Agriculture and Natural Resources 51 (2017) 7–14

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

Original article

Evaluation of mating type distribution and genetic diversity of three *Magnaporthe oryzae* avirulence genes, *PWL-2*, *AVR-Pii* and *Avr-Piz-t*, in Thailand rice blast isolates

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ARTICLE INFO

Article history:
Received 30 May 2016
Accepted 4 August 2016
Available online 22 February 2017

Keywords:
Avirulence gene
Genetic diversity
Magnaporthe oryzae
Mating type
Rice blast disease

ABSTRACT

Rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*), has been ranked among the most important diseases of rice. The molecular mechanisms against this fungus follow the idea of "gene-for-gene interaction", in which a plant resistance (*R*) gene product recognizes a fungal avirulence (*Avr*) effector and triggers the defense response. However, the *Avr* genes have been shown to be rapidly evolving resulting in high levels of genetic diversity. This study investigated genetic diversity that is influenced by sexual recombination and mutation for the adaptation of rice blast fungus to overcome the defense response. Mating type distribution and the nucleotide sequence variation of three avirulence genes were evaluated—*PWL-2*, *Avr-Pii* and *Avr-Piz-t*. In total, 77 rice blast isolates collected from infected rice plants in northern, northeastern and central Thailand in 2005, 2010 and 2012, were used in the analysis with mating type and avirulence gene-specific primers. The results revealed that all the tested blast isolates belonged to the mating type *MAT1-2*, suggesting a lack of sexual recombination within the population. The successful rates of *PWL-2*, *Avr-Pii* and *Avr-Piz-t* gene-specific primer amplification were 100%, 60% and 54%, respectively. Base substitution mutation was observed in coding regions of the *Avr-Pii* and *Avr-Piz-t* genes. Although these results showed a low level of genetic diversity in Thai rice blast isolates, non-synonymous mutations did occur which revealed common mechanisms of selective pressure that are prone to adaptation of *Avr* genes. The information on nucleotide sequence variation and the genetic diversity of *Avr* genes obtained from this study could be useful for planning novel strategies in the development of rice breeding programs in Thailand.

Introduction

Magnaporthe oryzae (anamorph *Pyricularia oryzae*), a filamentous ascomycete fungus, is an important plant pathogen, causing blast disease (Couch and Kohn, 2002). Rice blast disease is one of the most distributed plant diseases being reported world-wide (Zeigler, 1998). Blast fungus has a hemibiotrophic phase in its life cycle (Koeck et al., 2011), which can infect many growth stages of rice and causes up to 100% loss of rice production in infected areas (Zeigler et al., 1994). The genome of *M. oryzae* has been sequenced, with a predicted 11,109 genes, with a frequency of 1 gene for every 3.5 kb in the rice blast genome (Dean et al., 2005).

Previous studies revealed that rice blast fungus has a high level of genetic instability and evolves rapidly in nature (Couch and Kohn, 2002; Huang et al., 2014). It can adapt itself to new resistance genes and selection pressures within a few years after being released (Huang et al., 2014). The fungal adaptation usually involves sexual recombination and genetic mutation and genetic recombination through sexual reproduction plays an important role by increasing genetic diversity (Debuchy and Berteaux-Lecellier, 2010). The fusion of mycelia between strains of opposite mating types leads to the production of sexual spores (ascospores), which sometimes generates new virulence forms of the fungus (Zeigler

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http://dx.doi.org/10.1016/j.anres.2016.08.005

et al., 1994). The mating type of rice blast fungus is controlled by a single locus with two alleles, *MAT1-1* and *MAT1-2* (Debuchy and Berteaux-Lecellier, 2010).

The rice and rice blast fungus system has been developed as an effective model for the study of gene-for-gene interactions. The first evidence to support this concept was revealed by the direct interaction between the *Pita* protein and the *Avr-Pita* effector more than a decade ago (Jia et al., 2000). To date, 25 *Avr* genes of *M. oryzae* have been genetically mapped in the rice blast genome (Dioh et al., 2000). Among them, 11 *Avr* genes (*PWL1*, *PWL2*, *AVR-Pita*, *AVR1-CO39*, *ACE1*, *AVR-Pizt*, *AVR-Pia*, *AVR-Pii*, *AVR-Pik/km/k*, *AVR-Pi9* and *Avr-Pib)* have been cloned and characterized (Kang et al., 1995; Sweigard et al., 1995; Orbach et al., 2000; Farman et al., 2002; Fudal et al., 2005; Li et al., 2009; Miki et al., 2009; Yoshida et al., 2009; Wu et al., 2014).

PWL2 (pathogenicity toward Weeping Love grass) is a member of the family PWL, which consists of four genes—*PWL1*, *PWL2*, *PWL3* and *PWL4*—(Sweigard et al., 1995). The *PWL2* gene encodes a glycine-rich, hydrophobic protein that appears to localize in the biotrophic interfacial complex (BIC), a novel interfacial structure associated with the first hyphal cell to invade the host cell (Khang et al., 2010). Amino acid alterations in the *PWL2* gene of the *PWL2D* mutant have been shown to have an important role in changing fungal virulence (Schneider et al., 2010). *AVR-Pii* encodes a small 70 amino acid protein, which can be recognized by the *Pii* resistance gene product inside cells of rice plants (Silva et al., 2004; Yoshida et al., 2009). Previous studies revealed that a loss of the *AVR-Pii* gene led to a change from an avirulent to a virulent fungal strain (Silva et al., 2004; Yasuda et al., 2006). In addition, the *AVR-Pii* gene is located on chromosome 7 in a highly unstable chromosome segment (Yasuda et al., 2006; Yoshida et al., 2009). Silva et al. (2004) and Rehmeyer et al. (2006) reported that *AVR-Pia* and *AVR-Pii* were located in unstable genomic regions which suggested a high possibility of gene loss and horizontal transfer events. *AvrPiz-t* is recognized by *Piz-t*. It encodes a 108 amino acid polypeptide which functions to suppress the pathogen-associated molecular pattern (PAMP) and triggers the immune system by inhibiting the ubiquitin ligase activity (Li et al., 2009; Park et al., 2012). The insertion of the *Pot3* transposon in the promoter region of the *AvrPiz-t* gene in the GUY11 strain led to a lack of its virulent function (Li et al., 2009). These *Avr* genes are important for the recognition of host resistance gene product in order to activate a host defense response.

The objective of this study was to evaluate the genetic diversity of three avirulence genes—*PWL2*, *Avr-Pii* and *Avr-Piz-t*—and the frequency of the different mating types of Thai rice blast isolates using gene specific markers. The data obtained from this study can be used to prepare a rice-blast-resistance breeding program for Thailand in the future.

Materials and methods

Fungal materials

In total, 77 rice blast isolates were obtained from the rice blast fungus genetic stock at the National Center for Genetic Engineering and Biotechnology (BIOTEC, Bangkok, Thailand), the Department of Agronomy, Kasetsart University (Bangkok, Thailand) and King Mongkut's Institute of Technology Ladkrabang (Samut Prakan, Thailand) as shown in Table 1 These rice blast isolates were collected from the infected leaves of rice with typical blast disease symptoms from central, northern and northeastern Thailand in 2005, 2010 and 2013 (Fig. 1).

Fungal culture and storage

Each fungal isolate was cultured in a Petri dish containing rice flour agar medium (RFA; 2.0% rice flour, 2.0% agar and 0.2% yeast extract and 1 L dH_2O) at room temperature under fluorescence lighting to produce mycelia. After 7 d incubation, the mycelia were transferred into a new RFA Petri dish whose surface was covered with filter paper for 7–14 d. The filter papers were dried in desiccators and were cut into 1 cm × 1 cm pieces. Each isolate was stored at −20 °C in a freezer for permanent stock and at 4 °C for working stock.

DNA preparation and extraction

Each rice blast isolate was cultured in a 50 mL plastic tube containing potato dextrose broth with shaking at 200 rpm and 28 °C for 7 d. Fungal mycelia were filtrated through Whatman no.1 filter paper. Total genomic DNA was extracted using liquid nitrogen and the cetyltrimethylammonium bromide (CTAB) method (10 mM Tris–HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 2% sodium dodecyl sulfate) and incubated at 65 °C for 60 min. The DNA was purified using the classic chloroform:isoamyl alcohol (24:1) method (Doyle and Doyle, 1987) and was precipitated by adding the same amount of cold iso-propanol and then incubated overnight at 4 °C. After incubation, the DNA was centrifuged at 12,000 rpm and 4 °C for 30 min and washed twice with 95% and 70% ethanol, respectively. RNAse (10 μg/mL) was added into the dissolved DNA to remove the RNA. Each DNA sample was quantified using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Mating-type determination by the polymerase chain reaction (PCR)

The DNA samples of the *M. oryzae* 70-15 *(MAT1-1)* and GUY11 *(MAT1-2)* strains were extracted and used as the reference strains to determine the mating type in this study. Screening of gene encoding for the mating-type was performed using polymerase chain reaction (PCR) with a gene-specific marker for the mating-type gene in rice blast fungus (Table 2). One microliter of 50 ng genomic DNA was used as the template in a 10 μL PCR. The PCR mixture contained 1 U of *Taq* DNA polymerase, 1 μL of 10× PCR buffer, 1 μL of 2.5 mM $MgCl_2$, 1 μL of 1 mM dNTPs and 0.5 μL of each 5 μM primer. The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 56 °C for 15 s, and 30 s of extension at 72 °C and a final extension at 72 °C for 5 min. The PCR products were resolved using 1.5% agarose gel electrophoresis in 0.5 × TBE (Tris-borate-EDTA) buffer and the sizes of the amplified fragments were estimated using a 100 bp Gene Ruler™ Express DNA ladder (Fermentas Inc., Glen Burnie, MD, USA), stained with ethidium bromide, visualized and photographed using an infinity 3000 gel photographic system (Vilber Lourmat, Eberhardzell, Germany).

Polymerase chain reaction amplification and DNA sequencing

One primer pair was used to identify the presence and the quality of rice blast fungus genomic DNA—IDMF: 5′-GACCTATGCAATCAC CAC-3′ and IDMR: 5′-CGTACTCGAGTGTAATCTCG-3′—which was designed from the rice blast fungus-specific DNA sequence (Kasetsomboon et al., 2013). The molecular characterization of *M. oryzae* isolates was performed using PCR with a gene-specific primer of the three avirulence genes (*PWL2*, *Avr-Pii* and *Avr-Piz-t*)

Table 1
Details of Thai rice blast isolates, mating-type alleles and presence/absence of three avirulence genes.

Code	Location	Year	Reference[a]	Mating-type[b]	*PWL-2*[c]	*AVR-Pii*[c]	*AVR-Piz-t*[c]
70-15	–	–	–	–	A	–	A
GUY11	–	–	–	+	A	–	A
BCC10100	Sa Kaeo	2006	BIOTEC	+	A	–	–
BCC10301	Si Sa Ket	2006	BIOTEC	+	A	A	–
BCC10302	Si Sa Ket	2006	BIOTEC	+	A	A	A
BCC10459	Lampang	2006	BIOTEC	+	A	A	–
BCC10551	Nakhon Ratchasim	2006	BIOTEC	+	A	A	–
BCC10552	Nakhon Ratchasim	2006	BIOTEC	+	A	–	A
BCC10576	Kalasin	2006	BIOTEC	+	A	A	A
BCC10577	Nakhon Ratchasim	2006	BIOTEC	+	A	A	–
BCC10578	Nakhon Ratchasim	2006	BIOTEC	+	A	A	A
BCC10581	Nakhon Ratchasim	2006	BIOTEC	+	A	A	–
BCC10652	Maha Sarakham	2006	BIOTEC	+	A	–	–
BCC10681	Roi Et	2006	BIOTEC	+	A	–	–
BCC10694	Nong Khai	2006	BIOTEC	+	A	A	–
BCC10732	Kamphaeng Phet	2006	BIOTEC	+	A	A	A
BCC10760	Kamphaeng Phet	2006	BIOTEC	+	A	A	–
BCC10812	Chiang Rai	2006	BIOTEC	+	A	A	–
BCC10837	Surin	2006	BIOTEC	+	A	A	–
BCC10873	Mae Hong Son	2006	BIOTEC	+	A	A	A
BCC10926	Phayao	2006	BIOTEC	+	A	–	–
BCC10927	Phayao	2006	BIOTEC	+	A	A	–
BCC10941	Lampang	2006	BIOTEC	+	A	A	–
BCC10945	Chiang Mai	2006	BIOTEC	+	A	–	–
BCC10971	Nan	2006	BIOTEC	+	A	A	–
BCC10985	Si Sa Ket	2006	BIOTEC	+	A	A	A
BCC10993	Buri Ram	2006	BIOTEC	+	A	–	–
BCC11100	Udon Thani	2006	BIOTEC	+	A	A	A
BCC11108	Ubon Ratchathani	2006	BIOTEC	+	A	A	–
BCC11109	Ubon Ratchathani	2006	BIOTEC	+	A	–	–
BAG1.1	Phitsanulok	2010	AKU	+	A	A	A
BAG2.3	Ubon Ratchathani	2010	AKU	+	A	A	A
BAG3.1	Phitsanulok	2010	AKU	+	A	A	A
BAG4.1	Phitsanulok	2010	AKU	+	A	A	A
BAG5.1	Khon Kaen	2010	AKU	+	A	A	A
BAG6.5	Khon Kaen	2010	AKU	+	A	A	A
BAG8.1	Ubon Ratchathani	2010	AKU	+	A	–	A
BAG9.1	Nong Khai	2010	AKU	+	A	A	A
BAG11.1	Nong Khai	2010	AKU	+	A	A	A
BAG13.2	Nong Khai	2010	AKU	+	A	A	–
BAG14.2	Nong Khai	2010	AKU	+	A	A	A
BAG15.1	Udon Thani	2010	AKU	+	A	–	A
BAG16.1	Udon Thani	2010	AKU	+	A	–	A
BAG17.2	Chaiyaphum	2010	AKU	+	A	–	A
BAG18.1	Udon Thani	2010	AKU	+	A	A	A
BAG19.2	Udon Thani	2010	AKU	+	A	–	A
BAG20.2	Udon Thani	2010	AKU	+	A	A	A
BAG21.2	Udon Thani	2010	AKU	+	A	A	A
BAG22.1	Udon Thani	2010	AKU	+	A	A	A
BAG23.2	Udon Thani	2010	AKU	+	A	A	A
BAG24.1	Chaiyaphum	2010	AKU	+	A	–	A
BAG25.1	Udon Thani	2010	AKU	+	A	–	A
BAG26.1	Nong Khai	2010	AKU	+	A	–	A
BAG27.1	Nong Khai	2010	AKU	+	A	–	A
BAG28.1	Nong Khai	2010	AKU	+	A	A	A
BAG29.2	Udon Thani	2010	AKU	+	A	–	A
BAG32.1	Nong Khai	2010	AKU	+	A	–	A
SRN54001	Surin	2011	KKMITL	+	A	A	–
SRN54002	Surin	2011	KKMITL	+	A	A	A
SRN54005	Surin	2011	KKMITL	+	A	A	–
SRN54006	Surin	2011	KKMITL	+	A	A	–
SRN54007	Surin	2011	KKMITL	+	A	–	–
SRN54009	Surin	2011	KKMITL	+	A	A	–
CPM55001	Chaiyaphum	2012	KKMITL	+	A	–	–
CPM55002	Chaiyaphum	2012	KKMITL	+	A	–	–
CPM55003	Chaiyaphum	2012	KKMITL	+	A	A	–
NYK55001	Nakhon Nayok	2012	KKMITL	+	A	A	–
NYK55003	Nakhon Nayok	2012	KKMITL	+	A	–	–
RBR55001	Ratchaburi	2012	KKMITL	+	A	–	A
RBR55002	Ratchaburi	2012	KKMITL	+	A	A	A
RBR55003	Ratchaburi	2012	KKMITL	+	A	–	–
RBR55004	Ratchaburi	2012	KKMITL	+	A	–	–
CCOO56001	Chachoengsao	2013	KKMITL	+	A	–	A

(*continued on next page*)

Table 1 (*continued*)

Code	Location	Year	Reference[a]	Mating-type[b]	*PWL-2*[c]	*AVR-Pii*[c]	*AVR-Piz-t*[c]
CCOO56002	Chachoengsao	2013	KKMITL	+	A	–	A
CCOO56003	Chachoengsao	2013	KKMITL	+	A	A	–
CCOO56004	Chachoengsao	2013	KKMITL	+	A	–	–
BKK55001	Bangkok	2012	KKMITL	+	A	A	A
BKK55002	Bangkok	2012	KKMITL	+	A	–	A
BKK55003	Bangkok	2012	KKMITL	+	A	–	A

[a] BIOTEC = National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand; AKU = Department of Agronomy, Kasetsart University, Kampheang Saen, Thailand; KMITL = King Mongkut's Institute of Technology Ladkrabang, Samut Prakan, Thailand.
[b] (–) represents *MAT1-1*, (+) represents *MAT1-2*.
[c] Signal in PCR amplification: A = presence of avirulence gene; – = absence of avirulence gene.

as shown in Table 2. These specific primers were designed using the Launch NetPrimer program (http://www.premierbiosoft.com/netprimer/netpr-launch/Help/xnetprlaunch.html) to amplify the coding sequences. The PCR product was amplified in a total volume of 20 μL reaction mixture consisting of 50 ng of genomic DNA, 1 U of *Taq* DNA polymerase, 1 × Intron PCR buffer, 20 mM $MgCl_2$, 10 mM dNTPs and 1 μL of each 5 μM primer. The PCR cycling program consisted of initial denaturation for 2 min at 94 °C, 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing depending on each primer pair and 50 s of extension at 72 °C and a final extension at 72 °C for 5 min. Amplified products were separated using electrophoresis in 1.5% agarose gels and detected using ethidium bromide staining. The PCR products were purified with a Qiaquick gel extraction kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. After purification, the PCR products were submitted for sequencing by Macrogen Inc. (Seoul, Republic of Korea).

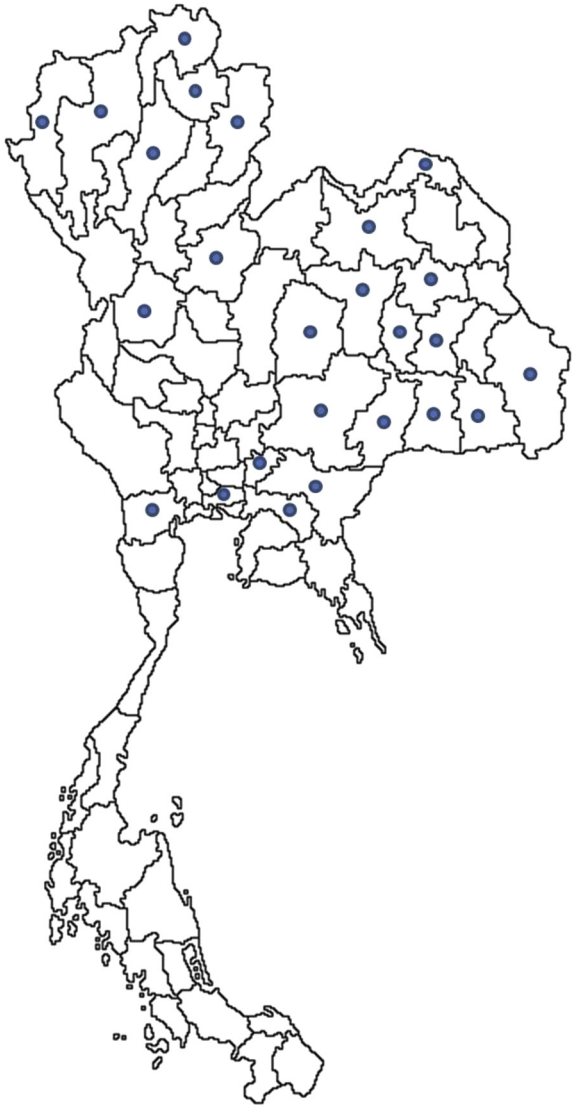

Fig. 1. Geographic distribution of *Magnaporthe oryzae* isolates from Thailand used in this study.

Data analysis

For sequence analysis, the nucleotide sequences were aligned using the ClustalW program (Thompson et al., 1994) with reference sequences obtained from the GeneBank/EMBL/DDBJ databases (accession numbers U26313.1, AB498874.1 and EU837058.1). Haplotype diversity was calculated with the DnaSP (DNA Sequence Polymorphism) version 5.0 program (Barcelona, Spain) to indicate the polymorphic site. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) in the MEGA program, version 6 (Kumar et al., 2004) using bootstrap analysis with 1000 replicates.

Results

Distribution of mating-type in Thai rice blast population

Among the 77 Thai rice blast isolates collected from rice variety KDML105 in central, northern and northeastern Thailand, there was no amplification by the *MAT1-1* primer pair except for the reference strain (70-15) with an amplicon size of 809 bp. All isolates were confirmed by *MAT1-2* primer pair amplification with an amplicon size of 940 bp. The results showed that only the 70-15 strain had mating type *MAT1-1*. In contrast, the *MAT1-2* allele was found in all 77 isolates and in the Guy11 strain (Fig. 2B and C). The results revealed that 100% of Thai rice blast isolates in this study exhibited the *MAT1-2* allele (Table 1).

Presence of PWL-2, Avr-Pii and Avr-Piz-t in M. oryzae from Thailand

To identify the diversity of the three avirulence genes, three specific primer pairs were designed and used in this study (Table 2). The coding sequences of the *PWL-2* gene (614 bp) were successfully amplified in all isolates. The coding sequences of the *Avr-Pii* gene (534 bp) and the *Avr-Piz-t* gene (642 bp) were able to amplify from 46 (60%) and 42 (54%) blast isolates, respectively (Table 1 and Fig. 2). One possible explanation for the PCR amplification failure was nucleotide substitution of the coding gene or deletion/insertion within the gene region or at the primer binding site. The gene frequency distribution in each year was calculated. The frequency distribution analysis showed the loss of the *Avr-Pii* gene frequency

Table 2
Gene-specific PCR primers used in this study.

Primer Name	Primer sequences (5′–3′)	Annealing temperature (°C)	Expected size (bp)	Reference
MAT1-1	F: TCAGCTCGCCCAAATCAACAAT R: ACTCAAGACCCGGCACGAACAT	56	809	Samanta et al., 2014
MAT1-2	F: GAGTTGCCTGCCCGCTTCTG R: GGCTTGGTCGTTGGGGATTGT	56	940	Samanta et al., 2014
IDM	F: GACCTATGCAATCACCAC R: CGTACTCGAGTGTAATCTCG	56	512	Kasetsomboon et al., 2013
PWL-2	F: TCTTCACAGCTCCCAATTAC R: CAGGCATACGTTGGAGAACC	60	614	In this study
AVR-Pii	F: TTATGCAGGCCCAAATCCG R: GAAATTCCCGCAATAGTCC	57	534	In this study
AVR-Piz-t	F: CCGTCACTTTCATTCTCCAGC R: TGGACCTAAGTCGCAAGCCTC	60	642	In this study

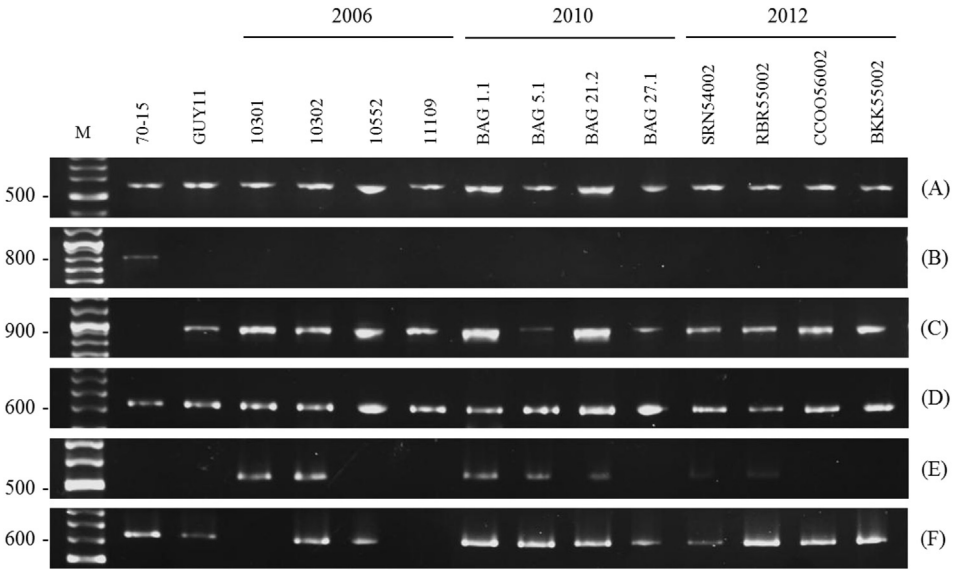

Fig. 2. Ethidium bromide-stained agarose gel showing representative results of polymerase chain reaction *Avr* gene product from genomic DNA amplified with IDM primer (A), *MAT1-1* primer (B), *MAT1-2* primer (C), *PWL-2* primer (D), *Avr-Pii* primer (E) and *Avr-Piz-t* primer (F).

from 2006 until 2012; 71% in 2006 (20 out of 28), 59% in 2010 (16 out of 27) and 45% in 2012 (10 out of 22) as shown in Table 3 and Fig. 3. The frequency distribution of the *Avr-Piz-t* gene has changed each year since being 96% in 2010 (26 out of 27).

Nucleotide sequence analysis of three avirulence genes in Thai rice blast isolates

To characterize the nucleotide variation, the coding sequences of the three avirulence genes (*PWL-2*, *Avr-Pii* and *AvrPiz-t*) were aligned with reference sequences—isolates 4392-1-6 for *PWL-2*, Ina168 for *Avr-Pii* and 81278ZB15 for *AvrPiz-t* (accession numbers U26313.1, AB498874.1 and EU837058.1, respectively). The alignment began from the first position at the start, to the stop codon. The results showed that the coding sequences of the *PWL-2* gene were identical in all 77 isolates. The coding sequence of the *Avr-Pii* gene from 46 isolates revealed three different haplotypes with 11 nucleotide polymorphic sites (55, 56, 58, 65, 75, 88, 90, 91, 92, 103 and 106), including one major haplotype (44 out of 46 isolates) and two minor haplotypes with a single isolate each. The haplotype diversity index (0.0842) indicated that the number of polymorphic sites was due to the differences between two minor haplotypes. The 11 nucleotide polymorphic sites of the *Avr-Pii* gene can be classified into nine non-synonymous sites that led to amino acid substitution and two synonymous substitutions. These results revealed a strong signal of positive selection within this gene.

The coding sequence alignment of the *Avr-Piz-t* gene from 42 isolates revealed nine nucleotide polymorphic sites (4, 6, 212, 240, 261, 270, 291, 312 and 318) resulting in four different haplotypes including one major haplotype (39 out of 42 isolates) and three

Table 3
Frequency of presence/absence and haplotype diversity in three avirulence genes on 77 Thai rice blast isolates.

Avr gene	Absence of avirulence gene	Presence of avirulence gene (%)				Haplotype diversity
		2006	2010	2012	Total	
PWL-2 (614 bp)	–	28 (36%)	27 (35%)	22 (29%)	77 (100%)	1
AVR-Pii (534 bp)	31	20 (26%)	16 (21%)	10 (13%)	46 (60%)	3
AVR-Piz-t (642 bp)	35	8 (10%)	26 (34%)	8 (10%)	42 (54%)	4

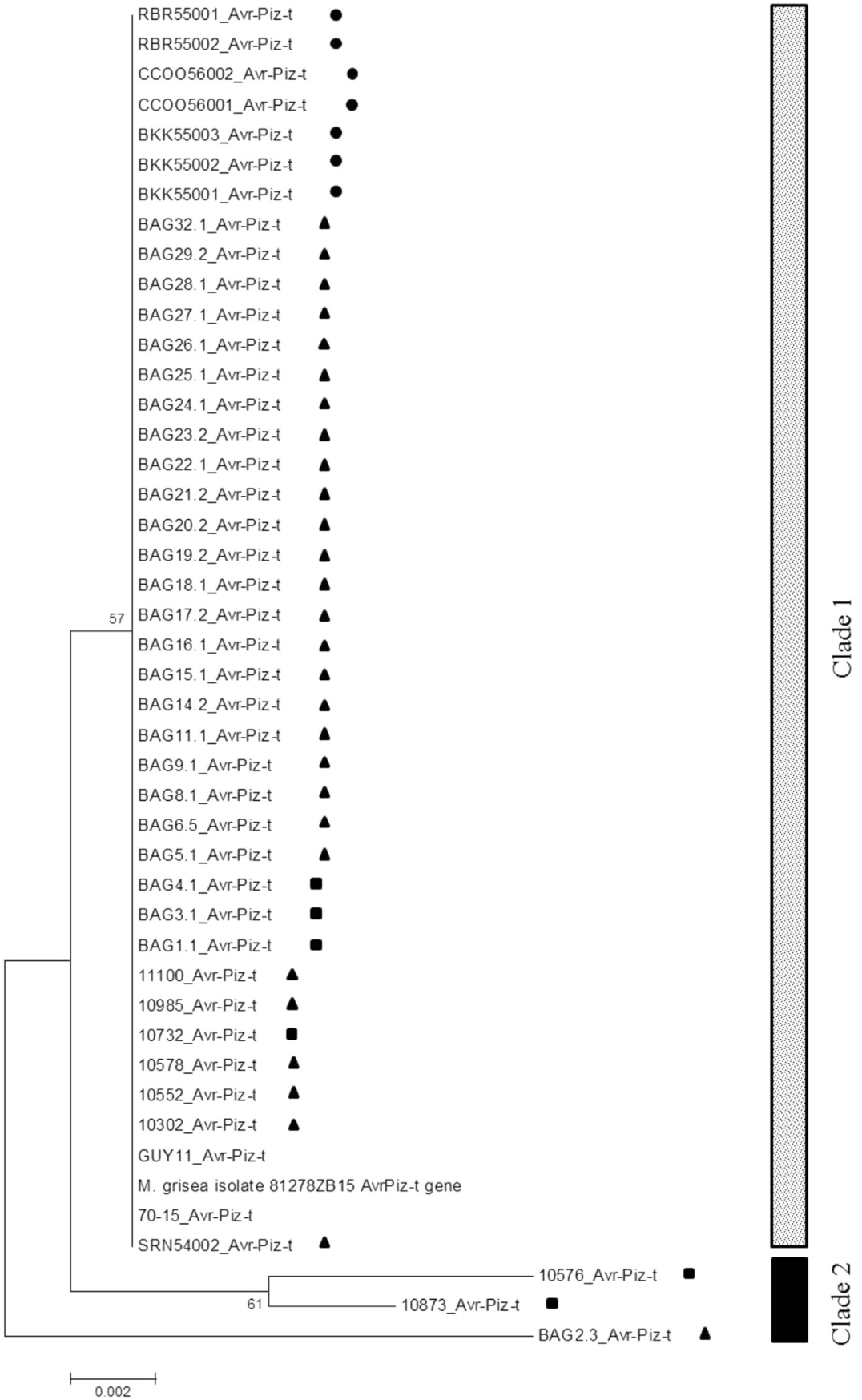

Fig. 3. Neighbor-joining tree of *Avr-Piz-t* gene of the 42 rice blast isolates constructed from nucleotide coding sequences with reference sequence using 1000 bootstraps. Circles (●) represent blast isolates from central of Thailand, squares (■) represent blast isolates from northern Thailand, triangles (▲) represent blast isolates from northeastern Thailand.

minor haplotypes with a single isolate each. The haplotype diversity index (0.1303) showed a low level of genotypic diversity. Only three non-synonymous mutations were found. The ratio between non-synonymous and synonymous polymorphic sites was less than 1, which indicated that the *Avr-Piz-t* gene was not under selection pressure. All the results indicated a low level of genetic diversity of the *Avr-Pii* and *Avr-Piz-t* genes in the Thai rice blast isolates.

Phylogenetic analysis of the M. oryzae isolates

To clarify the genetic diversity relationships among the 77 blast isolates in Thailand, a phylogenetic tree was constructed using neighbor-joining statistical analyses based on the coding sequences of two avirulence genes (*Avr-Pii* and *Avr-Piz-t*). The *PWL2* gene was not used for the analysis because of the lack of polymorphism. The results showed that the phylogenetic tree based on the *Avr-Pii* sequences of the 46 blast isolates were mostly clustered together in one group except for two isolates from Nong Kai in 2010 (data not shown). The phylogenetic tree based on the *Avr-Piz-t* sequences of the 42 blast isolates revealed two clusters and one out group, which was an isolate from Ubon Ratchathani in 2010. One cluster contained 39 isolates and the reference sequence and the other cluster was composed of two isolates from Lampang and Mae Hong Son in 2005. The phylogenetic analysis of Thai rice blast isolates based on the *Avr-Pii* and *Avr-Piz-t* sequences confirmed that the rice blast isolates included in this study had a low level of genetic diversity based on either sample collection date or geographical location. There were 31 and 35 rice blast isolates for *Avr-Pii* and *Avr-Piz-t*, respectively, that could not be amplified in this study. This suggested that either these isolates did not contain *Avr-Pii* and *Avr-Piz-t* genes or the primer binding sites of these isolates had been changed by mutation.

Discussion

Blast disease caused by *M. oryzae* is one of the most devastating diseases of rice worldwide. One of the most effective strategies to control this disease is the utilization of appropriate resistant cultivars that have major resistance genes against and recognize *Avr* genes following the “gene for gene concept” (Flor, 1971). However, the *Avr* genes have been shown to be rapidly evolving resulting in a high level of genetic diversity because of sexual recombination and mutation. Thus, mating type distribution and mutation analysis in blast fungal populations are important in understanding the genetic diversity.

This study showed that Thai rice blast isolates exhibited only one mating type namely, *MAT1-2*, which indicated a low level of genetic diversity. These results were similar to Park et al. (2008) and Urak et al. (2008), who revealed only one mating type namely, *MAT1-1*, in 254 Korean rice blast isolates and 160 North Central California isolates, respectively. In addition, Notteghem and Silue (1992) found that all 467 rice blast isolates from 34 countries in Europe and Africa were the *MAT1-1* mating type. These results indicated that rice blast fungus in the field lacks sexual reproduction and recombination, which means that the pathogen reproduces through asexual reproduction. Thus, mutation was the only factor that affected the genetic diversity of these populations. However, Samanta et al. (2014) found both mating types from 46 Indian rice blast isolates with 70% *MAT1-1* and 30% *MAT1-2* alleles. Bao-Hua et al. (2004) reported that 150 isolates from Fujian, China showed 83% *MAT1-2* and 17% *MAT1-1*. Therefore, the factors of genetic diversity in these populations were a consequence of both sexual recombinations and mutations.

Evaluation of genetic variation is a major molecular mechanism for understanding the evolution of *Avr* genes and the co-evolution of *M. oryzae* and rice. Previous studies have reported the high instability of several *Avr* genes, which are closely located to unstable chromosomes in telomere regions including *Avr-Pita*, *Avr-Pik*, *Avr-Pia* and *Avr-Pii* (Yoshida et al., 2009; Dai et al., 2010; Chuma et al., 2011). Moreover, the insertion of a transposable element at either the promoter or coding region led to new virulence alleles. For example, a 1.9 kb MINE retrotransposon was inserted in the last exon of the *ACE1* gene (Fudal et al., 2005). The Pot3 element was inserted in the *Avr-Piz-t* promoter region and in *Avr-Pita* in both the promoter and coding regions (Kang et al., 2001; Li et al., 2009). However, the current results revealed that the *PWL-2* gene in Thai rice blast isolates had no genetic variation or genetic diversity. Similar results from 62 Chinese blast isolates showed close genetic diversity and no differences in geographical locations (Huang et al., 2014). The low level of genetic variation of the *PWL-2* gene was probably due to the important function of the *PWL-2* gene product, which plays a role in rice cell infection and fungal movement from an infected cell to the neighboring un-invaded cells (Khang et al., 2010). The loss of *Avr-Pii* frequency in Thai rice blast isolates indicated changes of the genome in the fungal population. Previous studies revealed that *AVR-Pii* had a high level of genetic variation due to insertion of multiple translocations in the genome (Yoshida et al., 2009; Chuma et al., 2011). *Avr-Piz-t* revealed that the loss/gain of gene frequency could be a consequence of arms-race evolution, suggesting *Avr-Piz-t* has co-evolved with resistance genes corresponding to the gene-for-gene concept.

From the analysis of the coding sequences of three *Avr* genes (*PWL-2*, *Avr-Pii* and *Avr-Piz-t*), the current results showed that the Thai rice blast isolates had a low level of genetic diversity. These findings were similar to those reported by Chen et al. (2014) which revealed low diversity in the *Avr-Piz-t* ORF region in Chinese blast isolates. Although both *Avr-Pii* and *Avr-Piz-t* were revealed to have low levels of nucleotide polymorphism, *Avr-Pii* showed high numbers of non-synonymous substitutions—more so than synonymous sites—for which Ka > Ks, indicating that *Avr-Pii* was under strong, positive selection and these variations might be responsible for new adaptations, while *Avr-Piz-t* was found only in three non-synonymous substitutions indicating that it was not under positive selection. This result suggested that selective pressure was a common mechanism for rapid adaptation and genetic variation of *Avr* genes. Similarly a recent study by Kasetsomboon et al. (2013) reported high genetic diversity of the *Avr-Pita* coding region containing 15 haplotypes among 30 Thai blast isolates was under positive selection pressure. From these findings, it is suggested that the differences in the *Avr* genes might result in different adaptation rates based on the role of selection occurring in nature.

In conclusion, this study demonstrated a low distribution rate of sexual recombination and genetic diversity in Thai rice blast isolates. These findings presented a different pattern of genetic diversity within each *AVR* gene. Lastly, this information will be crucial for understanding the diversity of pathogenic populations and pathogenic selection, which can lead to novel strategic development for a co-evolutionary relationship between the *R* and *Avr* genes in rice breeding programs.

Conflict of interest

There is no conflict of interest.

Acknowledgements

The authors thank Mr. R. Harman II for critically and grammatically reviewing the manuscript. This project was supported by

the Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand. Miss Thanyaluk Sirisathaworn received financial support to conduct part of this experiment from the International Affairs Division, Kasetsart University, Bangkok, Thailand.

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