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Screening for Rice Blast Resistant Genes, *Pi9*, *Pita*, *Pigm(t)* and *Pi54* in Landrace Rice Varieties of Northeastern Thailand

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ABSTRACT

Rice blast disease, caused by *Magnaporthe oryzae*, is one of the most frequent and significant impediments to sustainable rice production and the largest impediment to sustainable rice production. Effective control of this disease largely depends on identifying the resistant genes. In our study, 37 Thai landrace rice varieties (18 white-grain rice varieties and 13 colored-grain rice varieties) along with 6 improved white-grain rice varieties were screened for four rice blast resistant genes, *Pi9*, *Pita*, *Pigm(t)* and *Pi54* by PCR technique using the gene-specific makers. The results showed that 20 rice varieties (54.0%) contained the rice blast resistant gene *Pi9*, 11 rice varieties (29.7%) contained the rice blast resistant gene *Pita*, 22 rice varieties (59.4%) contained the rice blast resistant gene *Pigm(t)* and 23 rice varieties (62.1%) contained the rice blast resistant gene *Pi54*. All 31 Thai landrace rice varieties carried at least one rice blast resistant gene(s). Four white-grain landrace rice varieties, four colored-grain landrace rice varieties and five improved rice carried the highest

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number of rice blast resistant genes at three genes, which can be used as the resistant donor in the rice breeding program. Two white-grain landrace rice varieties; Jao Dæng 2 and Mak Yom, contained one examined rice blast resistant genes. Two colored-grain landrace rice varieties; Nieow Dam (skn 3) and Kee Nok, contained all examined rice blast resistant genes. Five improved rice varieties contained three rice blast resistant genes. The information generated from this study is useful for parental selection in developing the new resistant rice varieties and breeding.

INTRODUCTION

Rice blast disease, caused by an ascomycete *Magnaporthe oryzae*, is one of the most destructive diseases of rice production worldwide [1]. It has been reported that this disease caused about 10 to 20% yield loss in regular seasons and as high as 100% yield loss in years with blast epidemics [2]. More than 100 rice blast resistant genes are mapped on different rice chromosomes, but only 22 resistant genes have been successfully cloned, namely; *Pib, Pita, Pid2, Pi9, Pi2, Piz-t, Pi36, Pi37, Pikm, Pi5, Pit, Pid3, Pi21, Pish, Pb1, Pik, Pikp, Pikh, Pia, Pil, Pi64* and *Pi50* [3]. The *Pi9* resistant gene is resistant to more than 100 Philippines rice blast isolates and 43 rice blast isolates from 13 countries [4]. The *Pita* and *Pita2* resistant genes were allelic and mapped near the centromere of chromosome 12 [5]. The *Pita* resistant gene was found in wide landrace rice varieties, including Tadukan in the Philippines, Tetep in Vietnam and Katy and Drew in the southern United States [6]. The *Pigm(t)* resistant gene confers broader-spectrum resistance to rice blast isolates from different rice regions in China [7]. The *Pi54* resistant gene was first identified in an Indian rice cultivar HR22 and later was cloned from an indica type rice cultivar Tetep [6]. Many resistant genes have been identified in the landrace rice varieties [7].

Rice landraces have been recognized as valuable genetic resources for improving the resistance level of modern rice cultivars against biotic diseases [8]. The landrace rice varieties, originating from nine diverse rice ecologies of India, harbored a range of five to nineteen rice blast-resistant genes with a frequency varied from 4.96% to 100% [9]. Rice blast epidemics have occurred in most provinces in the north and northeast Thailand, where the most effective management and control of rice blast disease is the use of resistant rice varieties [10]. There are more than 100,000 landraces and improved and elite rice varieties in Thailand, and many exhibits resistant reactions to the rice blast disease. Villa et al. [11] reported that the rice blast resistant genes, *Pi2*, *Pi9*, *Piz-t*, *Pigm(t)*, *Pid2*, *Pid3*, *Pia*, *Pik*, *Pi54* and *Pita* were presented in Thai landrace

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rice germplasm. The objective of this study was to investigate the four-rice blast resistant genes, *Pi9*, *Pita*, *Pigm(t)* and *Pi54,* in 37 Thai rice varieties, including 18 white-grain and 13 colored-grain landrace rice varieties and 6 improved rice varieties from northeastern Thailand using the gene specific primers. The results provided a list of the rice blast resistant genes in each examined Thai rice varieties and the information obtained from this study is useful for identifying the parental lines with the rice blast resistant genes in the future rice breeding programs.

MATERIALS AND METHODS

Plant materials

In total, 37 Thai rice varieties from northeastern Thailand, consisting of 18 white-grain landrace rice varieties, 13 colored-grain landrace rice varieties and 6 improved rice varieties from Sakonnaknon Rice Research Center, Puparn Royal Development Study Center for the conservation Thailand, were used in this study (Table 1, Figure 1-3). KDML105 and Nipponbare rice varieties were used as the susceptible control (Negative Control) and IRRI-inbred blast resistant lines (IRBLs) from the International Rice Research Institute (IRRI), IRBL12 (*Pita*) and IRBL22 (*Pi9*) were used as the resistant control (Positive Control).

Genomic DNA extraction

The genomic DNA extraction method was modified from Yadav et al. [12]. The leaves were ground in liquid nitrogen using a pre-cooled sterile mortar and pestle. Total DNA was isolated using the DNA extraction buffer (including 2% CTAB, 0.1 M Tris-HCL pH 8, 1.4 M NaCl and 0.02 M EDTA, pH8) and incubated at 65℃ for 90 minutes. After that, the solution was added with Chloroform: Iso-amyl alcohol (24:1) and mixed by using a vortex. Next, the mixture was centrifuged at 12,000 rpm for 15 min and transferred its upper phase to a new tube. After that 2/3–1 volume of Isopropanol was added and mixed by inverting. Next, the mixture was centrifuged at 12,000 rpm for 40 min, the supernatant was discarded, the pellet was washed in 70% ethanol, and the mixture was centrifuged at 12,000 rpm for 5 min. After that, the dried DNA pellet was then suspended in 100 μl of TE buffer (including 0.01M Tris-Cl pH 7.5 and 1mM EDTA) and frozen at -80°C for storage. DNA quality was measured using NANODROP 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The DNA was migrated on 1% agarose. Stained in ethidium bromide, then visualized on UV transilluminator.

Polymerase chain reaction amplification of genes

Thirty-seven Thai rice varieties were screened for four major rice blast resistant genes, *Pi9*, *Pita*, *Pigm(t)* and *Pi54,* using gene-specific DNA markers (Table 2). PCR amplifications were performed using *Taq* polymerase (Apslagen, Thailand) and ingredients by using the following PCR condition: initial denaturation for 2 min at 94°C, followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing depending on each marker (Table 2), 30 s of extension at 72°C and a

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final extension at 72°C for 5 min. The PCR products were determined using a stained photographic system (Vilber Lourmat, Eberhardzell, Germany) of 1% agarose gel electrophoresis at 100 V for 30 min.

DNA sequencing and sequence analysis

The PCR products were purified by GF-1 AmbicClean kit (PCR and Gel) (Vivantis Technologies Sdn. Bhd., Malaysia) according to the manufacturer's protocol. After purification, the PCR products were sent out for sequencing; by Sequence analysis of purified fragments was done by BGI tech in Hong Kong to confirm the sequence of the resistant genes. The nucleotide sequence was aligned with the reference sequences from GenBank databases with the MAFFTv7.380.

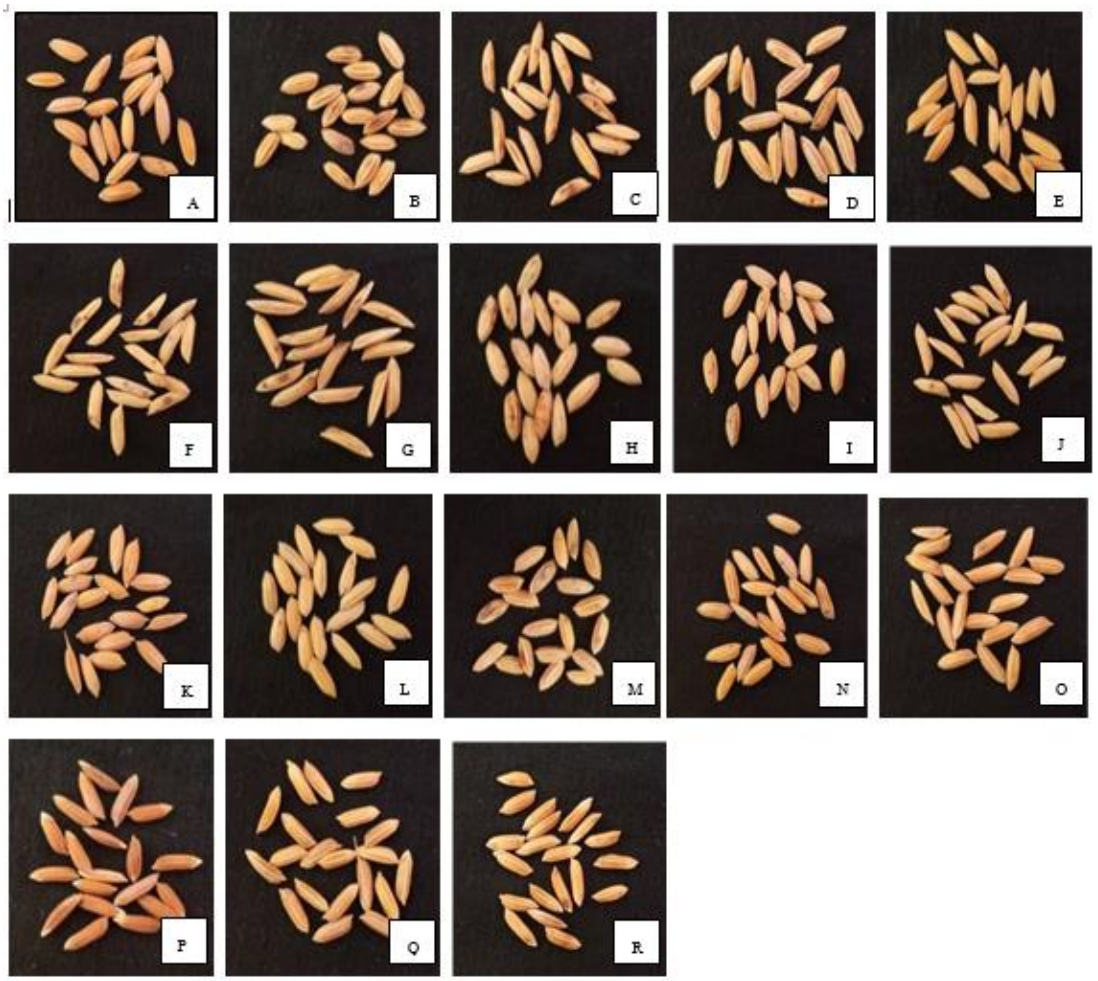

Figure 1 Eighteen white-grain landrace rice varieties; A (Leung Boonma), B (Hom Puang Ton Kieow), C (Leung khaw), D (Hom pla siw, E (Mak Khaek) , F (Khaw Hom Mai), G (Hom Daeng), H (Hom Puang Ton Muang), I (Ham Thung), J (Rai Dok Mai), K (Do Sakon), L (Tab Mei Khaw), M (Hom Jam Pla), N (Jao Dæng 2), O (Tab Mei), P (Mak Yom), Q (Phạk Hin), R (E rai)

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Figure 2 Thirteen colored-grain landrace rice varieties; A (Kee Nok), B (MayomNakhon Phanom), C (kee tom Hang Nak), D (Kæn Khu), E (Hom Nan Pon), F (Tab Mei Dom), G (Kab Yang 1), H (E mud), I (Kee Tom Ngan), J (Nieow Dam (skn 3)), K (Gom Na Nok Khea Na), L (Mae Phueng), M (Mafi Dam)

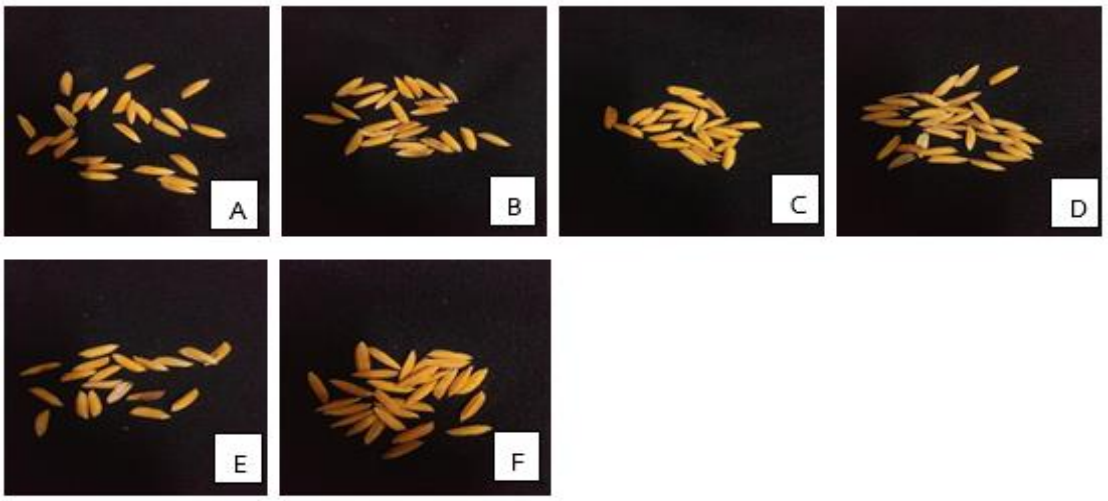

Figure 3 6 Improved rice varieties; A (Phu Phan 1 (75)), B (Phu Phan 1 (57)), C (Phu Phan 2), D (Phu Phan 1 (59)), E (Phu Phan 1 (58)), F (KD 6)

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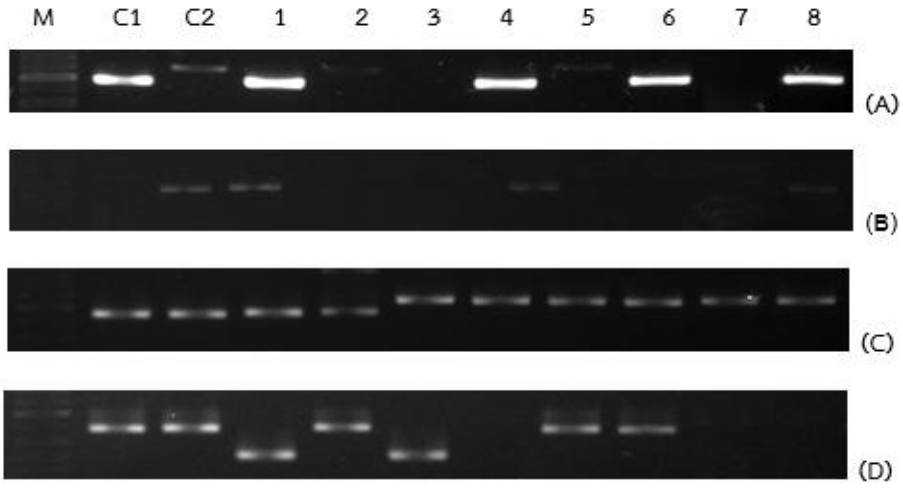

Figure 4 Gel electrophoresis detection of rice blast resistant allele using four *R* gene-specific primers, pB8 primer (A), *Pi54* MAS primer (B), S29747 primer (C) and YL155/YL8 primer (D), 1-8 represented individual rice sample; C1 Nipponbarae (Negative Control), C2 IRBL22 resistance line with *Pi9* (Positive Control)

Table 1 Thirty-seven Thai rice varieties used in the rice blast resistant gene investigation

No.	Local Name	Varieties (Seed Color)	*Pi9* gene	*Pita* gene	*Pigm(t)* gene	*Pi54* gene	Total *R* gene(s)
1.	Leung Boonma	Landrace (W)	+	+	-	+	3
2.	Hom Puang Ton Kieow	Landrace (W)	-	-	-	-	0
3.	Leung khaw	Landrace (W)	-	-	+	+	2
4.	Hom pla siw	Landrace (W)	+	-	+	-	2
5.	Mak Khaek	Landrace (W)	-	+	+	-	2
6.	Khaw Hom Mai	Landrace (W)	+	-	+	-	2
7.	Hom Daeng	Landrace (W)	-	-	+	-	1
8.	Hom Puang Ton Muang	Landrace (W)	+	-	+	-	2
9.	Ham Thung	Landrace (W)	+	+	+	-	3
10	Rai Dok Mai	Landrace (W)	+	+	-	+	3
11.	Do Sakon	Landrace (W)	+	+	-	-	2
12.	Tab Mei Khaw	Landrace (W)	+	-	+	-	2
13.	Hom Jam Pla	Landrace (W)	-	-	-	-	0
14.	Jao Dæng 2	Landrace (W)		+	-	-	1
15.	Tab Mei	Landrace (W)	+	-	+	-	2
16.	Mak Yom	Landrace (W)	-	+	-	-	1

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No.	Local Name	Varieties (Seed Color)	*Pi9* gene	*Pita* gene	*Pigm(t)* gene	*Pi54* gene	Total *R* gene(s)
17.	Phạk Hin	Landrace (W)	-	-	-	-	0
18.	E rai	Landrace (W)	-	+	+	+	3
19.	Kee Nok	Landrace (Br)	+	-	-	+	2
20.	MayomNakhon Phanom	Landrace (Bl)	-	-	+	+	2
21.	kee tom Hang Nak	Landrace (Br)	-	-	+	+	2
22.	Kæn Khu	Landrace (Br)	+	-	-	+	2
23.	Hom Nan Pon	Landrace (Br)	+	-	+	+	3
24.	Tab Mei Dom	Landrace (Br)	-	-	+	+	2
25.	Kab Yang 1	Landrace (Br)	-	-	+	+	2
26.	E mud	Landrace (Bl)	+	+	-	+	3
27.	Kee Tom Ngan	Landrace (Br)	+	+	-	+	3
28.	Nieow Dam (skn 3)	Landrace (Br)	-	-	-	+	1
29.	Gom Na Nok Khea Na	Landrace (Bl)	-	-	+	+	2
30.	Mae Phueng	Landrace (Br)	-	+	+	+	3
31.	Mafi Dam	Landrace (Br)	-	-	-	+	1
32.	Phu Phan 1 (75)	Improve (W)	+	-	+	+	3
33.	Phu Phan 1 (57)	Improve (W)	+	-	+	+	3
34.	Phu Phan 2	Improve (W)	+	-	-	+	2
35.	Phu Phan 1 (59)	Improve (W)	+	-	+	+	3
36.	Phu Phan 1 (58)	Improve (W)	+	-	+	+	3
37.	KD 6	Improve (W)	+	-	+	+	3
Total			20	11	22	23	

* W (Write grain), Br (Brown grain), Bl (Black grain), + (Resistance positive band, - (No or negative band)

Table 2 Gene-specific PCR primers used in the identification of the rice blast resistant genes

R gene	DNA Makers	Sequence primer (5'>>>3')	AT (°C)	ES (bp)	Reference
Pi9	pB8	F: CCCAATCTCCAATGACCCATAAC	56	500	Liu et al.,
		R: CCGGACTAAGTACTGGCTTCGATA			2002
Pi54	*Pi54* MAS	F: CAATCTCCAAAGTTTTCAGG	55	R: 261	Ramkumar et
		R: GCTTCAATCACTGCTAGACC		S: 359	al., 2011
Pigm(t)	S29747	F: CAGTGAAACGAACGCTATG	56	R: 555	Deng et al.,
		R: AATAGGAAGGGTTGATGTTG		S: 461	2006

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R gene	DNA Makers	Sequence primer (5'>>>3')	AT (°C)	ES (bp)	Reference
Pi-ta	YL155/YL87	F: AGCAGGTTATAAGCTAGGCC	58	1024	Jia et al.,
		R: CTACCAACAAGTTCATCAAA			2002

**R* gene (rice blast resistance gene); AT, Annealing temperature; ES, Expected size

Table 3 Distribution of of four blast resistant genes, *Pi9*, *Pita*, *Pigm(t)* and *Pi54*, in 37 Thai rice varieties in Thai rice varieties

Rice set	Total	*Pi9*	*Pita*	*Pigm(t)*	*Pi54*
white-grain landrace rice	18	9 (50.0%)	8 (44.4%)	10 (55.6%)	4 (22.2%)
colored-grain landrace rice	13	5 (38.5%)	3 (23.1%)	7 (53.8%)	13 (100%)
Improved rice	6	6 (100%)	0 (0%)	5 (83.3%)	6 (100%)
Total	37	20 (54.0 %)	11 (29.7 %)	22 (59.4%)	23 (62.1%)

RESULTS AND DISCUSSION

Twenty Thai rice varieties (54.0%) and IRBL22 (the rice line with the *Pi9* resistant allele) showed a positive band of 500 bp (Figure 4). These 20 rice varieties included nine white-grain landrace rice varieties, five colored-grain landrace rice varieties and six improved rice varieties (Table 1, 3). Fourteen PCR samples of the landrace rice varieties that produced the positive DNA band were randomly selected for purification and sequencing. The obtained sequence showed a 100% sequence identity to the *Pi9* reference sequence (accession no. DQ285630.1).

Eleven rice varieties (29.7%), including eight white-grain landrace rice varieties and three colored-grain landrace rice varieties, showed a positive 1,024 bp DNA fragment. All improved rice could not amplify with *Pita*-specific primer (Table 1, 3) (Figure 4). To confirm the presence of the *Pita* resistant allele, the PCR product of a rice variety HomNanPon was sequenced and compared with the *Pita* reference sequence from NCBI GenBank (accession no. AF207842.1) and the results showed 100% sequence identity.

The *Pigm(t)* gene-specific Indel marker was used to detect the *Pigm(t)* resistant allele. The resistant allele of the *Pigm(t)* showed the 555 bp amplicon and the susceptible allele showed the 461 bp amplicon. Twenty-two rice varieties (59.4%), including 10 white-grain landrace rice varieties and seven colored-grain landrace rice varieties and five improved rice varieties, gave a 555 bp DNA fragment of the resistant allele (Table 1, 3) (Figure 4). The sequence confirmation of the *Pigm(t)* resistant allele from a rice variety Kean Khu showed 100% identity

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to the reference sequence from NCBI GenBank (accession no. AF207842).

In the *Pi54* gene-specific Indel marker, the resistant allele of the *Pi54* gene showed the 261 bp amplicon and the susceptible allele showed the 359 bp amplicon. The results showed that twenty-three rice varieties (62.1%), including four white-grain landrace rice varieties, 13 colored-grain landrace rice varieties and six improved rice varieties, carried the *Pi54* resistant allele with PCR product of 261 bp (Table 1, 3) (Figure 7). To confirm the presence of the *Pi54* resistant allele, a PCR product from rice variety Mafi Dam was sequenced and compared with the reference sequence from NCBI GenBank (accession no HE586202.1.) and the results showed 99.78% nucleotide sequence identity and 100% amino acid sequence identity. One base substitution was changed from GTC to GTT, causing silent mutation.

Two white-grain landrace rice varieties, Jao Dæng 2 and Mak Yom contained four examined rice blast resistant genes. Two colored-grain landrace rice varieties, Nieow Dam (skn 3) and Kee Nok contained, all examined rice blast-resistant genes. Five improved rice varieties contained three rice blast-resistant genes.

CONCLUSION

Rice blast disease is one of the most limiting serious factors for rice production worldwide. Landrace rice varieties have been recognized as valuable genetic resources for improving the resistance level of modern rice cultivars against biotic diseases [14]. The results from this study validated that Thai landrace rice varieties of northeastern Thailand were a source of four major rice blast resistant genes, *Pi9, Pita, Pigm(t) and Pi54*. The distribution of resistant genes ranged from 29.7% to 62.1%. All examined landrace rice varieties contained at least one rice blast resistant gene, with many having more. Our result agreed with previous reports, including Koide et al. [13] that reported that 159 from 226 Thai rice cultivars had at least one rice blast resistant gene, *Pid3*, *Pi54* and *Pigm*. Four cultivars had three resistant genes. Similar results were reported by Kobayashi et al. [14], which screened the rice blast resistant genes in the rice germplasm of Manipur, India and found that wide rice varieties contained 2 - 7 rice blast resistant genes. Chaipanya et al. [15] reported that 84 Korean rice varieties possessed more than three positive bands of the eight-rice blast resistant genes. Similar results were reported by Wattanaporn et al. [16], which screened the resistance gene *Pita*, Pib and Pi2t were found in 78 Thai landrace rice varieties. *Pita* is present in 29 Thai landrace rice varieties. Similar results were reported by Agrawal et al. [17]; it has been reported that *Pi9* is present in 64 Thai landrace rice varieties and 1 among them was southern landrace Thai rice, 16 varieties from northern, and 47 varieties from north-eastern Thailand regions and *Pigm(t)* is present in 201 Thai landrace rice varieties. In the *Pi54* gene-specific Indel marker, the resistant allele of the *Pi54* gene showed the 261 bp amplicon and the susceptible allele showed the 359 bp amplicon.

The results showed that twenty-three rice varieties. Similar results were reported by Liu et al. [18], *Pi54* MAS showed a PCR product size of 359 bp. Twenty Thai rice varieties (54.0%) and IRBL9-w (the rice line with the *Pi9* resistant allele) showed a positive band of 500 bp. Similar results were reported by Liang et al. [19], reported 203 landrace rice samples were tested for *Pi9* blast resistance gene using the pB8 DNA marker, 64 cultivars were able to be quantified, *Pi9* marker the resulted showed a PCR product size of a 500 bp. These results indicated that the landrace rice germplasm is a valuable source of the rice blast resistant genes for rice breeding programs.

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