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Pathogen detection and gut bacteria identification in *Apis cerana indica* in Thailand

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Pathogen infection of honeybees can lead to economic losses in apiculture. The earlier the pathogen contamination can be found the better it will likely be for the treatment of the infected colony and prevention of the spread of the pathogen within and between colonies. A total of 50 colonies of *Apis cerana* were sampled in Samut-Songkhram (five colonies) and Chumphon (45 colonies) provinces in the central and the south of Thailand, respectively. Diagnostic multiplex polymerase chain reaction (PCR) revealed that 20, 6, 4, 20 and 0% of the samples were infected by *Paenibacillus larvae*, *Nosema ceranae*, *Nosema apis*, *Ascosphaera apis* and Sacbrood virus (*Morator aetatulus*), respectively. Positive amplified PCR products of target genes were sequenced. A phylogenetic tree was constructed by the neighbor-joining (NJ) distance based, using the Phylogenetic Analysis Using Parsimony (PAUP 4.0b10) program. The phylogenetic relationship of each pathogen, based on the partial sequence of the *16S rRNA* of *P. larvae*, *N. ceranae* and *N. apis*, and of the *5.8S rRNA* of *A. apis*, revealed a significant difference from the non-Thai isolates of these pathogens, but no significant geographical isolation between the different Thai apiaries, although it separated some into closely related clusters. In order to reduce the use of antibiotics in an apiary, bacteria in the gut of healthy bees were focused. Interestingly, *Bifidobacterium* species, *Lactobacillus* species, *Bacillus* species, *Lactobacillus* spp. and other lactic acid bacteria, were isolated from larvae and adult workers, but gave conflicting preliminary identities based on their biochemistry-morphology versus sequence analysis of a partial fragment (1.4 kb) of their *16S rRNA*.

Key words: *Apis cerana indica*, bee pathogens, gut bacteria, multiplex polymerase chain reaction (PCR), *16S rRNA*.

INTRODUCTION

Honey bees (*Apis* species) are environmentally and economically important insects, with the latter being crop pollination and essential products used for food and traditional medicine (Moritz et al., 2010). However, there

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is a diverse array of pathogen-caused bee diseases, some of which can cause serious morbidity and mortality to bee colonies and lead to the economic loss to bee farmers. Thus, it would be much better if pathogen infections could be detected at an early stage.

After *Apis cerana indica* foragers return to a hive from foraging, they will dance to communicate about the food source (Oldroyd and Wongsiri 2006). Also, other bees inside the nest perform a cleaning behavior in order to remove contaminating objects including mites and some microorganisms in the flower pollen. However, contaminated microorganisms within the bees and in the nectar stored in forager's honey crop still remain (Anderson et al., 2013).

For decades, a diverse array of antibiotics and chemicals has been used to try to control bee disease. For example, tetracycline has been used to inhibit *Paenibacillus larvae* growth to limit American foulbrood (AFB) and *Melissococcus plutonius* growth to limit European foulbrood (EFB) (Martel et al., 2006). For microsporidia (*Nosema*) infections, treatment with fumagillin B has been found to be very effective, including in the control of infections by treating the hive box with fumagillin B (Higes et al., 2011). For chalkbrood disease, which is caused by *Ascosphaera apis*, many reagents have been used to eliminate the spores, such as sodium propionate, sorbic acid (Taber et al., 1975), citral and geraniol (Gochnauer et al., 1979).

However, the use of antibiotics and antimycotics to treat bee diseases has decreased in general due to public concern over the residual antimicrobial levels in the bee products and bacterial resistance to these chemicals (Kochansky et al., 2001). Many alternative sources were reported. For example, botanical compounds from plant extracts, such as cinnamon oil, bay oil, clove oil and origanum oil, have been used to control *A. apis* infections and prevent chalkbrood disease. The most effective compounds in these plant extracts (oils) were reported to be azadirachtin, thymol and α-terpinene (Calderone and Shimanuki, 1994).

In addition, three strains of *Flourensia* (*Flourensia tortuosa*, *Flourensia riparia* and *Flourensia fiebrigii*) isolated from *Andiroba* and *Copaiba* plant extracts were recently found to inhibit the *in vitro* growth of *P. larvae*, as also could the essential oils from these plants (Reyes et al., 2013). However, as an alternative approach, it is viewed that effective probiotics from the bees themselves or their products that can inhibit their own pathogens would be a better option. Prebiotics, probiotics and balance of intestinal flora in bee gut are capable of preventing and combating disease. Their imbalance results in abnormal function of the intestine and easily causes illness (Patruica and Mot, 2012). Along these lines, five strains of *Bacillus* and 11 phylotypes of lactic acid bacteria (LAB) from the genera *Lactobacillus* and *Bifidobacterium,* isolated from the honey and gut of *Apis mellifera*, were found to be able to inhibit the *in vitro* growth of 17 strains of *P. larvae* (Alippi and Reynaldi, 2006; Forsgren et al., 2010). In addition, the feeding of this LAB mixture to bee larvae caused a significant decrease in the number of infected colonies (Alippi and Reynaldi 2006; Forsgren et al., 2010).

In this research, *A. cerana*, which is an economic and native honey bee species to Thailand, was used. The diagnosis and distribution of some pathogens known to cause serious bee diseases were screened for using diagnostic multiplex polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR), and their phylogenetic relationships were evaluated. In addition, the bacteria residing in the gut of healthy bee larvae and adult workers were isolated and preliminarily identified, since they may include probiotic isolates.

MATERIALS AND METHODS

Sample collection

In total, 10 apiaries were selected and were sampled in May and November, 2012. One apiary was in Samut-Songkhram province (Central Thailand) and the other nine were in Chumphon province (Southern Thailand). In each apiary, five colonies were chosen at random and sampled where, for each colony, 50 each of larvae, pupae and adults were randomly collected. However, for the analysis of gut bacteria, three colonies within the apiary in Samut-Songkhram province only were selected at random and 50 each of healthy larvae and adults were collected for analysis.

Disease diagnosis by morphology

The presence of abnormal morphological characters of larvae and adults were observed externally, including the color and body shape. Additionally, the presence or absence of Varroa mite was observed at mites adhering to the bee's body (Genersch, 2010).

Pathogen detection by diagnostic multiplex PCR

DNA and RNA extraction

DNA from the larva, pupa and adults was extracted using a DNA extraction mini kit (cat. # 51304, QIAgen), while RNA was extracted from those samples using a RNeasy mini kit (cat. # 74104, QIAgen), both as per the manufacturer's instructions. Eluted samples were kept at -20°C until used. The concentration of DNA or RNA in each sample was calculated from the absorbance at 260 nm and the purity of the sample was estimated from the ratio of absorbance at 260 and 280 nm. The quality of DNA and RNA was observed by 0.8% (w/v) agarose-Tris-borate-EDTA (TBE) gel electrophoresis or 1.2% (w/w) formaldehyde/agarose gel electrophoresis, respectively, followed by ethidium bromide (EtBr) staining and ultraviolet (UV)-transillumination.

Primer design

Total DNA was amplified by multiplex PCR using the designed primers shown in Table 1. In order to detect *P. larvae* infection, the

Table 1. Primers used for the multiplex PCR or RT-PCR(*) detection of pathogens.

Primer name	Sequences (5' → 3')	Target	Product size (bp)
PL5-F1 PL4-R1	TCAGTTATAGGCCAGAAAGC CGAGCGGACCTTGTGTTTCC	*P. larvae*	700
Cytb-F2 Cytb-R2	TGATAAAAGAAATATTTTGA TGAAACAAATATATAAATTG	Host *Cytb*	500
MITOC-F3 MITOC-R3	CGGCGACGATGTGATATGAAAATATTAA CCCGGTCATTCTCAAACAAAAAACCG	*N. ceranae*	218
APIS-F4 APIS-R4	GGGGGCATGTCTTTGACGTACTATGTA GGGGGGCGTTTAAAATGTGAAACAACTATG	*N. apis*	321
RpS5-F5 RpS5-R5	AATTATTTGGTCGCTGGAATTG TAACGTCCAGCAGAATGTGGTA	Host *RpS5*	115
SBV-F6* SBV-R6*	AAGGAACTATAGTATGGCGAA CTGTTGCTGGTCTCTTGT	SBV	200
28S-F7* 28S-R7*	AAAGATCGAATGGGGAGATTC CACCAGGTCCGTGCCTCC	Host 28S rRNA	358
As.apis-F8 As.apis-R8	TGTCTGTGCGGCTAGGTG CCACTAGAAGTAAATGATGGTTAGA	*As. apis*	500

causative agent of AFB, each reaction used the PL5-F1/PL4-R1 primer pair to amplify a fragment of the *16S rRNA* of *P. larvae* (accession # DQ 079621.1) in conjunction with the Cytb-F2/Cytb-R2 pair to amplify a fragment of the *A. cerana cytochrome b* gene (accession # ACR 55919.1) as an internal control (Piccini et al., 2002). DNA from *P. larvae* spore which was used as a positive control was a gift from Dr. P. Chantawannakul, Chiang Mai University, Thailand. In order to detect the microsporidian *Nosema* species infections, two pairs of primers were designed from the SSU *rRNA*, the first (MITOC-F3/MITOC-R3) for *Nosema ceranae* (accession # FJ 425736.1), and the second (APIS-F4/APIS-R4) for *N. apis* (accession # JX 860435.1). These were used in conjunction with the primer pair RpS5-F5/RpS5-R5 to amplify a fragment of the host ribosomal protein S5 (*RpS5*) (designed from *A. mellifera*; accession # XM 393226.3) as an internal control (Hamiduzzaman et al., 2010).

For the detection of *A. apis*, the specific primer pair *A. apis*-F8/*A. apis*-R8 was designed from the *5.8S rRNA* of *A. apis* (accession # HQ 905552.1) and used in conjunction with the RpS5-F5/RpS5-R5 primer pair as an internal control (James and Skinner, 2005).

For the detection of Sacbrood virus (SBV), the specific primer pair SBV-F6/SBV-R6 designed from the polyprotein (*pol*) of SBV (accession # HG 779873.1) was used in conjunction with the 28S-F7/28S-R7 primer pair to amplify a fragment of the *28S rRNA* of *A. cerana* (designed from *A. mellifera*; accession # X 93388.1) as an internal control (Yang et al., 2012).

PCR amplification

The PCR reactions were performed in a final volume of 25 µl containing 12.5 µl of Emerald Amp GT PCR master mix (2x Premix, cat. # RR310Q, Takara), 5 µl of DNA template (200 ng), 1 µl of 10 µM of each primer and 0.5 µl of 10 mM deoxyribonucleotide triphosphate (dNTP mix).

For the detection of *P. larvae*, the PCR was performed with an initial 94°C for 1 min followed by 35 cycles of 94°C for 1 min, 45°C for 1 min and 64°C for 2 min before a final 72°C for 7 min. For *Nosema*, the PCR conditions were an initial 94°C for 2.5 min, 10 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 45 s, followed by 20 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 50 s, and then a final 72°C for 7 min. For the detection of *A. apis*, the PCR was performed at 94°C for 1 min, followed by 30 cycles of 94°C for 45 s, 45°C for 1 min and 72°C for 1 min, and then a final 72°C for 7 min. After PCR amplification, all PCR products were observed following resolution by 0.8% (w/v) agarose gel electrophoresis, EtBr staining and UV-transillumination.

RT-PCR amplification

Since SBV is a RNA virus, it was detected via RT-PCR. Reactions were performed in a final volume of 25 µl composed of 12.5 µl of Emerald Amp GT PCR master mix (2x Premix), 5 µl of RNA template (200 ng), 400 nM of each primer, 0.5 µl of 200 µM dNTP mix and 0.5 µl of AMV Reverse Transcriptase (cat. # A1250, Promega). The thermal cycling was performed at an initial 48°C for 45 min for RT-mediated cDNA synthesis, and then PCR amplified at 94°C for 2 min followed by 30 cycles of 93°C for 1 min, 50°C for 30 s and 72°C for 1 min before a final 72°C for 7 min. After the amplification was done, the RT-PCR products were observed by

0.8% (w/v) agarose-TBE gel electrophoresis, EtBr staining and UV-transillumination.

Phylogenetic analysis

The targeted PCR product was purified using a PCR product purification kit (cat. # 28104, Qiagen) and then commercially direct sequenced by AIT biotech (Singapore). The obtained nucleotide sequence was then searched for similar sequences in the NCBI GenBank database using the BLASTn algorithm. The obtained nucleotide sequences and the similar ones from GenBank along with the selected outgroup sequences were aligned using the Clustal X program.

The phylogenetic relationship among the different isolates of each pathogen was analyzed by the neighbor-joining (NJ) distance based, using the Phylogenetic Analysis Using Parsimony (PAUP 4.0b10) program (Swofford 2000). The support for the nodes was evaluated by bootstrap analysis with 1,000 times.

Gut bacteria in healthy bees

Bacterial culture

The isolation of gut bacteria from healthy appearing larvae or adult *A. cerana* bees followed the method of Yoshiyama and Kimura (2009). In brief, the larval body and the dissected gut of adult bees were surface sterilized by immersion into 10 ml of 70% (v/v) ethanol for 1 min, followed by 10 ml of 6% (w/v) sodium hypochlorite (NaOCl) for 1 min, and then, rinsed with 10 ml of sterile distilled water for 1 min. After that, either three larvae or the guts from three healthy bees were homogenized in 700 µl of phosphate buffer saline (PBS) pH 7.2, and then clarified by centrifugation at 2,817× g for 1 min at room temperature. The supernatant was harvested, serially diluted and each serial dilution was spread at 200 µl per plate onto a duplicate set of triplicate Brain Heart Infusion (BHI) agar (cat. # 241830, Difco) plates. The first set of triplicate plates was then cultured for aerobic bacteria at 37°C for 18 to 24 h, whilst the second set of triplicate plates was cultured for anaerobic bacteria by incubation in an anaerobic jar with a gas pak (cat. # AN 25US, Oxoid) at 37°C for four to five days.

Bacterial colony morphology

The colony morphology, in terms of the color, luster and smooth, convex or jagged edges, was observed and recorded. The number of colonies (Nc) was counted from three randomly selected 1 cm^2 boxes on each 63.6 cm^2 plate and the colony forming units (CFUs)/ml was then evaluated as:

CFU/ml = [(Nc) × (the area of plate (cm^2)) × (the serial dilution factor) / 0.6].

In addition, Gram staining of bacteria from representative colonies of which each with different morphology was performed.

Biochemical characterization (substrate fermentation) of bacterial colonies

The ability of three representative colonies from each different morphological bacterial isolate (section 2.5.2) to ferment different substrates was observed using the API 20A test (cat. # 20300, Biomerieux) for anaerobic bacteria and the API 50 CH test (cat. # 50300, Biomerieux) for aerobic bacteria.

Sequence analysis of the 16S rRNA gene

For representative isolates from each morphologically different colony type (section 2.5.3), colony PCR was performed. A single colony was picked into 20 µl of 10 mM TE buffer (pH 7.5) and subjected to three cycles of -20°C for 30 min followed by incubated 96°C for 5 min and vortexing. A 2 µl aliquot (prediluted to ~100 ng/µl) was then PCR amplified using the eu27F (5'-GAGAGTTTGATGCTGGCTCAG -3') and eu1495R (5'-CTACGGCTACCTTGTTACGA -3') primer pair (Yoshiyama and Kimura, 2009) in a final volume of 25 µl containing 12.5 µl of Emerald Amp GT PCR master mix (2x Premix), 8.5 µl of nuclease free distilled water and 1 µl of 10 µM of each primer. The PCR was performed by an initial 95°C for 1 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and then a final 72°C for 7 min. After PCR, the amplified product was checked by 0.8% (w/v) agarose-TBE gel electrophoresis with EtBr staining and UV-transillumination. The expected size of PCR product was about 1.4 kb.

RESULTS

Disease diagnosis

Bee morphology

The morphology of randomly sampled bee larvae, pupae and adults from 50 colonies was observed. In overall, an abnormal black and brown body color was found in a large number of larvae, whilst the pupa was a more prevalent infection target for mites. In contrast, neither mites (*Varroa* or any species, parasitic or also phoretic) nor an abnormal body color was observed in the sampled adults.

Multiplex PCR or RT-PCR

After each multiplex PCR, the control (host gene amplicon) bands of the expected size (Table 1) were observed in each case (Figure S1 in supplementary information). Interestingly, polymorphism of detected pathogens could be observed. The percentage of infected bees could then be estimated (Table 2). From the five colonies (one apiary) sampled at Samut-Songkhram province (Central Thailand), *P. larvae*, *N. ceranae* and *N. apis* infections were found in 2 (40%), 3 (60%) and 1 (20%) of the colonies, respectively, although even in the infected colonies, they were at a low frequency of individuals (1.5 to 2%). However, no *A. apis* or SBV infections were found. For the 45 colonies (five apiaries) sampled in Chumphon province (Southern Thailand), *P. larvae*, *N. apis* and *A. apis* infections were detected in 8 (17.8%), 1 (2.2%) and 10 (22.2%) of the colonies, respectively, although these also were at a typically low prevalence in each infected colony (0 to 5.5%). However, no *N. ceranae* infections or SBV infections were found, and so no SBV infections were detected in this study at all.

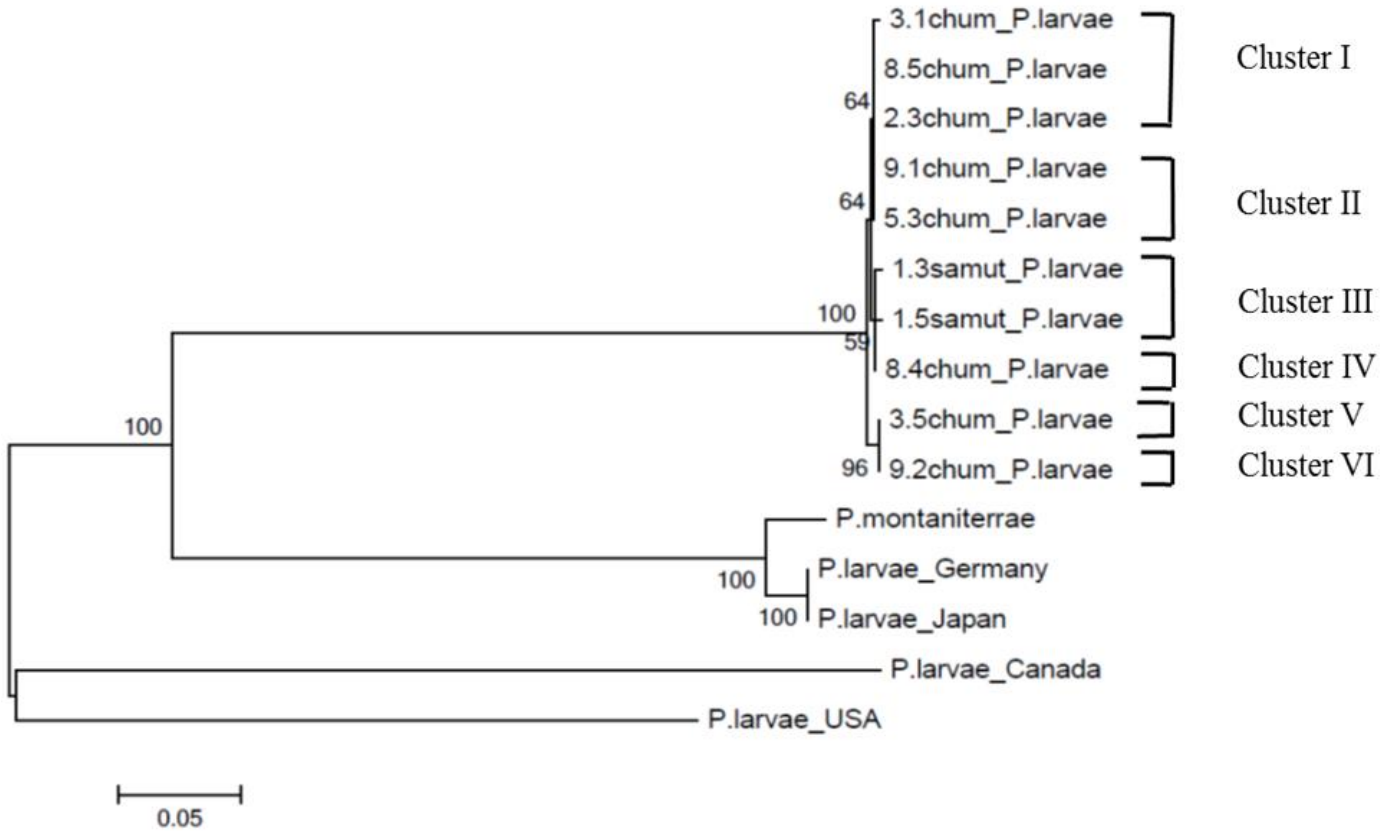

Figure 1. The NJ tree of the aligned 497 bp nucleotide data set of the *16S rRNA* gene fragment sequences from *P. larvae*. The *16S rRNA* gene sequence of *P. montaniterrae*, *P. larvae*_Germany, *P. larvae*_Japan, *P. larvae*_Canada and *P. larvae*_USA were used as the outgroups for the analysis. CP and SS were abbreviated from Chumphon and Samut-Songkhram. The number under branches indicates the percentage of bootstrap support from 1,000 replications. Scale bar represents substitution or site.

Phylogenetic analysis of the bee pathogens

Similarity of partial DNA sequence

The partial sequences of the obtained *16S rRNA* gene fragments obtained from the PCR amplifications for the 10 isolates of *P. larvae*, 3 isolates of *N. ceranae* and 2 isolates of *N. apis*, and that for the *5.8S rRNA* gene of the 10 isolates of *A. apis* were searched against the GenBank database. The highest DNA identities to AY030079.1 for *P. larvae*, KC708007.1 for *N. ceranae*, U76706.1 for *N. apis* and HQ905552.1 for *A. apis* with 99 to 100% sequence identity. This supports the likely correct PCR amplification and ascribed infection status.

Phylogenetic tree construction

The sequences of the amplified pathogen gene fragment (*16S* or *5.8S rRNA*), along with the most identical sequences from GenBank and that for the selected outgroups, were aligned using Clustal, manually checked and after removal of ambiguous aligned bases, were used to construct the respective pathogen phylogenetic trees by the NJ.

The aligned 497 bp fragments of the *16S rRNA* gene from *P. larvae* from the infected bees in both Samut-Songkhram and Chumphon provinces revealed 74 differences of which 5 sites were parsimonious informative. Using the sequences from *P. montaniterrae* (accession code AB295646.1), *P. larvae*_Germany (accession code DQ079621.1), *P. larvae*_Japan (accession code NR_112166.1), *P. larvae*_Canada (accession code AF478106.1) and *P. larvae*_USA (accession code NC_022577.1) as outgroups, the obtained phylogeny revealed that the Thai *P. larvae* sequences were closely related to the recorded *Paenibacillus* genus from elsewhere (Figure 1), and all together formed six closely related clusters (Clusters I to VI). The samples from Samut-Songkhram fell into clusters III and IV whilst those from Chumphon fell into their own groups (clusters I, II, V and VI). The isolates of

Table 2. Prevalence of pathogen infections in *A. cerana* in Thailand.

Province	Number of colonies	Number of colonies infected by			
		P. larvae **(bee stage)**	***N. ceranae*** **(bee stage)**	***N. apis*** **(bee stage)**	***A. apis*** **(bee stage)**
Samut-Songkhram	5	2 (Pupa 1, adult 2)	3 (Larva 2, adult 3)	1 (Adult 1)	0
Chumphon	45	8 (Larva 8, pupa 8, adult 6)	0	1 (Adult 1)	10 (Larva 8, pupa 8, adult 3)
Total	50	10 (20%)	3 (6%)	2 (4%)	10 (20%)

No Sacbrood virus (SBV) infected individuals were detected in all samples.

P. larvae in clusters I to VI originated from bees in different geographical areas, where the dominant plant species also differed, being palm, rambutan, longan, pineapple, coconut, and rubber trees, respectively.

However, these six clusters are neither strongly divergent, nor are they well supported by bootstrap analysis (50 to 78% bootstrap support). Thus, *P. larvae* are not resolved by this gene fragment into separate geographical regions, which may reflect its recent pandemic spread or lack of geographic isolation.

With respect to the *16S rRNA* gene fragment sequences of *N. ceranae*, the 202 bp contained 21 different sites of which 0 were parsimonious informative. Using *N. bombycis* (accession code AB093018.2) and *N. ceranae*_Indonesia (accession code FJ789802.2), *N. ceranae*_Argentina (accession code FJ227957.1), *N. ceranae*_USA (accession code EF584420.1), *N. ceranae*_Poland (accession code JX860434.1), *N. ceranae*_Australia (accession code EU045844.1), *N. ceranae*_France (accession code DQ374655.1), *N. ceranae*_Germany (accession code DQ374656.1), *N. ceranae*_Hiroshima (accession code AB562584.1), *N. ceranae*_Mie (accession code AB562583.1), *N. ceranae*_Saga (accession code AB562585.1) and *N. ceranae*_Spain (accession code DQ673615.1) as the outgroups, the obtained phylogeny revealed that the sequences of the three isolates from Samut-Songkhram province were in clusters I and II (Figure 2).

With respect to the *16S rRNA* gene fragment sequences of *N. apis*, the 251 contained 114 different sites of which 0 were parsimonious informative. Using *Encephalitozoon cuniculi* (accession code NC_003242.2), *N. apis*_Canada (accession code EF584425.1), *N. apis*_USA (accession code U26534.1), *N. apis*_Spain (accession code DQ235446.1), *N. apis*_Australia (accession code FJ789798.1), *N. apis*_Tasmania (accession code FJ789792.1), *N. apis*_Nova Scotia (accession code EU545140.1), *N. apis*_Poland (accession code JX860435.1), *N. apis*_Island (accession code FJ536221.1), *N. apis*_Queensland (accession code FJ789793.1), *N. apis*_Ireland (accession code JX213662.1), *N. apis*_UK (accession code JX213660.1) and *N. apis*_China (accession code EU864522.1) as the outgroups, the obtained phylogeny revealed that the sequences of the two isolates, one each from Samut-Songkhram and Chumphon provinces, were in cluster I (Figure 3).

Finally, the *5.8S rRNA* fragment sequences of *A. apis*, the 338 bp fragments contained 78 different sites of which 21 were parsimonious informative. Using *Beauveria bassiana* (accession code AF293968.1), *A. apis*_California (accession code EF156415.1), *A. apis*_Belgium (accession code AARE01003486.1), *A. apis*_Denmark (accession code HQ905552.1), *A. apis*_USA (accession code EU327581.1) and *A. apis*_Canada (accession code U18362.1) as the outgroup, the obtained phylogeny revealed that the sequences of the 10 isolates from Chumphon province are as shown in Figure 4. The isolates from Chumphon province fell into four distinct groups (Clusters I-IV).

Cluster I contained the sequence of *A. apis* infecting bees found in palm trees in the La-mae district, whilst clusters II, III and IV contained *A. apis* isolates from bees found in rubber, cashew nut and mango trees, respectively. However, the relevance of the tree-geographic location upon the *A. apis* genotype and population genetics is not addressed due to insufficient samples to allow any meaningful inferences.

Gut bacteria in healthy bees

The potential use of natural probiotics as an alternative way to control bee pathogens is increasingly interesting challenging (Evans and Lopez, 2004). Here, the aerobic and anaerobic bacteria in the guts of healthy (asymptomatic) *A.*

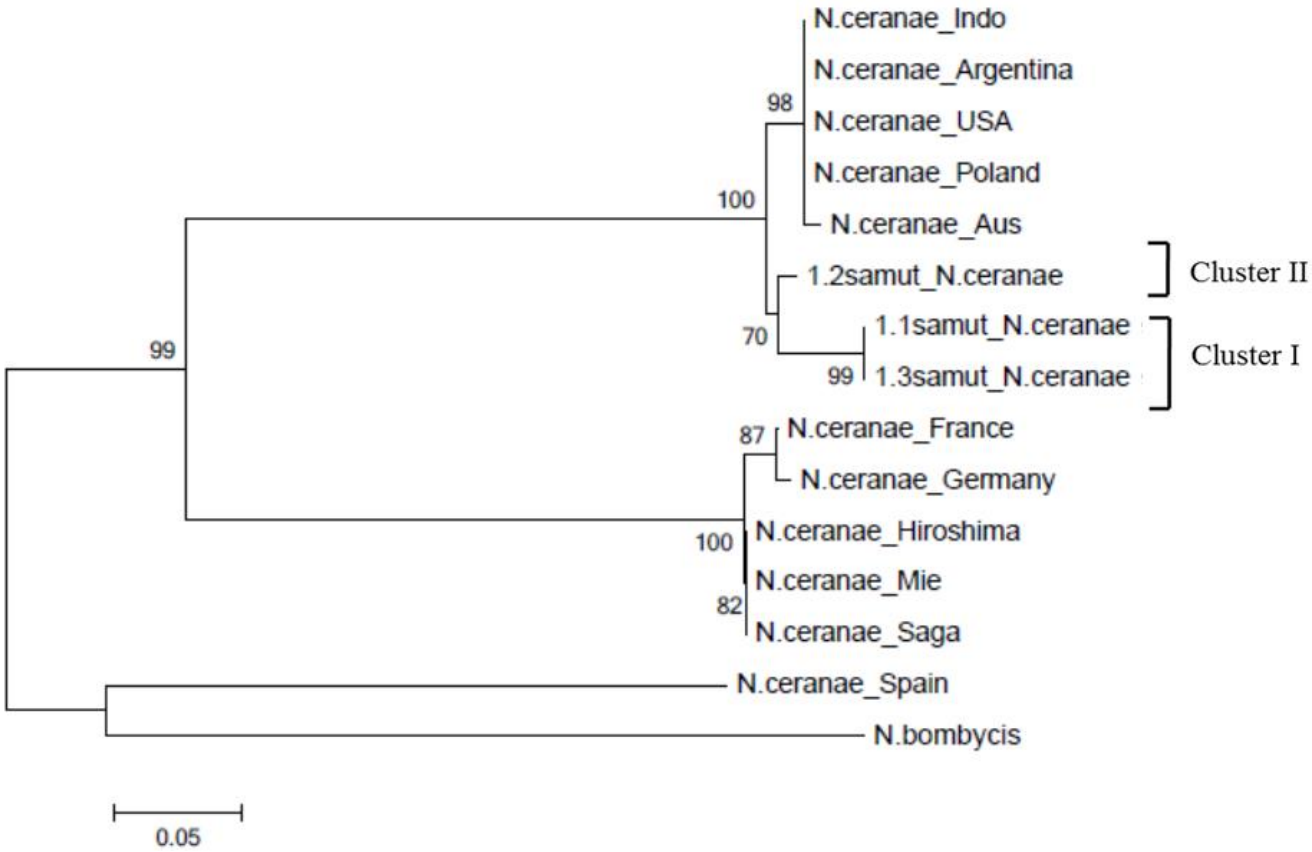

Figure 2. The NJ tree of the aligned 202 bp nucleotide data set of the *16S rRNA* gene sequences from *N. ceranae*. The *16S rRNA* gene sequence of *N. bombycis*, *N. ceranae*_Indonesia, *N. ceranae*_Argentina, *N. ceranae*_USA, *N. ceranae*_Poland, *N. ceranae*_Australia, *N. ceranae*_France, *N. ceranae*_Germany, *N. ceranae*_Hiroshima, *N. ceranae*_Mie, *N. ceranae*_Saga and *N. ceranae*_Spain were used as the outgroups for the analysis. SS was abbreviated from Samut-Songkhram. The number under branches indicates the percentage of bootstrap support from 1,000 replications. Scale bar represents substitution or site.

cerana bees were isolated and then characterized by their colony morphology, biochemistry (Gram stain and substrate fermentation) and analysis of the partial *16S rRNA* gene sequence (Table 3). For the *16S rRNA* sequence analysis, the nucleotide identity of the obtained sequences to the recorded ones in GenBank ranged between 90 and 99% over the 981 bp region examined.

However, the identification of just over half (10/17; ~59%) of the colonies was clearly different at the genus and higher taxonomic levels between the sequence analysis and the morphological-biochemical analysis, although it seemed that molecular analysis was more reliable. Nonetheless, the same class of taxonomy could be obtained by both methods.

DISCUSSION

The deleterious bee diseases that are frequently found in apiaries are AFB caused by *P. larvae*, microsporidosis caused by *N. ceranae* and *N. apis*, chalkbrood disease caused by *A. apis,* and sacbrood disease caused by the SBV. In order to detect these pathogen infections in bee colonies at an early stage prior to the onset of acute or more severe chronic symptoms, PCR (and RT-PCR for RNA viruses) have been used to detect low (asymptomatic) pathogen levels as well as to confirm the diagnosis and infection levels in both the bees and in their products, such as honey. Nowadays, several diverse applications of PCR have been introduced to bee farm management. For example, the detection of SBV contamination in *A. mellifera* adults using a one-step RT-PCR loop-mediated isothermal amplification primer set (RT-LAMP), where the SBV-specific primers were designed based on the SBV *pol* gene sequence. This method is simple, rapid and can be visualized by a simple color change from orange to green (Yang et al., 2012). Furthermore, a portable RT-PCR thermal cycler has been successfully developed to work *in situ* in apiaries that can be applied to detect many different types of bee pathogens

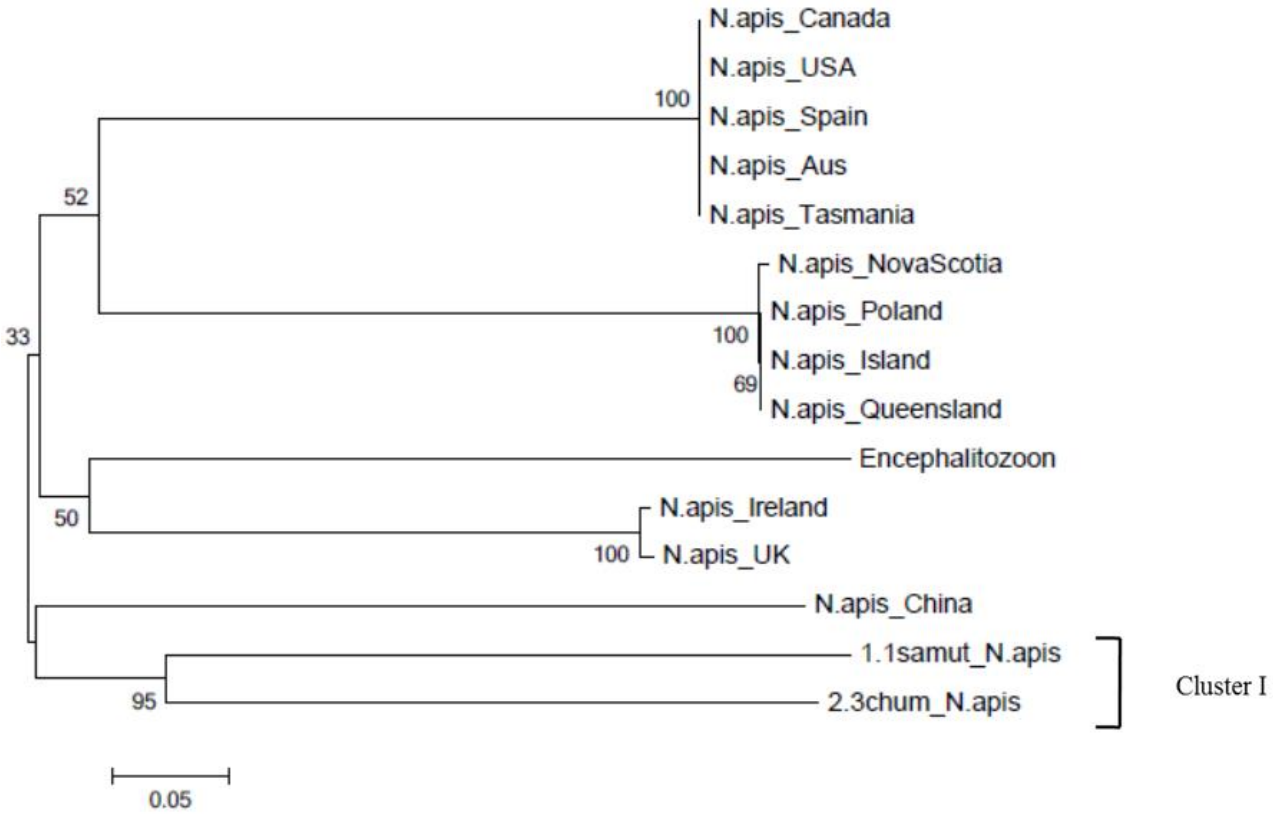

Figure 3. The NJ tree of the aligned 251 bp nucleotide data set of the *16S rRNA* gene sequences from *N. apis*. The *16S rRNA* gene sequence of *Encephalitozoon cuniculi*, *N. apis*_Canada, *N. apis*_USA, *N. apis*_Spain, *N. apis*_Australia, *N. apis*_Tasmania, *N. apis*_Nova Scotia, *N. apis*_Poland, *N. apis*_Island, *N. apis*_Queensland, *N. apis*_Ireland, *N. apis*_UK and *N. apis*_China were used as outgroups for the analysis. CP and SS were abbreviated from Chumphon and Samut-Songkhram. The number under branches indicates the percentage of bootstrap supported with 1,000 replications. Scale bar represents substitution or site.

in less than 8 min (Han et al., 2008).

Distribution and screening of pathogens in *A. mellifera* have been reported in many countries but very few in Thailand. However, no report in Thailand was focused on *A. cerana* although they are economically important as well. In this research, apiaries in Samut-Songkhram province and Chumphon province were the target. An apiary in Samut-Songkhram province is a natural apiary. A bee farmer has never used any antibiotics or chemicals at all so we used *A. cerana* in this location as control. In contrast, for the rest of apiaries, bee apiaries in Chumphon province are managed. Antibiotics or chemicals have been used. In addition, *A. cerana* are very popular for bee farmers in Chumphon province which led us to collect samples easier.

In this study of Thai, *A. cerana indica* apiaries by diagnostic multiplex PCR, the number of colonies infected with *P. larvae*, *N. ceranae*, *N. apis* and *A. apis* were 4, 6, 2 and 0%, respectively, in Samut-Songkhram province and 16, 0, 2 and 20%, respectively, in Chumphon province. No SBV infections were found in all samples from either province. Thus, the numbers of infected colonies and individual bees in infected colonies found in this research was low, and lower than that which reported 91.1 and 57.1% *P. larvae* infected *A. mellifera* honey and colonies, respectively (Lauro et al., 2003). Whilst it is possible that the pathogen susceptibility depends on the bee species, it is also likely to depend upon local population genetics and demography of host-parasite interactions. Whilst *A. mellifera* is very good at foraging and can be managed well in an apiary, it has a relatively weak disease resistance. In contrast, the native (to Thailand) *A. cerana* is less efficient at foraging and less easy to manage in an apiary since it absconds from the allocated hive easily, but it is very tolerant to diseases, which might reflect that it has co-evolved to the local pathogens (Chen et al., 2000).

Varroa mites can cause a serious problem to bee colonies and can also transmit or enhance susceptibility to other pathogens. Of the seven known pathogenic bee

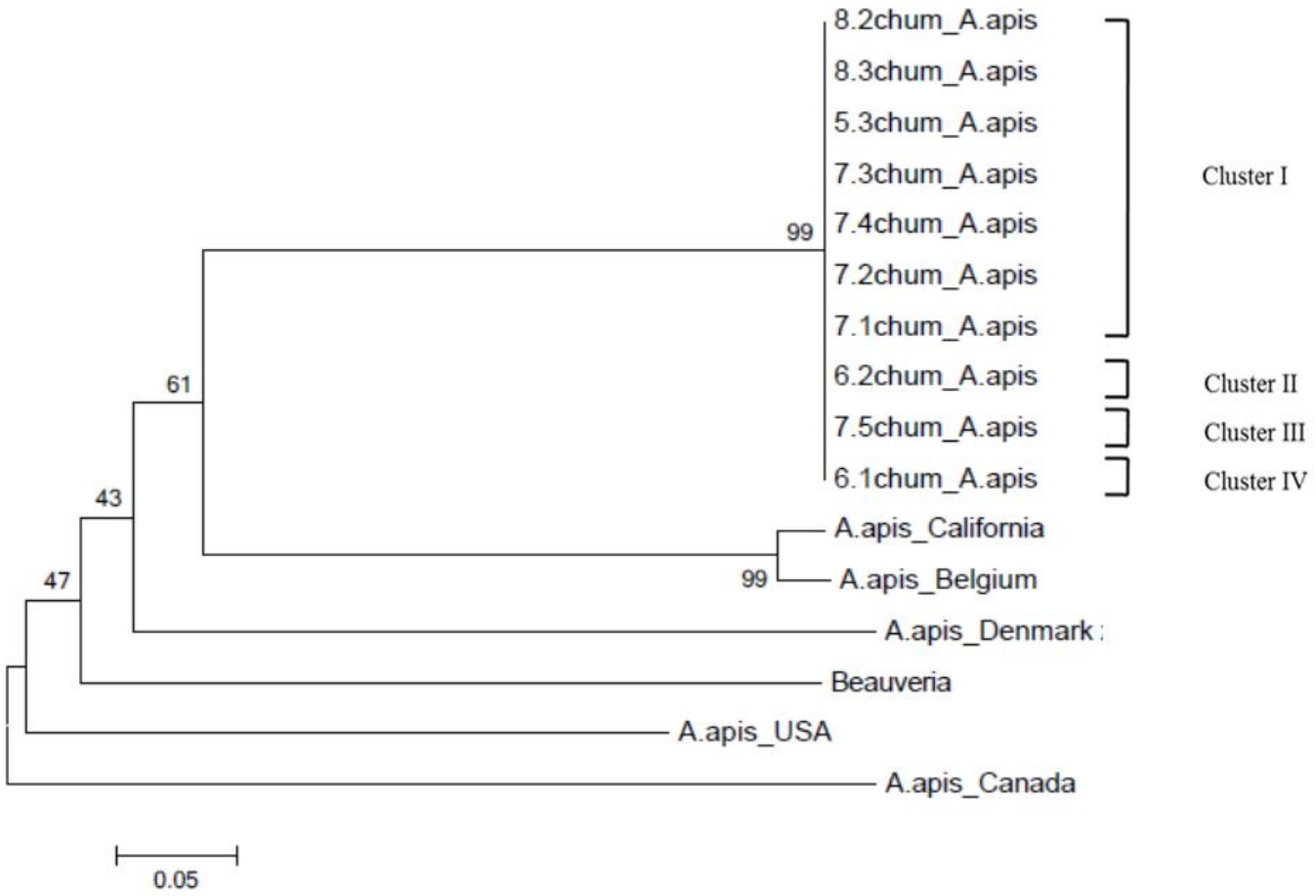

Figure 4. The NJ tree of the aligned 338 bp nucleotide data set of *5.8S rRNA* gene sequences from *A. apis* and related species. The *5.8S rRNA* gene sequence of *Beauveria bassiana*, *A. apis*_California, *A. apis*_Belgium, *As. apis* Denmark, *A. apis*_USA and *A. apis*_Canada were used as outgroups for the analysis. CP was abbreviated from Chumphon. The number under branches indicates the percentage of bootstrap support with 1,000 replications. Scale bar represents substitution or site.

viruses, five were detected in just 20 *Varroa destructor* mites by RT-PCR (Chantawannakul et al., 2006). These were SBV, Kashmir bee virus (KBV), acute paralysis virus (APV), deformed wing virus (DWV) and black queen cell virus (BQCV). Thus, the transfer of these viruses between colonies and bee species is possible.

Subject to the limitation of the very small sample size of infected individuals and geographically sampled locations, *N. ceranae* and *N. apis* appeared allopatric being restricted to the central and southern region, respectively. Although it is possible that the developmental stage of the bee, in terms of larva, pupa and adult worker, was also an important determinant in the type of infection found, that is the different bee life stage have different susceptibilities, or survival rates or immunity to the different pathogens, the number of infected individuals of each developmental stage was too low in this study to allow any such reliable analysis. Moreover, the infection prevalence may depend on the geographic region. For example, a higher *Nosema* species infection level was previously reported for bees from the northern part of Thailand than in this research. Investigation of the *N. ceranae* and *N. apis* infection level in *Apis dorsata*, *A. cerana*, *Apis florea* and *A. mellifera* in apiaries and forests in northern Thailand revealed that 77.5% of *A. mellifera* were infected by *N. ceranae* compared to 22.2, 45.4 and 37.5% in the native honeybees *A. cerana*, *A. florea* and *A. dorsata*, respectively (Chaimanee et al., 2010). However, infection by *N. apis* was not found. In general, microsporidia infections (*Nosema* species) in bees are a severe global problem. High infection frequencies of *N. ceranae* in *A. cerana* have been found in China (61%), Taiwan (73%), Japan (75%), Indonesia (2.63%), Vietnam (2.63%), South Korea (2.63%) and the Solomon islands (21%), respectively (Chen et al., 2009; Botías et al., 2012).

The phylogenetic analysis of bee pathogens has been investigated in several regions. For example, the genetic

Table 3. Identification and classification of targeted bacteria in the gut of healthy *A. cerana* bees from three colonies in Samut-Songkhram.

Bee stage	Bacteria type	No. of bacteria (CFU/ml)	***16S rRNA*** (% identity)	Gram	Shape	Fermentation
Larva	Aerobic	2,939 ± 166.86	*Pantoea* spp. (94-98%)	-	Rod	*Pantoea* spp.
		475 ± 34.45	*Shigella* spp. (95-99%)	-	Rod	*Shigella* spp.
	Anaerobic	4,052 ± 43.79	*Enterococcus* spp. (98-99%)	+	Rod	*Bifidobacterium* spp.
		3,551 ± 124.22	*Staphylococcus* spp. (94-98%)	+	Coccus	*Clostridium* spp.
Worker	Aerobic	11,316 ± 81.21	*Pantoea* spp. (92-96%)	-	Rod	*Pantoea* spp.
		1,405 ± 5.03	*Klebsiella* spp. (92-96%)	-	Rod	*Klebsiella* spp.
		3,133 ± 13.07	*Bacillus* spp. (91-95%)	+	Rod	*Brevibacillus* spp.
		5,150 ± 2.00	*Bacillus* spp. (92-94%)	+	Rod	*Bacillus* spp.
		185 ± 1.15	*Bacillus* spp. (91-95%)	+	Rod	*Aneurinibacillus* spp.
		1,950 ± 18.26	*Azotobacter* spp. (89-92%)	-	Rod	*Photobacterium* spp.
	Anaerobic	1,218 ± 57.33	*Escherichia* spp. (95-99%)	-	Rod	*Bacteroides* spp.
		14,197 ± 629.10	*Bacillus* spp. (90-93%)	+	Rod	*Actinomyces* spp.
		5,000 ± 46.23	*Bacillus* spp. (94-98%)	+	Rod	*Bifidobacterium* spp.
		7,400 ± 4.00	LAB (93-97%)	+	Rod	*Bifidobacterium* spp.
		5,733 ± 17.32	*Azotobacter* spp. (88-90%)	-	Rod	*Fusobacterium* spp.
		2,717 ± 35.16	*Lactobacillus* spp. (90-94%)	+	Rod	*Staphylococcus* spp.
		917 ± 3.51	*Lactobacillus* spp. (92-96%)	+	Rod	*Lactobacillus* spp.

diversity and likely origin of *N. ceranae* in *A. mellifera* from Japan, as derived from the analysis of the partial sequences of the small sub unit (*16S*) *rDNA* of *N. ceranae*, has been reported (Yoshiyama and Kimura, 2011). This analysis revealed two groups of *N. ceranae* from different geographic regions and it was assumed that the pathogen was derived from imported honeybee products and queens.

With respect to the phylogenetic placement of the *16S rRNA* gene fragments for the *P. larvae*, *N. ceranae* and *N. apis* isolates, and that for the *5.8S rRNA* gene fragments from *A. apis* isolates of this study, these Thai samples formed six, two, one and four closely related clusters, respectively, with no evidence of separate local races. They were, however, significantly different from other geographical isolates obtained from bees outside of Thailand. The control of infections by antimicrobial treatment is often easier when the pathogens are closely related, since the drug resistance is typically less polymorphic within the pathogen population. For example, of the four different haplotypes (A to D) of *P. larvae* found in North America, 38.4% of haplotype A isolates from Alberta could resist 5 µg of tetracycline, while this was higher at 48% for haplotype B from New York and South Dakota (Evans, 2003). Note, however, that any given drug resistance or resistance to a pathogen can evolve separate from and at a different rate to mitochondrial or unlinked nuclear markers.

The Thai *A. cerana indica* colony infection level with *A. apis* reported here at Chumphon (20%) was not that dissimilar to the 13% infected *A. mellifera* honey samples from Argentina (Reynaldi

et al., 2003). However, these Thai *A. apis* isolates formed six different clusters (I to VI), with cluster III the most prevalent, and so the potential differential resistance to treatment of these *A. apis* isolates may be of some concern.

In this study, no SBV infections were detected across all the sampled bees, which contrasted to other studies in different bee species or geographical regions. For example, the low prevalence (0.90%) of detected SBV infections in *A. mellifera* in Germany (Hedtke et al., 2011), and the very high SBV infection frequencies in *A. cerana* in China (Ai et al., 2012), at 86% for adults, 33% for pupae and 79% for larvae, and in South Korea at 60.5% in larvae and 30.7% in adults (Choe et al., 2012).

It is also possible that the infection level depends on the bee developmental stage, but the number of infected bees in this study was too low to allow any meaningful discrimination of the infection frequency in different developmental life stage at each geographic location. With this caveat in mind, in this study, the frequency of infection by *Nosema* was slightly lower in larvae than in adults, *A. apis* infection was more prevalent in larvae and pupae than in adults and infection by *P. larvae* was the highest in the pupae. Elsewhere, as already mentioned earlier, SBV infection frequencies in *A. cerana* in China was higher in adults (86%) and then larvae (79%) than in pupae (33%) (Ai et al., 2012), whilst in South Korea the SBV infection frequency in *A. cerana* was also higher in larvae (60.5%) than in adults (30.7%) (Choe et al., 2012). Furthermore, pupae of *A. cerana* were highly susceptible to infection by *P. larvae* vegetative cells, whilst 1-d-old larvae were the most susceptible to infection by *P. larvae* spores (Chen et al., 2000).

The severity of pathogen infection has been reported to depend on the bee larval instar and form of the pathogen (Bamrick, 1967). Spores of *P. larvae* could infect 100 and 90% of intermediate (21 h old) and older (45 h old) *A. mellifera* larvae, respectively, but only 60% of younger (3 h old) larvae. In contrast, the vegetative cells of *P. larvae* could highly infect younger (77.5%) and intermediate (75%) larvae to a higher frequency than older larvae (55%). Moreover, 1-day-old larva could be infected by about 10 *P. larvae* spores compared to the millions of spores needed to infect 2-day-old larva (Alippi and Aguilar 1998).

In order to find a safe way to control pathogen infections in bees, the use of probiotics is an interesting but challenging possibility that is gaining interest. Thus, the cultivable aerobic and (facultative) anaerobic bacteria from the gut of healthy bees (larvae and adult workers) were isolated and characterized by the colony morphology, biochemistry (substrate fermentation ability and Gram stain) and analysis of a *16S rRNA* fragment sequence. The discrepancy between the genus, and sometimes even higher taxonomic levels, classification of just over half (10/17) of the isolates between the biochemical and molecular classification is of concern and awaits resolution, but it is likely that *Bacillus* species were the most often obtained isolates. For example, from the molecular based classification of the 17 different morphologically discriminated colonies, six were *Bacillus* spp. (35.3%) (1/4 in larvae and 5/13 in adult workers) and they accounted for 44.5% of all CFUs (36.8 and 45.9% in larvae and adult workers, respectively). This finding agrees with Yoshiyama and Kimura (2009) who found mostly *Bacillus* spp. from the gut of *A. cerana japonica*, although Wu et al. (2013) found 11 different strains of *Bifidobacterium* species from the gut of *A. cerana japonica*.

In addition, members of the Firmicute, Actinobacteria, Alphaproteobacteria and Gammaproteobacteria have been isolated from the gut of *A. cerana cerana* in China (Jiang et al., 2013). In related bee species, 15 strains of *Bacillus* were isolated from *A*. *mellifera* gut and honey (Sabaté et al., 2009), whilst *Bifidobacterium*, *Lactobacillus*, *Gluconacetobacter*, *Simonsiella*, *Serratia* and *Bartonella* were isolated from the gut of worker *A. mellifera capensis* and *A. mellifera scutellata* bees (Jeyaprakash et al., 2003). Overall, broadly similar bacterial families have been isolated from different honey bee species. Although previously, the bacteria from the gut of bees has been found to exert antimicrobial activity against *P. larvae* (Evans and Armstrong, 2006) and antibiotic resistant bacteria (Aween et al., 2012), the potential probiotic activity, if any, of these 17 isolates from *A. cerana indica* in Thailand remains to be established.

Conflict of Interests

The authors have not declared any conflict of interests.

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SUPPLEMENTARY FIGURE

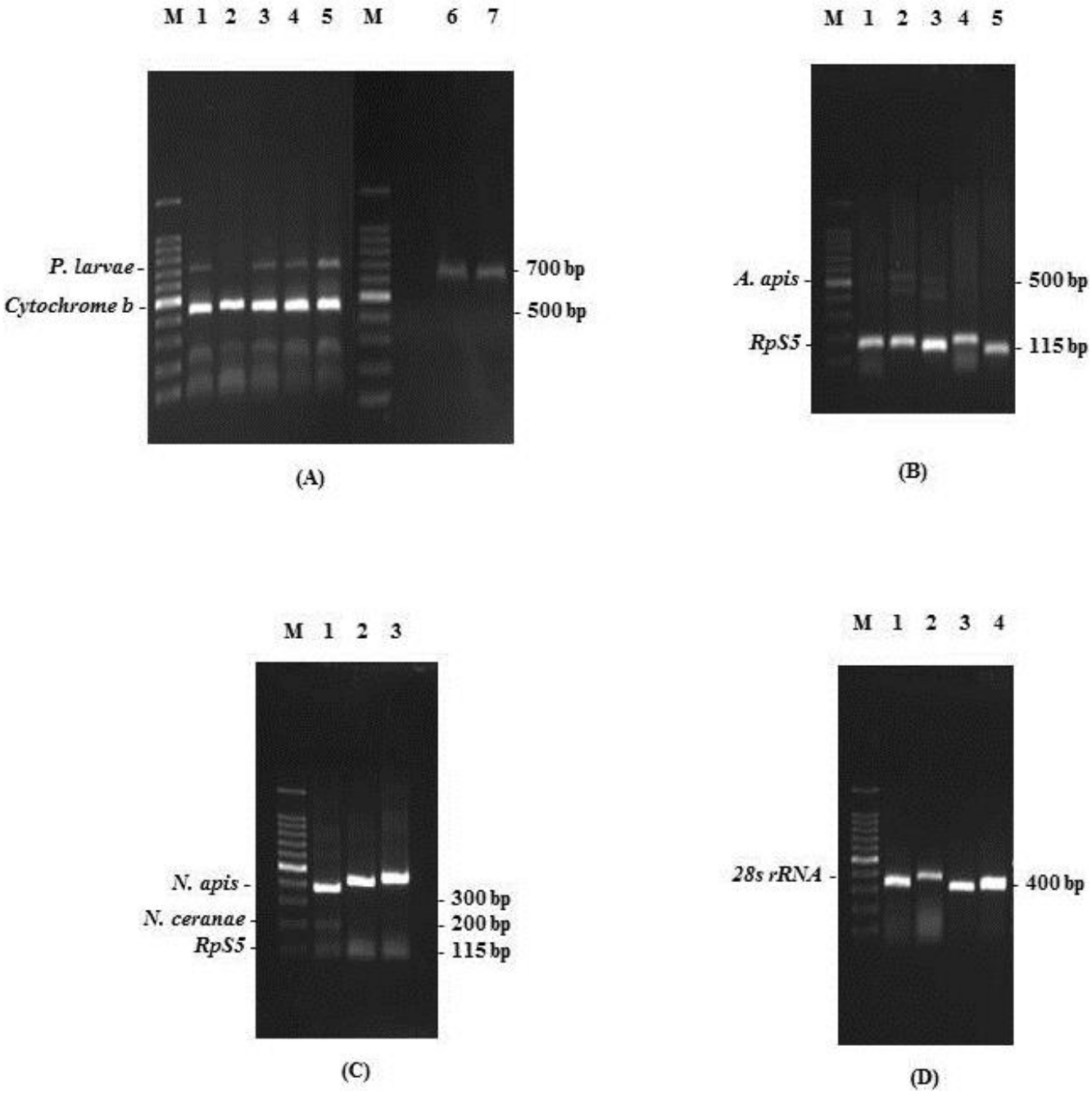

Figure S1. Multiplex PCR presenting *P. larvae* **(A)**, *A. apis* **(B)**, *N. ceranae* and *N. apis* **(C)**, and *pol* **(D)** infection. Lane M in all figures was 100 bp DNA ladder. For (A), lanes 1-5 contained *cytochrome b* amplified products (500 bp) as control and lanes 1, 3-5 contained *P. larvae* amplified products (700 bp). Also, lanes 6-7 contained spores of *P. larvae* amplified products (700 bp) as positive control. For (B), lanes 1-5 contained *RpS5* amplified products (115 bp) and lanes 2-3 contained *As. apis* amplified products (500 bp). For (C), lanes 1-3 contained *RpS5* amplified products and *N. apis* amplified products (300 bp). Furthermore, lane 1 contained *N. ceranae* amplified products (200 bp). For (D), lanes 1-4 contained *28S rRNA* amplified products (400 bp) as control, but no *pol* amplified products (200 bp).