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ORIGINAL ARTICLE

Sequence variation of avirulence gene *AVR-Pita1* in rice blast fungus, *Magnaporthe oryzae*

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Abstract The interaction between rice, *Oryza sativa*, and rice blast fungus, *Magnaporthe oryzae*, is triggered by an interaction between the protein products of the host resistant gene, and the pathogen avirulence gene. This interaction follows the 'gene-for-gene' concept. The resistant gene has effectively protected rice plants from rice blast infection. However, the resistant genes usually break down several years after the release of the resistant rice varieties because the fungus has evolved to new races. The objective of this study is to investigate the nucleotide sequence variation of the *AVR-Pita1* gene that influences the adaption of rice blast fungus to overcome the resistant gene, *Pi-ta*. Thirty rice blast fungus isolates were collected in 2005 and 2010 from infected rice plants in northern and northeastern Thailand. The nucleotide sequences of *AVR-Pita1* were amplified and analyzed. Phylogenetic analysis was conducted using the MEGA 5.0 program. The results showed a high level of nucleotide sequence polymorphisms and the positive genetic selection pressure in Thai rice blast isolates. The details of sequence variation analysis were described in this article. The information from this study can be used for rice blast resistant breeding program in the future.

Keywords Nucleotide variation · Rice blast disease · Avirulence gene · Rice blast fungus

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (GenBank ID: JQ409300–JQ409329).

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Introduction

Rice blast disease, caused by the ascomycete fungal pathogen *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) (Couch and Kohn 2002; Kato et al. 2000), is generally considered the most important rice disease worldwide. The disease could result in substantial grain yield loss (Zeigler et al. 1994). The major methods currently used for disease control include fungicides, resistant varieties and cultural practice. The use of fungicides is effective but can cause environmental pollution. While cultural practice can be effective, it has limitations because the specific rice blast virulent isolates that infest in any particular year cannot be predicted. The use of host resistance remains the most cost-effective method for disease management strategy (Hulbert et al. 2001).

Rice blast resistant genes (*Pi* genes) have been identified and incorporated into rice cultivars for managing rice blast disease throughout the world (Zhou et al. 2007). The *Pi* genes are effective at protecting against infection of *M. oryzae* races that contain corresponding avirulence genes (*AVR* genes) (Silué et al. 1992). Thus far, more than 80 *Pi* genes for blast resistance have been reported and some of them have been used to control blast disease (Ballini et al. 2008). Eleven *Pi* genes have been cloned—namely, *Pi-ta* (Bryan et al. 2000), *Pi-b* (Miyamoto et al. 1996; Wang et al. 1999), *Pi-2*/*Pi-zt* (Zhou et al. 2006), *Pi-d2* (Chen et al. 2006), *Pi-9* (Qu et al. 2006), *Pi-36* (Liu et al. 2007), *Pi-37* (Lin et al. 2007), *Pikm* (Ashikawa et al. 2008), *Pi-d3* (Shang et al. 2009), *Pi-5* (Lee et al. 2009) and *Pi-t* (Hayashi and Yoshida 2009). These cloned *Pi* genes encode the putative cytoplasmic nucleotide binding site and leucine rich repeat (NBS-LRR) proteins with the exception of the *Pi-d2* product, which is a putative transmembrane β-lectin-kinase (Chen et al. 2006).

The resistant genes have effectively controlled rice blast disease; however, they usually break down several years after the release of resistant rice cultivars (Lauge and De Wit 1998, Tosa et al. 2005). It has been hypothesized that the ability of *AVR* genes to defeat *R* genes is caused by instability and/or high levels of variation of the *AVR* genes (Khang et al. 2008). To date, 25 *AVR* genes in *M. oryzae* have been described (Dioh et al. 2000) and nine *AVR* genes have been cloned and characterized—namely, *AVR-Pita* (Orbach et al. 2000), *AVR-CO39* (Farman and Leong 1998), *PWL1* (Kang et al. 1995), *PWL2* (Sweigard 1995), *ACE1* (Fudal et al. 2005), *AVR-Pizt* (Li et al. 2009), *AVR-Pia*, *AVR-Pii*, and *AVR-Pik*/*km*/*kp* (Yoshida et al. 2009). The *AVR-Pita1* gene, located in the telomeric region of chromosome 3 of *M. oryzae* genome, encodes for a putative zinc metalloprotease protein (Orbach et al. 2000). The *AVR-Pita1* gene product is recognized by the *Pi-ta* resistant gene product from rice (Jia et al. 2000). The *Pi-ta* resistant gene has been regularly used in rice breeding programs worldwide to control rice blast disease (Jia et al. 2004; Moldenhauer et al. 1990). It encodes the predicted putative cytoplasmic NBS-LRR protein which triggers the immune response that follows the 'gene-for-gene' concept. However, epidemics of rice blast disease have occurred on the *Pi-ta* gene containing rice cultivars, suggesting that it has been defeated (McDowell and Woffenden 2003; Dai et al. 2010). Recently, many studies have shown that the structural variation of *AVR-Pita1* alters *Pi-ta*-specific recognition, for example, a frame-shift mutation in the first exon of *AVR-Pita1*, which creates a premature stop codon after the 41st amino acid; partial or complete deletion of *AVR-Pita1*; Pot3 transposon insertion in the coding region corresponding to the *AVR-Pita1* protease motif; and base substitutions in the *AVR-Pita1* coding sequence (Kang et al. 2001; Zhou et al. 2007; Takahashi et al. 2010). The objective of this present study was to examine the sequence variation of *AVR-Pita1* in Thai rice blast fungus isolates. Thirty isolates from northern and northeastern Thailand were collected in 2005 and 2010. The *AVR-Pita1* gene from Thai rice blast isolates was cloned and sequenced. Sixty *AVR-Pita1* sequences previously reported in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) were also downloaded and compared. The analysis of the polymorphism patterns based on the DNA sequences, their molecular evolution and the selective forces shaping the evolution of the *AVR-Pita1* gene in *M. oryzae* were reported.

Materials and methods

Infected rice sample collection

Khao Dawk Mali 105 (KDML105) is the most popular Thai aromatic rice variety known by consumers worldwide. KDML105 rice, when cooked, has several distinct characteristics (e.g. highly aromatic, soft and delicious) but KDML105 is susceptible to all major diseases and insect pests including rice blast fungus (Bureau of Rice Research and Development, Rice Department, Thailand 2010). KDML105 is normally used as a susceptibility check variety for the rice blast resistant breeding program in Thailand as KDML105 does not contain any blast resistant genes: *Pita*, *Pib*, *Pi9,* etc. (Srikeaw 2011).

In 2005 and 2010, samples of KDML105 rice variety infected with blast fungus in rice production fields in Thailand were collected for blast fungal isolation. The collection sites were distributed throughout the northern and northeastern regions of Thailand as shown in supplemental Figure S1. Several diseased KDML105 leaf samples from each production field were used for fungal isolation but only one of the single spore isolates from each location was used for *Avr-Pita1* cloning, sequencing and sequence analysis.

Fungal isolates and culture

In total, 86 rice blast isolates were collected from the rice variety KDML105 from northern and northeastern Thailand during severe rice blast epidemics in commercial fields in 2005 and 2010 (Table 1). For isolation of single spores, the infected leaves of diseased plants were cut into small pieces and placed on moist filter paper in Petri dishes, then incubated under light for 24 h at 25 °C; single spores were picked with a fine glass needle under a binocular microscope. Each single spore was transferred in to rice flour agar (RFA) medium whose surface was covered with filter paper for 7–14 days. Each isolate was stored at −20 °C on desiccated filter paper and was grown at room temperature under fluorescence lighting to produce mycelia.

Table 1 Accessions of rice blast fungus, host, country of origins and their references

AS No.	Code	Host	Country of origins	Reference
DQ855953	G-1	*D. sanguinalis*	United States	Khang et al. 2008
DQ855954	G-223	*P. typhoideum*	Burkina Faso	Khang et al. 2008
DQ855955	G-78	*P. polystachyon*	Philippines	Khang et al. 2008
DQ855956	G-213	*D. smutsii*	Japan	Khang et al. 2008
AB607333	PO-02-7306	*Oryza sativa*	Indonesia (Jawa Barat)	Chuma et al. 2011
AB607335	Y93-245c-2	*Oryza sativa*	China (Yunnan)	Chuma et al. 2011
AB607337	Ken54-04	*Oryza sativa*	Japan (Gifu)	Chuma et al. 2011
AB607338	PO-12-7301-2	*Oryza sativa*	Indonesia (Lampung)	Chuma et al. 2011
AB607339	PO-02-7306	*Oryza sativa*	Indonesia (Jaw a Barat)	Chuma et al. 2011
AB607341	KANSV1-4-1	*Setaria italic*	Japan (Kanagawa)	Chuma et al. 2011
AB607344	Br29	*D. Horizontalis*	Brazil (Sao Paulo)	Chuma et al. 2011
AB607345	Br36	*Cenchus echinatus*	Brazil (Parana)	Chuma et al. 2011
FJ842861	91A38	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842862	80 F1	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842863	ZN41	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842864	ZN49	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842865	China 20	*Oryza sativa*	China	Dai et al. 2010
FJ842866	PTr24	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842867	85 M5	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842868	49D	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842869	C12	*Oryza sativa*	Colombia	Dai et al. 2010
FJ842870	ZN39	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842871	92 M3	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842872	ZN11	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842873	91 T59	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842874	75A19	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842875	A119	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842876	ZN27	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842877	SEF6	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842878	CG3	Crabgrass	U.S.A	Dai et al. 2010
FJ842879	X25	Crabgrass	U.S.A	Dai et al. 2010
FJ842880	X54	Crabgrass	U.S.A	Dai et al. 2010
FJ842881	ZN04	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842882	91A55	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842883	93A17	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842884	92A8	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842885	81 F3	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842886	SEF9	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842887	ZN25	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842888	93 L6	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842889	ZN62	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842890	RP44	*Oryza sativa*	Philippines	Dai et al. 2010
FJ842891	FC23	*Oryza sativa*	Colombia	Dai et al. 2010
FJ842892	RP9	*Oryza sativa*	Philippines	Dai et al. 2010
FJ842893	IN24	*Oryza sativa*	India	Dai et al. 2010
FJ842894	IN46	*Oryza sativa*	India	Dai et al. 2010
FJ842895	SEF10	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842896	SEF11	*Oryza sativa*	U.S.A	Dai et al. 2010
FJ842897	93 L29	*Oryza sativa*	U.S.A	Dai et al. 2010

Table 1 (continued)

AS No.	Code	Host	Country of origins	Reference
FJ842898	PTr25	*Oryza sativa*	U.S.A	Dai et al. 2010
EU055560	LP4	–	China	Ma et al., unpublished
EU055561	LP5	–	China	Ma et al., unpublished
EU055562	WJ1	–	China	Ma et al., unpublished
EU055563	WJ7	–	China	Ma et al., unpublished
EU055564	WJ22	–	China	Ma et al., unpublished
EU055565	WJ25	–	China	Ma et al., unpublished
EU055566	WJ32	–	China	Ma et al., unpublished
EU055567	YC21	–	China	Ma et al., unpublished
EU055568	YC26	–	China	Ma et al., unpublished
EU055569	YC35	–	China	Ma et al., unpublished
JQ409300	TS 2.3	*Oryza sativa*	Thailand (Ubonratchathani)	Agronomy, Kasetsart University
JQ409301	TS 13.2	*Oryza sativa*	Thailand (Sungkon, Nongkrai)	Agronomy, Kasetsart University
JQ409302	TS 16.1	*Oryza sativa*	Thailand (Pen, Udonthani)	Agronomy, Kasetsart University
JQ409303	TS 16.2	*Oryza sativa*	Thailand (Pen, Udonthani)	Agronomy, Kasetsart University
JQ409304	TS 17.2	*Oryza sativa*	Thailand (Jaturat, Chaiyapoom)	Agronomy, Kasetsart University
JQ409305	TS 19.2	*Oryza sativa*	Thailand (Gudjub, Udonthani)	Agronomy, Kasetsart University
JQ409306	TS 20.2	*Oryza sativa*	Thailand (Nonghan, Udonthani)	Agronomy, Kasetsart University
JQ409307	TS 21.2	*Oryza sativa*	Thailand (Swangdandin, Udonthani)	Agronomy, Kasetsart University
JQ409308	TS 22.1	*Oryza sativa*	Thailand (Tungfon, Udonthani)	Agronomy, Kasetsart University
JQ409309	TS 23.2	*Oryza sativa*	Thailand (Banphou, Udonthani)	Agronomy, Kasetsart University
JQ409310	TS 24.1	*Oryza sativa*	Thailand (Chaiyapoom)	Agronomy, Kasetsart University
JQ409311	TS 25.1	*Oryza sativa*	Thailand (Gumpawape, Udonthani)	Agronomy, Kasetsart University
JQ409312	TS 32.1	*Oryza sativa*	Thailand (Pornjarern, Nongkrai)	Agronomy, Kasetsart University
JQ409313	BCC100	*Oryza sativa*	Thailand (Srakaew)	BIOTEC Culture Collection, Thailand
JQ409314	BCC 301	*Oryza sativa*	Thailand (Srisaked)	BIOTEC Culture Collection, Thailand
JQ409315	BCC 302	*Oryza sativa*	Thailand (Srisaked)	BIOTEC Culture Collection, Thailand
JQ409316	BCC 459	*Oryza sativa*	Thailand (Lampang)	BIOTEC Culture Collection, Thailand
JQ409317	BCC 551	*Oryza sativa*	Thailand (Nakornratchasima)	BIOTEC Culture Collection, Thailand
JQ409318	BCC 581	*Oryza sativa*	Thailand (Nakornratchasima)	BIOTEC Culture Collection, Thailand
JQ409319	BCC 732	*Oryza sativa*	Thailand (Kampangpet)	BIOTEC Culture Collection, Thailand
JQ409320	BCC 760	*Oryza sativa*	Thailand (Kampangpet)	BIOTEC Culture Collection, Thailand
JQ409321	BCC 812	*Oryza sativa*	Thailand (Chiangrai)	BIOTEC Culture Collection, Thailand
JQ409322	BCC 837	*Oryza sativa*	Thailand (Surin)	BIOTEC Culture Collection, Thailand
JQ409323	BCC 941	*Oryza sativa*	Thailand (Lampang)	BIOTEC Culture Collection, Thailand
JQ409324	BCC 945	*Oryza sativa*	Thailand (Chiangmai)	BIOTEC Culture Collection, Thailand
JQ409325	BCC 971	*Oryza sativa*	Thailand (Nan)	BIOTEC Culture Collection, Thailand
JQ409326	BCC 985	*Oryza sativa*	Thailand (Srisaked)	BIOTEC Culture Collection, Thailand
JQ409327	BCC 993	*Oryza sativa*	Thailand (Buriram)	BIOTEC Culture Collection, Thailand
JQ409328	BCC 1108	*Oryza sativa*	Thailand (Ubonratchathani)	BIOTEC Culture Collection, Thailand
JQ409329	BCC 1109	*Oryza sativa*	Thailand (Ubonratchathani)	BIOTEC Culture Collection, Thailand

DNA preparation

Each rice blast isolate was grown in potato dextrose broth with constant shaking (200 rpm) for approximately 7 days at room temperature to produce mycelia. Fungal mycelia were harvested by filtration through Whatman no.1 filter paper, lyophilized and ground in liquid nitrogen. DNA was extracted from powdered mycelia using cetyltrimethylammonium bromide (CTAB) extraction buffer and incubated at 65 °C for 60 min. The solution was extracted with chloroform/isoamyl alcohol (24:1) and centrifuged at 12,000 rpm at 20 °C for 30 min. After centrifugation, the upper layer was removed to

a new tube. The nucleic acid was precipitated by adding the same amount of cold iso-propanol and then incubated at 4 °C for 30 min, centrifuged at 12,000 rpm at 20 °C for 30 min and washed twice with 95 % and 70 % ethanol, respectively. The pellet was dried and dissolved in Tris EDTA buffer. Each DNA sample was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

PCR amplification and DNA sequencing

Two pairs of primers with overlapping fragments were designed and synthesized based on the genomic DNA sequence of *AVR-Pita1* (GenBank ID: AF207841); F1: 5′-AGTGGACCCTTGTCCGATC-3′, F2: 5′-CGCCTTTTA TTGGTTTAATTCG-3′, R1: 5′-CCGAAATCGCAACG GTGTG-3′ and R2: 5′-CCTCCATTCCAACACTAACG-3′. These primers were used to amplify and sequence the existence of *AVR-Pita1*. One primer pair was used to identify the presence and the quality of rice blast fungus genomic DNA; IDMF: 5′-GACCTATGCAATCACCAC-3′ and IDMR: 5′-CGTACTCGAGTGTAATCTCG-3′. This primer was designed from rice blast fungus-specific DNA sequence (GenBank ID: FW343765). All PCR reactions were performed using I-Tag™ DNA polymerase (Intron Biotechnology, Seongnam-si, Kyunggi-do, Korea). Each PCR reaction consisted of the following components: 1U of Taq DNA polymerase, 1× Intron PCR buffer, 20 mM $MgCl_2$, 10 mM dNTPs, 1 μL of each 5 μM primer, 30–50 ng of fungal genomic DNA and distilled water in a final reaction volume of 20 μL. Reactions were performed in a GenePro thermal cycler (Bioer Technology, Binjiang, Hangzhou, China) with the following PCR program: one cycle at 94 °C for 2 min for initiation denaturation, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 50 s, and a final extension of 72 °C for 5 min. The PCR products were separated by 1.0 % agarose gel electrophoresis in 0.5× TBE (Tris-borate-EDTA) buffer, the size of the amplified fragment was estimated using 1 kb Gene Ruler™ Express DNA ladder (Fermentas Inc., Glen Burine, MD, USA), stained with ethidium bromide, visualized and photographed using an infinity 3,000 gel photographic system (Vilber Lourmat, Eberhardzell, Germany). PCR products were purified with a Qiaquick gel extraction kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. After purification, all PCR products were submitted for sequencing by the Pacific Science Co., Ltd. (Thailand).

AVR Pita sequence mining from the GenBank database and sequence analysis

Sixty *AVR-Pita1* sequences previously reported in the GenBank database were downloaded. Four sequences from Khang et al. (2008) were referred to as DQ sequences. Eight sequences from Chuma et al. (2011) were referred to as AB sequences. Thirty-eight sequences from Dai et al. (2010) were referred to as FJ sequences and ten sequences from unpublished data were referred to as EU sequences (Table 1 and Fig. 1). DNA sequences of *AVR-Pita1* were assembled and aligned by Bioedit software V.7 (Hall 1999) and manually edited by FinchTV V1.4.0 (http://www.geospiza.com/Products/finchtv.shtml). The number of nucleotide diversity per site was estimated as π and θ (Nei 1987; Watterson 1975). Genetic parameters, namely, Tajima's *D* test (Tajima 1989), Fu and Li's *D* test and Fu and Li's F test (Fu and Li 1993) and the sliding window analysis, were calculated using DnaSP 5.0 (Rozas et al. 2003). Neutrality and selection tests were performed using DnaSP 5.0. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.0 (Tamura 2011). The phylogenetic trees were constructed using the Maximum likelihood method and drawn using the MEGA program. The stability of tree was evaluated by bootstrap analysis with 1,000 replications.

Results

Nucleotide variation of AVR-Pita1

The rice blast resistant gene, *Pi-ta*, has been effective in preventing infection by races of *M. oryzae* containing the *AVR-Pita1*. Eighty-six rice blast isolates were collected from rice variety KDML105 in northern and northeastern Thailand during severe rice blast epidemics in commercial field. Rice blast fungus DNA specific primer was successfully used to verify the present of rice blast fungus genomic DNA. The *AVR-Pita1* sequences from 30 blast isolates were successfully amplified using a combination of *AVR-Pita1* primers and sequences were deposited in GenBank (GenBank ID: JQ409300–JQ409329). The failure of *AVR-Pita1* amplification from 56 blast isolates suggested that DNA sequence at some of these primer sites may have been significantly altered or part of the gene might be deleted. Similar results have been shown by Dai et al. (2010).

Multiple sequence alignment of 30 *AVR-Pita1* sequences from Thai blast isolates and 60 previously reported *AVR-Pita1* sequences from GenBank (for a total of 90 sequences), were analyzed for nucleotide variation. Sixty-four haplotypes were identified, with 219 segregating sites: 133 in the coding region and 52 in the noncoding region (Table 2). The previously reported *AVR-Pita1* sequences from Dai et al. (2010) had the highest number of haplotypes at 37 with 26 of these haplotypes located in the coding region (Table 2). The *AVR-Pita1* sequences from Thai blast isolates showed 15 haplotypes with 47 segregating sites; 26 in the coding region and 18 in the noncoding region (Table 2). The length of the complete alignment was 882 bp. Numbering began from the first position of the *AVR-Pita1* start codon.

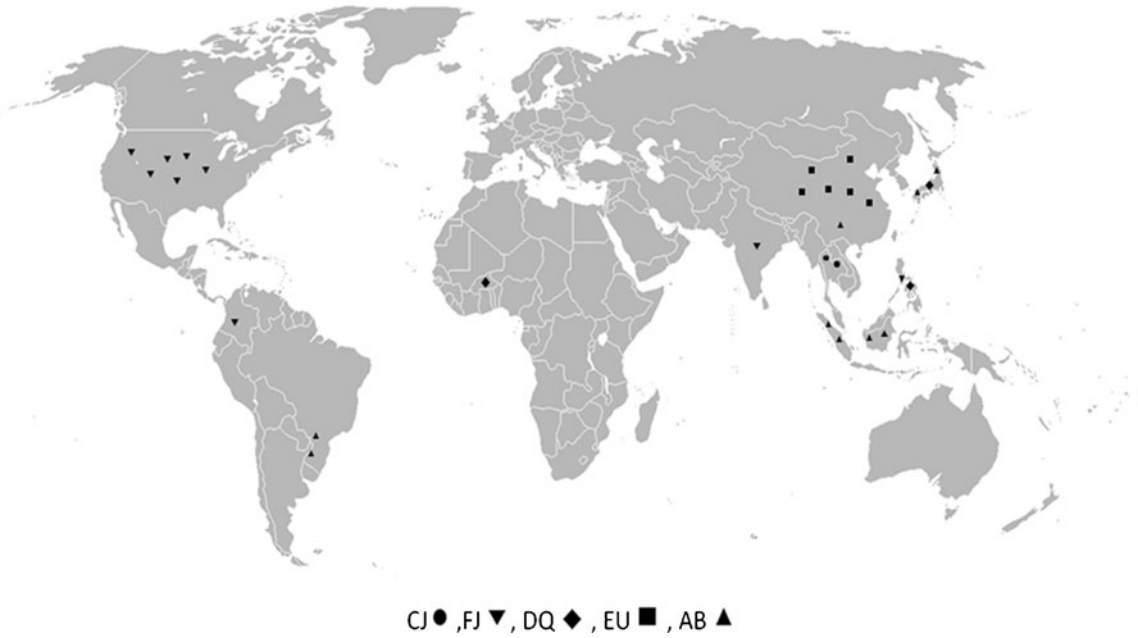

Fig. 1 Geographic distribution of the *Magnaporthe oryzae* isolates used in the study. Circle (●) represents blast isolates from Thailand (CJ); downward triangle (▼) represents blast isolates from Dai et al. (2010) (FJ); diamond (♦) represents blast isolates from Khang et al. (2008) (DQ); square (■) represents blast isolates from unpublished data (EU) and upward triangle (▲) represents blast isolates from Chuma et al. (2011) (AB) (Table 1)

Table 2 Polymorphism and neutral test of different groups of the *AVR-Pita1* gene

Coding sites	H	S	π	θ	Tajima's D	Fu and Li's D*	Fu and Li's F*
All Avr-Pita1							
Coding	47	133	0.01590	0.05031	−2.2983**	−5.1639**	−4.7444**
Non coding	16	52	0.01289	0.05471	−2.4737**	−5.1707**	−4.8932**
Entire gene	64	219	0.01548	0.05217	−2.3837**	−5.3085**	−4.8714**
CJ							
Coding	12	26	0.00378	0.01143	−2.3910**	−4.3022**	−4.3405**
Non coding	7	18	0.00577	0.02184	−2.5346***	−4.5233**	−4.5733**
Entire gene	15	47	0.00429	0.01426	−2.5979***	−4.8486**	−4.8486**
FJ							
Coding	26	26	0.00974	0.01082	−0.3439	−0.5539	−0.5717
Non coding	7	5	0.00498	0.00579	−0.3465	−1.6315	−1.4484
Entire gene	37	36	0.00891	0.00981	−0.3227	−0.6633	−0.6484
DQ							
Coding	4	50	0.04167	0.04371	−0.4857	−0.6129	−0.6263
Non coding	3	16	0.03964	0.04237	−0.6545	−0.6545	−0.6717
Entire gene	4	72	0.04261	0.04463	−0.4713	−0.6485	−0.6580
EU							
Coding	7	9	0.00288	0.00509	−1.9013*	−2.2187**	−2.4060**
Non coding	3	2	0.00193	0.00342	−1.4009	−1.5866	−1.7190
Entire gene	9	12	0.00272	0.00481	−1.9612*	−2.2986**	−2.4929**
AB							
Coding	8	107	0.05571	0.06603	−1.2446	−0.8507	−1.1610
Non coding	4	41	0.05488	0.07149	−1.5448	−1.2414	−1.5839
Entire gene	8	154	0.05593	0.06749	−1.3393	−0.9366	−1.2851

Abbreviations: *H* Number of haplotypes, *S* Number of segregating sites, π nucleotide diversity, θ Watterson's estimator, asterisk indicates significant statistics * $P<0.05$, ** $P<0.01$ and *** $P<0.001$

The nucleotide polymorphism (π) of the entire *AVR-Pita1* gene was 0.01548 (Table 2). This was caused by the high polymorphism in both coding region (π=0.01590) and non-coding region (π=0.01289) (Table 2). The level of nucleotide diversity of the entire *AVR-Pita1* gene from previously reported *AVR-Pita1* sequences from Chuma et al. (2011) and from Khang et al. (2008) (π=0.05593 and 0.04261, respectively) was much higher than that in Thai blast isolates reported in this study and those previously reported from Ma et al. (unpublished) and from Dai et al. (2010) (π=0.00429, 0.00272 and 0.00891, correspondingly) (Table 2).

Tests of neutral selection

Neutrality test of the *AVR-Pita1* sequences were examined with three statistical parameters—namely, Tajima's *D*, Fu and Li's *D*, and Fu and Li's F (Table 2). The results showed that all statistical parameters were negative. The test values of *AVR-Pita1* sequences from Thai blast isolates and from China were significantly deviated from neutrality. In contrast, the test values of previously reported *AVR-Pita1* sequences from Khang et al. (2008), Dai et al. (2010) and Chuma et al. (2011) were not significantly deviated from the neutral model (Table 2).

Sliding window analysis was used to characterize the pattern of polymorphism and divergence across the *AVR-Pita1* gene. There were substantial nonsynonymous polymorphisms detected in the coding region compared with nonsynonymous divergence in all partitions of AVR-Pita1 protein (Fig. 2). This result was supported by the ratio between nonsynonymous nucleotide polymorphism (π_{non}) and synonymous nucleotide polymorphism (π_{syn}) of the *AVR-Pita1* sequences (Table 3). The π_{non}/π_{syn} ratio of the entire *AVR-Pita1* gene and previously

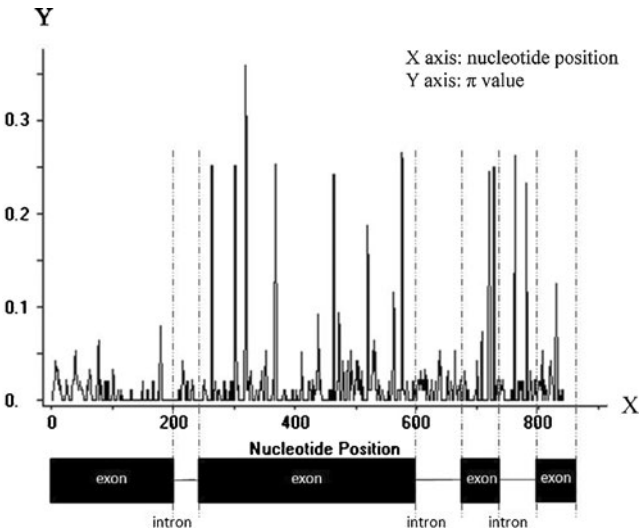

Fig. 2 Distribution of the *AVR-Pita1* allele variation (π value) using sliding window showing three introns and four exons of the *AVR-Pita1* gene

Table 3 Nonsynonynous over synonymous polymorphism and divergence of the *AVR-Pita* gene

Populations	π_{non}	π_{syn}	π_{non}/π_{syn} [a]	K_a	K_s	K_a/K_s [b]
CJ	0.00391	0.00245	1.599	0.00489	0.00123	3.995
FJ	0.00768	0.01910	0.399	0.00787	0.01721	0.455
DQ	0.04490	0.03949	1.141	0.03123	0.03215	0.971
EU	0.00311	0.00269	1.159	0.00739	0.02149	0.341
AB	0.05335	0.06622	0.798	0.03075	0.03991	0.766
Total groups	0.01523	0.02245	0.675	0.01046	0.01549	0.673

[a] The ratio of nonsynonymous (amino acid-altering) site diversity over synonymous site diversity of the *AVR-Pita*

[b] The ratio of nonsynonymous (amino acid-altering) changes per nonsynonymous site (Ka) to synonymous changes per synonymous site (Ks)

reported *AVR-Pita1* sequences (AB and FJ sequences) was smaller than 1. On the other hand, the π_{non}/π_{syn} ratio of *AVR-Pita1* sequences from Thai blast isolates and previously reported *AVR-Pita1* sequences (DQ and EU sequences) was greater than 1 (Table 3). The ratio of nucleotide substitutions that lead to amino acid replacements (nonsynonymous substitution, Ka) and nucleotide substitutions that do not lead to amino acid replacement (synonymous substitution, Ks) of all *AVR-Pita1* sequences were smaller than 1 except *AVR-Pita1* sequences from Thai blast isolates which was much larger than 1 (i.e., 3.995). This extremely high Ka/Ks ratio suggested that the *AVR-Pita1* sequences from Thai blast isolates were influenced by adaptive evolution and indicated strong selection for a novel protein function.

AVR-Pita1 amino acid diversification in blast isolates from Thailand

AVR-Pita1 is known to have three introns and four exons in the open reading frame (ORF) (Orbach et al. 2000). Thirty sequences of *AVR-Pita1* from Thai rice blast isolates were translated in to amino acid sequences. The amino acid sequences were aligned and compared with AVR-Pita1 protein of the Chinese isolate O-137 (Orbach et al. 2000). Amino acid alignments were predicted to produce 16 functional proteins including the original AVR-Pita1 protein from O-137 (Table 4). Among these 16 proteins, amino acid variations were predicted to occur at 35 positions including a deletion/insertion. All variations occurred throughout the entire protein (Table 4).

Phylogenetic analysis of AVR-Pita1

The maximum-likelihood method was used to generate a dendrogram, based on Dice's similarity, for the genetic

Table 4 Protein variation among 30 *AVR-Pita* sequences from Thai blast isolates

Protein[a]	Allele[b]	3	5	[c]	8	12	13	14	15	16	17	20	21	25	154	168	180	183	187	191	194	195	198	199	200	201	204	206	212	213	214	215	216	217	222	223
AVR-Pita1	n/a	F	S	-	F	V	A	I	S	A	F	I	G	H	P	G	H	L	K	Y	D	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	C
I	1	F	S	L	F	V	A	I	S	G	F	I	G	R	P	G	H	L	K	C	H	G	K	L	D	S	S	Q	V	I	F	A	Q	C	Y	C
II	2, 7-10, 16	F	S	L	F	V	A	V	S	G	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	V	I	F	A	Q	C	Y	C
	18-20, 23-24	F	S	L	F	V	A	V	S	A	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	C
	26-27	F	S	L	F	V	A	V	S	A	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	C
III	3	F	S	L	F	V	A	I	S	A	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	L	A	Q	C	Y	S
IV	4	F	S	L	F	V	A	I	S	A	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	S
V	5	F	A	L	S	V	A	V	P	G	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	S
VI	6	F	S	L	F	V	A	I	S	D	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	S
VII	11	F	S	L	F	V	A	I	S	A	F	I	G	H	P	G	H	L	K	C	H	V	K	L	D	S	C	K	A	W	I	V	Q	N	P	R
VIII	12	F	S	L	F	V	A	I	S	A	F	I	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	L	A	Q	C	Y	S
IX	13	F	S	L	F	V	A	I	S	D	F	I	G	H	P	G	H	L	K	C	H	G	N	F	Y	G	S	K	A	I	F	A	Q	C	Y	S
X	14, 29	F	S	L	F	V	A	I	S	A	F	I	G	H	P	G	H	L	K	Y	D	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	S
XI	15, 28, 30	F	S	L	F	V	A	I	S	A	F	I	G	H	P	G	H	L	K	Y	D	G	K	L	D	S	S	K	A	I	F	A	Q	C	N	S
XII	19	F	S	L	F	V	A	V	S	A	F	I	G	H	R	V	H	L	K	Y	D	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	S
XIII	21	F	S	L	F	A	A	I	S	A	F	I	N	H	P	G	H	L	R	C	H	G	K	L	D	S	S	K	A	I	F	A	H	C	Y	S
XIV	22	L	S	L	F	V	P	I	S	A	S	N	G	H	P	G	H	L	K	C	H	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	S
XV	25	F	S	L	F	V	A	I	S	A	F	I	G	H	P	G	N	I	K	C	H	G	K	L	D	S	S	K	A	I	F	A	Q	C	Y	S

Different amino acids and their positions. Number indicates a position of amino acid in the AVR-Pita1 protein (AF207841)

[a] Groups of *AVR-Pita1* variants based in amino acid sequences

[b] Groups of *AVR-Pita1* variants based on nucleotide sequences

[c] Leucine insertion

relationship of 90 *AVR-Pita1* sequences (Fig. 3). The phylogenetic analysis revealed two major clades, grouped by country of origin of sequences. The *AVR-Pita1* sequences from Thai rice blast isolates mostly clustered together in one clade, separated out from the *AVR-Pita1* sequences downloaded from public database, except one isolate (TS 16.2) from Pen, Udonthani which was placed in the other clade. Among 30 blast isolates from Thailand, isolates from 2005 and 2010 are grouped together showing no difference between the times of sample collection. In this clade, it is worth noting that blast isolates from Thailand were placed together with several blast isolates from south and southeast Asian countries including FJ842892 and FJ842890 from the Philippines, AB607338 from Indonesia and FJ842893 and FJ842894 from India. The other clade comprises the *AVR-Pita1* sequences mainly originated from China and from the USA. Interestingly, two isolates from Brazil and one isolate from Japan did not group with other *AVR-Pita1* sequences in the phylogenetic tree. The result from phylogenetic analysis suggested that blast isolates from Thailand, south and southeast Asia are more closely related to each other and more diverse from rice blast isolates from other parts of the world especially from China and the USA.

Discussion

Resistance to *M. oryzae* in rice follows a gene-for-gene specificity where major resistant *R* genes are effective in controlling infection by races of *M. oryzae* possessing corresponding avirulence (*AVR*) genes (Flor 1971; Correll et al. 2000). The *Pi-ta* gene is one of the most effective *R* genes deployed for blast resistance worldwide (Orbach et al. 2000). The effectiveness of the *Pi-ta* gene relies on the ability to recognize the pathogen's corresponding avirulence gene *AVR-Pita1*. The processed AVR-Pita1 protein from the rice blast fungus was demonstrated to interact directly with the translated product of the host *R* gene *Pi-ta* in rice triggering resistance (Bryan et al. 2000; Jia et al. 2000). The resistant gene, *Pi-ta*, is located at 10.6 Mb near the centromere of chromosome 12; a region that often associates with recombination suppression (Bryan et al. 2000). The *Pi-ta* gene contains two exons interrupted by a single intron and is predicted to be a cytoplastic protein with 928 amino acids with nucleotide-biding site and leucine-rich-repeat domain at the carboxyl terminus (Jia et al. 2009). A single amino acid, alanine at position 918 of the *Pi-ta* protein, determines its resistance specificity (Bryan et al. 2000; Jia et al. 2000). The genotype variation and resistant/susceptible phenotype at the *Pi-ta* locus of wild rice (*Oryza rufipogon*), the ancestor of cultivated rice (*O. sativa*), was surveyed in 36 locations worldwide to examine the molecular evolution and functional adaption of the *Pi-ta* gene. The results found that low nucleotide polymorphism of the *Pi-ta* gene in *O. rufipogon* was similar to that of *O. sativa*, but greatly differed from what has been reported for other *O. rufipogon* genes (Huang et al. 2008). The *AVR-Pita1* gene of *M. oryzae* is located at a telomeric region of chromosome 3 of rice blast fungus (Orbach et al. 2000). The existence of the *AVR-Pita1* variants was recently examined in races of *M. oryzae* from the southern US (Jia et al. 2009). The frequent generation of new virulent races or pathotypes leads to the short life of many newly released blast resistant cultivars (Skamnioti and Gurr 2009; Tharreau et al. 2009). Recent studies on the variations of *AVR-Pita1* gene have provided insight into the mechanism of blast genetic variations and instability (Lee et al. 2005). Multiple genetic mutation events and genetics recombination have been found to be the main driving force to creation of new virulent races that overcame major *R* genes. For example, the study of genetic variation of *AVR-Pita1* gene demonstrated that partial deletion, complete deletion, frame-shift mutation and sequence variation have occurred in the *AVR-Pita1* sequences among field isolates of *M. oryzae* from various rice producing countries (Dai et al. 2010). This mention is in agreement with our results which showed the high level of sequence variation of the *AVR-Pita1* (Table 2). This result could be explained by the different origins and the different host plant species of blast isolates from Khang et al. (2008) and Chuma et al. (2011), AB and DQ sequences (Table 1).

The statistical tests of neutrality showed the significant negative values of Tajima's D, Fu and Li's D and Fu and Li's F statistics of the entire gene; coding and noncoding regions. These results indicated that the *AVR-Pita1* gene possesses diversified sequence structures and is under positive selection pressure in nature. Our results are consistent with previous findings that the population structure of the *AVR-Pita1* gene is deviated from neutral model (Kang et al. 2001; Zhou et al. 2007; Dai et al. 2010; Takahashi et al. 2010). Since the statistical tests of neutrality of CJ and EU sequences were significantly deviated from neutrality (Table 2), it suggested that rice blast isolates from Thailand (CJ sequences) and China (EU sequences) are possibly exposed to higher level of selection in nature. This observation was supported by the results of divergence analysis that the π_{non}/π_{syn} ratio of CJ and EU sequences was greater than 1 and the Ka/Ks ratio of only CJ sequences (Thai blast isolates) was much larger than 1 (i.e., 3.995) (Table 3). Based on the very high level of nonsynonymous mutation in the coding region of *AVR-Pita1* gene, this suggests that the *AVR-Pita1* sequences from Thai blast isolates may be influenced by adaptive evolution and indicate strong selection for a novel protein function. Nevertheless, in this study we examined the mutation events at the microspore level of a single spore isolate. The mutations at the microspore level frequently occur in blast fungus and thus the mutation rate

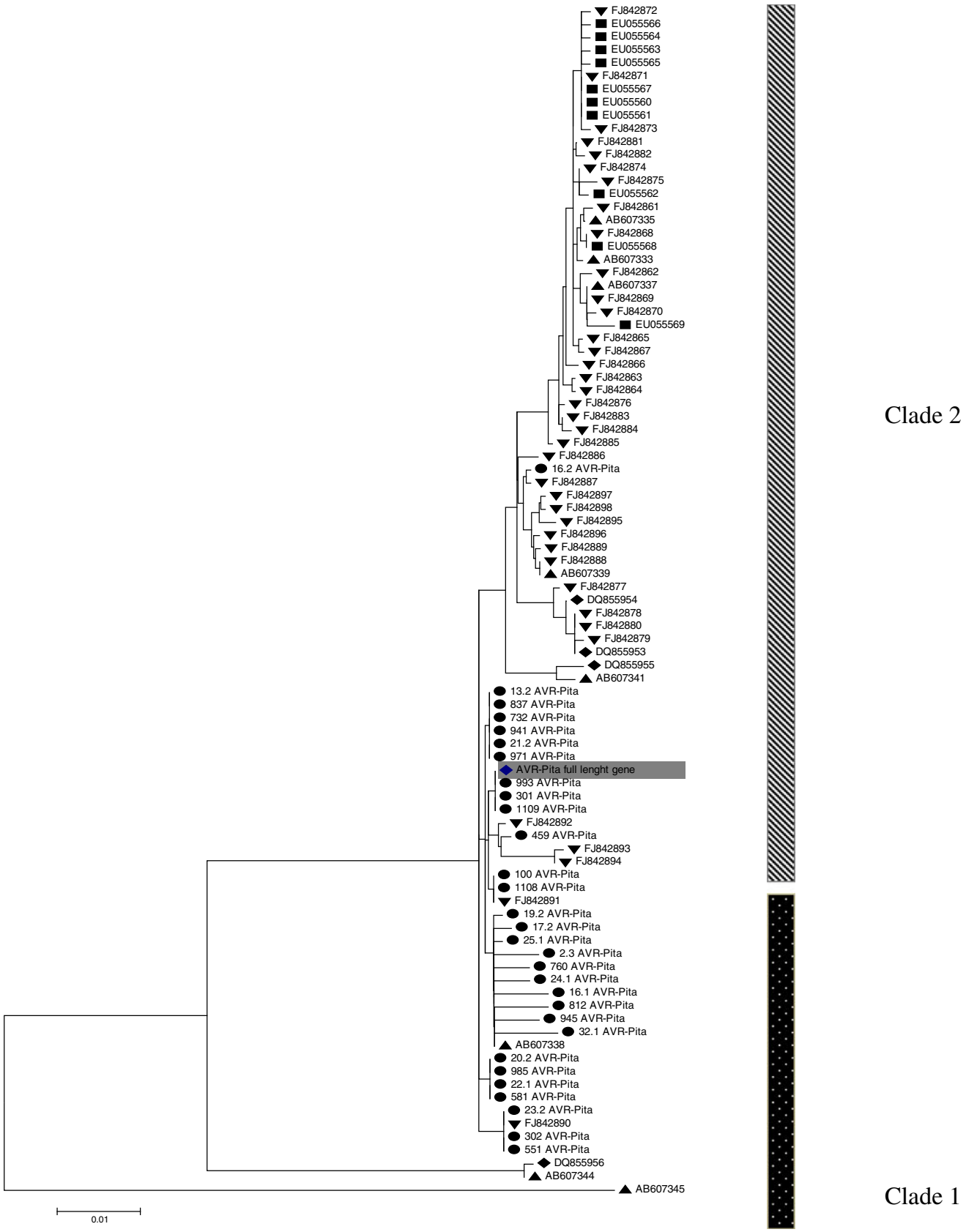

Fig. 3 Maximum-likelihood tree of the complete DNA sequence of the *AVR-Pita1* alleles with bootstrap value of 1,000 replications

obtained from this study might be higher than the actual mutation rate occurring in nature.

KDML105 is a non-glutinous rice variety which is sensitive to photoperiod so it can only be cultivated once a year. KDML105 has been cultivated in the rain-fed areas of northern and northeastern Thailand since 1969. More than 60 cycles of KDML105 crop have been cultivated. In the past three decades rice blast fungus has been the major cause of disease epidemic in Thailand (Smitamana et al. 2000; Rice Department and Thailand 2009). Farmers have been using pesticides for example: isoprothiolane (Fuji- 1 40 % EC), edifenphos (Hinosan 30 % EC) and tricclazole (Beam 75 % WP) to protect their rice production fields (Bureau of Rice Research and Development, Rice Department, Thailand 2010). The long history of KDML105 cultivation and the heavy use of pesticides are corresponding to our finding that the *AVR-Pita1* sequences from Thai blast isolates were under the positive selection pressure.

The phylogenetic analysis of the *AVR-Pita1* sequences was consistent with the previous report by Khang et al. (2008). This result suggested that Thai blast isolates might have been shaped by an older selective sweep within or near the gene, and other isolates were recently derived from Thai blast isolates (Fig. 3). Our finding suggested that evolutionary mechanism of the *AVR-Pita1* gene may have been caused mainly by recurrent selective sweeps.

In the current study, our finding showed high level of nucleotide sequence polymorphisms and the positive genetic selection pressure of the *AVR-Pita1* sequences in Thai rice blast isolates. It was also observed by the phylogenetic analysis of the *AVR-Pita1* sequences that Thai rice blast isolates were different from blast isolates from other part of the world. The information from this study could draw an attention to the *AVR-Pita1* diversification in this part of the world (southeast Asia) where is the origin of species for blast fungus and is rich in genetic diversity of blast fungus isolates.

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