Prevalence of *Nosema* from Managed Honey Bee Colonies in South Dakota and New York[1]

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ABSTRACT Despite a number of studies on the prevalence of *Nosema* across the United States, limited information exists regarding comparative rates of *N. ceranae* and *N. apis* infection within individual states. We conducted a study to detect *N. ceranae* and *N. apis* using molecular diagnostics on managed honey bee colonies from New York and South Dakota. A total of 1200 apiaries were visited by New York state bee inspectors in 2009, and samples were collected from 4300 honey bee colonies. A total of 1023 apiaries were sampled from South Dakota in 2009. Microscopy was used to detect *Nosema* spore presence and to estimate the intensity of infection by estimating spore counts in positive samples. *Nosema* was detected in 528 (44%) of the New York and 300 (29%) of the South Dakota sampled apiaries. A total of 464 New York and 290 South Dakota *Nosema*-positive apiary samples were subjected to molecular diagnostic polymerase chain reaction (PCR) analysis. For the New York samples, 250 (54%) were PCR-positive for *Nosema*, with 96.8% being *N. ceranae*, 0.8% *N. apis,* and 2.4% both *N. ceranae* and *N. apis*. For the South Dakota samples, 122 (42%) were PCR-positive for *Nosema*, all of which were *N. ceranae*.

KEY WORDS *Apis mellifera*, *Nosema ceranae*, *Nosema apis*, molecular diagnostics, PCR

Nosema apis Zander and *N. ceranae* Fries (Nosematidae), are microsporidian pathogens of honey bees, *Apis mellifera* L., causing nosemosis in their hosts (Bailey & Ball 1991, Fries 1997). Nosemosis affects only adult bees by infecting epithelial cells lining the midgut and causing dysentery (Bailey 1955). The disease is likely distributed by mailing and transporting honey bees (Jay 1966) and infective beekeeping materials (Klee et al. 2007) combined with migratory beekeeping practices (Giersch et al. 2009). Although *N. apis* has long been widespread in the United States (Matheson 1993), natural infections of *N. apis* rarely cause serious damage to infected honey bee colonies (Bailey & Ball 1991). In addition, treatment of *N. apis* is available with fumagillin (Moffet et al. 1969) in the United States. Another *Nosema* species, *N. ceranae*, was first found in the Asian honey bee, *Apis cerana* F., by Fries et al. (1996), and then reported in *A. mellifera* in 2004 (Higes et al. 2006). Further studies revealed that *N. ceranae* has been present in the United States since at least 1995 (Chen et al. 2008), and

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has been in Europe since at least 1998 (Paxton et al. 2007). *Nosema ceranae* is highly pathogenic in honey bees (Higes et al. 2008), and it is associated with reduced honey production and increased winter mortality (Higes et al. 2006).

Distinguishing spores of *N. apis* and *N. ceranae* morphologically is difficult, however, identification is possible using molecular diagnostics. Molecular diagnostics using polymerase chain reaction (PCR) primers specific for *N. ceranae* and *N. apis* are well established and have been conducted on honey bees from several countries, including Turkey (Whitaker et al. 2011), Argentina (Medici et al. 2012), the United States (Chen et al. 2008, Chapon et al. 2009, Traver & Fell 2011), and Canada (Williams et al. 2008). *Nosema ceranae* has been detected in many states in the United States, including Oregon, California, Hawaii, Idaho, North Dakota, Minnesota, Texas, Ohio, Tennessee, Connecticut, Maryland, Florida (Chen et al. 2008); Nebraska, South Dakota, Wisconsin (Chapon et al. 2009); and Virginia (Traver & Fell 2011).

The objectives of this study were to determine the prevalence of *N. apis* and *N. ceranae* in honey bee samples from South Dakota and New York, and to determine the state-wide distribution of *Nosema* based on spore counts.

Materials and Methods

New York honey bee samples were collected by New York State apiary inspectors from May to October 2009. A total of 4300 honey bee colonies from 1200 apiaries from New York were sampled, representing 59% of the apiaries and 6% of the honey bee colonies in the state. For each apiary in New York, a sample consisted of 10 to 20 foraging worker honey bees collected from one to five hives and placed in a plastic liquid scintillation vial containing 90% ethanol. For each apiary in South Dakota, a sample consisted of 20 to 30 foraging worker honey bees collected from one to five hives within each apiary and placed in a re-sealable plastic storage bag containing 70% ethanol. A total of 1023 apiaries were sampled during 2009 in South Dakota. Samples were then shipped to the USDA-ARS Bee Research Laboratory in Beltsville, MD for diagnosis by microscopy.

Fifteen adult bees from each sample were macerated in 15 ml of distilled water with a mortar and pestle. The suspension was decanted to remove coarse material and examined by phase contrast microscopy for the presence of *Nosema* spores. Infection intensity was characterized by estimating *Nosema* spore counts in positive samples. Spore counts were made using a haemocytometer per Cantwell (1970). Once samples with a positive spore count were identified, half of the remaining honey bees from the New York samples and all of the remaining honey bees from the South Dakota positive-spore-count samples were sent to the Insect Genetics Lab, University of Arkansas, Fayetteville, AR, for molecular diagnostic analysis.

The Puregene DNA extraction kit (Gentra, Minneapolis, MN) was used to obtain DNA from two individual worker honey bee abdomens from each sample with a positive spore count using a protocol from Whitaker et al. (2011). Extracted DNA was re-suspended in 50 μl of Tris: EDTA buffer (pH 8.0), and stored at -20°C until PCR analyses were done. PCRs were conducted using primer pairs that amplify a portion of the small subunit 16S rRNA gene specific for *N. ceranae* (*N. ceranae* F: 5′-CGGATAAAAGAGTCCGTTACC-3′ and R: 5′-TGAG-CAGGGTTCTAGGGAT-3′) (Chen et al. 2008) and *N. apis* (*N. apis*-sense: 5′-

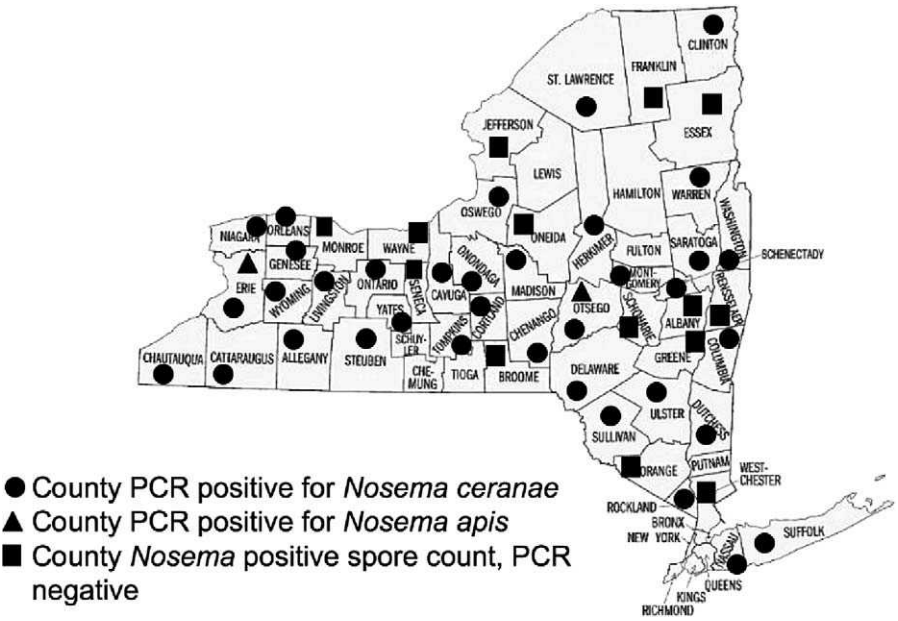

Fig. 1. Counties in New York where apiaries have been found infected with *N. ceranae* and *N. apis*.

CCATTGCCGGATAAGAGAGT-3′ and *N. apis*-antisense: 5′-CCACCAAAAACT-CCCAAGAG-3′) (Chen et al. 2009). These primers were used together for a multiplex PCR reaction and resulted in a 250-bp amplicon for *N. ceranae* and a 269-bp amplicon for *N. apis*. Morphologically identified and PCR-confirmed positive controls consisted of *N. ceranae* from worker honey bees collected from Nebraska, and *N. apis* from worker honey bees collected from Beaverlodge, Alberta, Canada. In addition, a negative control consisting of ultrapure water was included for all PCR runs. Concentration of PCR reagents followed Szalanski et al. (2004), with the exception that 1 μl of each primer was used, and the volume of water was reduced to maintain a final volume of 50 μl. The thermocycler profile consisted of an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 60 s, with a final extension of 5 min at 72°C. The PCR products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide, and products were visualized under UV light (BioDoc-It, UVP, Upland, CA). Amplicon sizes were estimated by comparison to a 50-bp ladder. Statistical analysis was conducted using a Z-test of equal proportions (Sokal & Rohlf 1969).

Results and Discussion

A total of 528 (44%) and 300 (29%) of the New York and South Dakota apiary samples had positive spore counts, respectively. This represents 54 of the 62 counties in the state of New York, and 26 of the 66 South Dakota counties (Figures 1

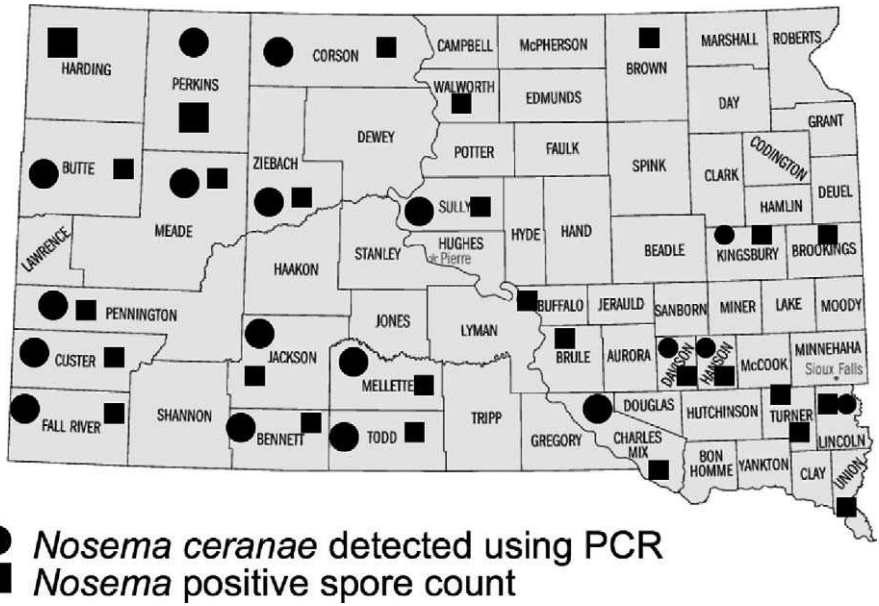

Fig. 2. Counties in South Dakota where apiaries have been found infected with *N. ceranae*.

& 2). Positive spore counts for the New York samples ranged from 200,000 to 19 million spores per worker honey bee. A total of 464 New York and 290 South Dakota positive-spore-count samples were subjected to PCR analysis, with 56% of the New York samples and 42% of the South Dakota samples (overall, $n = 372$) successfully amplifying for *Nosema*. From the PCR-positive New York samples, a total of 242 samples (96.8%) were positive for *N. ceranae* and two (0.8%) were positive for *N. apis*. Additionally, six of the samples (2.4%) were positive for both *N. ceranae* and *N. apis*. All of the South Dakota PCR-positive samples ($n = 122$) were *N. ceranae*.

This is the first study to document the occurrence and distribution of *N. ceranae* in honey bees from New York and South Dakota using molecular diagnostics. New York can now be added to the list of 16 states where the presence of *N. ceranae* has been documented (Chen et al. 2008, Chapon et al. 2009, Traver & Fell 2011). Traver & Fell (2011) documented the prevalence of *Nosema* in honey bees from Virginia using real time PCR. Out of 586 sampled colonies 220 (37.5%) were found to have *Nosema* infections, of which 97.3% were *N. ceranae* and the remainder were *N. apis* (Traver & Fell 2011). This level of *Nosema* infection was similar to what we observed in South Dakota (29% of apiaries) and in New York (44% of apiaries), although *Nosema* occurrence in South Dakota was lower ($P < 0.05$, Z-Test of Equal Proportions). The overall prevalence of *Nosema* is similar to levels observed in studies prior to the documentation of *N. ceranae* in the United States. For example Doull & Eckert (1962) observed 37.7% of 61 apiaries in California sampled from 1960 to 1961 to be infected with *Nosema*. The high level of *N. ceranae* relative to *N. apis* observed in Virginia (Traver & Fell 2011) was similar to what we observed in both New York (96.8% *N. ceranae*) and South Dakota (100% *N. ceranae*) ($P > 0.05$, Z-Test of

Equal Proportions). The relative dominance of *N. ceranae* over *N. apis* in recent surveys also has been documented in Buenos Aires Province, Argentina, where 100% of *Nosema*-positive samples were *N. ceranae* and 2.6% were co-infected with *N. apis* (Medici et al. 2012).

Whereas, Traver & Fell (2011) were able to successfully amplify *Nosema* DNA from all of their positive-spore-count samples, our success rate was considerably lower. This discrepancy could be due to the greater sensitivity of real-time PCR used by Traver & Fell (2011) over the conventional PCR technique that we used in this study.

Nosema ceranae infection has a range of different effects. In Spain, Higes et al. (2008) found that high spore counts were correlated with colony collapses. Spore loads peaked in spring followed by a summer drop-off (or "false recovery") and then another peak in the fall before colonies collapsed (Higes et al. 2008). Conversely, it has been reported that many beekeepers in New York have sustained no abnormal loss of colonies as of November 2009, despite spore counts in the millions per individual worker honey bee (P. Cappy, unpublished data). Data on late-fall spore counts in New York are lacking because it is extremely disruptive to open colonies in this climate when the weather is cold. Other studies have documented that *Nosema* is not correlated with colony collapse disorder (vanEngesdorp et al. 2009, Genersch et al. 2010).

The study by Traver & Fell (2011) found that 51.1% of 90 samples that had a negative *Nosema* spore count were positive based on real-time PCR. This provides evidence that the levels of infection of *Nosema* may be underestimated in the United States, and that the real-time PCR method may be superior to conventional PCR for the detection of *Nosema*. Our results highlight the need for continued research on the distribution and phenology of *N. ceranae* and *N. apis* in the United States.

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