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GENETIC VARIATION AND DISTRIBUTION OF DIFFERENT BLAST RESISTANCE GENES IN LANDRACE RICE OF THAILAND

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Abstract

Magnaporthe oryzae (also known as *Pyricularia oryzae*) is a plant pathogenic fungus causing blast disease in rice. While rice can resist *M. oryzae* via *R* or *Pi* genes, one *R* gene is not enough to provide long-term resistance as *M. oryzae* has a short lifespan, allowing it to quickly adapt to new environments and resistant rice. This study aimed to screen *R* gene resources from landraces for a rice breeding program using specific gene primers. Of eight *R* genes among 17 rice varieties, the *Pi54* gene had the highest frequency of 29.17%, while the *Pik* gene was found in only one landrace rice with a frequency of 2.08%. Phylogenetic trees showed the *Pi9*, *Pi54* and *Pib* genes were the most diverse, with their nucleotide alignment revealing indels and point mutations. Among the rice varieties, Tubtim ChoomPhoo contained five resistance genes and is therefore an appropriate germplasm resource for polygenic traits to resist *M. oryzae*. The studied results should be ideal for using parental lines to improve new rice varieties that are blast-resistant.

Key words: Blast disease, Blast resistant gene, *Oryza sativa*, Landrace, DNA marker.

Introduction

Rice (*Oryza sativa*) is the result of *O. rufipogon* originated in Asia, and it has three major subspecies cultivated in Asia: Japonica, Indica and Javanica (Chang, 1976; Khush, 1997; Hour *et al*., 2020). Additionally, Asian rice can be classified as wild, cultivated or landrace. Landrace rice is important for household consumption because it is primitive rice that has been developed in a specific region for many years and can resist many local biotic stresses, making landraces a key source of genetic diversity (Song *et al*., 2014; Thakur *et al*., 2015). Thailand is a resource of rice genetic diversity since there are various rice varieties; landrace, local varieties and improved varieties moreover, rice can be grown in different regions such as lowland, soil submerge, upland or on slopes (Fongfon *et al*., 2021).

A common goal of rice breeding is to improve disease resistance to reduce yield losses. Blast disease is produced by *Magnaporthe oryzae*, a fungal pathogen that produces avirulence (*avr*) proteins to invade rice. Some rice varieties produce blast resistant (*R*) or *Piricularia* (*Pi*) genes to resist the *avrs* by gene-to-gene interaction (Ali *et al*., 2016; Nyuget *et al*., 2019). Of more than 100 *R* genes, only 27 were cloned and characterized: *Pia, Pb1, Pi1, Pi2, Pi5, Pi9, Pi21, Pi25, Pi33, Pi36, Pi37, Pi50, Pi65(t), Pib, PiCO39, Pid2, Pid3, Pid3–A4, Pii, Pikm, Pita, Pizt, Pit, Pish, Pik, Pikh, and Pikp*. Blast resistance in rice is regulated by at least one dominant or recessive gene and/or minor genes, and rice carrying only one *R* gene is only blast resistant for up to a few years since *M. oryzae* has a short lifespan and unstable genetics (Rath & Padmanahan, 1972; Padmanabhan, 1965; Higashi & Saito, 1985; Ali *et al*., 2016; Ying *et al*., 2022).

Previous studies stated polygenic genes that means the *R* genes link together to resist *M. oryzae* (Deng *et al*., 2006; Imam *et al*., 2014). The Chinese variety, GM4, was investigated for polygenic genes to blast resistance, and the result represented that there are three *R* genes, namely *Pigm*(*t*), *Pi2* and *Pi9*, linking together in a broad-spectrum resistant gene (Deng *et al*., 2006). In 2016, rice germplasms from various regions of Europe and Asia were screened for the nine *R* genes and the results represented all samples showing polygenic combination to resist rice blast resistance. To improve rice blast resistance, polygenic genes should be used to obtain variable resistance against diverse *M. oryzae*. Therefore, this study aimed to investigate *R* genes from landraces in Buriram, Thailand as potential germplasm resources for future rice development.

Material and Method

Sample collection: A total of fourteen landraces and one inbred line (Table 1) were collected from Buriram province in Northeastern Thailand, a fertile volcanic area where a variety of landraces are cultivated. Khao Dawk Mali 105 (KDML105) was a negative control that is susceptible to blast disease, whereas RD43 is resistant to blast disease obtained from the Chai Nat Rice Research Center: KDML105 is a pure line selection from a Chacheongsoa landrace, while RD43 is an inbred line between Suphanburi1 and Kaojoahom suphanburi1.

DNA extraction: One gram of young leaves of all samples was collected from three-week-old seedlings. Total genomic DNA extraction was done by the cetyltrimethylammonium bromide method (CTAB). DNA samples were separated on 0.8% agarose gel for 20 min and stained with ethidium bromide before being placed on a gel documentation system. The total genomic DNA of all samples was diluted with 1x Tris-EDTA buffer to 10 ng/µl and kept at –20°C for later study.

Rice blast resistance gene amplification: Primers (Table 2) were used to analyze blast resistance genes in rice. The PCR mixture contained 10 µl of 2x Tiangen PCR mixture, 10 ng of template DNA and 1 µl of each primer (10 mM), and then ddH_2O was added to 20 µl for a final volume. The

PCR thermal cycle was one cycle of initial duration at 95°C for 5 min and then 30 cycles of 3-step process at 95°C for 30 s for denaturation, 55°C for 30 s for annealing, 72°C for 1 min for extension and one cycle of the last extension step at 72°C for 10 min. The PCR products were examined using 2% agarose gel electrophoresis, and then the gels were stained with ethidium bromide before visualization on a gel documentation system, and the PCR products were sequenced by ATGC Co. Ltd., Thailand with specific primers for each gene.

Table 1. The Thai landrace and cultivated varieties used in the blast resistance gene analyze.

Variety	Type
RD43 (positive control)	Cultivated, inbred line
KDML105 (negative control)	Cultivated
Hang Yee71	Inbred line
Hom Nin	Landrace
NangSao-Thai	Landrace
Tubtim ChoomPhoo	Landrace
Maled-Lek	Landrace
KhaoNeou Suphan	Landrace
KhaoNeou Dum	Landrace
Riceberry	Landrace
Khao Tah Haeng	Landrace
Leoum Pua	Landrace
JaoHom Deang	Landrace
Jib	Landrace
PhuKhaoPhrie KhokMuang	Landrace
Ja-Jaab	Landrace
Thansirisa	Landrace

Phylogenetic tree construction: The nucleotide sequences of each gene were aligned using the ClustalW program (Bioedit program). Phylogenetic trees were created using Mega X software (www.megasoftware.net) with a neighbor-joining program. A thousand replicates were performed, and bootstrap values were calculated to represent the node stability and support the inferred clusters. Bootstrap values of 50 to 74% indicated weak support, 75 to 84% indicated moderate support and 85 to 100% indicated strong support (Richardson *et al*., 2000).

Results

Blast resistance gene analysis: Fifteen landrace samples, one positive control and one negative control, were evaluated with PCR using *Pi9, Pigm(t), Pia, Pita, Pi54, Pi50, Pik* and *Pib* primers (Table 3). The positive control (RD43) had two resistance genes, while all the landrace samples had at least one blast resistance gene, except Thansirisa and Leoum Pua, which had a single resistance gene, *Pi54* and *Pib*, respectively, and KDML105 did not discover any resistance genes. Hom Nin was the only one with the *Pik* gene, while Khao Tah Haeng and JaoHom Daeng were the only two varieties with the *Pita* gene; moreover, *Pi50* was found in NangSoa-Thai and Maled-Lek. While no landrace carried all the resistance genes, there was only Tubtim ChoomPhoo having five resistance genes, indicating that they could be valuable germplasm resources for blast resistance.

Pi54 is one of the blast resistance genes with the highest frequency of 29.17% and was present in fourteen samples, including a positive control (Table 3). *Pi9* and *Pib* had the next highest frequency of 16.67%, followed by *Pigm(t)* and *Pia*, all of which were found in 14.58% and 12.5% of the varieties tested, respectively. *Pi50* and *Pita* were 4.17%, while *Pik* had the lowest frequency, approximately 2.08%.

Genetic diversity of rice blast resistance genes: Phylogenetic trees were constructed for five resistance (*R*) genes (*Pia, Pib, Pigm(t), Pi9* and *Pi54),* but could not be constructed for the *Pi50, Pik* and *Pita* genes because they were not found in enough varieties for phylogenetic tree construction. While *Pi54* had the highest frequency of 29.17%, the gene could not be classified into groups. There was one major group, which divided Ja-Jaab into another clade, with a strong bootstrap value of 95, whereas PhuKhaoPhrie KhokMuang and Jib were outliers (Fig. 1). The *Pi54* nucleotide alignment of Ja-Jaab, PhuKhaoPhrie KhokMuang and Jib were particularly distinct sequences due to the indels and point mutations identified that conformed to the sequence differently than the others (Fig. 2) and caused the evolutionary rates of HangYee71, Ja-Jaab, JaoHom Deang and KhaowNeou Dum to be the fastest compared to the others.

Table 2. List of rice blast resistance gene primers and annealing temperatures.

Primer	Sequence (5′ → 3′)	Annealing temperature (°C)	Reference
Pi9_F	CCCAATCTCCAATGACCCATAAC	56	Liu *et al*., 2002
Pi9_R	CCGGACTAAGTACTGGCTTCGATA		
Pigm(t)_F	CAGTGAAACGAACGCTATG	56	Deng *et al*., 2006
Pigm(t)_R	AATAGGAAGGGTTGATGTTG		
Pia_F	GAGCAATGCCCAATCTCCAG	60	Suksiri & Parinthawong, 2020
Pia_R	TTTACCGTTCACTGACGCAG		
Pita_F	AGCAGGTTATAAGCTAGGCC	58	Jia *et al*., 2002
Pita_R	CTACCAACAAGTTCATCAAA		
Pi54_F	CAATCTCCAAAGTTTTCAGG	55	Ramkumar *et al*., 2011
Pi54_R	GCTTCAATCACTGCTAGACC		
Pi50_F	CTTGACATCCAAACCGCACC	60	Xiao *et al*., 2017
Pi50_R	TAGGCCTAGCCAATTTTTGCC		
Pik_F	GGAAAGCTGATATGTTGTCG	58	Suksiri &Parinthawong, 2020
Pik_R	ACTCGGAGTCGGAGAGTCAG		
Pib_F	ATCAACTCTGCCACAAAAATCC	57	Cho *et al*., 2007
Pib_R	CCCATATCACCACTTGTTCCCC		

Table 3. Observed blast resistance genes (*R* genes) and gene distribution in 15 rice varieties and 1 positive controls.

	Rice cultivar	*Pi9*	*Pi54*	*Pia*	*Pi50*	*Pigm(t)*	*Pita*	*Pik*	*Pib*	Total *R* genes
1.	RD43 (positive control)	-	+	-	-	+	-	-	-	2
2.	Hang Yee71	-	+	-	-	+	-	-	-	2
3.	Hom Nin	+	+	-	-	+	-	+	-	4
4.	NangSao-Thai	-	+	+	+	+	-	-	-	4
5.	Tubtim ChoomPhoo	+	+	+	-	+	-	-	+	5
6.	Maled-Lek	-	+	-	+	+	-	-	+	4
7.	KhaoNeou Suphan	+	+	+	-	-	-	-	+	4
8.	KhaoNeou Dum	+	+	+	-	-	-	-	+	4
9.	Riceberry	-	-	+	-	+	-	-	+	3
10.	Khao Tah Haeng	-	+	-	-	-	+	-	+	3
11.	Leoum Pua	-	-	-	-	-	-	-	+	1
12.	JaoHom Deang	+	+	-	-	-	+	-	+	4
13.	Jib	+	+	-	-	-	-	-	-	2
14.	PhuKhaoPhrie KhokMuang	+	+	-	-	-	-	-	-	2
15.	Ja-Jaab	+	+	+	-	-	-	-	-	3
16.	Thansirisa	-	+	-	-	-	-	-	-	1
17.	KDML105 (negative control)	-	-	-	-	-	-	-	-	0
	Total	8	14	6	2	7	2	1	8	48
	Gene distribution (%)	16.67	29.17	12.50	4.17	14.58	4.17	2.08	16.67	100

Note: Dashes indicate no *R* gene, and pluses indicate *R* gene

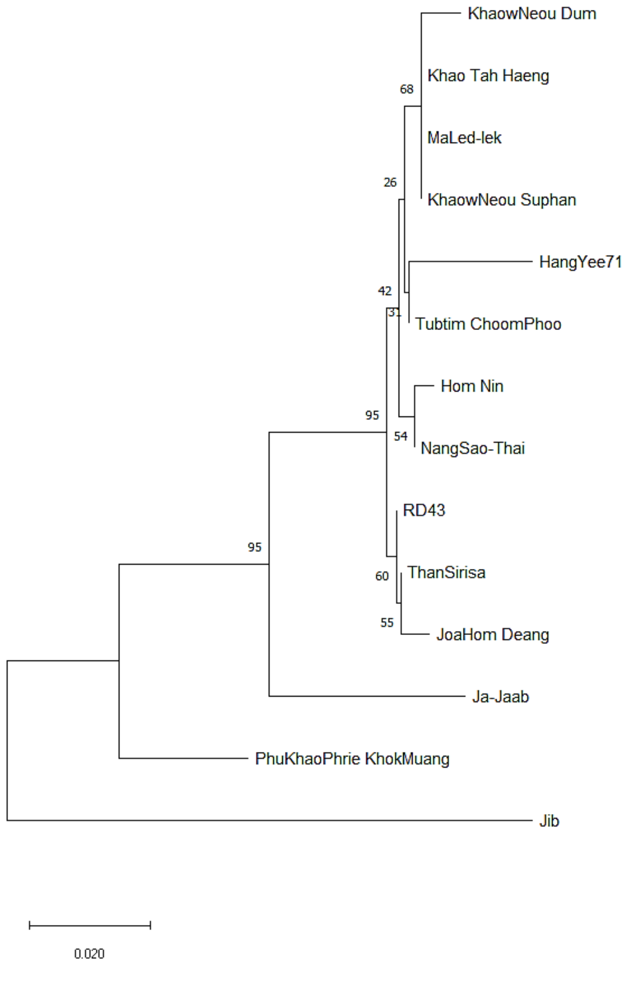

Fig. 1. Unrooted tree phylogeny was created with the neighbor-joining method based on sequences of the *Pi54* gene. The phylogenetic tree shows the relationship between rice varieties.

Pi9 had a 16.67% frequency, and its phylogenetic tree classified samples into two groups (Fig. 3). As for the first group, Tubtim ChoomPhoo and Hom Nin were in the same clade as PhuKhaoPhrie KhokMuang and Ja-Jabb, whereas Jib was an outlier. The others, glutinous rice (KhaoNeou Suphan and KhaowNeou Dum) and JaoHom Deang, were in the same cluster, but JaoHom Deang was an outlier. Both glutinous rice varieties had faster evolution rates of the *Pi9* gene than the others, while JaoHom Deang had the slowest evolution rate. According to the nucleotide alignment, all samples contained point mutations, especially glutinous rice, which showed diverse sequence variation, and the indels mutation (Fig. 4).

The frequency of *Pib* was moderate (16.67%) that was similar to *Pi9* however, *Pigm(t)* (14.58%) and *Pia* (12.5%) were also relatively moderate. However, the phylogenetic tree of *Pib* differed from *Pi9* and was classified into one major group, including six landraces, while JaoHom Deang was separated into a distinct cluster due to nucleotide sequences (Fig. 5). The sequence alignment showed diverting nucleotides, indels and point mutations in landrace rice especially JaoHom Deang resulting in an outlier and faster evolution rate than the others (Fig. 6). Although, the nucleotide sequence of JaoHom Deang was strongly divert, it was similar to *Pib* with 90% similarity.

Pigm(t) was found in seven rice samples, including a positive control, with a frequency of 13.46%. The phylogenetic tree classified one major group, RD43, Hom Nin, HangYee71 and NangSao-Thai and there was a clade of Tubtim ChoomPhoo and Maled-Lek whereas Riceberry was out of the groups (Fig. 7). The nucleotide alignment showed a few different positions including one indels mutation (Fig. 8). The evolution rates of RD43 and Riceberry were the fastest and followed by Hom Nin.

The *Pia* gene was presented in seven varieties and classified into two clusters. The first cluster contained Riceberry, NangSao-Thai and Tubtim ChoomPhoo, while the other contained KhaowNeou Suphan, and Ja-Jaab whereas KhaowNeou Dum was separated from its own clade (Fig. 9) and the evolution rate was quite consistent. The nucleotide alignment showed transition and transversion of some different nucleotides (Fig. 10).

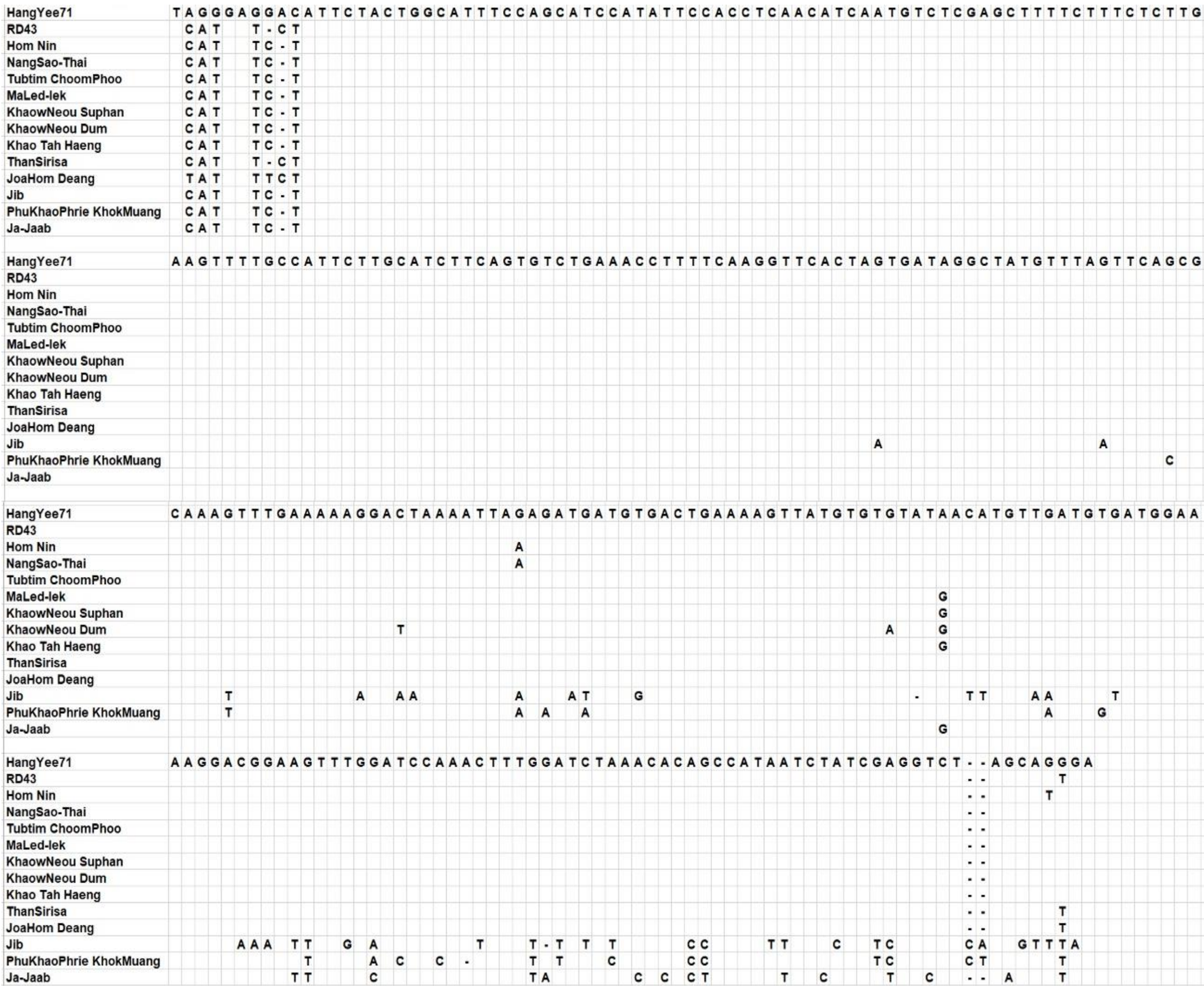

Fig. 2. *Pi54* nucleotide alignment using the ClustalW program. Blank boxes represent consensus nucleotides and dashes represent nucleotide deletions in the gene.

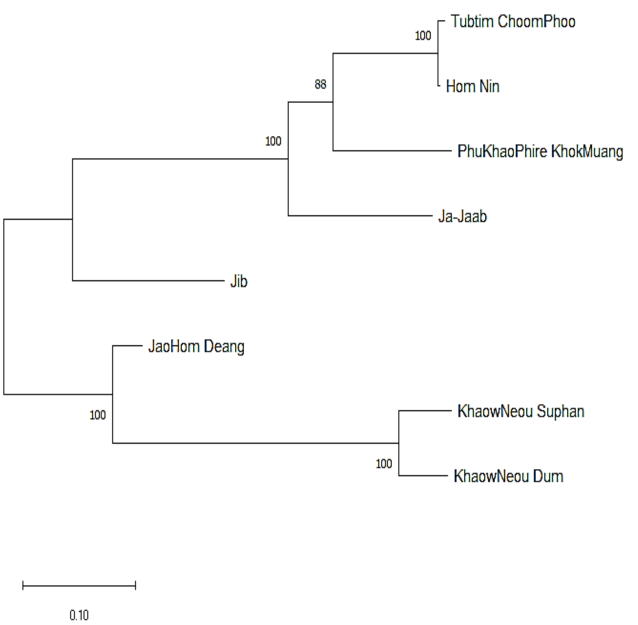

Fig. 3. Unrooted tree phylogeny was created with the neighbor-joining method based on sequences of the *Pi9* gene. The phylogenetic tree shows the relationship between rice varieties.

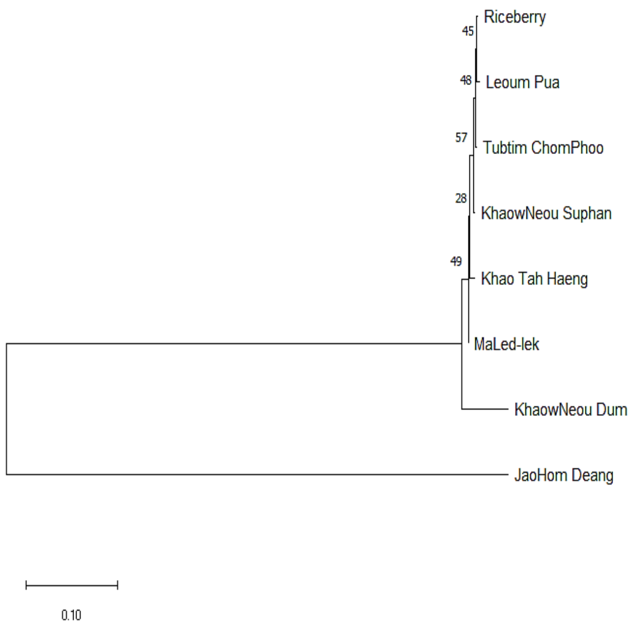

Fig. 5. Unrooted phylogenetic tree was constructed with the neighbor-joining method based on sequences of the *Pib* gene. The phylogenetic tree shows the relationship between rice varieties.

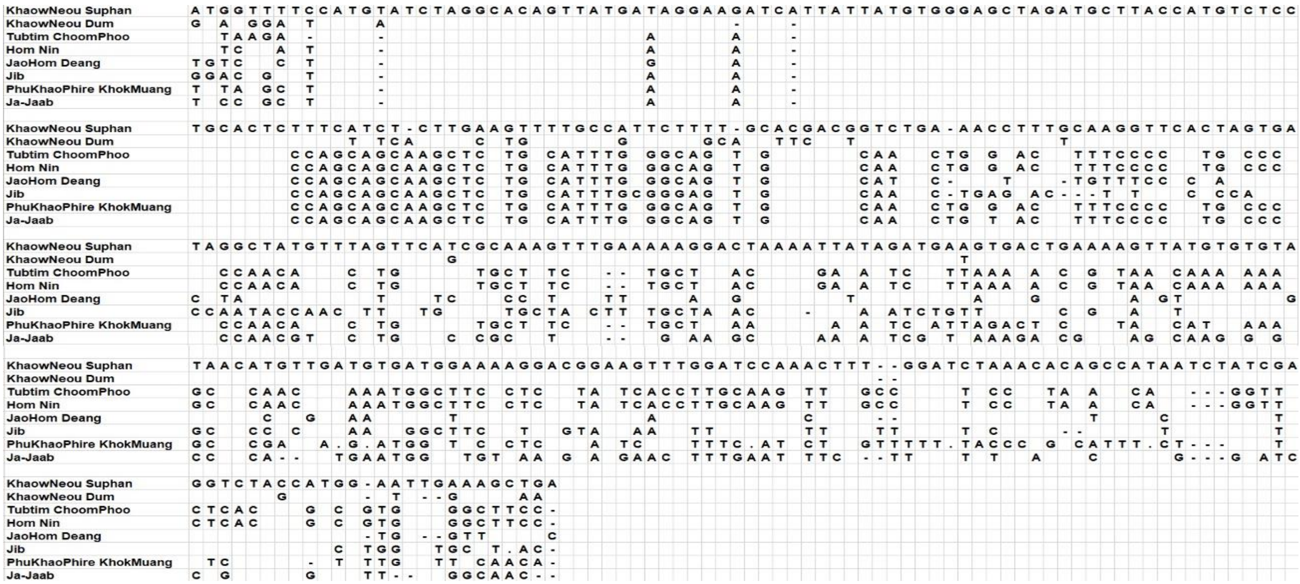

Fig. 4. *Pi9* nucleotide alignment using the ClustalW program. Blank boxes represent consensus nucleotides and dashes represent nucleotide deletions in the gene.

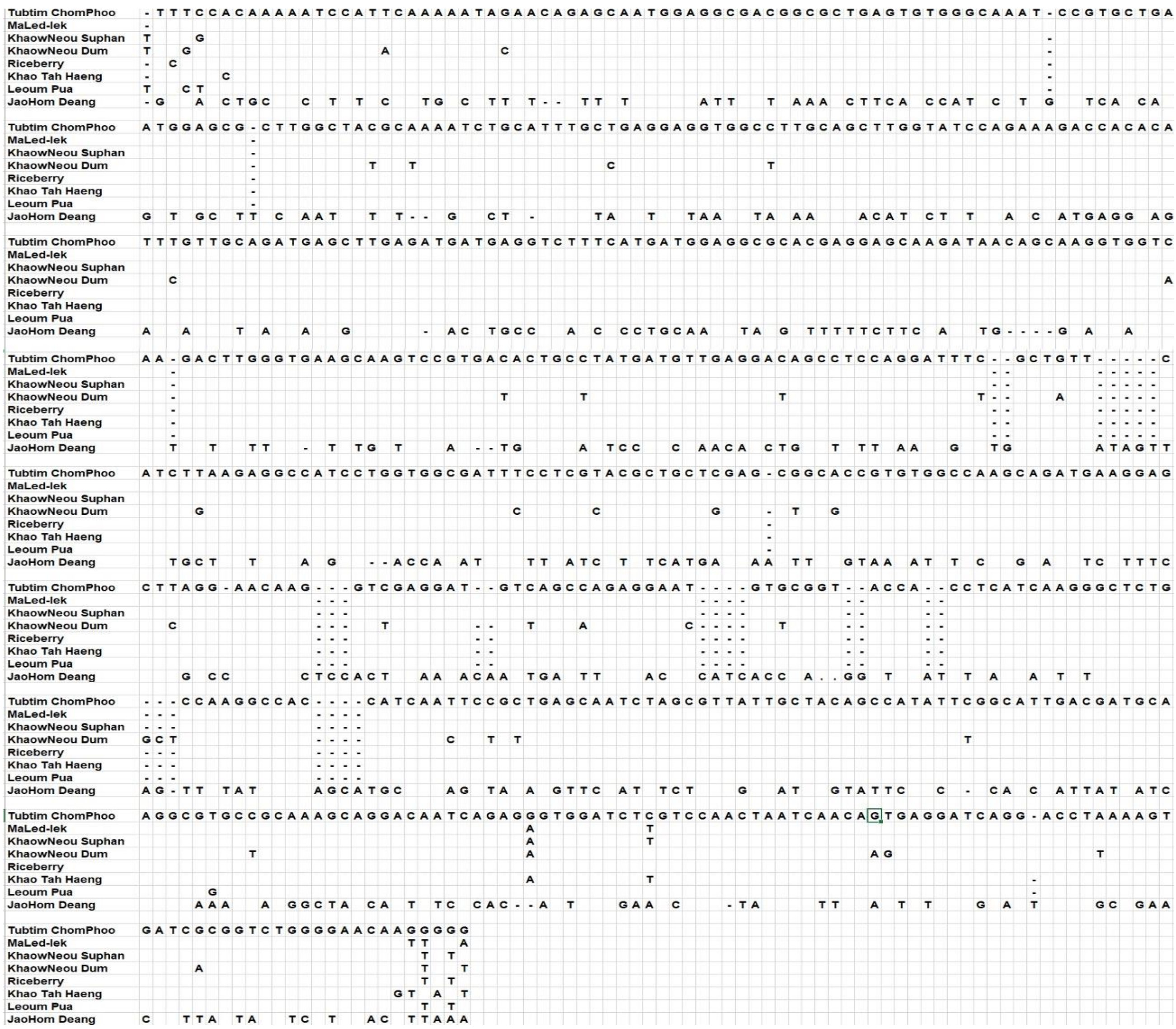

Fig. 6. *Pib* nucleotide alignment using the ClustalW program. Blank boxes represent consensus nucleotides and dashes represent nucleotide deletions in the gene.

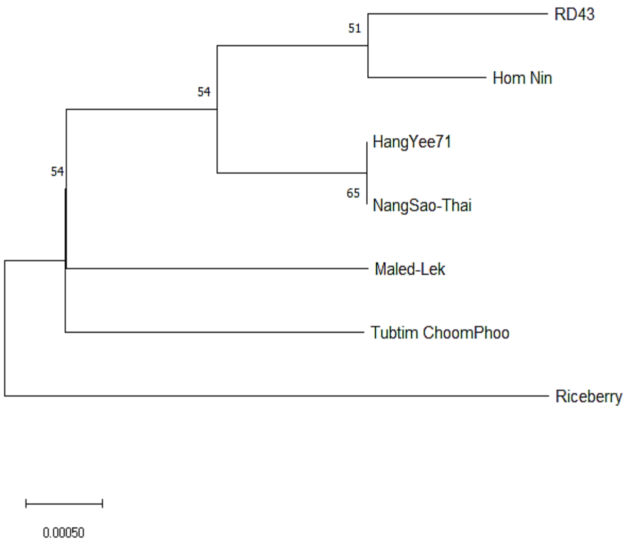

Fig. 7. Unrooted phylogenetic tree was constructed with the neighbor-joining method based on sequences of the *Pigm(t)* gene. The phylogenetic tree shows the relationship between rice varieties.

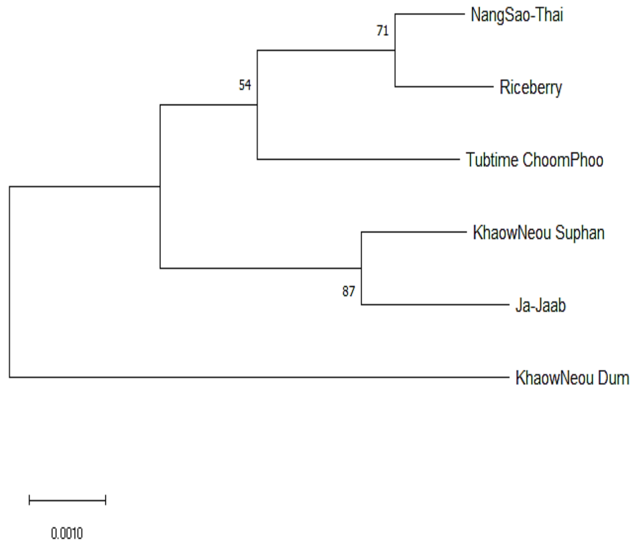

Fig. 9. Unrooted phylogenetic tree was constructed with the neighbor-joining method based on sequences of the *Pia* gene. The phylogenetic tree shows the relationship between rice varieties.

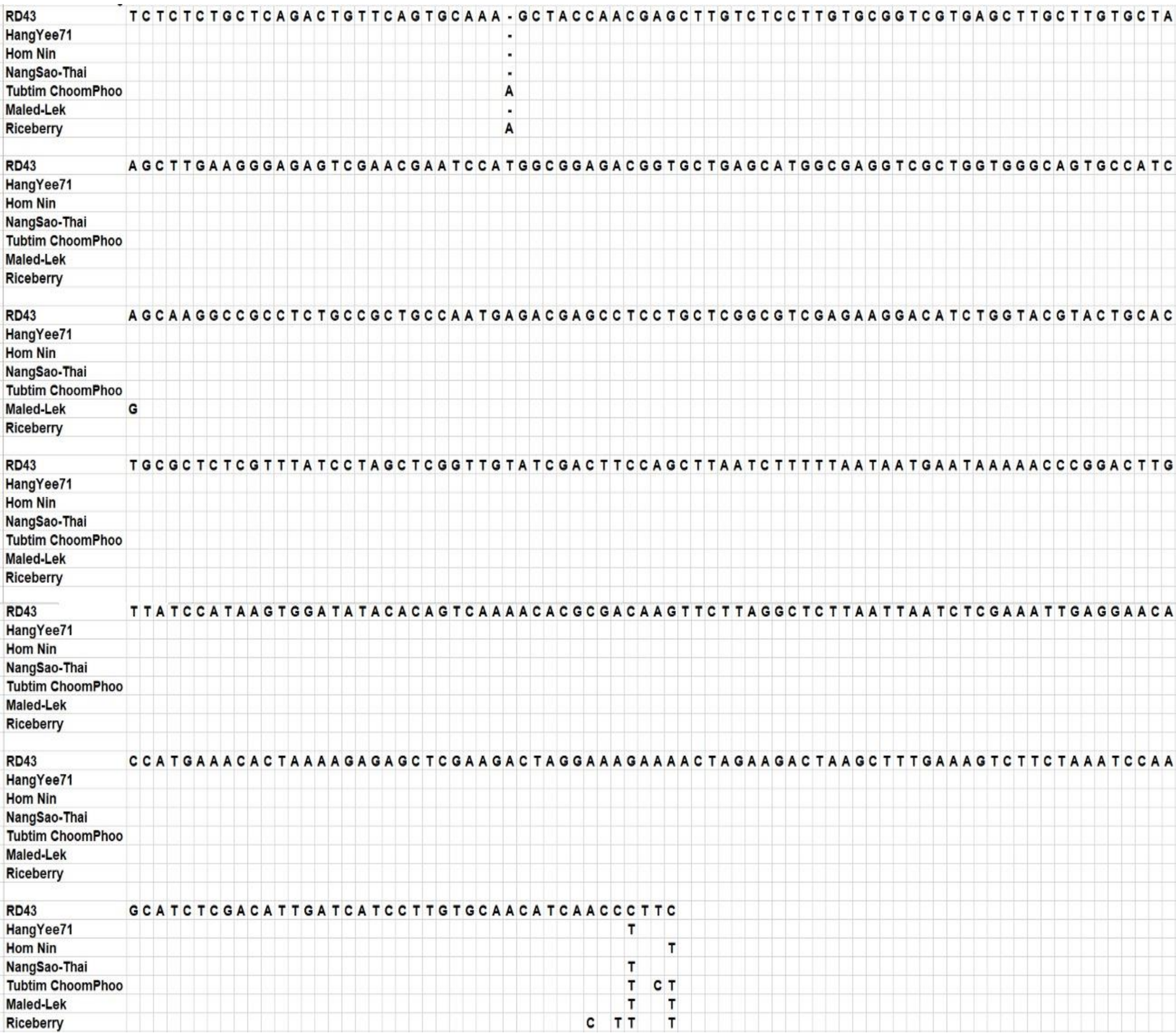

Fig. 8. *Pigm(t)* nucleotide alignment using the ClustalW program. Blank boxes represent consensus nucleotides and dashes represent nucleotide deletions in the gene.

Fig. 10. *Pia* nucleotide alignment using the ClustalW program. Dots represent consensus nucleotides and dashes represent nucleotide deletions in the gene.

Discussion

Rice is a staple food across Thailand, with sticky or waxy (glutinous) rice planted in specific areas, such as Northeastern Thailand. The necessity for rice continuously grows with population growth, but yields are limited by biotic stresses, such as rice blast disease affected by *Magnaporthe oryzae* (Kato *et al*., 2000; Couch & Kohn, 2002). *M. oryzae* is a hemibiotrophic fungus, for which the necrotrophic period is an important period because it produces *avr* proteins to damage the host tissue (Kankanala *et al*., 2007; Marcel *et al*., 2010). To date, greather than 25 *avr* genes have been determined, but only eleven have been cloned for molecular observation (Sharma *et al*., 2012). Rice can resist blast disease via blast resistance (*R*) genes. Of greather than 100 *R* genes, only 27 were cloned (He *et al*.,2022). Almost all *R* genes are located on chromosomes 6, 11 and 12, with respective frequencies of approximately 24%, 14% and 15%, respectively (Sharma *et al*., 2012). For example, *Pib* is located on chromosome 2, while *Pi2/ PiZ Pi8*, *Pi9*, *Pi13(t)*, *Pi50* and *Pigm (t)* are on chromosome 6. *Pia, Pif, Pik, Pi1, Pi7(t), Pi18(t), Pi44(t)*, and *Pi54* are located on chromosome 11 and *Pi6(t), Pi12(t), Pi19(t), Pi31(t), Pi32(t)* and *Pita*, are on chromosome 12 (Wang *et al*., 1994; Chen *et al*., 2005; Qu *et al*., 2006; Lin *et al*., 2007; Jantasuriyarat & Kate-ngam, 2009; Koide *et al*., 2009; Sharma *et al*., 2010; Bryan *et al*., 2013). Of the *R* genes, 51% and 45% are found in *O. indica* and *O. japonica* cultivars, whereas 4% are found in wild rice (Wang *et al*., 2014).

Landraces are important germplasm to preserve since they have been developed for generations to adapt to specific environments and resist yield-reducing insects and pathogens. In this study, eight resistance genes were investigated with specific primers using PCR. All landraces tested contained at least one resistance gene, as found in previous studies of Thai landraces (Phaitreejit *et al*., 2011; Poosin & Parinthawong, 2020). As noted in other research (Jia *et al*., 2004; Koide *et al*., 2009; Imam *et al*., 2014), landraces are significantly important resources for genetic improvement because they have been continuously and naturally improved to resist pathogens, resulting in *R* genes that have spontaneously evolved by several genetic mechanisms, including deletion, insertion (indels), single nucleotide polymorphisms, and genetic drift. This is supported by the level of diversity identified in the *Pi54*, *Pi9* and *Pib* genes (Figs. 1 to 6), which could be beneficial for rice breeding to improve blast resistance.

Rice with a single *R* gene (monogenic trait) is not stable enough to maintain resistance against diverse *M. oryzae* (Bonman *et al*., 1992; Zhou *et al*., 2006; Nyuget *et al*., 2019), while rice with various *R* genes via gene pyramiding has durable resistance to prevent a range of virulent strains. Some *R* genes can be related together to prevent rice blast via polygenic control, such as *Pi2* and *Pi9* linked to *Pigm*(*t*) in broad-spectrum resistant Chinese rice (Bonman *et al*., 1992; Deng *et al*., 2006). Gene pyramiding is an effective strategy to support long-term resistance against a broad spectrum of *M. oryzae*. This study identified germplasm resources to produce polygenic traits: Tubtim ChoomPhoo, followed by Hom Nin, NangSao-Thai, Maled-Lek, KhaowNeou Dum, KhaowNeouSuphan and JaoHom Deang (Table 3).

Conclusion

This research should be concluded that landraces are germplasm resources for genetic variation because they have been naturally selected over many years to survive biotic stresses and environmental changes. Rice yields are limited by blast disease, and environmentally friendly, cost-effective rice breeding programs are needed to enhance rice blast disease resistance. Because one *R* gene is not enough to provide long-term resistance, gene pyramiding using the landraces of Tubtim ChoomPhoo is a promising breeding strategy.

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