

Original Research Paper

Screening of Blast Resistance Genes from South Tapanuli Rice Cultivars, North Sumatra, Indonesia

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Abstract: Rice blast disease caused by a phytopathogenic fungus, *Magnaporthe oryzae*, has been considered as a major threat to global rice productivity, including Indonesia. South Tapanuli is a regency in North Sumatra, known for its intensive and significant rice production activities. A collection of 13 rice cultivars originating from the region, is claimed for its resistances against the rice blast disease based on the practices by the local farmers. Molecular screening of blast resistance (R) genes was initiated to the 13 accessions using the Polymerase Chain Reaction (PCR) targeting five blast resistance (R) genes, *Pup1*, *Pi-37*, *Pi-d2*, *Pi-ta²*, *Pib* and *Pi-kh*. Out of the 13 accessions, the *Pi-d2*, *Pi-ta²* and *Pib* were detected in all cultivars, showing 100% of R gene frequency. The second dominant, *Pup1* and *Pi-37* gene frequencies were 76.9 and 61.5%, respectively while the least dominant, *Pi-kh* gene frequency was 15.4%. The number of R genes detected among cultivars was ranged between 3 to 6 genes. The genetic associations among thirteen germplasms were determined using unweighted pair group method with arithmetic mean (UPGMA) analysis. Cluster analysis revealed that the least blast-resistant cultivars: Putri Kembar, Silatian and Siganteng, were grouped into cluster 1, showing polymorphism for *Pi-d2*, *Pi-ta²* and *Pi-b*. The high-resistant cultivars, Martabe-Sicondong and Sayuti, Sitolas, Pulo Raja, Pulo Pandan, Siporang and Pulo Manggis, were grouped into cluster 2 and 3, respectively. The medium blast-resistant cultivars, IR 64 and Sitampan were grouped into cluster 4, showing polymorphism for *Pup1*, *Pi-d2*, *Pi-ta²* and *Pi-b*. These results indicated that the utilization of blast-resistant cultivars in North Tapanuli was supported by the presence of R genes.

Keywords: Blast Resistance Gene, Sumatra, PCR, Rice

Introduction

Rice (*Oryza sativa* L.) is one of the essential agricultural commodities in the world and becoming a staple food to almost all populations in the tropical region. The increasing demands upon rice agricultural practices are related to the increasing population in Asia, Africa and Latin America (Wang and Li, 2005).

In Indonesia, intensive research and development have been efforted to yield a higher rice productivity annually as well as developing a stable or higher production in a dynamic environment modified by the presence of abiotic and biotic stressors (Panuju *et al.*,

2013). Indonesia's rice production is constantly threatened by several biotic stressors, from important pathogenic diseases to disruptive physical damages by insect pests. Rice blast disease is one of the most defective conditions with a fatality of plant deaths, caused by *Pyricularia grisea* (Cooke) Sayc., (syn.: *P. oryzae* Cavara), or the teleomorph *Magnaporthe oryzae* (Hebert) Barr. (Rossman *et al.*, 1990; Couch and Kohn, 2002; Choi *et al.*, 2013).

The substantial loss of global rice productivity impacted by the diseases, may reach 30% or equivalent to a food supply for 60 million people (Nalley *et al.*, 2016). Moreover, the presence of rice

blast disease directly decreased the rice yields along with increased production costs, which become one of the largest challenge in increasing rice production (Skamnioti and Gurr, 2009).

Control and management of blast diseases in rice fields incorporated the use of chemical pesticides, fungicides and fertilizers which are considered highly cost and detrimental to the environment, involved in the presence of residual toxins and pollution (Sha *et al.*, 2016). Furthermore, resistant populations of the pathogen may also emerged due to extensive use of antibiotics or fungicides (Chen *et al.*, 2019). In the brink of inconsistencies, the utilization of blast-resistant varieties is considered as the most economical, efficient, effective and environmental-friendly method to prevent the rice blast disease incidence (Ghazanfar *et al.*, 2009; Idowu *et al.*, 2013; Mau *et al.*, 2018).

Blast-resistant rice cultivars are genetically structured by numerous R genes and QTLs responsible for blast disease resistances. The genes are priorly identified through molecular screening and marker-assisted selections to produce superior hybrids for field application (Fukuoka *et al.*, 2014; Yan *et al.*, 2017). The functional traits may be studied thoroughly by preliminary screening of R genes and associated genes within the wild species and local cultivars.

Indonesia is rich in local cultivars and varieties, produced independently by local farmers, breeders and researchers (Vasudevan *et al.*, 2014). According to the International Rice Genebank Collection Information System, approximately 10,205 rice accessions are currently reported in database which are originating from Indonesia (IRGCIS, 2020). The prospect upon finding potential blast-resistant cultivars is progressively conducted due to the richness of local species which has not been fully revealed across provinces and regions, especially in North Sumatra.

Rice farming in North Sumatra province was ranked third in the Sumatra region, with the yield of 2,078,901.59 tons in 2019 (ISA, 2019). In 2016, South Tapanuli was placed 7th among regencies, producing 173,444 tons of rice with a trend of increasing productivity year to year (ISA, 2017). The local farmers utilized the highlands as planting areas for 13 indigenous rice cultivars, including the oldest IR 64 cultivar, which were claimed to be considerably resistant toward blast rice disease. There are still limited informations on the genetic basis of blast resistance among the collection of 13 rice germplasms originating from this region. This study then aimed to analyze the genetic relationship of blast resistance genes (R) in the local rice cultivars.

Materials and Methods

Plant Materials

Thirteen rice germplasms including the local cultivars and standard cultivar, IR 64 originating from various sites in South Tapanuli regency were selected, based on the claims upon their medium to high resistance to blast disease (Table 1). The seeds were collected and germinated in the dark on moistened filter papers for 2 days at 30°C in an incubator. The germinating seeds were then planted in plastic pots at maintained temperature, 27-30°C with daily sunlight exposure for 12 h. The seedlings were grown for 3 weeks prior genomic DNA isolation.

DNA Isolation

Principle of genomic DNA isolation was based on Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method (Doyle and Doyle, 1987). Rice leaves (0.1 g) were collected from plants and cut into smaller fragments. Fragments were crushed manually using pestle in 700 µL of pre-heated (65°C) 2% CTAB extraction buffer, consisted of 20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB and 0.4% β-Mercaptoethanol. Crude samples were inserted into 1.5 mL tube and heated at 65°C for 45 min, added with 500 µL phenol-chloroform-isoamyl alcohol (25:24:1) solution and vortexed. Sample solutions were centrifuged (Eppendorf® Centrifuge 5430 R, Germany) at 12,000 rpm for 10 min at 4°C. Supernatants were collected and mixed with a new 500 µL phenol-chloroform-isoamyl alcohol (25:24:1) solution.

The supernatants were collected and mixed with 700 µL cold isopropanol and incubated in a freezer for 2 h. Solutions were centrifuged at 12,000 rpm for 10 min at 4°C. Pellets were collected and suspended in 500 µL 70% ethanol with further centrifugation at 12,000 rpm for 10 min at 4°C. Pellets were air-dried and suspended into 50 µL ddH₂O and incubated at 37°C for 1 h.

DNA Marker Analysis

The rice germplasms or accessions were genetically screened for the presence of six major blast resistance (R) genes, *Pup1*, *Pi-37*, *Pi-d2*, *Pi-ta²*, *Pib* and *Pi-kh* using PCR markers with details of information listed in Table 2. All of the synthesized primers were obtained from Macrogen, Inc. South Korea. The PCR analyses were conducted in a thermal cycler (SensoQuest GmbH, Germany) with PCR reaction set as follows: 5 µL GoTaq® Green Master Mix solution, 3 µL nuclease-free water, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL DNA template.

Table 1: List of the 13 rice germplasms cultivated in South Tapanuli region, North Sumatra

No.	Accession	Code	Origin (District, Regency)
1.	IR 64	Ir	West Angkola, South Tapanuli
2.	Sicondong	Co	West Angkola, South Tapanuli
3.	Siganteng	Ga	Padangsidimpuan (City)
4.	Silatian	La	Sipirok, South Tapanuli
5.	Martabe	Ma	Batang Angkola, South Tapanuli
6.	Putri Kembar	Pk	Batang Angkola, South Tapanuli
7.	Pulo Manggis	Pm	Marancar, South Tapanuli
8.	Siporang	Po	Sipirok, South Tapanuli
9.	Pulo Pandan	Pp	Sipirok, South Tapanuli
10.	Pulo Raja	Pr	Sipirok, South Tapanuli
11.	Sitampan	Ta	Padangsidimpuan (City)
12.	Sitolas	To	Marancar, South Tapanuli
13.	Sayuti	Sa	Padangsidimpuan (City)

Table 2: Details of PCR primers for the amplification of six blast resistance (R) genes

Gene	Chr	Forward (5'-3')	Reverse (5'-3')	AT (°C)	ES (bp)	Reference
<i>Pup1</i>	12	tcaaaaattcttcaggtatgtactcc	ttgggtgatcagctttcaga	58	1010	Heuer <i>et al.</i> (2009)
<i>Pi-37</i>	1	ttgggtgatcagctttcaga	cgaacagtggctggtatctc	58	1149	Sun (2012)
<i>Pi-d2</i>	6	ttgctatcatagggctcc	atttgaagcgtttgcgtaga	58	1057	Chen <i>et al.</i> (2006)
<i>Pi-ta²</i>	12	agcaggttataagctaggcc	ctaccaacaagttcatcaaa	56	1042	Jia <i>et al.</i> (2002; 2004)
<i>Pi-b</i>	2	gactcggctcgaacattcgcc	atcaggccagccagatttg	54	388	Hayashi <i>et al.</i> (2004)
<i>Pik-h</i>	11	catgagttcatttactattcctc	acattggtagtagtgcaatgtca	52	1500	Sharma <i>et al.</i> (2005)

Chr, Chromosome; AT, Annealing temperature; ES, Estimated size

PCR amplification was performed with following specification: 94°C for 1 min, annealing at different temperature (Table 2) for 45 s, 72°C for 2 min and 72°C for 5 min. All PCR reactions for each sample were repeated three times. PCR products were separated by electrophoresis on 1.2% agarose gels in 1× TAE buffer at 70 V for 60 min. The gels were stained with ethidium bromide solution and visualized under UV transilluminator (Fire-Reader XS-D56, England). The DNA amplicons were recorded as absence (0) or presence (1) accordingly.

Data Analysis

All data sets were arranged using Microsoft Excel. Cluster analysis was performed using the Multivariate Statistical Package (MVSP) ver. 3.1A to produce a dendrogram of relationship among rice cultivars based on the binary matrix or Sørensen–Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) analysis (Kovach, 2007).

Results

Six R primers were used to amplify and detect the presence or absence of blast resistance genes and associated R genes from the thirteen South Tapanuli germplasms. All the 13 accessions possessed at least three to six R genes as revealed by the bands for different markers in agarose gel electrophoresis. The ‘Sicondong’ and ‘Martabe’ cultivars showed DNA bands in all rice blast resistance genes, *Pup1*, *Pi-37*, *Pi-*

d2, *Pi-ta²*, *Pi-b* and *Pi-kh*. Five others were positive for five markers. Three cultivars possessed four markers, while two cultivars showed positive bands for three markers (Fig. 1).

PCR results of the thirteen accessions were determined for the presence of one or more different R genes (Table 3). *Pup1* rice blast resistance gene was visualized by the amplicons of 1010 bp and was detected in 10 accessions (76.9%), in exception to ‘Siganteng’, ‘Silatian’ and ‘Putri Kembar’ cultivars. *Pi-37* gene was visualized by the amplicons of 1149 bp and was detected in 8 accessions (61.5%), in exception to ‘IR 64’, ‘Siganteng’, ‘Silatian’, ‘Putri Kembar’ and ‘Sitampan’. Three R genes, *Pi-d2*, *Pi-ta²* and *Pi-b* genes were detected in all thirteen accessions (100%) visualized by the amplicons of 1057, 1042 and 388 bp, respectively. *Pik-h* gene showed positive bands to only two accessions (15.4%), ‘Sicondong’ and ‘Martabe’ with the amplicon of 1500 bp.

Dendrogram analysis revealed the relationship among South Tapanuli cultivars based on the binary data of DNA amplicons (Fig. 2). Three cultivars, ‘Putri Kembar’, ‘Silatian’ and ‘Siganteng’ were placed into cluster 1, with a similarity coefficient of 0.66. Two cultivars, ‘Martabe’ and ‘Sicondong’ were placed into cluster 2, with a coefficient of 0.79. Six cultivars, ‘Sayuti’, ‘Sitolas’, ‘Pulo Raja’, ‘Pulo Pandan’, ‘Siporang’ and ‘Pulo Manggis’ were placed into cluster 3, with a coefficient of 0.83. The cultivar, ‘Sitampan’ was placed with ‘IR 64’ into cluster 4 with a coefficient of 0.83 as well.

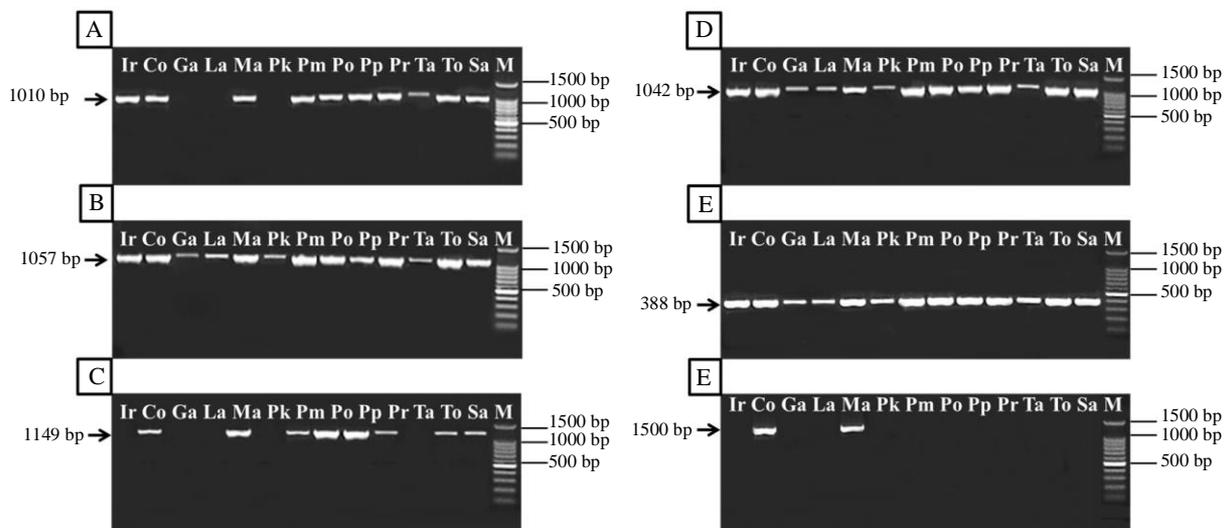


Fig. 1: Agarose gel electrophoretic pattern of 13 accessions from South Tapanuli by using specific markers for blast resistance: (A) *Pup1*, (B) *Pi-d2*, (C) *Pi-37*, (D) *Pi-ta²*, (E) *Pi-b*, (F) *Pik-h*, where M is 100 bp DNA size marker

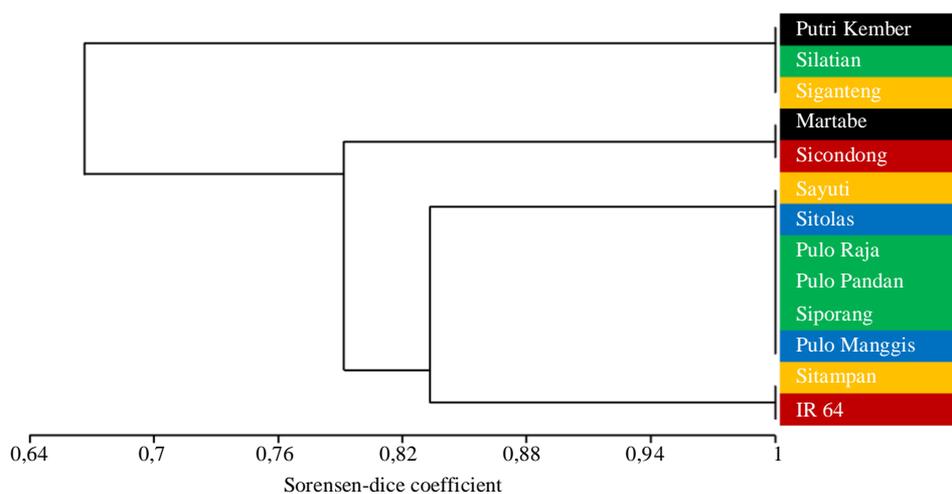


Fig. 2: Dendrogram based on UPGMA clustering analysis of 13 rice germplasm colored to represent different districts: ■ (West Angkola), ■ (Padangsidempuan), ■ (Marancar), ■ (Sipirok), ■ (Batang Angkola)

Table 3: Presence of six blast resistance (R) genes in 13 rice germplasm

Accession	<i>Pup1</i>	<i>Pi-37</i>	<i>Pi-d2</i>	<i>Pi-ta²</i>	<i>Pi-b</i>	<i>Pik-h</i>	Total genes
IR 64	1	0	1	1	1	0	4
Sicondong	1	1	1	1	1	1	6
Siganteng	0	0	1	1	1	0	3
Silatian	0	0	1	1	1	0	3
Martabe	1	1	1	1	1	1	6
Putri Kembar	0	0	1	1	1	0	3
Pulo Manggis	1	1	1	1	1	0	4
Siporang	1	1	1	1	1	0	5
Pulo Pandan	1	1	1	1	1	0	5
Pulo Raja	1	1	1	1	1	0	5
Sitampan	1	0	1	1	1	0	4
Sitolas	1	1	1	1	1	0	5
Sayuti	1	1	1	1	1	0	5
R gene frequency (%)	76.9	61.5	100.0	100.0	100.0	15.4	

Discussion

Utilization of medium to high blast-resistant rice cultivars in agricultural practices needs to consider the genetic stability of the germplasms. In addition, rice fields planted with only a monoculture of even high-resistant cultivar may lead to the evolution of a more virulent strain of *Magnaporthe oryzae* (Vasudevan *et al.*, 2016). Therefore, screening of new blast resistance (R) genes from local cultivars and wild relatives are still relevant until now to maintain the availability of blast-resistant germplasms.

Contribution of finding new blast resistance genes involved in Pathogen Associated Molecular Pattern (PAMP), is to introduce new traits to hybrid cultivars with superior resistance against blast disease through genetic engineering or introgression (Bao-hua *et al.*, 2017). Moreover, the genetic diversity of the most rice accessions has not been fully investigated despite the abundant source of germplasms, especially across regions in Indonesia (McCouch *et al.*, 2012).

In this study, we performed genotyping to the 13 South Tapanuli rice cultivars, by detecting the presence of six R genes, *Pup1*, *Pi-37*, *Pi-d2*, *Pi-ta*, *Pi-b* and *Pi-kh* with the genetic frequencies ranged from 15.4 to 100.0% or at least three positive bands out of the genes. Our results were similar to the study by Kim *et al.* (2010) in 84 aromatic rice accessions from Korea, possessing at least three positive bands out of eight R genes (37.5%). Imam *et al.* (2014) reported the lowest presence of one positive bands out of nine R genes (11.1 %) in 32 rice accessions from North East and Eastern India. Singh *et al.* (2015) reported the R genetic frequencies from 19.79 to 54.69% in 192 rice accessions from India with at least one positive bands detected out of 10 R genes (10.0%).

In a more recent study, Yan *et al.* (2017) reported the genetic frequencies of 11 R genes, ranging from 9.4 to 100.0% in 32 Chinese rice germplasms while Yadav *et al.* (2019) reported the genetic frequencies ranged between 4.96 and 100.0% in 161 Indian rice landraces. Prior to this study, Hannum *et al.* (2018) have screened 15 rice accessions originating from other districts in North Sumatra and reported higher genetic frequencies from 47.0 to 100.0%. Despite the genetic variability of the studied rice germplasms across regions, selection of blast resistance genes will produce variable results in genetic frequencies yet to be confirmed in the field tests.

The 13 local rice cultivars have been claimed for its blast resistance based on the field trials by the local farmers. The results can then be regarded as a bottom-up evaluation to prove the scientific basis of the resistance. The existence of R genes in our study may indicate a possibility of finding other local germplasms with a larger number of blast resistance genes from other regions in North Sumatra (Vasudevan *et al.*, 2014).

The 13 accessions possessed at least three to six blast resistance genes in which *Pi-d2*, *Pi-ta²* and *Pi-b* were detected in all cultivars. *Pi-d2* is located in chromosome 6, which encodes for a receptor-like kinase involved in PAMP-Triggered Immunity (PTI) responses (Chen *et al.*, 2006). *Pi-ta* or *Pi-ta²* are two tightly-associated blast resistance genes located in chromosome 12 (Rybka *et al.*, 1997). Recently, a novel broad spectrum R protein was reported to be encoded by *Pi-ta²* although the varieties studied by International Rice Research Institute (IRRI) were relatively low in frequency despite its high potential for future breeding of blast resistant varieties (Meng *et al.*, 2020). *Pi-b* is located in chromosome 2 and a member of the Nucleotide Binding Site - Leucine-Rich Repeats (NBS-LRR) class of blast resistance gene (Wang *et al.*, 1999). However, previous study reported that the detection of *Pi-b* produced none effect or even a susceptibility to *M. oryzae* based on the field study (Imam *et al.*, 2014).

Pup1 and *Pi-37* were detected as second dominant R genes in our study. *Pup1* or phosphorus uptake 1 locus is located in chromosome 12, which encodes major Quantitative Trait Locus (QTL) for phosphorus (P) tolerance (Heuer *et al.*, 2009). Chithrameenal *et al.* (2018) stated that majority of the modern rice cultivars lacked *Pup1* loci, yet highly susceptibility to P starvation. Therefore, genetic introgression of both P tolerant gene and blast resistance gene into new cultivars is a strategy to yield a higher rice productivity in the future. Meanwhile, *Pi-37* is a member of NBS-LRR which is also responsible for the blast resistance (Lin *et al.*, 2007). In addition, Yan *et al.* (2017) reported that both *Pi-36* and *Pi-37* were less likely detected from rice accessions.

Pi-kh was the least detected R gene with only two cultivars in our study. *Pi-kh* was a former name for *Pi54*, cloned from rice line Tetep which only induced in response to pathogen infections (Sharma *et al.*, 2010). The over-expression of *Pi-kh* with a strong promoter CaMV 35S, has proven to improve rice tolerance to *M. oryzae* blast disease (Azizi *et al.*, 2016).

Based on the dendrogram, it can be seen that there was no spatial effect to the detection of R genes in different districts. The result suggested that tolerance of the cultivars against pathogenic fungus were not based on environmental condition of rice fields. Moreover, pathogenicity assay is needed to confirm the interaction between associated rice R genes with disease incidences in field trials since less frequent detected R genes may be related to the increased susceptibility of plants to *M. oryzae* (Imam *et al.*, 2014). In the future, a large scale analysis of detecting multiple R genes may be investigated as well as identifying other rice cultivars originating from North Sumatra, as a hidden source of blast-resistant cultivars in the future.

Conclusion

Thirteen rice cultivars from South Tapanuli, North Sumatra previously claimed to be moderately or highly resistant to blast disease, were proven to possess at least three R genes, with the most frequent genes, *Pi-d2*, *Pi-ta²* and *Pi-b* (100.0%), followed with *Pi-37* (61.5%), *Pup1* (76.9%) and *Pi-kh* (15.4%) detected from all cultivars. Although our study may be considered as the first to reveal the genetic basis of the indigenous rice cultivars, more efforts are needed to confirm the contribution of single or multiple R genes involved in resistance on the field or through pathogenicity assay.

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Author's Contributions

Saleha Hannum: Conceived the original idea, designed the study and manuscript approval.

Hesti Wahyuningsih: Helped supervise the project.

Riyanto Sinaga: Designed research methodology, Conducted field sampling and data interpretation.

Ummu Kulsum Hasibuan: Data collection.

Adrian Hartanto: Literature search and manuscript writing.

Ethics

No potential conflict of interest among authors.

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