	*2*
PT12	Macan Sapac	Philippines	*0*	*0*	*0*	*1*	*1*
PT13	Minanok	Philippines	*0*	*0*	*0*	*0*	*0*
PT14	Nala	Philippines	*0*	*0*	*0*	*1*	*1*
PT15	Purtok	Philippines	*0*	*1*	*1*	*1*	*3*
PT16	Wagwag	Philippines	*1*	*0*	*1*	*1*	*3*
PT17	Wagwag Raois	Philippines	*1*	*0*	*1*	*1*	*3*
PM01	NSIC Rc120 (Matatag 6)	Philippines	*0*	*0*	*1*	*1*	*2*
PM02	NSIC Rc128 (Mabango 1)	Philippines	*0*	*0*	*1*	*1*	*2*
PM03	NSIC Rc146 (PJ7)	Philippines	*0*	*0*	*1*	*1*	*2*
PM04	NSIC Rc160 (Tubigan 14)	Philippines	*0*	*1*	*1*	*1*	*3*
PM05	NSIC Rc184 (Salinas 2)	Philippines	*0*	*0*	*1*	*1*	*2*
MT1	Malinja	Malaysia	*0*	*1*	*1*	*1*	*3*
MT2	Mahsuri	Malaysia	*0*	*1*	*1*	*1*	*3*
MT3	Ria	Malaysia	*0*	*1*	*1*	*1*	*3*
MT4	Murni	Malaysia	*0*	*1*	*1*	*1*	*3*
MT5	Jaya	Malaysia	*0*	*1*	*1*	*1*	*3*
MT6	Sri Malaysia 1	Malaysia	*0*	*1*	*1*	*1*	*3*
MT7	Sri Malaysia 2	Malaysia	*0*	*1*	*1*	*1*	*3*
MT8	Pulut Malaysia 1	Malaysia	*0*	*1*	*0*	*0*	*1*
MT10	Sekencang	Malaysia	*0*	*0*	*0*	*1*	*1*
MT11	Sekembang	Malaysia	*1*	*1*	*1*	*1*	*4*
MT12	Kadaria	Malaysia	*1*	*1*	*1*	*1*	*4*
MT13	Pulut Siding	Malaysia	*0*	*1*	*1*	*1*	*3*
MT14	Manik	Malaysia	*0*	*1*	*1*	*1*	*3*
MT15	Muda	Malaysia	*0*	*1*	*0*	*0*	*1*
MT16	Seberang	Malaysia	*0*	*1*	*1*	*1*	*3*
MT17	Makmur	Malaysia	*0*	*1*	*1*	*1*	*3*
MT18	MR 81	Malaysia	*1*	*1*	*1*	*1*	*4*
MT19	MR 103	Malaysia	*0*	*0*	*0*	*0*	*0*
MT20	MR 106	Malaysia	*0*	*0*	*0*	*0*	*0*
MM1	MR84	Malaysia	*0*	*1*	*1*	*1*	*3*
MM2	MR219	Malaysia	*0*	*1*	*1*	*1*	*3*
MM3	MR253	Malaysia	*0*	*1*	*1*	*1*	*3*
MM4	MR263	Malaysia	*0*	*0*	*1*	*1*	*2*
MM5	MR269	Malaysia	*0*	*1*	*1*	*1*	*3*

0: Absent of resistant allele
1: Present of resistant allele

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