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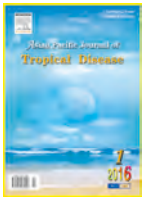

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Molecular epidemiology and geographical distribution of *Nosema ceranae* in honeybees, Northern Thailand

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ABSTRACT

Objective: To determine the contamination levels of *Nosema ceranae* in honeybees and its molecular linkages in different geographical areas of Northern Thailand.
Methods: Seventy-eight apiaries in Northern Thailand were chosen at random. The detection was accomplished both by microscopic examination and multiplex PCR. *Nosema* positive samples were evaluated by PCR sequencing and phylogenetic analysis.
Results: Of the samples subjected to microscopic examination, 11.54% were found to be positive for *Nosema* while 29.49% of the samples evaluated by PCR were found to be positive for the disease. Honeybees from four of the six provinces surveyed in Northern Thailand were positive for *Nosema*, with the highest prevalence in Chiang Mai Province (48.57%). There was a high diversity of *Nosema* strains in some locations, while the same strain of pathogen was identified in many locations in Northern Thailand.
Conclusions: This is the first report about the contamination levels and distribution pattern of nosemosis in Thailand. The study found the same group of *Nosema* in different locations, and different groups of *Nosema* in the same location. This pattern of distribution will be an advantage for disease control in the future.

1. Introduction

The honeybee industry is now more important than it had been in the past. In Thailand, there are about 1 556 apiaries, of which 702 (45.12%) are in Northern Thailand. The main types of honeybees raised are *Apis cerana* (*A. cerana*), *Apis mellifera* (*A. mellifera*), *Apis dorsata* and *Apis florae*. They collect nectar for honey production from a variety of flowers including longan, lychee, sunflower, rubber tree, sesame, *etc*. Total honey production in Thailand is more than 10 000 tons per year, royal jelly is more than 200 tons per year and bee pollen is more than 100 tons per year. However, the honeybee industry may suffer from many problems such as environmental pollution, poor management and many diseases in the apiary caused by bacteria, viruses, protozoa and fungi. In the US in 2006, beekeepers' hives began to suffer from colony collapse disorder, a condition which may have resulted from pesticides, excessive inbreeding, parasitic varroa mites, invertebrate iridescent virus type VI as well as the parasitic microsporidian *Nosema ceranae* (*N. ceranae*) fungus[1-3]. Microsporidian fungi are microorganisms which can produce spores and are pathogenic in many species of insects. *Nosema* is a disease caused by both *Nosema apis* (*N. apis*) and *N. ceranae*, microsporidian fungi which are pathogenic in honeybees in North America, Europe, Australia and Asia[4-6]. The disease results in increased morbidity and mortality in apiaries. Adult honeybees become infected with the microsporidian fungus by orally ingesting spores on contaminated sources such as food, water, feces and hive material. The spores primarily enter midgut epithelial cells where they geminate quickly[7,8]. Inside the infected cells, the number and volume of spores increases in 6 to 10 days. The infected cell becomes filled with new spores until it bursts, releasing 30–50 million infective spores into the lumen of the honeybee's digestive tract. Defecation by infected honeybees inside the hive is the primary source of spread of the infection within the colony. When worker bees clean the hive, they become infected

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with the spores when they ingest fecal matter or contaminated food. Transmission of *Nosema* disease between hives can occur by transferring contaminated combs between hives, feeding bees with contaminated honey and honey robbing from infected hives, *etc*.

Nosema is caused by two described species of microsporidia fungi, *N. apis* and *N. ceranae*[6,8]. Originally, *N. apis* was found only in European honeybees (*A. mellifera*), while *N. ceranae* was found only in Asiatic honeybees (*A. cerana*). Later research found that European honeybees were infected with *N. ceranae* much more than *N. apis* and that the symptoms of the disease in European honeybees were more severe than in Asiatic honeybees[5,9,10]. Because this pathogen grows and multiplies in the mid part of the digestive tract of honeybees, the symptoms mostly occur in digestive system, *e.g.*, dysentery, extension and swelling of the abdomen of infected bees. These symptoms appear when the infection is very severe; when the beekeeper finds the symptoms, it is too late for treatment. To decrease the incidence of this disease in the apiary, beekeepers should diagnose the disease before the infection has progressed to the point that symptoms appear.

Recently, in Thailand, there were some studies about the phylogeny and infectivity of *N. ceranae* in many types of honeybee in Thailand, for example, *A. mellifera*, *A. cerana*, *Apis florae* and *Apis dorsata*[11,12]. But no previous research reports about the prevalence of *Nosema* in Thailand. The aim of this research was to investigate the presence of *Nosema* spp., its prevalence and distribution in Northern Thailand, by using light microscope examination and multiplex PCR which intended to improve surveillance of the disease in apiaries[13].

2. Materials and methods

2.1. Sample collection and microscopic examination

A. mellifera worker samples were collected from 78 apiaries in six provinces in Northern Thailand in April 2014: 35 in Chiang Mai, 19 in Chiang Rai, 7 in Lumphun, 2 in Phrae, 12 in Nan and 3 in Phayao (Figure 1). About 30 honeybee samples were collected from each individual hive, with three hives chosen at random in each of the apiaries. The honeybee samples were soaked in 70% ethanol, dried, then stored at -20 °C. Abdominal parts from ten honeybees from each hive were cut excised then soaked in liquid nitrogen before being ground. About 30 mg of the ground sample from each hive was combined in a test tube with samples from the other hives from the same apiary. The samples were then mixed together. Twenty microlitres of the suspension was placed on a slide with a cover-slip; microscopic examination was performed at 400× magnification through using bright-field optics.

2.2. Genomic DNA extraction

DNA was extracted from the suspension described above by using a DNeasy Plant mini kit (Qiagen, Germany). The final elution step was completed by using 100 μL of AE buffer. DNA concentration and DNA purification were measured by using Beckman Coulter® DU® 730 (Life Science UV/Vis spectrophotometer). Extracted DNA was diluted to a concentration of 50 ng/μL.

Figure 1. Distribution of *N. ceranae* collected from Northern Thailand in April 2014.
The stars refer to the location of the apiary with *N. ceranae* infection detected by PCR.

2.3. Nosema detection by PCR

The extracted DNA was analyzed by PCR to determine the presence of region in the 16S rRNA gene. The primers for *N. ceranae* were 218MITOC-FOR (5'-CGG CGA CGA TGT GAT ATG AAA ATA TTA A-3') and 218MITOC-REV (5'-CCC GGT CAT TCT CAA ACA AAA AAC CG-3'). The amplicon size was about 218 bp. The pair of primers used for amplification of *N. apis* were 321APIS-FOR (5'-GGG GGC ATG TCT TTG ACG TAC TAT GTA-3') and 321APIS-REV (53'-GGG GGG CTT TTA AAA TGT GAA ACA ACT ATG-3')[13]. The 20 μL PCR reaction volume contained the following: 0.2 μmol/L of each pair of primers, 1.5 mmol/L $MgCl_2$, 1× *Taq* buffer, 0.25 mmol/L deoxynucleotide triphosphates, 0.5 IU *Taq* polymerase and 100 ng of DNA template. The thermo cycler program was as follows: 95 °C (5 min); 35 cycles of 95 °C (30 s), 61.8 °C (30 s) and 72 °C (45 s); final extension at 72 °C (5 min). The molecular size of the PCR products was determined by electrophoresis in a 2% agarose Tris-borate-ethylene diamine tetra-acetic acid (TBE) gel in 0.5× TBE buffer, stained with ethidium bromide and visualized by using the gel documentation system. The nucleic acid sequences of the PCR products were determined and compared to the nucleotide sequences deposited in GenBank database.

2.4. Partial polar tube protein gene amplification, sequencing and phylogenetic analysis

2.4.1. Patial polar tube protein gene amplification by PCR

The DNA of *Nosema* positive samples was amplified by PCR using a pair of primers based on the hypothetical polar tube protein gene of *N. ceranae*. The sequences of the primers used for PCR were NcORF1664-FOR (5'-GAC AAC AAG GAA GAC CTG GAA GTG-3')

and NcORF1664-REV (5'-TGT GAA TAA GAG GGT GAT CCT GTT GAG-3')[11]. PCR was performed with 25 μL mixture containing 0.2 μmol/L of each pair of primers, 1 mmol/L $MgCl_2$, 1× *Taq* buffer, 0.2 mmol/L deoxynucleotide triphosphates, 1 IU *Taq* polymerase and 100 ng of DNA template. The thermo cycler program was 95 ˚C (2 min); 45 cycles of 95 ˚C (30 s), 51 ˚C (50 s) and 72 ˚C (50 s); and final extension at 72 ˚C (10 min). The molecular size of the PCR products was determined by electrophoresis in a 1% agarose TBE gel in 0.5× TBE buffer, stained with ethidium bromide and visualized using the gel documentation system. The amplicon size was about 838 bp. The PCR products were then purified and sequenced.

2.4.2. Phylogenetic analysis

The DNA sequences of polar tube proteins obtained in this study were aligned by using ClustalW method. After alignment, both ends of the sequences were trimmed and phylogenetic trees were constructed by using the MEGA6 program. *Encephalitozoon cuniculi* (*E. cuniculi*) (GenBank accession No. NM_001041403.1), a mammalian microsporidian species, was used as an out group to root the phylogenetic tree because the gene encoding this protein was closely linked to *E. cuniculi*. Phylogenetic trees were constructed with MEGA6 program by using UPGMA method[14]. The bootstrap consensus tree, inferred from 10 000 replicates[15], was taken to represent the evolutionary history of the taxa analyzed[15]. The evolutionary distances were computed by using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 670 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[16,17].

2.5. Statistic analysis

The prevalence of *Nosema* spp. contamination levels in honeybees from Northern Thailand was calculated by using descriptive statistics and a confidence interval (*CI*) of 95%.

3. Results

3.1. Microscopic examination

Spores of *Nosema* spp. were detected at 400× magnification under bright-field optics with a light microscope in samples from nine of 78 apiaries (11.54%). *Nosema* spores were oval shaped, 3–4 μm wide and 5–7 μm long (Figure 2).

3.2. PCR

Detection by PCR using 2 pairs of primers for *N. apis* and *N. ceranae* found that 23 of 78 apiaries (29.49%) were positive for *N. ceranae* (Figure 3), but no apiaries were positive for *N. apis*. Seventeen of the apiaries in Chiang Mai Province (48.57%) were *Nosema* positive, followed by Lumphun, Nan and Chiang Rai with prevalences of 42.85%, 16.67% and 5.26%, respectively (Table 1 and Figure 2). No *Nosema* was found in Phrae or Phayao. The nucleotide sequences of the amplification products from the *Nosema* positive honeybee samples were 100% identical with the *N. ceranae* sequence deposited in the GenBank database.

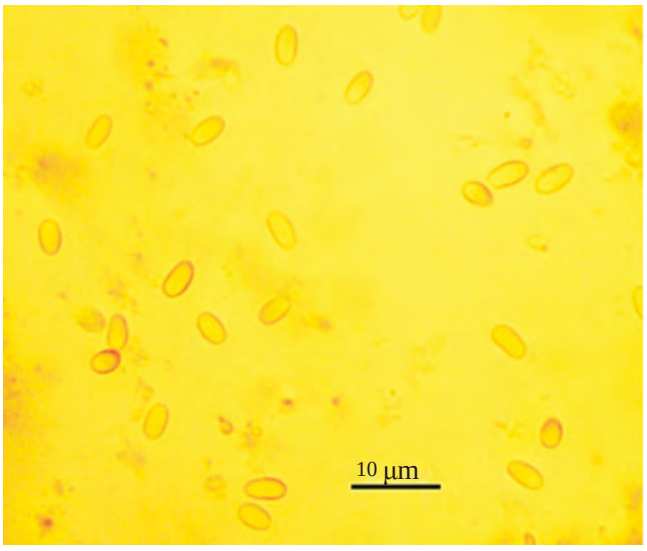

Figure 2. Spores of *N. ceranae* have an oval shape with a dark border detected by using microscopic examination with a light microscope at 400× magnification Axio®.

Table 1
Estimated contamination levels calculated by using the Excel program PHStat.

Province	No.	Positive (*n*)	Contamination level (%)	95% *CI*
Nan	12	2	16.67	0.00–37.75
Phayao	3	0	0.00	0.00
Chiang Rai	19	1	5.26	0.00–15.30
Chiang Mai	35	17	48.57	32.01–65.13
Lumphun	7	3	42.85	6.20–9.52
Phrae	2	0	0.00	0.00

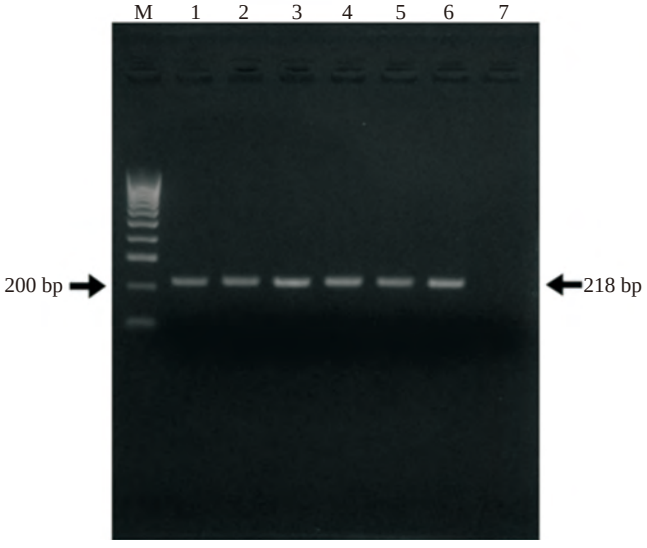

Figure 3. Agarose gel showing amplification of the part of small subunit of ribosomal RNA by using primer set 218MITOC (F & R) and 321APIS (F & R).
Lane M: DNA marker 100 bp ladder; Lanes 1–5: Honeybee sample positive for *N. ceranae*; Lane 6: PCR product of positive control with PCR product 218 bp for *N. ceranae*; Lane 7: No amplification in negative control.

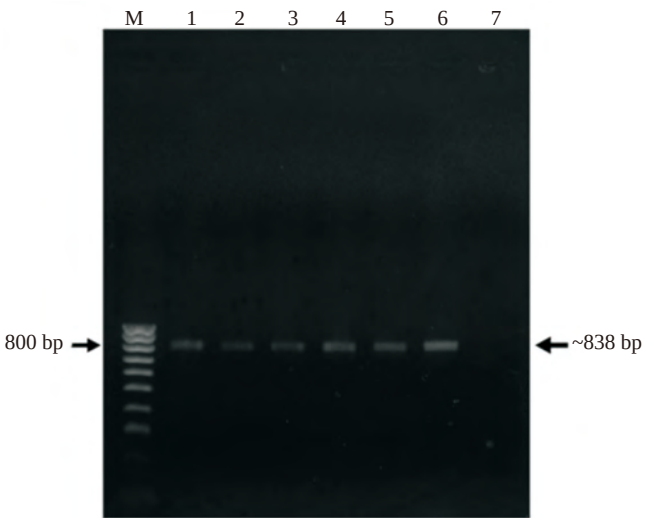

Figure 4. Agarose gel showing amplification of the partial polar tube protein-1 gene with primer set NcORF1664 (F & R).
The size of the PCR product was about 838 bp. Lane M: DNA marker 100 bp ladder; Lanes 1–5: Honeybee sample positive for polar tube protein; Lane 6: PCR product of positive control; Lane 7: No amplification in negative control.

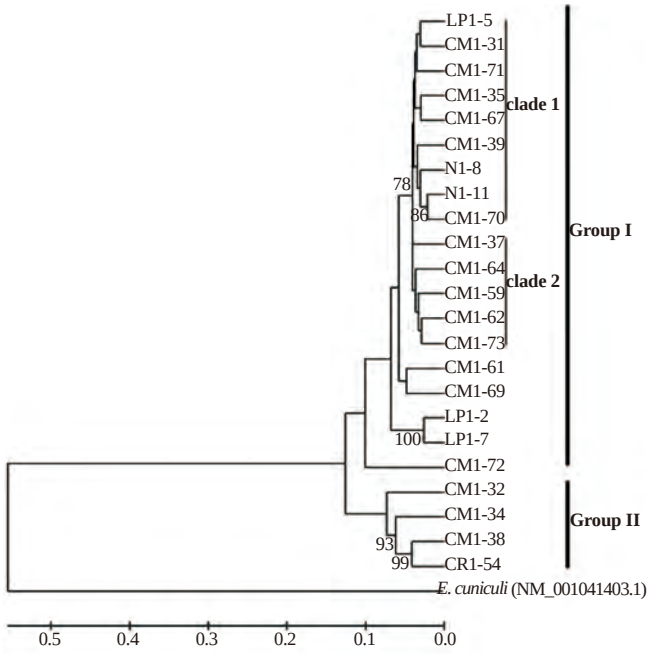

Figure 5. Phylogenetic tree of *N. ceranae* isolated based on the partial part of polar tube protein-1 gene sequence.
CM: Chiang Mai; CR: Chiang Rai; LP: Lumphun; N: Nan.

3.3. Partial sequencing of polar tube protein genes and phylogenetic analysis

All of 23 *Nosema* positive samples were amplified for the partial part of the polar tube protein gene which was about 838 bp in size (Figure 4). After purification and DNA sequencing, the program MEGA6 was used for phylogenetic analysis (Figure 5). The evolutionary history was inferred by using the UPGMA method[14]. The optimal tree with the sum of branch length of 2.14842681 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) were shown next to the branches[15]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed by using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 670 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[16,17].

The samples were divided into two groups. There were 2 clades in Group 1 samples which collected from Chiang Mai, Lumphun and Nan. The samples in Group 2 were collected from Chiang Mai and Chiang Rai. Two samples collected from Nan (N1-8 and N1-11) were in the same clade (clade1) in Group 1. Sample from Chiang Rai (CR1-54) was in Group 2. There were 3 samples from Lumphun in which 2 samples (LP1-2 and LP1-7) were out of clade in Group 1 and another sample (LP1-5) was in clade 1, Group 1. Samples from Chiang Mai were distributed in Group 1 and Group 2.

4. Discussion

Our results showed that *N. ceranae* was the only *Nosema* species infecting honeybees in Northern Thailand, although both *A. mellifera* or the European honeybee and *A. cerana* or the Asiatic honeybee are found in Thailand. It is surprising that although the prevalence of *Nosema* in Northern Thailand is 29.89%, local beekeepers do not know much about this disease and they thought it is not important (personal communication, April 2014). That may be because signs of colony weakness were not evident until the queen could no longer replace the loss of infected bees. The long incubation period can explain the absence of evident symptoms prior to colony collapse[2]. The beekeepers see no clinical signs or no deaths from the disease, so they do not pay attention to it. They were not aware that there was *Nosema* spp. in their apiaries. It is very difficult to control or prevent this disease because of its relatively long asymptomatic period[18]. The prevalence found in this research was lower than that in China. In China, the bee samples were collected from 9 provinces in Northern China and 10 provinces from Southern China[6]. In this study, honeybee samples were collected from 6 provinces in Northern Thailand. The prevalence of *N. ceranae* in northern part of China was 40% whereas the prevalence was 29.89% in this research.

Recently, there were many researches about *N. ceranae* in Thailand, such as, phylogeny of *N. ceranae* from honeybee in Northern Thailand[11], infectivity of *N. ceranae* in honeybee species[12]. But no research reported about the prevalence and distribution of *N. ceranae* in Thailand. So, this is the first report which surveyed the prevalence and distribution of *N. ceranae* in honeybee in Northern Thailand.

From microscopic examination, the prevalence of *Nosema* in Northern Thailand was calculated to be 11.54%, while the prevalence

determined by PCR was 29.49%. Fourteen of 55 microscopically negative samples were found to test positive with multiplex PCR, confirming the advantage of the increased sensitivity and specificity molecular diagnostic methods. Another disadvantage of microscopic examination is that it cannot differentiate *N. ceranae* from *N. apis*. Even though the Office International Des Epizooties Terrestrial Manual mentions differences between these two species, in practice it is very difficult to identify the species by microscopic examination[13]. Different methods have been developed to distinguish *N. apis* from *N. ceranae* including multiplex PCR which can clearly identify both pathogens[13].

Phylogenetic analysis found the same group of *Nosema* in different locations, and different groups of *Nosema* in the same location. For example, the Group 1 samples were from Chiang Mai (CM1-31, CM1-35, CM1-37, CM1-39, CM1-59, CM1-61, CM1-62, CM1-64, CM1-67, CM1-69, CM1-70, CM1-71 and CM1-73), Lumphun (LP1-2, LP1-5 and LP1-7) and Nan (N1-8 and N1-11). Whereas, the samples from Chiang Mai (CM1-32, CM1-34 and CM1-38) were in Group 2. The partial part of polar tube protein-1 gene sequence was used in our research because it is a reliable marker for revealing genetic relationships within species[11]. Furthermore, we found that there were spread of *N. ceranae* between the provinces in Northern Thailand. It might be because of the transportation of the pathogen across the provinces for example, in the longan's flower bloom season, many apiaries in Northern Thailand moved to Chiang Mai and Lumphun province because there were many longan gardens.

However, continually study about the epidemiological surveillance and phylogenetic analysis will help to reveal the presence of mutations and it will provide comprehension in the contamination level of this pathogen.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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