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Research article

Analyzing sequence variation of the Avirulence *Avr-Pita1* gene of rice blast isolates, *Magnaporthe oryzae* in Vietnam

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Abstract

Rice blast disease caused by a filamentous fungus (*Magnaporthe oryzae*) has been one of the most devastating of all cereal diseases worldwide. The interaction between rice plant, *Oryza sativa* and the rice blast fungus, *M. oryzae* is activated by the interaction between protein products of rice resistant genes and fungal avirulence genes. The resistant genes can protect rice from infection of rice blast fungus. However, the resistant genes are usually broken down after several years due to the evolution of new fungal races. Thus, finding rice blast isolates containing the avirulence *Avr-Pita1* gene and analyzing the sequence variation of this gene are necessary for further research in reducing rice blast infection. The avirulence *Avr-Pita1* gene of 25 rice blast isolates collected from northern, middle and southern Vietnam were amplified and analyzed. The phylogenetic trees were constructed using neighbor-joining and maximum likelihood methods in the MEGA 6.0 program. The results showed that 18 rice blast isolates had the *Avr-Pita1* gene and most of them were from middle and southern Vietnam. The results of phylogenetic analysis and polymorphism analysis also showed the diversity of the nucleotide sequence of the *Avr-Pita1* gene among the 18 rice blast isolates and differences between *Avr-Pita1* of Vietnamese isolates and those from other countries.

Introduction

Rice blast disease caused by *Magnaporthe oryzae* has been evaluated as the most devastating agriculture disease in the world (Talbot, 2003). Rice blast was known as a rice fever disease and first discovered in China in 1637 (Wang and Valent, 2009). It is reported to be present in at least 85 countries resulting in significant economic losses and affecting global food security (Wang and Valent, 2009). Numerous yield losses including 8%, 10%, and 14% have been reported in Korea, India and China, respectively, and the yield loss may be up to 85% as reported in the Philippines (Leung, 2013). In Vietnam, rice blast disease has been considered as the second most serious disease and is known to have a huge impact on Vietnamese agriculture as this disease is dominant over a wide environmental range and can be spread by wind-borne spores; therefore it is easily transmitted in both tropical and temperate areas (Nguyen et al., 2015). Currently, some methods are used to control this disease such as fungicides and resistance varieties (Hulbert et al., 2001). Using fungicides is an effective method, but it can cause environmental pollution; hence, genetic engineering has been considered as effective and environmentally friendly method that can improve the disease resistance in crop agriculture (Kasetsomboon et al., 2013). To prevent rice blast disease, different rice varieties are used that contain rice blast resistance genes in their genome (Huang et al., 2014). At least 85 rice blast resistance genes have been identified in the plant genome, but they usually break down after several years due to the diversity and variation in the *M. oryzae* genome (Huang et al., 2014). The resistance gene in the plant genome (the R gene) can only prevent the infection of *M. oryzae* strains containing appropriate avirulence genes (*AVR*) (Silue et al., 1992). According to the gene-for-gene hypothesis, a single product of plant R gene can be recognized by a unique protein encoded by the *AVR* gene that leads to the immediate activation of

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the plant immune system to prevent rice blast infection (Silue et al., 1992). Studies have identified 25 avirulence (AVR) genes in *M. oryzae* with the well-known 9 *AVR* genes consisting of: *AVR-pita*, *AVR-Pizt*, *AVR-Pia*, *AVR-pii*, *AVR-Pik/km/kp*, *AVR-CO39*, *PWL1*, *PWL2* and *ACE1* (Kasetsomboon et al., 2013). In particular, *Avr-Pita1* has been evaluated as a nonstable gene containing different mutations such as deletion, point mutation and insertion of transposon that can inactivate the activity of plant resistance genes (Khang et al*.,* 2008). The *AVR-Pita* gene is a member of a gene family composed of functional and nonfunctional *AVR* genes and was renamed to *AVR-pita1* (Zhou et al., 2007; Khang et al., 2008). In addition, *AVR-Pita2* and *AVR-Pita3* belonging to this gene family were characterized based on distribution, avirulence activity and genomic context (Khang et al., 2008). Therefore, the genetic variation data of the *AVR-Pita1* gene will provide useful information in preventing rice blast disease. The current research used specific primers to detect the *AVR-Pita1* gene in 25 Vietnam rice blast isolates. Furthermore, the *AVR-Pita1* of Vietnamese rice blast isolates were compared with other isolates from Thailand, China, India and the USA to identify variation in nucleotide sequences using the MEGA 6.0 and DnaSP 5.0 programs.

Materials and Methods

Fungal isolation and genomic DNA isolation of M. oryzae isolates

25 *M. oryzae* isolates were collected from different areas of Viet Nam and isolated using single spore technique (Choi et al., 1999). The spores were picked up directly from surface lesions caused by *M. oryzae* and transferred onto a water agar plate using sterilized forceps. Spores were checked every 24 hr until germination. A small piece of agar containing single germinated spores was cut and transferred to a potato dextrose agar (PDA) plate and incubated at 28°C until the colony diameter was about 2 cm. Then, the mycelia of the fungus were subcultured to obtain a pure fungal culture.

Genomic DNA of the 25 fungal samples was extracted from mycelia using the cetyltrimethylammonium bromide (CTAB) method including 1mM ethylenediaminetera acetic acid (EDTA), 10mM Tris-HCl pH8, 2% sodium dodecyl sulfate and 100mM NaCl (Doyle and Doyle, 1987). All rice blast isolates were cultured on rice flour agar for 2 wk at 28°C. The fungal mycelia were harvested and homogenized in liquid nitrogen using a mortar and pestle. Then, 1 g of the fungal mycelia were transferred into a 1.5 mL Eppendorf tube and 700 µL CTAB extraction buffer was added with 2 µL β-mercaptoethanol and incubated at 65°C for 60 min. The solution was extracted using chloroform:isoamyl alcohol (24:1) and centrifuged at 12,000 revolutions per minute (rpm) at room temperature for 10 min. After centrifugation, the upper layer was transferred to a clean 1.5 mL Eppendorf tube. The nucleic acid was precipitated by adding the same amount of isopropanol and then incubated overnight. Samples were centrifuged at 12,000 rpm at room temperature for 10 min and washed with 70% ethanol. The pellet was dried and dissolved in TE buffer (1mM Tris, 1mM ethylenediaminetetraacetic acid, pH8.0). The concentration of DNA samples was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.; Waltham, MA, USA).

Polymerase chain reaction amplification, DNA sequencing and phylogenetic trees

A Vivantis kit was used to amplify the polymerase chain reaction (PCR) products. To evaluate the quality of extracted DNA samples, a specific pair of primers was used for *M. oryzae* iDM_F: 5′-GACCTATGCAATCACCAC-3′ and iDM_R: 5′-CGTACTCGAGTGTAATCTCG-3′ (Kasetsomboon et al., 2013). Another pair of primers was used to identify the presence of the full length of *AVR-Pita1* gene: Pita1_F: 5′-CAGGCATACATTGGAGGCC-3′and Pita1_R:5′-CCCTCCATTCCAACACTAAC-3′ (unpublished data). Each PCR reaction included 0.2 µL of Taq DNA polymerase, 2 µL of buffer S, 2 µL of 7mM dNTP, 1 µL of primer and 1 µL of DNA template (50 ng/µL). The PCR procedure was performed as: 2 min at 94°C, then 35 cycles of 30s denaturation at 94°C, 30s annealing at 56°C for the iDM primer and at 58°C for the *AVR-Pita1* primer, 1 min extension at 72°C and a final incubation for 5 min at 72°C. The PCR products were separated using 1% agarose gel electrophoresis in 0.5×TBE (Tris-borate-EDTA) buffer and the size of the amplified fragment was estimated using a 1 kb Gene ruler Express DNA ladder (Fermentas Inc; Glen Burnie, MD, USA) stained with red safe, visualized and photographed using an infinity 3000 gel photographic system (Vilber Lourmat; Eberhardzell, Germany). The PCR products were sent for sequencing by Macrogen (Seoul, Korea). The sequences were then compared with selected 53 *AVR-pita1* sequences from Thailand (JQ409313.1; JQ409314.1; JQ409316.1; JQ409316.1; JQ409317.1; JQ409318.1; JQ409319.1; JQ409320.1; JQ409321.1; JQ409322.1; JQ409323.1; JQ409324.1; JQ409325.1; JQ409326.1; JQ409328.1), China (EU055560.1; EU055561.1; EU055562.1; EU055563.1; EU055564.1; EU055565.1; EU055566.1; EU055567.1; EU055568.1; EU055569.1), India (HE651028.1; HE657550.1; HE657549.1; HE657548.1; HE657547.1; HE657546.1; HE657545.1; HE657544.1; HE657543.1; HE650798.1; HE650796.1; HE650795.1; HE650794.1; HE650793.1), USA (FJ842898.1; FJ842897.1; FJ842896.1; FJ842895.1; FJ842894.1; FJ842893.1; FJ842892.1; FJ842891.1; FJ842890.1; FJ842889.1; FJ842888.1) in the GeneBank database using BLAST (version BLASTN 2.2.18; National Center for Biotechnology Information; Bethesda, MD, USA). The phylogenetic tree of these sequences was constructed using the neighbor-joining and maximum likelihood procedures in the MEGA 6.0 software and bootstrapped for 1,000 replicates (Tamura and Kumar*,* 2013). Polymorphism analysis was done using DNASP 5.0 program (Librado and Rozas, 2009).

Results

Molecular identification of rice blast isolates in Vietnam

In total, 25 fungal isolates were collected from northern, middle and southern Vietnam as shown in Table 1. They were cultured on PDA medium for mycelia growth and extracted for genomic DNA. The agarose gel electrophoresis of PCR products using iDM primers showed the presence of the single band of 548 bp indicating that the 25 fungal isolates were the rice blast fungus, *M. oryzae* (Fig. 1).

Identification of AVR-pita1 gene in rice blast isolates

A specific pair of primers was used to detect *AVR-Pita1*. The results of the PCR assay indicated that 18 *M. oryzae* isolates displayed a single DNA fragment of which the expected size was 1549 bp, while no amplification was observed in the seven remaining *M. oryzae* isolates (M11, M13, M53, M54, M67, CBCC, LCT) as shown in Fig. 2. The presence of the *AVR- Pita1* gene in the 18 *M. oryzae* isolates was used for DNA sequencing and genomic variation analysis.

Table 1 List of 25 rice blast isolates in Vietnam

Sample name	Host rice variety	Sampling location	Infected site	Year
M11	Lua xi	Nghe An province	Leaf	2014
M13	Bac Thom 7	Thai Binh province	Leaf	2014
M14	Khang Dan	Hanoi city	Leaf	2014
M15	Lúa nếp	Ninh Giang district, Hai Duong province	Leaf	2014
M42	Chiem rau	Bac Ninh province	Leaf	2014
M46	Tam thom	Nam Dinh province	Leaf	2014
M53	CR203	Ha Nam province	Leaf	2014
M54	Khang Dan	Nam Truc district, Nam Dinh province	Leaf	2014
M67	Q5	Nam Phong district, Nam Dinh province	Leaf	2014
M70	C70	Huu Lung district, Lang Son province	Leaf	2014
CBTA	OM6976	Tan An district, Long An province	Panicle	2014
CBCC	Da Lai	Cu Chi district, Ho Chi Minh City	Panicle	2014
LVT1	Lua lai	Tan Thanh district, Vung Tau province	Leaf	2014
LVT2	Lua lai	Tan Thanh district , Vung Tau province	Leaf	2014
RGCC	Lua co	Cu Chi district, Ho Chi Minh city	Leaf	2014
CLCC	Lua da lai	Cu Chi district, Ho Chi Minh city	Collar	2015
LTT	Nang hoa	Can Duoc district, Long An province	Leaf	2015
LCT	IR50404	Chau Thanh district, Tien Giang province	Leaf	2015
LLH	Nang hoa	Can Duoc district, Long An province	Leaf	2015
NHBL	Nang hoa	Ben Luc district, Long An province	Leaf	2015
LPY2	Phu Yen	Tay Hoa district, Phu Yen province	Leaf	2015
LBTS	Ma Lam 48	Ham Thuan Bac district, Binh Thuan province	Leaf	2015
LBT	Ma Lam 48	Ham Thuan Bac district, Binh Thuan province	Leaf	2015
LBT1	Ma Lam 48	Ham Thuan Bac district, Binh Thuan province	Leaf	2015
LBT2	Ma Lam 48	Ham Thuan Bac district, Binh Thuan province	Leaf	2015
CBTA	OM6976	Tan An district, Long An province	Panicle	2014

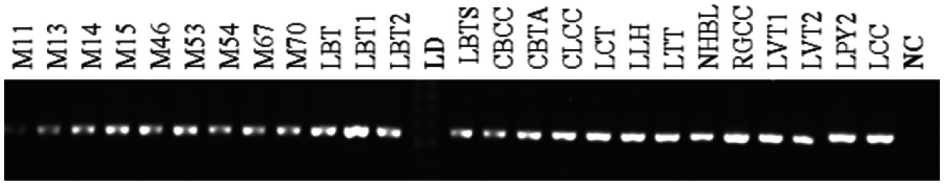

Fig. 1 Polymerase chain reaction products of 25 rice blast isolates using iDM primer on agarose gel 1%, where LD = 1kb ladder; NC = negative control without DNA template

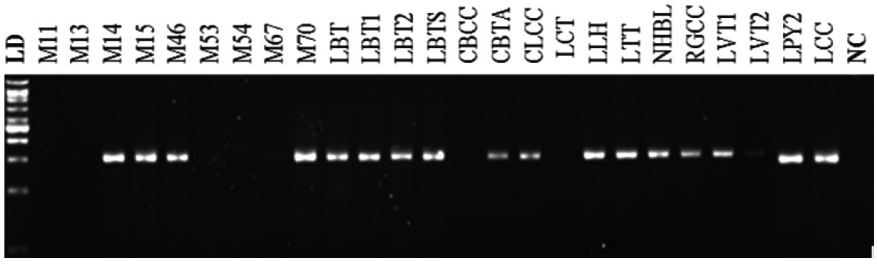

Fig. 2 Polymerase chain reaction product of 25 rice blast isolates using *AVR-pita1* primer on agarose gel 1%, where LD = 1kb ladder; NC = negative control without DNA template

Phylogenetic and genetic differentiation analyses

To analyze the genetic diversity and relationship between the 18 *AVR-Pita1* sequences and *AVR-Pita1* sequences in the Genebank database, the neighbor-joining and maximum likelihood procedures were used to build the phylogenetic tree (Tamura and Kumar, 2013). In this study, at least 52 *AVR-Pita1* sequences of *M. oryzae* isolates from Thailand, China, India and the USA were used for comparative analysis. The phylogenetic analysis of 70 *AVR-Pita1* sequences (52 *AVR-Pita1* sequences from other countries and 18 sequences from Vietnam) showed a significant difference between the Vietnamese *AVR-Pita1* sequence and those from China, India, Thailand and the USA (Fig. 3). As shown in Fig. 3A using the neighbor-joining method for phylogenetic analysis, the *AVR-Pita1* sequences of two *M. oryzae* isolates (LBT2 and RGCC) and two other rice blast isolates (LCC, LVT2) were closely related to those from Thailand and India, respectively. Another 14 *AVR-Pita1* sequences of Vietnamese rice blast isolates were classified into one separate clade in the phylogenetic tree indicating the great difference in the *AVR-Pita1* sequence of Vietnamese *M. oryzae* isolates compared to those form the other countries (Fig. 3A). However, using the maximum-likelihood method, the *AVR-Pita1* sequences of five (LVT1, RGCC, LBTS, LBT2, LBT) and four (LVT2, LTT, PY2, M14*) M. oryzae* isolates were highly similar to Thai and Indian *M. oryzae* isolates, respectively (Fig. 3B). The remaining 9 *M. oryzae* isolates were highly different from each other (Fig. 3B). Therefore, the phylogenetic trees constructed using the neighbor-joining and maximum-likelihood methods had high correlation and showed variation of the *AVR-Pita 1* sequences of *M. oryzae* isolates in Thailand and India, but significant differences to others from China and the USA. Additionally, polymorphism analysis of the 70 *AVR-Pita1* sequences from *M. oryzae* isolates in Vietnam, Thailand, China, India and the USA also revealed that the *AVR-Pita1* sequences of *M. oryzae* isolates in Vietnam had the highest number of segregation sites (S), haplotype diversity (Hd), nucleotide diversity (K) and average number of nucleotide differences (Pi) compared with those in the other countries (Table 2). These results demonstrated the high diversity and variation of the *AVR-Pita1* sequences of rice blast isolates in Vietnam.

Discussion

AVR-Pita1 can encode a putative neutral zinc metalloprotease having 223 amino acids functioning as an avirulence gene (Orbach et al., 2000; Jia et al., 2016). *AVR-Pita1* is evaluated as the most valuable gene that can control rice blast infection effectively (Kasetsomboon et al., 2013). The interaction of the *AVR-Pita1* gene, known to be located at the telomere of chromosome 3 of *M. oryzae*, and the resistance gene (R gene) in rice can activate a plant immune response to prevent the blast invasion (Orbach et al., 2000). *AVR-Pita1* alleles also have a high rate of mutations in nucleotide sequences such as point mutation, insertion of the transposon, deletion and duplication causing the breakdown of blast resistance in the rice plant. In fact, the *AVR Pita1* gene contains two exons and functional structures that are easily mutated due to the insertion of single intron or insertion of one amino acid such as alanine resulting in a change in the specific resistance capability of the *AVR-Pita1* gene (Jia et al., 2000). Additionally, the insertion of the Pot 3 transposon into the coding region of *AVR-Pita1* can also cause inactivation of avirulence activity (Zhou et al., 2007). The *AVR-Pita1* locus has an important function in the virulence of the rice blast fungus, *M. oryzae* (Jia et al., 2016). In the current study, seven *M. oryzae* isolates (M11, M13, M53, M54, M67, M70, CBCC, LCT) did not show the amplified product of *AVR-Pita1*. This suggested that these seven *M. oryza*e strains may not contain *AVR-Pita* or some genetic variation happened on the *AVR-Pita1* locus such as insertion of a transposon or deletion translocation of a nucleotide in the *AVR-Pita1* sequences. The current study examined 25 *M. oryzae* strains in Vietnam of which seven strains were considered to have mutations at the *AVR-Pita1* locus (28%). This result demonstrated the high frequency of variation of *AVR-Pita1* in the Vietnamese *M. oryzae* strains resulting in breakdown resistance of the different Vietnam rice varieties and the rapid expansion of the rice blast fungus in several years.

The knowledge of sequence variation of both the *Pita* and *AVR-Pita* genes revealed the most effective method to control rice blast disease (Jia et al., 2016). Using the neighbor -joining and maximum-likelihood methods for comparative analysis of the *AVR-Pita1* sequences of *M. oryzae* isolates in Vietnam, Thailand, India, China and the USA indicated that maximum-likelihood provided a more reliable result than the neighbor-joining method for building the phylogeny using sequence data. The genetic diversity of the *AVR-Pita1* locus in different countries was described using the number of segregating sites (S) or the total number of mutations and the haplotype diversity (Hd) known as the probability differences between two random sequences (Nei, 1987). Additionally, the nucleotide diversity (Pi) is the average number of nucleotide differences per site between two sequences (Nei, 1987) and nucleotide diversity (K) was calculated as the average proportion of nucleotide differences between the five populations of Vietnam, Thailand, India, China and the USA (Tajima, 1983). Based on S, Hd, K and Pi, significant differences were revealed between the *AVR-Pita1* sequence of Vietnam and those of the other countries. Both phylogenetic and polymorphism analyses demonstrated that 18 rice blast isolates from Vietnam had high diversity in the *AVR-Pita1* sequences and significant difference with *M. oryzae* isolates from the other countries. This finding was consistent with other studies reporting that the high genetic diversity of the *AVR-Pita1* locus is due to its location in the telomere which is known as a highly unstable region (Chuma et al., 2011; Starnes et al., 2012). According to the gene-for-gene hypothesis, the *AVR-Pita1* protein in rice blast fungus can be recognized by the *Pita* protein in rice varieties, leading to a plant immune response. Hence, to prevent rice blast disease, the *Pita* gene in different rice varieties should be considered. The Vietnamese rice variety IR46 was reported to contain the *Pita* gene in its genome and this has been used to prevent *M. oryzae* strains with the highly variable *AVR-Pita* gene so that a wide range of rice varieties have the *Pita* gene in their breeding programs due to its frequency of resistance to rice blast fungus (Nguyen et al., 2015).

In conclusion, sequence analysis of the *AVR-Pita1* gene is still the most informative tool for identification of genetic diversity and the phylogeny of *M. oryzae* in populations from different countries. Vietnam isolates possessed high diversity in the nucleotide sequence of the *AVR-Pita1* gene. The results obtained in this study have provided useful data for further research in gene engineering, especially on the *AVR-Pita1* gene.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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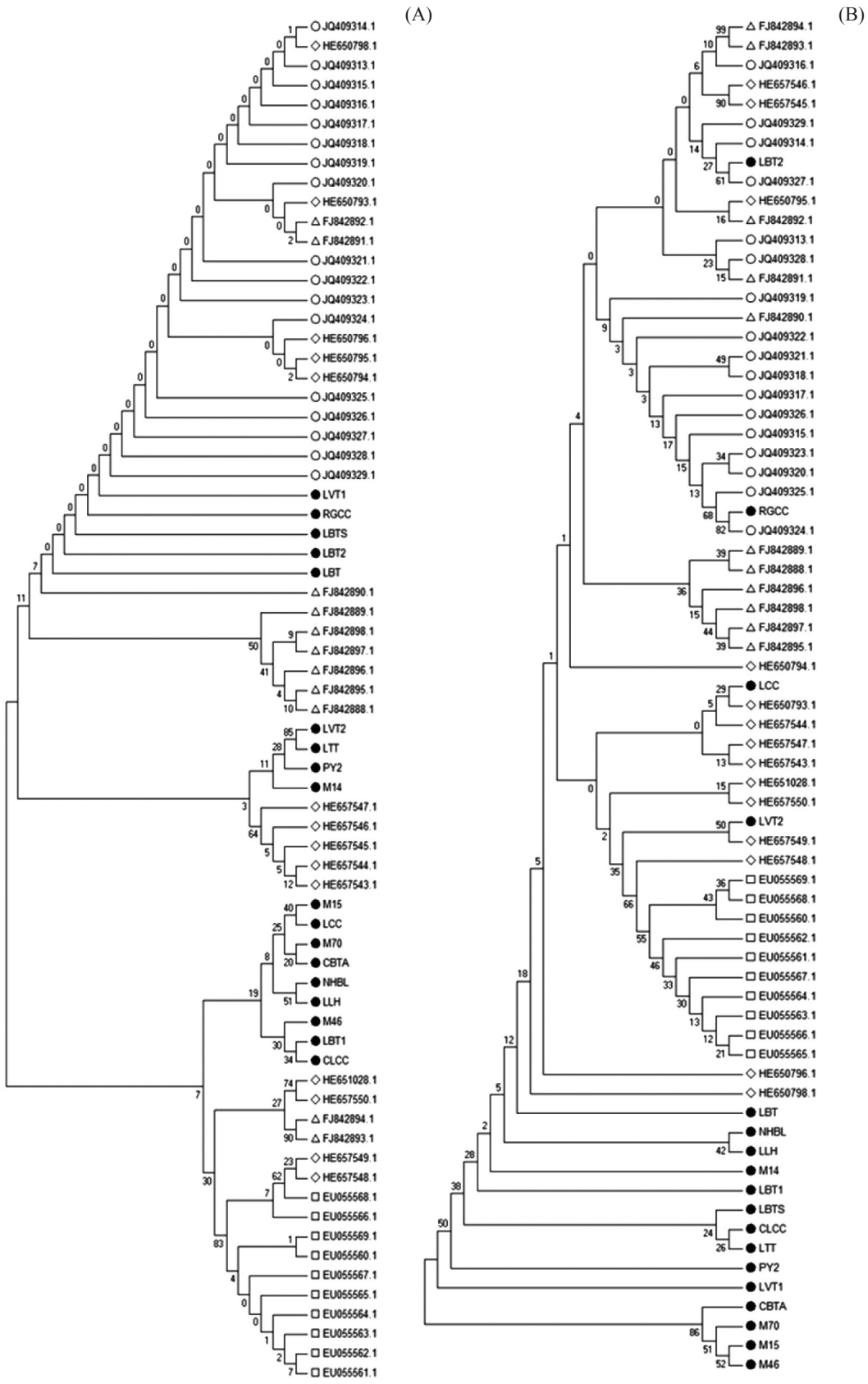

Fig. 3 Phylogenetic tree of 70 *AVR-pita1* sequences from Vietnam, India, China and the USA: (A) phylogenetic tree using neighbor-joining method; (B) phylogenetic tree using maximum likelihood. ● Vietnam, ○ Thailand, □ China, ◇ India, △ the USA

Table 2 Genetic differentiation of 70 *AVR-pita1* sequences collected from China, India, Thailand, the USA and Vietnam using Dnasp 5.0 software

Population name	N	S	h	Hd	K	Pi
Vietnam_AVR_pita1	18	78	12	0.92157	14.23529	0.03381
Thailand_AVR_pita1	17	13	4	0.33088	1.52941	0.00363
China_AVR_pita1	10	5	5	0.66667	1.00000	0.00238
India_AVR_pita1	14	6	4	0.75824	1.98901	0.00472
USA_AVR_pita1	11	5	4	0.74445	1.70909	0.00406
Total data	70	101	25	0.83727	5.84306	0.01388

N = number of sequences; S = number of segregation sites; h = number of haplotypes; Hd = haplotype diversity; K = average number of nucleotide differences; Pi = nucleotide diversity.

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