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Short Communication

Nosema ceranae is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States

Yanping Chen, Jay D. Evans, I. Bart Smith, Jeffery S. Pettis *

USDA-ARS Bee Research Laboratory, Bldg. 476, BARC-East, Beltsville, MD 20705, USA

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Abstract

Honey bee samples collected between 1995 and 2007 from 12 states were examined for the presence of *Nosema* infections. Our results showed that *Nosema ceranae* is a wide-spread infection of the European honey bee, *Apis mellifera* in the United States. The discovery of *N. ceranae* in bees collected a decade ago indicates that *N. ceranae* was transferred from its original host, *Apis cerana* to *A. mellifera* earlier than previously recognized. The spread of *N. ceranae* infection in *A. mellifera* warrants further epidemiological studies to identify conditions that resulted in such a widespread infection.
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Keywords: *Nosema* disease; Honey bee; *Apis mellifera*; *Apis cerana*; *Nosema ceranae*; *Nosema apis*; rRNA

Nosema disease is one of the most prevalent adult honey bee diseases (Bailey, 1981; Matheson, 1993) and is caused by two described species of microsporidia, *Nosema apis* and *Nosema ceranae*. Transmission of *Nosema* in honey bee colonies is mainly via the fecal-oral route in which pathogens are spread by transferring feces of diseased hosts to uninfected hosts via ingestion. Adult bees ingest *Nosema* spores when they are eating contaminated food and when they are cleaning up fecal material from infected bees. The spores geminate within the midgut and release polar tubes that transfer their sporoplasm into midgut epithelial cells where they generate more spores. Millions of new spores can be found inside of a bee's midgut a few weeks after initial infection (Bailey and Ball, 1991) and the spores excreted with feces become new sources of the infection in the colonies. Although infected bees do not exhibit obvious external disease symptoms, infection of *Nosema* causes digestive disorders, shortened life spans of honey bees, decrease of population size of honey bee colonies (Hassanein, 1953; Rinderer and Sylvester, 1978; Malone et al., 1995), and reduction of honey production and crop products that rely on bees for pollination (Anderson and Giacon, 1992; Fries et al., 1984; Goodwin et al., 1990).

Since late 2006, a mysterious illness, termed "Colony Collapse Disorder" (CCD), has been devastating massive numbers of honey bee colonies and threatens the beekeeping industry in the United States. As part of our efforts to identify the causes of sudden disappearances and extensive die-offs of colonies in the hope of designing appropriate control strategies to safeguard bee health in the future, the status of *Nosema* infections in honey bees collected from states affected by CCD was also examined. While our investigation did not show a direct correlation between *Nosema* infection and CCD, the results revealed that *N. ceranae*, a species of *Nosema* originally found in the Asian honey bee, *Apis cerana* (Fries et al., 1996), was the only *Nosema* species found in infected European honey bees (*Apis mellifera*) and had a wide geographical distribution in the United States. For years, *Nosema* disease of the European honey bee was exclusively attributed to *N. apis*. However, two recent reports showed that *N. ceranae* has been discovered

* Corresponding author. Fax: +1 301 504 8736.
E-mail address: Jeff.Pettis@ars.usda.gov (J.S. Pettis).

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in the European honey bee in Taiwan and Spain (Higes et al., 2006; Huang et al., 2007). In addition, Klee et al. (2007) reported that *N. ceranae* was a widespread infection of the European honey bee worldwide. In light of the discovery that *N. ceranae* is a widespread infection of European honey bees, we hypothesized that *N. ceranae* might have been present in the European honey bees in the United States long before we realized. To clarify this, honey bee samples collected a decade ago were examined for the presence of *N. ceranae*. Here we present evidence that *N. ceranae* is a long-established and wide-spread infection of the European honey bee in the United States.

Bee samples were collected from the field and sent to our laboratory by beekeepers. The samples came from different geographic regions of the United States from the years 1995–2007. All the bee samples were stored in 70% ethanol at room temperature prior to examination. Ten bees were randomly selected from each group and a total 180 bees were examined for the presence of *Nosema*. Genomic DNA was extracted from individual bees using a DNA purification kit, DNAzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The complete sequences of rRNA including small subunit ribosomal RNA gene, internal transcribed spacer, and large subunit ribosomal RNA gene, for both *N. apis* (GenBank Accession No: U97150) and *N. ceranae* (GenBank Accession No: DQ486027) were retrieved from GenBank and aligned with the MegAlign (DNASTAR Lasergene software program, Madison, WI). Based on the alignment of rRNA, a set of generic primers, *Nosema* F (5′-ggcagttatgggaagtaaca-3′)/*Nosema* R (5′-ggtcgtcacatttcatctct-3′) was designed in the region conserved in both *N. apis* and *N. ceranae* to allow for amplification of rRNA in both species. To differentiate between two the *Nosema* species, one set of primers, *N. ceranae* F (5′-cggataaaagagtccgttacc-3′)/*N. ceranae* R (5′-tgagcagggttctagggat-3′) was designed in the region complimentary only to *N. ceranae*, while another set of primers, *N. apis* F (5′-ccattgccggataagagagt-3′/*N. apis* R (5′-cacgcattgctgcatcattgac-3′) was designed in the region complimentary only to *N. apis*. DNA from individual bees was amplified with three sets of *Nosema* primers separately. Invitrogen recombinant TaqDNA polymerase was used for PCR amplification and the reaction mixture as well as PCR thermal cycling profiles was performed according to the manufacturer's recommended protocol. The specificity of PCR amplification of each pair of primers was confirmed by purifying PCR fragments from agarose gels using Wizard PCR Prep DNA Purification System (Promega, Madison, WI), sequencing the PCR fragments, and comparing sequence results with sequences deposited in the GenBank database, NCBI, NIH. DNA extracted from *Nosema* infected bees (provided by Dr. Stephen F. Pernal from Agriculture Agri-Food Canada) that were confirmed to be positive for both *N. ceranae* and *N. apis* by PCR method and sequencing analysis was used as a positive control. Negative controls (H_2O) were also included in each run of PCR amplification.

Our results showed that *N. ceranae* is the only *Nosema* species found to infect European honey bees from our widespread geographic collections in the United States. *N. ceranae* infected bees were found in samples collected from each of 12 states including Oregon, California, Hawaii, Idaho, North Dakota, Minnesota, Texas, Ohio, Tennessee, Connecticut, Maryland and Florida, representing the Northeast, Southeast, Midwest, Southwest, and the West regions of the U.S. Among 180 bees examined for *Nosema*, 16% of the bees (28/180) were positive for *N. ceranae*. The results of PCR amplification with generic *Nosema* primer pair perfectly matched the results of amplification with specific *N. ceranae* primer pair. However, the results showed that all examined bees were negative for *N. apis* when amplified with specific *N. apis* primer pair.

In an attempt to investigate the historical incidence of *N. ceranae* infection in European honey bees, we found infections of *N. ceranae* in samples collected during the period from 1995 to 2007 in the United States (Fig. 1). The detection of *N. ceranae* in bees collected a decade ago indicated that *N. ceranae* is not a new emerging pathogen for European honey bees and in fact had transferred from its original host to *Apis mellifera* earlier than previously recognized.

Our studies of *N. ceranae* infection in European honey bees in the U.S. confirm and extend the previous observations by Fries et al. (2006), Higes et al. (2006, 2007) and Huang et al. (2007) that infection of *N. ceranae* was not restricted to its original host. Detection of a pathogen in bees stored over a long period of time suggests that *N. ceranae* has adapted to and established an infection in European honey bees for quite a while and also demonstrates the power of PCR technique in handling historical samples for pathogen infections. However, the discovery of *N. ceranae* in European honey bees raises several questions. First, assuming *A. cerana* was the original host of *N. ceranae*, when was the exact time that *N. ceranae* expanded its host range from *A. cerana* to *A. mellifera*? Which transmission pathway(s) provided opportunities for *N. ceranae* to infect new host populations? And while host specificity is a characteristic of microsporidia and the host range of most microsporidia is relatively narrow (Solter and Becnel, 2003; Solter et al., 2005), how did *N. ceranae* overcome the species barrier to expand its host range and establish infection in a new host? What pathogenic traits of *N. ceranae* provide *N. ceranae* with the ability to take over the *N. apis* and to become a predominant infection in *A. mellifera* in the U.S. populations? In addition, when did this displacement occur, or for how long has *Nosema* disease in the U.S. been misclassified as arising solely from *N. apis*. Indeed, is it possible that the parasite identified genetically as *N. ceranae* (Fries et al., 1996) is in fact the historical source of this disease in the U.S. The data presented in this study indicate a strong need for epidemiological and pathogenetic studies to identify the conditions that results in widespread infection of *N. ceranae* in European honey bees.

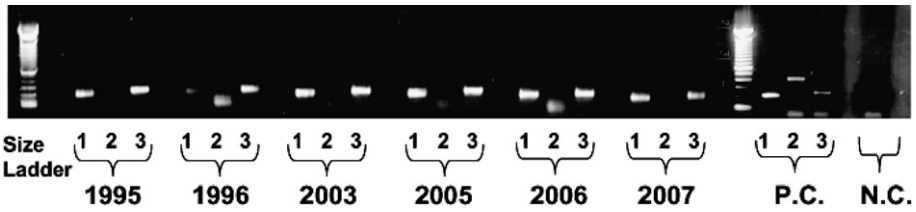

Fig. 1. PCR amplification of representative bee samples collected from 1995 to 2007 in the United States. For *x*-axis, No. 1 indicates that DNA was amplified with generic primers, *Nosema* F/R; No. 2 and 3 indicates DNA was amplified with specific primers, *N. apis* F/R and *N. ceranae* F/R, respectively. The primer pairs, *Nosema* F/R, *N. apis* F/R, and *N. ceranae* F/R generated PCR fragments of 208, 401, and 250 bp, respectively. DNA that was extracted from bees collected from Canada and identified to be positive for both *N. ceranae* and *N. apis* was used as a positive control (P.C.). Water was used as a negative control (N.C.). The PCR amplification bands are seen in samples amplified both with primer pairs *Nosema F*/*F* and *N. ceranae F*/*R* but in samples amplified with primer pair *N. apis F*/*R*.

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