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ORIGINAL ARTICLE

Molecular characterization of lepidopteran-specific toxin genes in *Bacillus thuringiensis* strains from Thailand

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Abstract

A total of 511 local isolates of *Bacillus thuringiensis* from different geographical regions of Thailand were analyzed for the presence of the *cry1A, cry1B, cry2A, cry9*, and *vip3A* genes encoding for lepidopteran-specific toxins. PCR results revealed that 94.32% (482/511) of *B. thuringiensis* isolates harbored at least one of the detected genes, of which the *cry1A, cry1B, cry2A, cry9*, and *vip3A* genes were detected at frequencies of 90.61%, 89.63%, 76.32%, 40.70%, and 48.18%, respectively. Nineteen gene-combination profiles were discovered among 482 *B. thuringiensis* isolates, of which the most frequently detected profile contained the *cry1A, cry1B, cry2A*, and *vip3A* genes. Sixty-one isolates (12.66%), which harbored all of the detected insecticidal toxin genes, were further detected for the exochitinase (*chi36*) gene and chitinase activity. The results revealed that all 61 isolates contained the *chi36* gene and exhibited chitinase activity. Insect bioassays showed that five isolates were highly toxic (more than 80% mortality) against second instar larvae of *Spodoptera litura*, of which the highest insect mortality (93%) was obtained from the *B. thuringiensis* isolates 225-15 and 417-1. Scanning electron microscopy revealed that the crystal morphologies of the five effective isolates were bipyramidal and cuboidal shapes. SDS-PAGE analysis of the spore–crystal mixture showed major bands of approximately 65 and 130 kDa. These five effective strains are alternative candidates for use as a microbial insecticide for the control of the *S. litura* pest.

Keywords *Bacillus thuringiensis* · Bacterial toxin · Entomopathogenic bacteria · Microbial insecticide

Introduction

Intensive use of chemical insecticides to control the outbreak of insect pests has caused major public health and environmental problems in several countries including Thailand (Negatu et al. 2016; Panuwet et al. 2012; Tawatsin et al. 2015). Microbial insecticides are attractive alternatives to chemical insecticides as the former are safe for humans and non-target organisms, and are environmentally friendly.

Bacillus thuringiensis, a Gram-positive, endospore-forming bacterium has become one of the most successful microbial insecticides for the control of the larvae of insect pests and vectors belonging to the Lepidoptera, Coleoptera, and Diptera (Palma et al. 2014). The outstanding ability of this bacterium is its production of two major groups of insecticidal proteins including the Cry and Vip toxins. Cry proteins are produced during the sporulation phase and accumulated as parasporal crystalline inclusions (Palma et al. 2014). To date, 308 holotype Cry proteins have been reported under 75 major classes (Cry1–Cry75) (http://www.btnomenclature.info/). Cry proteins have been used worldwide for the control of insect pests in the form of crystal protein–spore mixture or heterologous expression in transgenic plants. The Cry1, Cry2, and Cry9 proteins were reported toxic against larvae of various lepidopteran insect pests (Herrero et al. 2016; Seifinejad et al. 2008). Vegetative insecticidal proteins (Vip) are produced during the vegetative phase of growth and are secreted into the culture medium (Palma et al. 2014). More than 30 holotype Vip proteins have been identified and separated into four classes (Vip1–Vip4) (http://www.

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btnomenclature.info/). The Vip1 and Vip2 proteins form a binary toxin that is highly toxic against some coleopteran insect larvae and certain hemipteran species (Chakroun et al. 2016; Palma et al. 2014). Vip3 proteins have been reported as toxic against the larvae of various lepidopteran insect pests including those reported to have decreasing susceptibility to Cry1A toxins (de Escudero et al. 2014; Zhang et al. 2015). However, very little information is known about Vip4 protein (Chakroun et al. 2016).

Cry toxins exhibit their insecticidal activity after proteolytic activation and then binding to specific receptors on the epithelial cells of the insect midgut and forming non-selective pores permeable to inorganic ions, amino acids, and sugars leading to cell disruption and insect death (Vachon et al. 2012). Even though there is no structural homology to Cry proteins, Vip3 toxins exhibited their larvicidal activity with the same sequence of events including proteolytic activation, receptor binding, and pore formation (Chakroun et al. 2016). However, Vip and Cry toxins have been reported do not share binding sites (Gouffon et al. 2011; Lemes et al. 2017). A combination of the use of toxins with diverse modes of action or different binding sites for insect pest control has been proposed as an efficient strategy to broaden the insecticidal spectrum and delay the onset of insect resistance (Gouffon et al. 2011). For example, a mixture of Cry and Vip3Aa toxins demonstrated a synergistic effect and broadened the insect spectrum (Baranek et al. 2017). In addition, the combined use of toxins by co-expression of more than one *B. thuringiensis* toxin gene (the pyramid strategy) has been increasingly reported in the new generation of transgenic plants to overcome the resistance of insect pests (Chen et al. 2017; Naqvi et al. 2017). Application of *B. thuringiensis* in microbial insecticides may be limited due to the narrow host range, low larvicidal activity to the targeted pests, and the resistance from insects after long-term exposure to a single toxin. Therefore, screening new effective isolates and screening for novel *B. thuringiensis* toxins have been performed continuously (Jain et al. 2017; Reyaz et al. 2017; Sauka and Benintende 2017). Since the presence of specific toxin genes correlates with the corresponding specific larvicidal activity, detection of specific toxin genes using polymerase chain reaction (PCR) were performed worldwide to screen and characterize the effective *B. thuringiensis* isolates (Sauka and Benintende 2017; Thammasittirong and Attathom 2008; Yu et al. 2011).

In addition to insecticidal toxins, *B. thuringiensis* also produces a chitinase enzyme that can hydrolyze chitin, a necessary component of the peritrophic membrane of the insect midgut (Kelkenberg et al. 2015), thus allowing bacterial toxins greater access to the receptors on epithelial cells (Sampson and Gooday 1998). Chitinase has been used as a synergistic agent to increase the larvicidal activity of *B. thuringiensis* toxins (González-Ponce et al. 2017; Juarez-Hernandez et al. 2015). *Spodoptera litura* is one of the most damaging insect pests that cause huge losses in many economically important crops in tropical and subtropical areas. In this work, we performed molecular characterization of the lepidopteran-specific toxin genes, *cry1A, cry1B, cry2A, cry9*, and *vip3A* in 511 local *B. thuringiensis* isolates to determine the frequency and distribution of these genes in different geographical regions of Thailand. *B. thuringiensis* containing all the detected genes were assayed for their toxicity against *S. litura* larvae. In addition, analyses of morphology and molecular mass of crystal proteins of the effective *B. thuringiensis* isolates were also performed.

Materials and methods

B. thuringiensis isolates and reference strains

The 511 bacterial isolates collected from soil samples from different geographical regions of Thailand, which were preliminary identified as *B. thuringiensis* base upon their crystal protein production were kindly provided by Prof. Dr. Tipvadee Attathom, Department of Entomology, Kasetsart University, Thailand. *B. thuringiensis* serovar *kurstaki* HD1 and serovar *israelensis* supplied by the Thailand Institute of Scientific and Technological Research (TISTR), the Ministry of Science and Technology (MOST), Thailand, were used as reference strains in this study. *Serratia marcescens*, used as a reference strain for chitinase activity assay, was supplied by the Department of Microbiology, Faculty of Liberal Arts and Science, Kasetsart University, Thailand.

Total DNA extraction and PCR analysis

Total DNA was extracted from *B. thuringiensis* isolates and *B. thuringiensis* serovar *kurstaki* HD1 following Thammasittirong and Attathom (2008). PCR was performed to screen the lepidopteran-specific toxin genes, *cry1A, cry1B, cry2A, cry9* and *vip3A*, and to detect *chi36* chitinase gene. Each 25-µl reaction contained 1.25 U *Taq* polymerase (RBC Bioscience, Taiwan), 1× polymerase buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM of forward and reverse primers, and 1 µl (approximately 50 ng) of each total DNA. PCR amplifications were carried out using the MultiGene OptiMax Thermal Cycler (Labnet, USA) with the following thermal cycling parameters: initiation denaturation at 94 °C for 4 min; followed by 30 cycles of denaturation (94 °C for 1 min), annealing (T_a of each primer pair in Table 1, 1 min), and extension (72 °C for 1 min); and a final extension at 72 °C for 10 min. PCR products were electrophoresed through 1% agarose gel and stained with ethidium bromide. PCR products of each gene were purified

Table 1 Primers used for detection of *cry1A, cry1B, cry2A, cry9, vip3A*, and *chi36* genes

Target gene(s) recognized	Primer name	Sequence 5′ → 3′	Product size (bp)	T_a (°C)	References
cry1Aa, cry1Ab, cry1Ac cry1Ad, cry1Ae, cry1Af, cry1Ag	Cry1A-f	ATTCGCTAGGAACCAAGC	398	55	Thammasittirong and Attathom (2008)
	Cry1A-r	AATCCGGTCCCCATACAC			
cry1Ba, cry1Bb, cry1Bc, cry1Bd, cry1Be, cry1Bf	Cry1B-f	CAGAAACAACAGAACGACC	921	57	Thammasittirong and Attathom (2008)
	Cry1B-r	CACTTCCCCACCATCCAT			
cry2Aa, cry2Ab, cry2Ac, cry2Ad	Cry2A-f	TACCTTTATTTGCACAGGCA	1170	54	Thammasittirong and Attathom (2008)
	Cry2A-r	CTACCGTTTATAGTAACTCG			
cry9A, cry9B, cry9C, cry9D, cry9E	Cry9-f	CACATGAGTTTTCTTCCTAT	440	54	Thammasittirong and Attathom (2008)
	Cry9-r	AGATACGATGCTTGTTGTAA			
vip3A	Vip3A-f	GGATTTGCCACTGGTATCAAAG	1591	53	The current work
	Vip3A-r	TTGCTTTCCACGGCTCTA			
chi36	Chi36-f	GATGTTAAACAGGTTCAA	1083	50	Arora et al. (2003)
	Chi36-r	TTATTTTTGCAAGGAAAG			

using GF-1 AmbiClean Kit (Vivantis, Malaysia) and sent for sequencing (First BASE Laboratories, Malaysia). The obtained nucleotide sequences were compared to the GenBank database using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Chitinase activity assays

Colloidal chitin was prepared according to Wang et al. (2014). *B. thuringiensis* isolates containing all the detected lepidopteran-specific genes (*cry1A, cry1B, cry2A, cry9, vip3A*) and chitinase gene were point inoculated on colloidal chitin agar and incubated for 96 h at 37 °C. The zone of clearance due to chitin hydrolysis and the diameters of colonies were measured. *S. marcescens* was used as the positive control.

Insecticidal activity assays

To obtain larvicidal activities against the second instar larvae of the common cutworm (*Spodoptera litura*), surface contamination bioassays were performed according to Lone et al. (2016) with modification. The second instar larvae of *S. litura* were supplied by the Ministry of Agriculture and Cooperatives, Thailand. A loopful of the selected *B. thuringiensis* isolates was inoculated into 5 ml of nutrient broth (NB) and incubated overnight at 37 °C with shaking at 100 rpm. Two percent of the seed inoculum was used to inoculate 100 ml of NB in 250 ml flask and incubated for 10 h at 37 °C with shaking at 100 rpm. A 1 ml sample of cell suspension was aliquoted and the culture supernatant was collected to obtain the secreted proteins. The remaining culture continued to incubate under the same conditions until it reached the sporulation phase at 48 h. The optical density at a wavelength of 600 nm (OD_{600}) was measured using a spectrophotometer (GENESYS 6, Thermo Scientific, USA). The suspension mixtures containing cells, spores, and inclusion proteins at 10 OD_{600} units were harvested using centrifugation at 12,000×*g* at 4 °C for 10 min. The mixture pellets were resuspended in 1 ml of culture supernatant collected at 10 h after incubation and spread onto 2 × 2 cm^2 of *Ricinus communis* L. leaf. NB was used as a control. After drying for approximately 1 h, each piece of leaf coating with each spore–crystal mixture was used to feed 10 larvae of *S. litura* in a plastic box. The treated larvae were left for 10 h and then they were fed new fresh leaves without the coating of the spore–crystal mixture. The percentage mortality was recorded after 7 days of incubation at room temperature (approximately 32 °C) and a 14 h (light): 10 h (dark) photoperiod. Three replications were performed for each *B. thuringiensis* isolate. *B. thuringiensis* serovar *kurstaki* HD1 was used as a reference strain.

In addition to lepidopteran insects, the selected *B. thuringiensis* isolates were assayed for toxicity against the larvae of a dipteran insect, *Aedes aegypti*, according to Thammasittirong et al. (2017). *A. aegypti* larvae were hatched from eggs supplied by the Ministry of Public Health, Thailand. The 10 OD_{600} units of suspension mixtures of each *B. thuringiensis* containing cells, spores, and inclusion proteins were harvested using centrifugation at 12,000×*g* at 4 °C for 10 min. The mixture pellets were resuspended in 10 ml of sterile distilled water. The larvicidal activity assays were performed in 48-well plates containing 1 ml of the suspension mixtures and 10 *A. aegypti* larvae per well. Distilled water without toxins was used as a control. *B. thuringiensis* serovar *israelensis* was used as a reference strain. In total, 100 larvae were assayed per *B. thuringiensis* isolate. The larvae mortality was recorded after 24 h of incubation. Three

independent experiments were performed for each *B. thuringiensis* isolate.

Characterization of high larvicidal activity *B. thuringiensis* isolates

Crystal protein morphology analysis

Effective *B. thuringiensis* isolates were cultured on nutrient agar at 37 °C for 48 h. The sporulated cells were spread on a glass cover slip, dried overnight, and coated with carbon. The crystal morphology of each isolate was observed and imaged using a scanning electron microscope (SEM-Hitachi SU8020, Hitachi, Japan) operated at 1.5 kV.

Determination of molecular mass of proteins

A loopful of each effective *B. thuringiensis* isolate was inoculated into 5 ml of NB and incubated overnight at 37 °C with shaking at 100 rpm. Two percent of the seed inoculum was used to inoculate 50 ml of NB in 250 ml flask and incubated for 48 h at 37 °C with shaking at 100 rpm. After incubation, the absorbance at OD_{600} was measured and the culture medium containing the spore–crystal mixture with an OD_{600} of one was centrifuged at 13,000×*g* at 4 °C for 10 min using a MX-307 centrifuge (Tomy, Japan). The pellet of the spore–crystal mixture was resuspended in 70 µl of diH_2O and mixed with 30 µl of sample buffer (62.5 mM Tris hydrochloride, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue), then boiled for 5 min. After centrifugation at 13,000×*g* for 20 min, a 10 µl of sample was separated by sodium dodecyl sulfate (10% w/v) polyacrylamide gel electrophoresis (SDS−PAGE) and stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA). The molecular masses of the resolved proteins were estimated by comparison with the prestained protein standard (BLUeye prestained protein ladder, GeneDireX Inc., Taiwan). Inclusion proteins of *B. thuringiensis* serovar *kurstaki* HD1 and *B. thuringiensis* serovar *israelensis* were used as references for protein molecular weight sizes.

Results

Frequency and distribution of lepidopteran-specific toxin genes

We investigated lepidopteran-specific toxin genes (*cry1A, cry1B, cry2A, cry9*, and *vip3A*) encoding for their corresponding lepidopteran-specific toxins in 511 local *B. thuringiensis* isolates using PCR-based detection. The amplification products of target genes were analyzed using agarose gel electrophoresis (Fig. 1). The PCR products were confirmed as their corresponding genes using DNA sequencing and NCBI-BLAST analysis. *B. thuringiensis* isolates that showed the amplification products of desired sizes were consider positive for respective genes. The results showed that 94.32% (482/511) of the *B. thuringiensis* isolates contained at least one of the detected genes, whereas only 5.68% (29/511) did not show any of the PCR products of the detected genes. The *cry1A, cry1B*, and *cry2A* genes were detected with high frequencies of 90.61% (463/511 isolates), 89.63% (458/511), and 76.32% (390/511 isolates), respectively (Fig. 2), whereas the *cry9* and *vip3A* genes were detected with medium frequency at 40.70% (208/511 isolates) and 47.55% (243/511 isolates), respectively.

The *cry1A* and *cry1B* genes were detected at high frequency (more than 90%) in *B. thuringiensis* from every region of Thailand except the East (Fig. 3), where it was found in less than 20% of isolates. The *cry2* gene was highly distributed in the North-East and Central regions (more than 80%) but moderately in the North, South, and West regions.

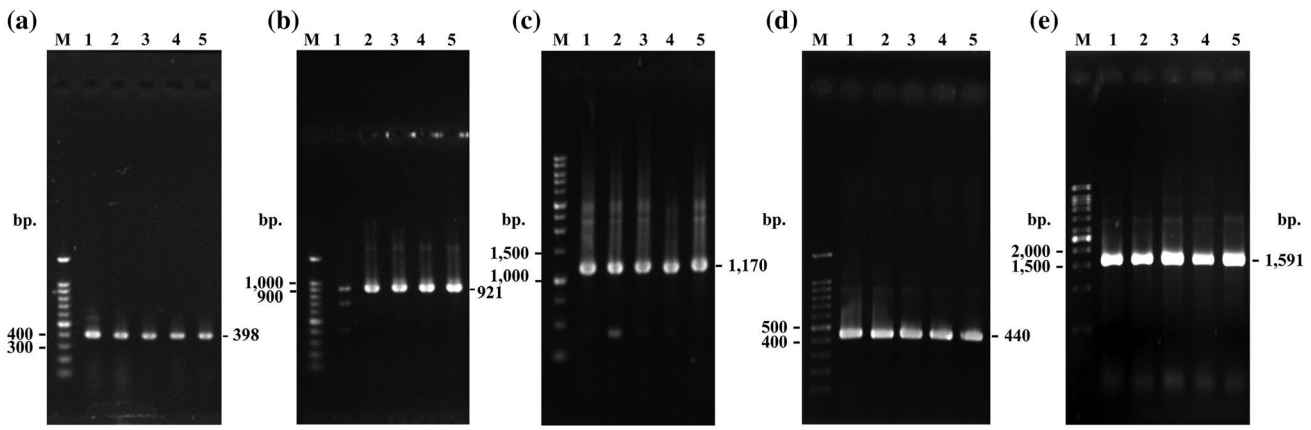

Fig. 1 PCR amplification products of indigenous *B. thuringiensis* **a** *cry1A*, **b** *cry1B*, **c** *cry2A*, **d** *cry9*, **e** *vip3A*; (Lane M: DNA Marker, 1: *B. thuringiensis* 225-15, 2: *B. thuringiensis* 349-4, 3: *B. thuringiensis* 417-1, 4: *B. thuringiensis* 831-2, 5: *B. thuringiensis* 834-1)

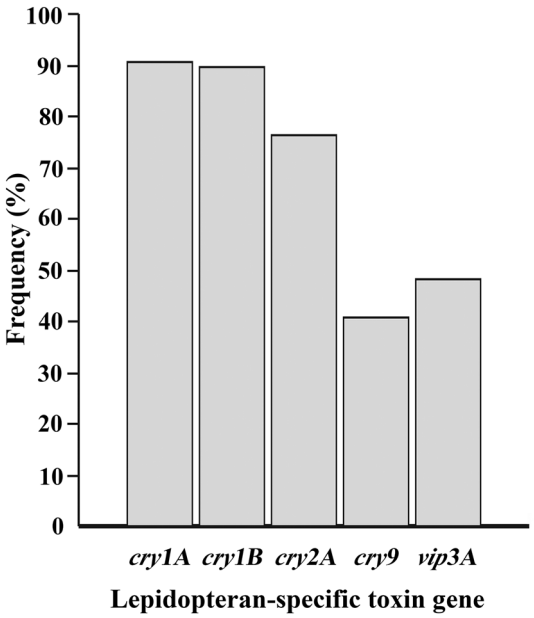

Fig. 2 Insecticidal toxin gene composition of *B. thuringiensis* in Thailand

The *cry9* and *vip3A* genes were detected at moderate to low frequency in almost every region except for the West, where *cry9* was present at a high frequency (more than 80%).

Among the 482 isolates harboring insecticidal toxin genes, 19 gene combination profiles were observed in these isolates (Table 2). There were 471 *B. thuringiensis* isolates (97.72%) that harbored more than one lepidopteran-specific gene, whereas only 11 isolates (2.28%) contained only one of the detected genes. The most frequent pattern contained the *cry1A, cry1B, cry2A*, and *vip3A* genes in 118 isolates (24.48%), whereas 61 *B. thuringiensis* isolates (12.66%) contained all the detected genes.

Chitinase assays

Sixty-one isolates containing all the detected genes (*cry1A, cry1B, cry2A, cry9* and *vip3A*) were further detected for the *chi36* gene encoding exochitinase. Amplification products of the target gene were analyzed using agarose gel electrophoresis (Fig. 4). The results revealed that all 61 isolates contained the *chi36* gene as they provided amplicons of the expected size (1083 bp). In addition, the exochitinase activity of 61 isolates was analyzed by measuring the hydrolysis zones around their colonies, for which the highest activity was obtained from the *B. thuringiensis* 312-4 (Table S1 and Fig. S1).

Insecticidal activity against *Spodoptera litura*

Twenty-five isolates of *B. thuringiensis* containing *cry1A, cry1B, cry2, cry9, vip3A*, and *chi36* genes were selected for further larvicidal activity assays against second instar larvae of *S. litura*. The results revealed that all the isolates displayed larvicidal activity with different level of toxicities (Fig. 5) in a range between 30 and 93% mortality. It

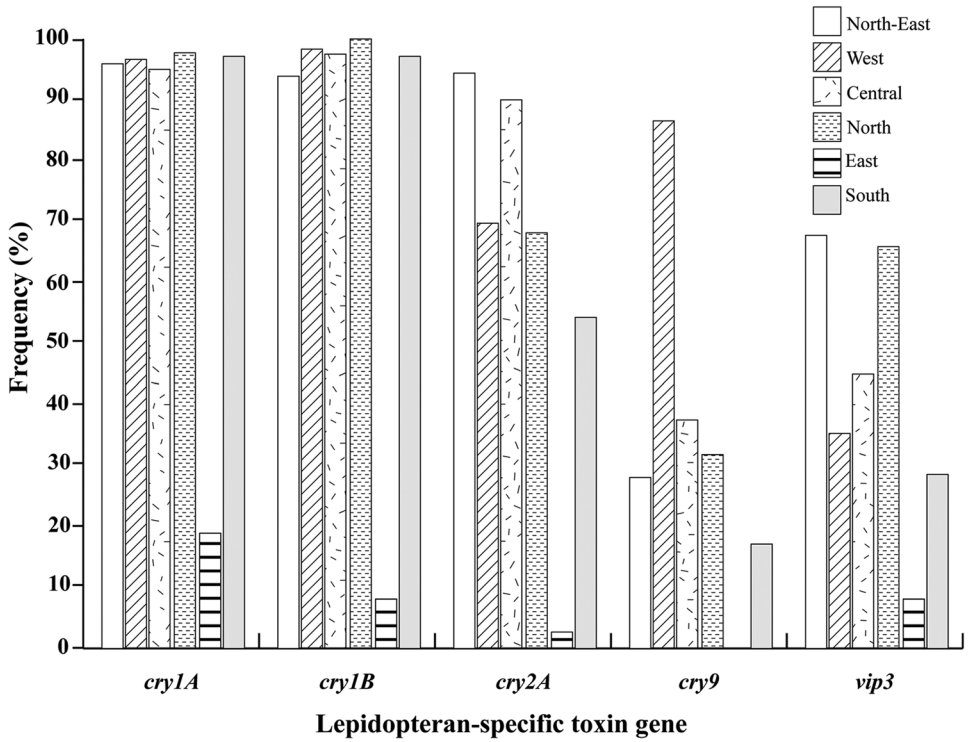

Fig. 3 Insecticidal toxin gene distribution in different regions of Thailand

Table 2 Insecticidal toxin gene combination profiles of *B. thuringiensis* in Thailand

Profile	Gene composition	No. of isolate	%
1	*cry1A* + *cry1B* + *cry2* + *vip3A*	118	24.48
2	*cry1A* + *cry1B* + *cry2*	94	19.50
3	*cry1A* + *cry1B* + *cry2* + *cry9*	88	18.26
4	*cry1A* + *cry1B* + *cry2* + *cry9* + *vip3A*	61	12.66
5	*cry1A* + *cry1B* + *cry9*	41	8.51
6	*cry1A* + *cry2*	23	4.77
7	*cry1A* + *cry1B* + *cry9* + *vip3A*	11	2.28
8	*cry1A* + *cry1B* + *vip3A*	11	2.28
9	*cry1B* + *cry2* + *cry9*	7	1.45
10	*cry1B* + *cry2*	6	1.24
11	*cry1A*	5	1.04
12	*cry2*	5	1.04
13	*cry1A* + *cry2* + *cry9*	4	0.83
14	*cry1A* + *cry2* + *vip3A*	2	0.41
15	*cry1A* + *cry9*	2	0.41
16	*cry1B* + *cry2* + *vip3A*	1	0.21
17	*cry1B* + *cry9*	1	0.21
18	*cry1A* + *cry1B*	1	0.21
19	*cry1B*	1	0.21
	Total	482	100

is interesting to note that five isolates (*B. thuringiensis* 225-15, 349-4, 417-1, 831-2, and 834-1) showed high larvicidal activity that caused more than 80% mortality of *S. litura* larvae which was comparable to that of the reference strain *B. thuringiensis* serovar *kurstaki* HD1.

Characterization of high lepidopteran-toxic *B. thuringiensis* isolates

SEM analysis of crystal morphologies

Crystal protein morphologies of five effective isolates (*B. thuringiensis* 225-15, 349-4, 417-1, 831-2, and 834-1) were characterized using SEM. The SEM images revealed that the bipyramidal and cuboidal crystal proteins were present in all isolates (Fig. 6).

SDS-PAGE analysis

The molecular mass of the protein compositions of the crystal proteins from the five effective isolates was determined using SDS-PAGE analysis. SDS-PAGE investigation of the spore–crystal mixture revealed that all isolates contained major bands with molecular mass of approximately 130 and 65 kDa that were similar to those of *B. thuringiensis* serovar *kurstaki* HD1, which was used as a reference (Fig. 7), whereas the protein pattern of *B. thuringiensis* serovar *israelensis* showed major bands at approximately 27, 70, and 130 kDa.

Insecticidal activity against *Aedes aegypti*

The larvicidal activity of five isolates (*B. thuringiensis* 225-15, 349-4, 417-1, 831-2, and 834-1), which were highly toxic to *S. litura* larvae, was assayed against a non-target organism, *A. aegypti* larvae (a dipteran insect). The bioassay results demonstrated that these isolates showed very low toxicity (less than 20% mortality) against second instar larvae of *A. aegypti* compared with the reference strain *B. thuringiensis* serovar *israelensis* that provided 100% mortality (Fig. 8).

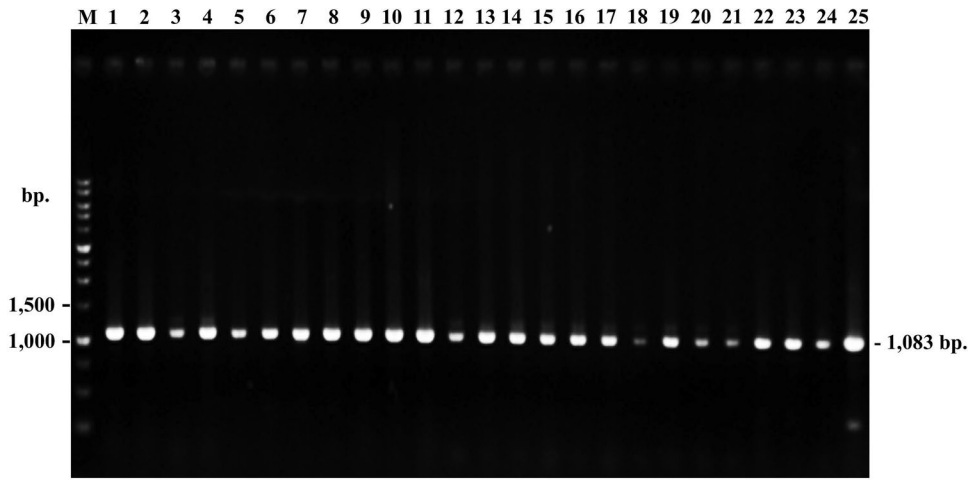

Fig. 4 Examples of PCR amplification products of *chi36* gene in *B. thuringiensis* (Lane M: DNA Marker, 1–25: *B. thuringiensis* isolates)

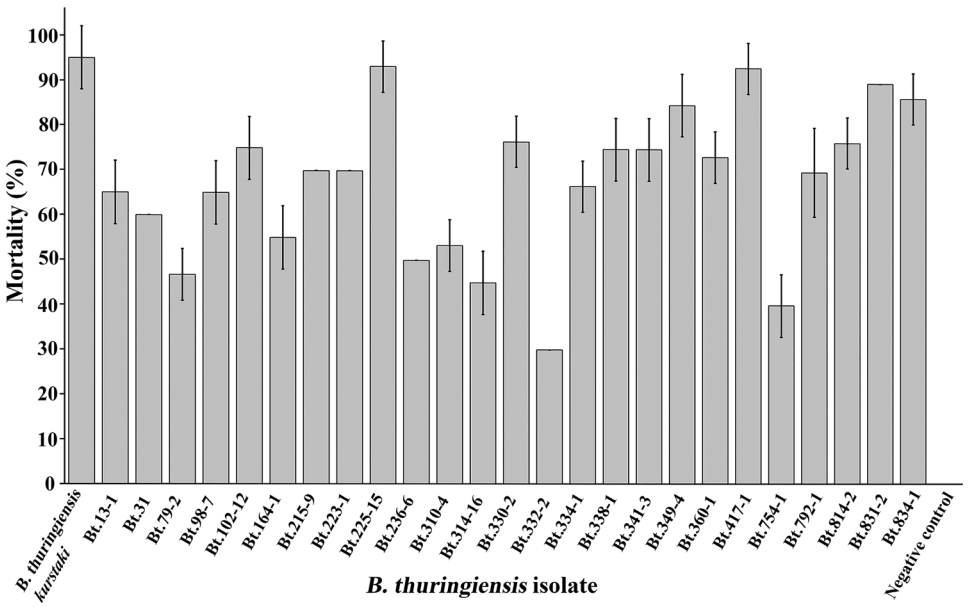

Fig. 5 Larvicidal activity of *B. thuringiensis* against *S. litura* larvae. Error bar indicates ± standard error for three experiments

Discussion

Isolation and characterization of novel *B. thuringiensis* strains may provide known or novel insecticidal proteins with higher larvicidal activity or broaden the insect spectrum. Therefore, analysis for native effective *B. thuringiensis* isolates was intensively performed, leading to an increase in the *B. thuringiensis* collection worldwide. We previously analyzed the *cry* gene composition in 134 *B. thuringiensis* isolates from 6 geographical regions of Thailand and reported that the *cry1A* and *cry1B* genes were found at moderate frequencies of approximately 50%. *cry2A* was found at high frequency of 80.6%, whereas 37.3% of *B. thuringiensis* isolates contained *cry9* gene (Thammasittirong and Attathom 2008). In the present study, a higher number of local *B. thuringiensis* isolates (511 isolates from the same geographical regions of Thailand) were analyzed for the presence of the *cry1A, cry1B, cry2A, cry9*, and the additional *vip3A* genes encoding for lepidopteran-specific toxins. The isolated *B. thuringiensis* isolates from the same soil sample those shared the same morphology of inclusion proteins, protein pattern and the insecticidal toxin gene profile were considered as twin strains. The twin strains were excluded from this analysis to obtain a real estimate of the genetic diversity of the sampled areas. The results revealed that the *cry2A* and *cry9* genes were detected with almost similar frequencies to the previous study, with the exceptions being *cry1A* and *cry1B* whose frequencies (approximately 90%) were higher in this *B. thuringiensis* collection. The results showed that even using the same primers and conditions for PCR detection and the same geographical regions of samples, differences in gene frequencies could be obtained.

The *cry1A* gene was detected at high frequency in our *B. thuringiensis* collection comparable with those of *B. thuringiensis* in Argentina and Egypt (Table 3). It was interesting to note that *cry1B* was not present in *B. thuringiensis* from Argentina and Iran and was found at low-medium frequency in China and Egypt compared with the high frequency in our collection (Table 3). The *cry2A* gene was detected with high frequencies in our collection and in *B. thuringiensis* from Argentina and China; however, this gene was detected at medium frequency in Iran and Egypt (Table 3). The *cry9* gene was detected with low frequency in Argentina, Iran, and China compared with medium frequency in our collection. Finally, the *vip3A* gene was detected in our collection at a frequency comparable to those of India and China; however, higher frequencies of *vip3A* were reported for the *B. thuringiensis* collections from Argentina and Iran (Table 3). This information confirmed that the frequencies of the *cry* and *vip* genes varied depending on each *B. thuringiensis* collection. The diversity of *B. thuringiensis* in each collection may provide novel *B. thuringiensis* isolates with higher larvicidal activity or broaden the target insects suitable for use in combination with or as an alternative to the existing *B. thuringiensis* strains.

We previously reported that the *cry1A, cry1B*, and *cry2* genes were found in effective isolates at high frequencies and suggested that these toxins may contribute to high larvicidal activity against lepidopteran insects (Thammasittirong and Attathom 2008). In the current work, we observed higher frequencies of *cry1A* and *cry1B* in *B. thuringiensis* from every area except for *B. thuringiensis* from the East region. The results suggest that we might obtain a high number of *B. thuringiensis* isolates containing larvicidal activity against lepidopteran insect larvae. The results showed high

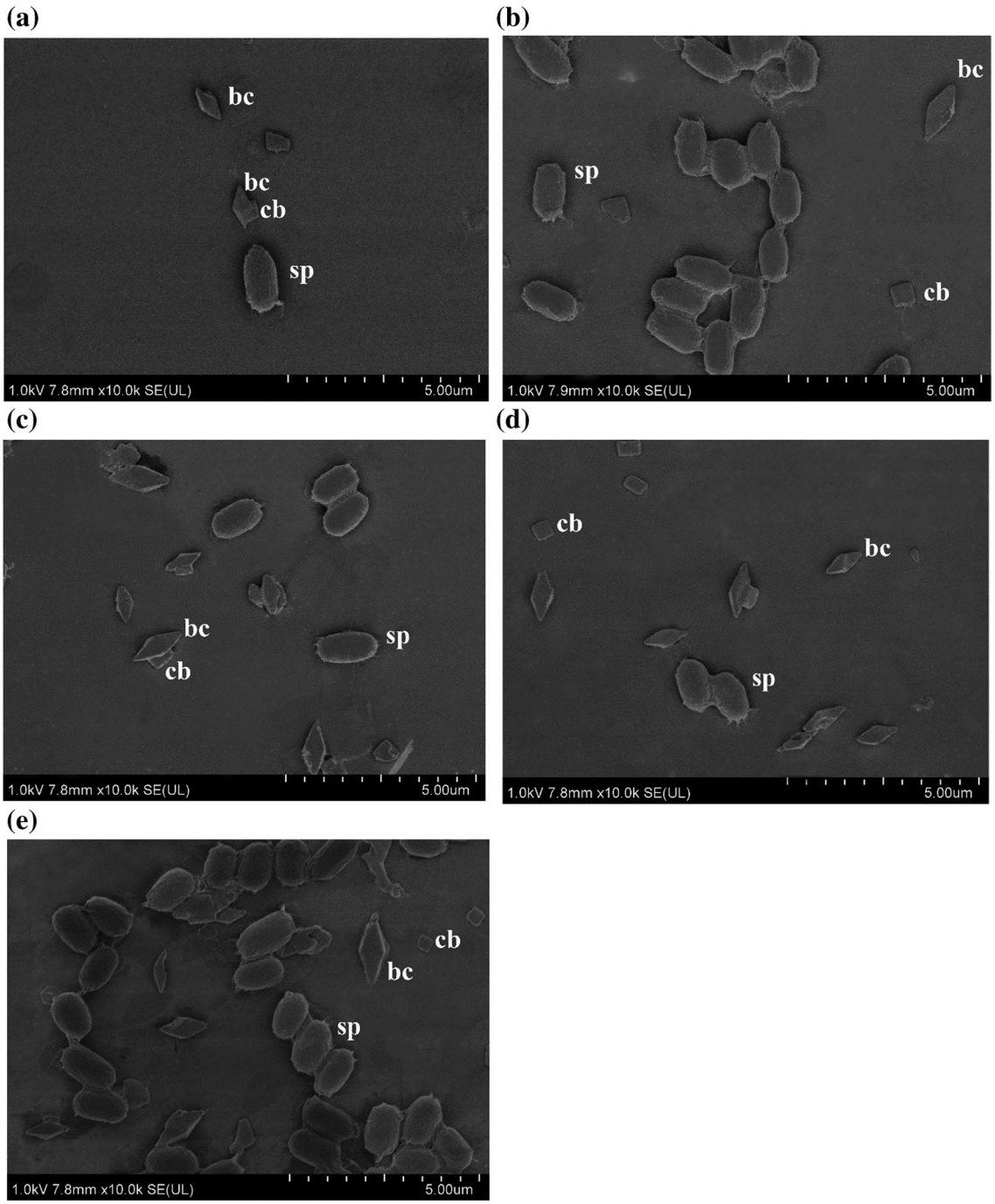

Fig. 6 Electron micrographs of crystals and spores of *B. thuringiensis* **a** *B. thuringiensis* 225-15, **b** *B. thuringiensis* 349-4, **c** *B. thuringiensis* 417-1, **d** *B. thuringiensis* 831-2 **e** *B. thuringiensis* 834-1. *sp* spore, *bc* bipyramidal crystal, *cb* cuboidal crystal

frequencies of gene combinations in our *B. thuringiensis* collection, as 19 gene profiles were observed which were higher than that reported in Argentina where 10 gene combination profiles were reported (Sauka and Benintende 2017). However, higher numbers of *cry* gene combination profiles (43 profiles) were reported for *cry1* subclasses of *B. thuringiensis* from China (Wang et al. 2003). The difference in gene combination profiles may be attributable to differences in the numbers of isolates in each *B. thuringiensis* collection, the type of the detected genes, and the diversity of *B. thuringiensis* isolates in the collection. Our results showed that *cry1A* was usually found with *cry1B* and *cry2*, with combination frequencies of 88.17% and 80.91%, respectively. High associations between *cry1* and *cry2* have been commonly

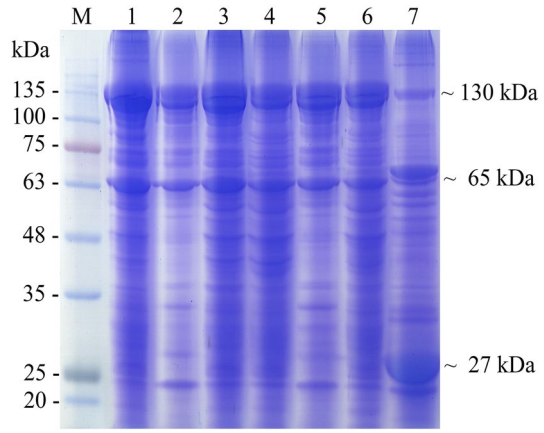

Fig. 7 SDS-PAGE of spore–crystal mixture of *B. thuringiensis*; (Lane M: prestained protein ladder, 1: *B. thuringiensis* serovar *kurstaki*, 2: *B. thuringiensis* 225-15, 3: *B. thuringiensis* 349-4, 4: *B. thuringiensis* 417-1, 5: *B. thuringiensis* 831-2, 6: *B. thuringiensis* 834-1, 7: *B. thuringiensis* serovar *israelensis*)

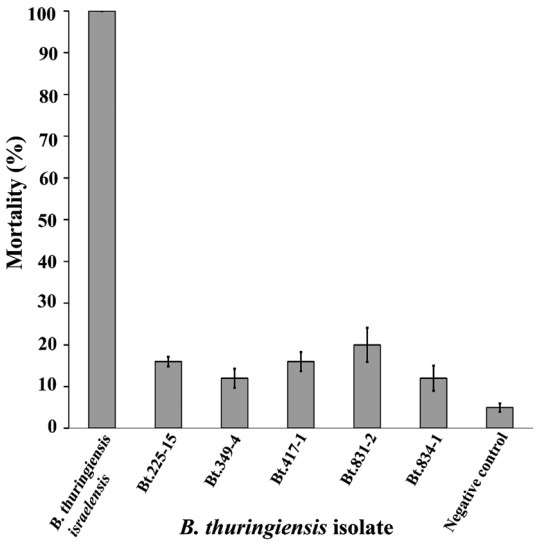

Fig. 8 Larvicidal activity of *B. thuringiensis* against *A. aegypti* larvae. Error bar indicates ± standard error for three experiments

reported in various *B. thuringiensis* strains including *B. thuringiensis* serovar *kurstaki* HD1 and various *B. thuringiensis* isolates (Rangeshwaran et al. 2014; Sauka and Benintende 2017; Wang et al. 2003).

As previous reports have shown that the larvicidal activity of Cry toxins was improved by the chitinase enzyme (Chen et al. 2015; Ding et al. 2008), the 61 isolates of *B. thuringiensis* containing all the detected genes in the current work were therefore detected for the exochitinase gene and chitinase activity. Exochitinase gene and chitinase activities were detected in all 61 isolates; the exochitinase may synergize the larvicidal activity of insecticidal toxins from these isolates. The Cry1B, Cry2, and Vip3A toxins have been reported as toxic toward *S. litura* larvae (Herrero et al. 2016; Lu et al. 2013; Reyaz et al. 2017), whereas Cry1Ab and Cry1Ac were reported as non-toxic against *S. litura* larvae (Lu et al. 2013). In addition, Cry9Ea1, which is a member of the *cry9* genes that presented at a low level in our *B. thuringiensis* collection, were reported as non-toxic against *S. litura* larvae (Wasano et al. 2005). Out of the 61 isolates containing all the detected genes and chitinase activity, 25 isolates were selected for larvicidal activity assay against *S. litura* larvae. The result revealed that only five isolates (*B. thuringiensis* 225-15, 349-4, 417-1, 831-2, and 834-1) showed high larvicidal toxicity (more than 80%) to *S. litura* larvae, implying that insecticidal activity may not rely only on the presence of the detected toxins and the chitinase enzyme but also on toxins, which were not detected in this work or the expression level of the protein toxins.

The SEM images showed bipyramidal and cuboidal crystal morphologies of *B. thuringiensis* 225-15, 349-4, 417-1, 831-2, and 834-1 that were similar to most of the *B. thuringiensis* samples in Argentina, of which 88.8% of isolates containing *cry1* and *cry2* genes exhibited bipyramidal and cuboidal crystals (Sauka and Benintende 2017). The inclusion morphologies of *B. thuringiensis* serovar *kurstaki* HD1 have been reported to be bipyramidal and cuboidal structures (Monnerat et al. 2007; Patel et al. 2012; Zorzetti et al. 2017); however, some publications reported that this strain

Table 3 Insecticidal toxin gene detection in different countries

Country of Bt collection	Gene frequency (%)					No. of isolate	References
	cry1A	*cry1B*	*cry2A*	*cry9*	*vip3A*		
Thailand	90.61	89.63	76.32	40.70	48.18	511	This study
Argentina	92.5	0	92.5	2.5	91.3	268	Sauka and Benintende (2017)
Iran	44.29	0	54.29	25.71	82.6	70	Seifinejad et al. (2008)
Egypt	83.33(*cry1*)	38.89	55.55	nt	0	18	Salama et al. (2015)
India	nt	nt	nt	nt	43.18	86	Lone et al. (2016)
China	nt	nt	nt	nt	67.4	2134	Yu et al. (2011)
China	67.7	12.9	70.0	15.5	nt	310	Wang et al. (2003)

nt not test

contained bipyramidal, cuboidal, and spherical structures (Azizoglu et al. 2015; Yılmaz et al. 2012). Molecular mass analysis using SDS-PAGE showed the major bands of five effective isolate at approximately 130 and 65 kDa proteins, which were consistent with the 130 and 65 kDa proteins of the bipyramidal crystals of Cry1 proteins and the cuboidal crystals of Cry2 proteins, respectively (Azizoglu et al. 2015; Patel et al. 2012; Zorzetti et al. 2017). Even Cry2 toxins which form cuboidal inclusion have been reported to exhibit dual toxicity against both lepidopteran and dipteran insects (Ribeiro et al. 2017). In this work, very low toxicity against *A. aegypti* larvae was observed, this may be due to the five effective *B. thuringiensis* isolates contain Cry2 toxin which do not show larvicidal activity to *A. aegypti* larvae. The Cry2Aa was reported to exhibit the toxicity to lepidopteran and dipteran insects including *A. aegypti*, whereas a closely related Cry2Ab toxin was shown to contain toxicity to lepidopteran insects and mosquitocidal activity against *Anopheles gambiae* but not *A. aegypti* larvae (McNeil and Dean 2011). Low toxicity against *A. aegypti* larvae implies that the five effective isolates exhibit their larvicidal activities with high specificity.

Conclusion

The frequencies and distribution of insecticidal toxin genes were analyzed in 511 local *B. thuringiensis* isolates. The results showed that the frequencies of insecticidal toxin genes depended on the diversity of the *B. thuringiensis* isolates in the collection. Five isolates containing all the detected insecticidal toxin genes and chitinase enzyme displayed high toxicity and specificity against *S. litura*. These five effective isolates are alternative candidates for use in combination or as alternatives to the existing *B. thuringiensis* strains for controlling outbreaks of *S. litura*.

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Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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