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Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees

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ABSTRACT

Globalization has provided opportunities for parasites/pathogens to cross geographic boundaries and expand to new hosts. Recent studies showed that *Nosema ceranae*, originally considered a microsporidian parasite of Eastern honey bees, *Apis cerana*, is a disease agent of nosemosis in European honey bees, *Apis mellifera*, along with the resident species, *Nosema apis*. Further studies indicated that disease caused by *N. ceranae* in European honey bees is far more prevalent than that caused by *N. apis*. In order to gain more insight into the epidemiology of *Nosema* parasitism in honey bees, we conducted studies to investigate infection of *Nosema* in its original host, Eastern honey bees, using conventional PCR and duplex real time quantitative PCR methods. Our results showed that *A. cerana* was infected not only with *N. ceranae* as previously reported [Fries, I., Feng, F., Silva, A.D., Slemenda, S.B., Pieniazek, N.J., 1996. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur. J. Protistol. 32, 356–365], but also with *N. apis*. Both microsporidia produced single and mixed infections. Overall and at each location alone, the prevalence of *N. ceranae* was higher than that of *N. apis*. In all cases of mixed infections, the number of *N. ceranae* gene copies (corresponding to the parasite load) significantly out numbered those of *N. apis*. Phylogenetic analysis based on a variable region of small subunit ribosomal RNA (SSUrRNA) showed four distinct clades of *N. apis* and five clades of *N. ceranae* and that geographical distance does not appear to influence the genetic diversity of *Nosema* populations. The results from this study demonstrated that duplex real-time qPCR assay developed in this study is a valuable tool for quantitative measurement of *Nosema* and can be used to monitor the progression of microsprodian infections of honey bees in a timely and cost efficient manner.

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1. Introduction

Microsporidia are opportunistic intracellular spore-forming parasites in nature infecting all animal phyla (Weber et al., 1994). *Nosema* is a genus of microsporidia and increasingly has been recognized as an important insect pathogen. Nosemosis is one of the most serious diseases of adult honey bees occurring in nearly every country with beekeeping practices (Bailey and Ball, 1991; Matheson, 1996) and is caused by two species, *Nosema apis* and *Nosema ceranae*. For years, nosemosis as a disease of the European honey bee, *A. mellifera*, was exclusively attributed to *N. apis* (Ellis and Munn, 2005; Zander, 1909). However, recent studies have showed that *N. ceranae*, a species of *Nosema* originally found in the Eastern honey bees, *Apis cerana* (Fries et al., 1996), is a disease agent of European honey bees as well (Fries et al., 2006; Higes et al., 2006; Huang et al., 2007) and is frequently related to colony losses (Faucon et al., 2002; Higes et al., 2008a; Martín-Hernández et al., 2007). Further studies indicated that *N. ceranae* is highly pathogenic to its new host and the disease caused by *N. ceranae* is far more prevalent than that caused by *N. apis* in European honey bees (Chen et al., 2008; Cox-Foster et al., 2007; Higes et al., 2007; Klee et al., 2007).

While *N. ceranae* has successfully invaded and become established in its new host, little is known about microsporidian infection in *A. cerana*, the original host of *N. ceranae*. In order to gain more insight into epidemiology of microsporidian parasitism in honey bees, we conducted studies to investigate the status of *Nosema* infection in Eastern honey bees and the possibility of host range expansion for *N. apis*. In this work, we developed a duplex TaqMan real time quantitative PCR (qPCR) assay for one-step differentiation and quantification of *N. apis* and *N. ceranae* infections in bees. We provide the first evidence that *N. apis* infects European

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honey bees as well as Eastern honey bees and this host-parasite association has existed for at least 16 years, even before *N. ceranae* was first identified in Eastern honey bees in 1996 (Fries et al., 1996). We also provide evidence that co-infection of *N. ceranae* and *N. apis* is present in Eastern honey bees, and that *N. ceranae* prevails over *N. apis* as shown by its greater infection rate and higher copy number in the host.

2. Materials and methods

2.1. Samples

Samples of *A. cerana* were collected from Taiwan, China, and Japan. Bees from Taiwan were collected in four different locations including Taizhong, Jilong, Lantou, and Miaopu in 1992 and had been stored in −80 °C freezer at Beltsville Bee Research Laboratory, USDA for 16 years prior to our analysis. Bees from Changsha, Hunan, China and Tsukuba, Japan were sampled in 2007 and 2008, respectively. Fifty bees were collected from each location and DNA was isolated from individual bees of *A. cerana* using a genomic DNA extraction kit, DNAzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.2. Primers and probes

The complete coding sequences of rRNA genes of *N. apis* (GenBank Accession No.: U97150) and *N. ceranae* (GenBank Accession No.: DQ486027) including small subunit ribosomal RNA (SSUrRNA), intergenic transcribed spacer (ITS), and large subunit ribosomal RNA (LSUrRNA) were retrieved from GenBank and aligned with the MegAlign (DNASTAR Lasergene software program, Madison, WI). Alignment of these two sequences revealed that sequence variations between *N. apis* and *N. ceranae* were located mostly in the region of SSUrRNA gene. As a result, the primers and probes for differentiation and quantification of *N. apis* and *N. ceranae* infections were selected from the regions of SSUrRNA. The primer pair, *N. apis*-sense (5′-CCATTGCCGGATAAGAGAGT-3′) and *N. apis*-antisense (5′-CCACCAAAAACTCCCAAGAG-3′) was used to generate a 269-bp amplicon of *N. apis*. The primer pair, *N. ceranae*-sense (5′-CGGATAAAAGAGTCCGTTACC-3′) and *N. ceranae*-antisense (5′-TGAGCAGGGTTCTAGGGAT-3′), was used to amplify a 250-bp fragment of *N. ceranae*. Fluorophores, FAM (6′-carboxyfluorescein) with excitation and emission wavelength were 495 nm and 515 nm, respectively, and VIC (a proprietary fluorescent reporter dye) with excitation and emission wavelength were 535 nm and 555 nm, respectively, were chosen for probe labeling because their absorption and emission wavelengths are well separated from each other. The probe specific for *N. apis* (5′-ATAGTGAGGCTCTATC ACTCCGCTG-3′) was labeled with VIC reporter dye at the 5′ end while the probe specific for *N. ceranae* (5′-CGTTACCCTTCGGG GAATCTTC-3′) was labeled with FAM reporter dye at the 5′ end to allow for duplex PCR reaction of two targets in the same tubes. Both probes were labeled with TAMRA (6-carboxytetramethylrhodamine), a quencher dye with sufficient spectral overlap (excitation and emission wavelength between 535 and 605 nm) with FAM and VIC, at the 3′ end. All primer and probe combinations were tested to make sure there was no potential for formation of primer-dimers or hairpins (http://primerdigital.com/Tools/PrimerAnalyser.html). The housekeeping gene, beta-actin, previously used as an *A. mellifera* internal control which was proved also to be applicable for *A. cerana* in our pilot studies, was included in the analysis as an internal control. The primers and probe of beta-actin were described in detail in our previous paper (Chen et al., 2004). The primers were synthesized by Invitrogen and FAM- or VIC-labeled TaqMan probes were purchased from Applied Biosystems.

2.3. Conventional PCR

DNA extracted from each bee was amplified with two sets of *Nosema* primers individually. Amplification was performed using Platinum Taq Polymerase High Fidelity (Invitrogen; Carlsbad, CA). The reaction mixture contained: 1× high fidelity PCR buffer, 0.1 mM each dNTP, 2 mM $MgSO_4$, 0.2 μM of sense primer, 0.2 μM of antisense primer, and 1 unit Platinum Taq High Fidelity polymerase and 500 ng genomic DNA in a total volume of 25 μl. The thermal cycling conditions were as follows: one cycle of initial denaturation at 95 °C for 2 min, 45 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 30 s. For each run of PCR amplification, a negative control containing no template DNA was included. The positive control was purposely not included in order to exclude the possible chance of contamination. The PCR product was analyzed by 1.2% Low Melting Point Agarose (Invitrogen) gel electrophoresis. The specificity of PCR amplification of each pair of primers was verified by purifying PCR fragments using Wizard PCR Prep DNA Purification System (Promega, Madison, WI), sequencing the PCR fragments, and performing homology searching using the BLAST server in the GenBank database, NCBI, NIH.

2.4. Recombinant plasmid DNA and standard curve

Purified *N. apis* and *N. ceranae* specific amplicons were individually incorporated into a pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) following manufacturer's protocol. Recombinant plasmid DNA was purified using Plasmid Mini Prep Kit (BIO-RAD, Hercules, CA). The copy number of plasmid DNA was calculated based on the molar concentration and molecular mass of the recombinant plasmid consisting of plasmid vector and the PCR insert. The recombinant plasmids containing *N. apis* or *N. ceranae* specific fragment were constructed individually and equal copy numbers of the two plasmids were mixed together and assayed in duplex. Each template of plasmid DNA was diluted to copy numbers of 1×10^0, 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9. The standard curves for both *N. apis* and *N. ceranae* were generated by amplifying the serially diluted plasmids in a duplex qPCR assay. The sensitivity of the duplex qPCR assay was determined by plotting the log initial quantities of 10-fold serial diluted plasmid mixture against corresponding threshold value (C_T). The amplification efficiency of each plasmid template was calculated from the slope of the standard curve according to the following formulas: $E = 10^{(-1/\text{slope})} - 1$ (Efficiency of PCR reaction, Stratagene Application Note: http://www.stratagene.com/lit_items/appnotes10:pdf).

2.5. Duplex real-time qPCR

Bees co-infected with *N. apis* and *N. ceranae* as identified by conventional PCR, were subjected to duplex real-time qPCR using the Stratagene Mx3005P™ Multiplex Quantitative PCR System. The final amplification mixture contained 0.3 μM of each primer, 0.15 μM of each TaqMan probe, 3 mM $MgSO_4$, 0.4 mM dNTP, 1 unit Platinum TaqDNA polymerase and 500 ng DNA, in a total volume of 50 μl. The cycling conditions were the same as for conventional PCR. A housekeeping gene, beta-actin, was amplified for each sample under the same conditions as described for *Nosema*. All samples were run three times. Amplification results were expressed as the threshold cycle (C_T) value, which was defined as the number of the amplification cycles needed to generate fluorescent signal above a predefined threshold after the noise filtering. The specificity of duplex qPCR was confirmed by electrophoresis and sequencing analyses to verify the correct size and specificity of PCR products.