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Short Communication

Phylogenetic analysis of *Nosema ceranae* isolated from European and Asian honeybees in Northern Thailand

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ABSTRACT

Nosema ceranae was found to infect four different host species including the European honeybee (*A. mellifera*) and the Asian honeybees (*Apis florea*, *A. cerana* and *Apis dorsata*) collected from apiaries and forests in Northern Thailand. Significant sequence variation in the polar tube protein (PTP1) gene of *N. ceranae* was observed with *N. ceranae* isolates from *A. mellifera* and *A. cerana*, they clustered into the same phylogenetic lineage. *N. ceranae* isolates from *A. dorsata* and *A. florea* were grouped into two other distinct clades. This study provides the first elucidation of a genetic relationship among *N. ceranae* strains isolated from different host species and demonstrates that the *N. ceranae* PTP gene was shown to be a suitable and reliable marker in revealing genetic relationships within species.

1. Introduction

Nosema genus is a parasitic microsporidia commonly infecting Lepidoptera (Tsai et al., 2003) and Hymenoptera (Fries et al., 1996; Higes et al., 2006) but has also been found in Orthoptera (Henry, 1971) and Amphipoda (Terry et al., 1999). *Nosema apis* and *Nosema ceranae* are the two microsporidian species that are reported as important honeybee pathogens. *N. apis* has long been known as a pathogen that infects the European honeybee, *Apis mellifera* (Zander, 1909). However recent studies have shown that *N. ceranae*, a species that was first known to infect the Asian honeybee, *Apis cerana* (Fries et al., 1996) has become the dominant species infecting honeybees worldwide (Fries et al., 2006; Higes et al., 2006; Chauzat et al., 2007; Cox-Foster et al., 2007; Huang et al., 2007; Klee et al., 2007; Paxton et al., 2007; Chen et al., 2008; Williams et al., 2008; Chaimanee et al., 2010). *N. ceranae* infection has impacts at both the individual honeybee (Paxton et al., 2007; Higes et al., 2009; Martín-Hernández et al., 2007) and colony level, and has been associated with honeybee colony losses in Spain (Higes et al., 2008, 2009).

The early classification of *Nosema* species was based on morphological characters (eg. spore size, shape and ultrastructure), life cycle and pathological symptoms within the host range. However, it is difficult to differentiate the closely related species using morphological characters (Weiss and Vossbrinck, 1999). The genes coding for the rRNAs of the small and large subunit of the ribosomes have been used in the taxonomic determination of microsporidia and in the differentiation of *Nosema* species (Ku et al., 2007; Huang et al., 2008; Williams et al., 2008; Chen et al., 2009). The rRNA genes have been widely used for identification, molecular characterization and as a molecular marker for phylogenetic analysis among eukaryotes. The multiple copies of rRNA are a common phenomenon of microsporidia (Tay et al., 2005; O'Mahony et al., 2007), then the rRNA genes may not be ideal choices for the phylogenetic analysis of microsporidia. Therefore, the study of phylogenetic relationships among species or strains of *Nosema* need to be based on non-repeated loci (Sagastume et al., 2011).

Recently, Chaimanee et al. (2010) reported that *N. ceranae* had been detected not only in the European honeybee (*A. mellifera*), but also in the cavity nesting Asian honeybee (*A. cerana*), the dwarf Asian honeybee (*Apis florea*) and the giant Asian honeybee (*Apis dorsata*) in Northern Thailand. However, the genetic relationship of possible *N. ceranae* isolates from different host species has not been determined. In this study, we conducted phylogenetic studies using sequences of the 16S rRNA gene and of the gene coding for the polar tube protein 1 (PTP 1) of *N. ceranae* independently to determine the genetic relationship of *N. ceranae* strains that were isolated from four different honeybee species.

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2. Materials and Methods

2.1. Samples

The samples of *Nosema* infected *A. mellifera* ($N = 10$), *A. cerana* ($N = 8$), *A. florea* ($N = 10$) and *A. dorsata* ($N = 10$), were collected from Northern Thailand in 2008–2009 and stored at −20 °C prior to molecular analysis.

2.2. DNA extraction

Genomic DNA was extracted from individual bees, homogenized with a pestle and the crude homogenate suspended in 400 μl of CTAB buffer (100 mM Tris–HCl, pH8.0; 20 mM EDTA, pH 8.0; 1.4 M sodium chloride; 2% (w/v) cetyltrimethylammonium bromide; 2% 2-mercaptoethanol). The 0.15 g of glass beads (425–600 μm, Sigma-Aldrich, St.Louis, MO) were added in the spore suspensions and the mixture shaken at the maximum speed for 4–5 min using a FastPrep cell Disrupter (Qbiogene, Carlsbad, CA). The mixture was incubated with proteinase K (200 μg/ml) at 55 °C overnight. DNA was extracted from suspensions using a DNA purification Kit, DNAzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The concentration of total DNA was determined by using the NanoDrop spectrophotometer (Thermo Scientific, USA).

2.3. PCR amplification and DNA sequencing

A region of the 16S rRNA gene was amplified using the primer set described by Chen et al. (2008). Two primer sets, *N. apis*-forward/*N. apis*-reverse (5′-CCATTGCCGGATAAGAGAGT-3′/5′-CACGCATTGCTGCATCATTGAC-3′) and *N. ceranae*-forward/*N. ceranae*-reverse (5′-CGGATAAAAGAGTCCGTTACC-3′/5′-TGAGCAGGGTTCTAGGGAT-3′), were individually used to amplify a 269 bp fragment of *N. apis* and a 250 bp product of *N. ceranae*, respectively. A pair of primers based on the hypothetic PTP gene of *N. ceranae* isolated from *A. mellifera* (XM_002995447) were designed to amplify a ~838 bp product. The sequences of the primers used for PCR were as follows: NcORF 1664-forward: 5′-GACAACAAGGAAGACCTGGAAGTG-3′and NcORF-1664 reverse: 5′-TGT GAATAAGAGGGTGATCCTGTTGAG-3′.

PCR amplification was carried out in a 25 μl reaction mixture containing 1 × high fidelity PCR buffer, 0.1 mM each dNTP, 2 mM $MgSO_4$, 0.2 μM of each primer, 1U of Platinum Taq High Fidelity polymerase (Invitrogen; Carlsbad, CA) and 500 ng of total genomic DNA. The amplication conditions were 95 °C for 2 min, 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s with a final extension at 72 °C for 10 min on a thermal cycler. The PCR product from each reaction was electrophosed on 1.0% low melting point agarose gel (Invitrogen). The specificity of amplified PCR products was verified by purifying PCR fragments using Wizard PCR Prep DNA Purification System (Promega, Medison, WI) according to the manufacturer's instruction and sequenced using both forward and reverse primers by the Center for Biosystems Research, University of Maryland Biotechnology Institute.

2.4. Phylogenetic analysis

The DNA sequences of PTP obtained from this study were aligned using the ClustalX version 1.83 program. After alignment the unaligned sequences at both ends were truncated. *Encephalitozoon cuniculi* (GenBank Accession No. NM_001041403.1), a mammalian microsporidian species, was used as an outgroup to root the phylogenetic tree since the gene encoding this protein is closely linked to that of *E. cuniculi*. Phylogenetic trees were constructed with the Mega 4 program (Tamura et al., 2007) using the maximum parsimony method (MP) and the close-neighbor-interchange algorithm with search level one in which the initial trees were obtained with the random addition of sequences. One thousand bootstrap replicates were assessed to test the robustness of the tree.

3. Results

3.1. Detection of N. ceranae in different honeybee species

When we amplified the partial 16S rRNA gene of *Nosema* using the primer set *N. apis*-forward/*N. apis*-reverse and *N.ceranae*-forward/*N.ceranae*-reverse, a 250 bp fragment specific for *N. ceranae* was amplified from all samples. No *N. apis* specific PCR products were genereated from any samples examined. This is consistant with our previous findings that *N. ceranae* but not *N. apis* could be isolated from four different honeybee species (Chaimanee et al., 2010). The primer set of NcORF-1664 forward/NcORF-1664 reverse amplifying a 808 bp-fragment of the PTP 1 gene yielded a PCR-product of the expected size (Fig. 1A). Sequence analysis further confirmed the specificity of PCR amplification by PTP primers.

3.2. Phylogenetic analysis

Based on the partial polar tube protein (PTP 1) sequences of *N. ceranae* isolates, no difference has been found amongst *N. ceranae* isolates from *A. mellifera* and *A. cerana*. We observed 22 and 7 nucleotide differences amongst the *N. ceranae* isolates from *A. florea* and *A. dorsata* and share 97% and 99% similarity, respectively with isolates from *A. mellifera* and *A. cerana*. No differences *N. ceranae* strains found within host species. Deletion occurrence in *N. ceranae* sequences that found in Thailand at 325th bp to 354th bp when compared to the *N. ceranae* sequence (XM_002995447) from USA (Fig. 2).

We analyzed the phylogenetic relationship amongst *N.ceranae* isolates found in *A. mellifera*, *A. cerana*, *A. florea* and *A. dorsata* based on the partial sequences of 16S rRNA and PTP genes separately. The phylogenetic tree based on the partial rRNA gene sequences resulted in *N. ceranae* from the four different honeybee species forming one clade. However, the phylogenetic tree based on the partial protein coding region (PTP 1) gene sequences showed three distinct clades. *N. ceranae* isolated from *A. mellifera* grouped into the same clade as *N. ceranae* isolated from *A. cerana* supported by a high bootstrap value (97%). However, *N. ceranae* isolated from *A. dorsata* and from *A. florea* formed district clades with bootstrap value 95% and 99%, respectively (Fig. 1B).

4. Discussion

Recently, it has been shown that *N. ceranae* is capable of infecting multiple host species including European honey bees, Asian honey bees, and bumble bees (Chaimanee et al., 2010; Plischuk et al., 2009). The wide host range of this parasite is of significant epidemiological concern. However, the host-parasite interactions that determine host susceptibility to the parasite remain poorly understood and the genetic relationship of different *N. ceranae* isolates from different host species had not been previously explored. While the phylogeny of the hosts should play an important role in host-parasite interactions and the establishment of successful infection, the phylogeny of the parasites may also aid in our understanding of host range expansion. In this study, we analyzed the phylogenetic relationships between *N. ceranae* isolated from four different honeybee species.