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Short Communication

First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and central USA

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Abstract

Nosema ceranae is an emerging microsporidian parasite of European honey bees, *Apis mellifera*, but its distribution is not well known. Six *Nosema*-positive samples (determined from light microscopy of spores) of adult worker bees from Canada (two each from Nova Scotia, New Brunswick, and Prince Edward Island) and two from USA (Minnesota) were tested to determine *Nosema* species using previously-developed PCR primers of the 16S rRNA gene. We detected for the first time *N. ceranae* in Canada and central USA. One haplotype of *N. ceranae* was identified; its virulence may differ from that of other haplotypes.
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Nosema apis (Zander, 1909) is a microsporidian parasite of European honey bees, *Apis mellifera*. *Nosema* affects adults only, infecting epithelial cells lining the midgut after spores are ingested (Bailey, 1955). *N. apis* can induce queen supersedure (Webster et al., 2004), reduce pollen collection (Anderson and Giacon, 1992), and shorten bee life span (Wang and Moeller, 1970).

Previously, nosemosis in European honey bees was attributed exclusively to *N. apis* (Ellis and Munn, 2005), with the recent exception of disease in regions of Asia and Europe that were ascribed to the closely-related *Nosema ceranae* (Higes et al., 2006; Huang et al., 2007). However, it appears that *N. ceranae* is an emerging pathogen that has increased its distribution in the past decade by jumping from Asian honey bees, *Apis cerana*, to European honey bees (Klee et al., 2007). *N. ceranae* has now been detected on four continents (Asia, Europe, North America, and South America) and it may be displacing *N. apis* in European honey bees (Klee et al., 2007). Originally isolated from Asian honey bees (Fries et al., 1996), *N. ceranae* is highly pathogenic when experimentally inoculated into European honey bees (Higes et al., 2007), and is associated with reduced honey production and increased winter mortality (Higes et al., 2006). Here, we describe the first detection of *N. ceranae* in European honey bees in Canada and central USA.

We collected adult worker bees from Minnesota, USA (in July 2006, 1 beekeeper and 10 colonies), and from the three Canadian Maritime Provinces (New Brunswick in September 2006, 3 beekeepers and 9 colonies; Prince Edward Island in September 2006, 10 beekeepers and 35 colonies; Nova Scotia in April 2007, 2 beekeepers and 2 colonies). Bees were kept at -20 °C until suspensions could be created by adding 10 ml of distilled water to the abdomens of 10 bees (from a single colony) that were crushed using a mortar and pestle (Rogers et al., 2003). Ratios of *Nosema*-positive hives in Minnesota, New Brunswick, Prince Edward Island, and Nova Scotia, determined from light microscopy, were 4/10, 3/9, 4/35, and 2/2, respectively. Two *Nosema*-positive suspensions were chosen from Minnesota, USA and each Canadian Maritime Province

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for molecular analyses. Each selected suspension represented a separate beekeeping operation, with the exception of bees collected in Minnesota, which originated from a single beekeeper.

For each of the eight selected suspensions, an aliquot of 10 μl was added to 10 μl of proteinase K (20 mg/ml), vortexed, and warmed to 37 °C. After 10 min, 100 μl of Chelex 100/Instagene matrix (Bio-Rad) was added, and the solution was boiled for 10 min, and then incubated at 56 °C overnight (Walsh et al., 1991). The solution was centrifuged for 3 min at 13,000*g*. The resulting supernatant was used as the DNA template in the subsequent polymerase chain reaction (PCR), which was performed on a TGradient thermal cycler (Biometra). The ~25 μl reaction mix consisted of 10.9 μl ultrapure H_2O, 5 μl 5× PCR buffer (Promega), 2.5 μl $MgCl_2$ (25 mM), 0.5 μl dNTPs (10 mM each), 2.5 μl primers (10 mM each), 1.25 U GoTaq polymerase (Promega), and 1 μl of template DNA. Primers NOS-FOR and NOS-REV (Higes et al., 2006) used to amplify the 16S rRNA gene were optimized using an annealing temperature gradient and then run under the following parameters: an initial 2-min denaturing period at 94 °C, followed by 40 cycles of 45 s denaturing at 94 °C, 45 s annealing at 58 °C, and a 1-min extension period at 72 °C. The cycling period was followed by an additional 72 °C extension period of 3 min. PCR products were visualized and purified from a 1% agarose gel in 1× TAE buffer using a gel extraction kit (Qiagen). The PCR contained a negative control, which was verified by confirming the absence of a band in the gel. Double-stranded sequencing was performed at Florida State University (Tallahassee) using a gene analyzer with capillary electrophoresis (Applied Biosystems).

The 16S rRNA fragment sequences were aligned using BioEdit v.7.0.5.3 (Hall, 1999) and confirmed by eye. The generated consensus sequences, created from both forward and reverse sequences, were compared to those of related species obtained from GenBank (Fig. 1). All newly-generated sequences were deposited in GenBank (Accession Nos. EF584418–EF584425).

The phylogenetic position of each *Nosema*-positive sample was determined using minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) methods, with 16S rRNA sequences from *Vairimorpha imperfecta* (Canning et al., 1999) used as an outgroup reference. Genetic distances among sequences for ME analysis were calculated using the Tamura-Nei model as selected by Modeltest v.3.06 (Posada and Crandall, 1998). The accompanying tree was constructed using the software package MEGA 3 (Kumar et al., 2004). The software package PAUP V.4.0b10 (Swofford, 2002) was used for both MP and ML analyses. For MP analysis, we implemented a full heuristic search with all characters unweighted and unordered, and we used the TrN+I model of nucleotide substitution for the ML analysis. Support for all the phylogenetic analyses was measured using 1000 bootstrap replicates.

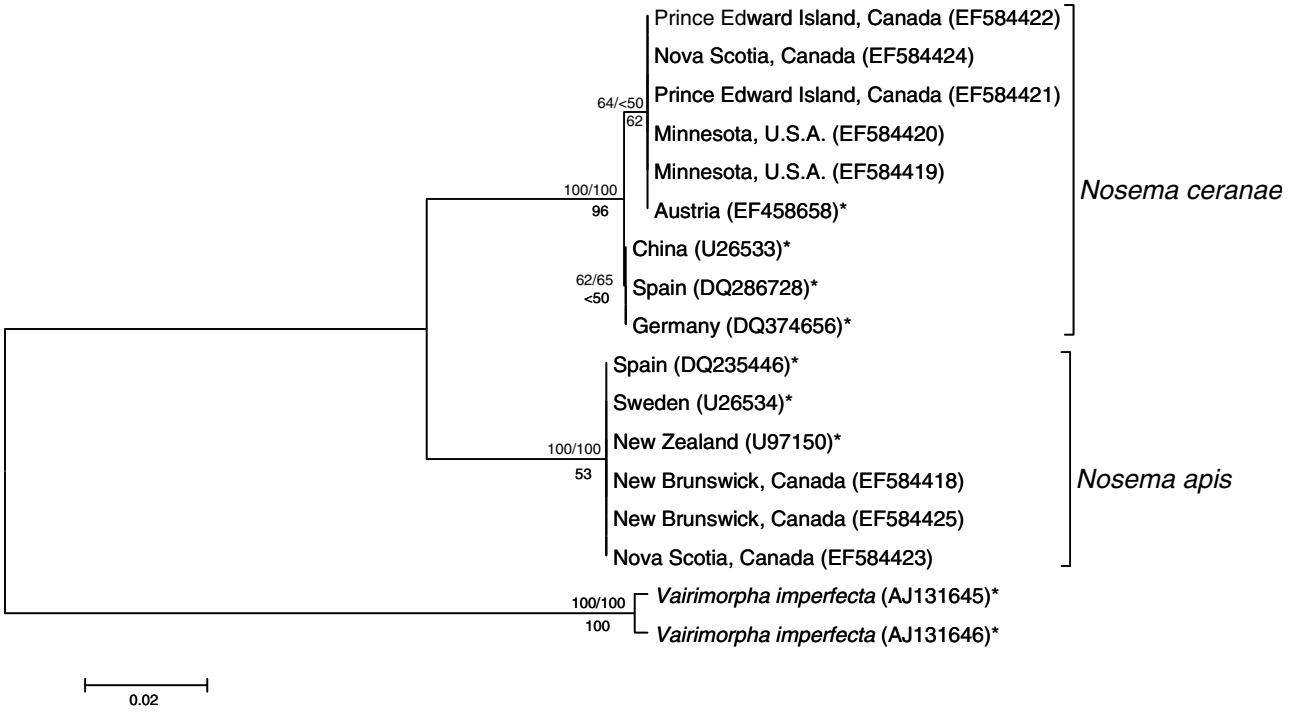

Fig. 1. Phylogenetic tree illustrating the position of *Nosema* collected from European honey bees (*Apis mellifera*) in the Canadian Maritime Provinces (Nova Scotia, New Brunswick, and Prince Edward Island) and Minnesota USA, with previous *Nosema* GenBank submissions (denoted by ∗). *Vairimorpha imperfecta* is used as the outgroup reference. GenBank accession numbers are in parentheses. Bootstrap support values above the branch (minimum evolution/maximum parsimony) are from 1000 replicates. Support for the maximum likelihood analysis, below the branch, has also been obtained from 1000 bootstrapped replicates.

Five out of eight samples produced high probability matches on GenBank to *N. ceranae* (99–100%), and the remaining three produced high probability matches to *N. apis* (100%). The amplified fragment lengths were 252 and 240 bp for *N. ceranae* and *N. apis*, respectively. Phylogenetic clustering using ME, MP, and ML analyses further confirmed our samples as either *N. ceranae* or *N. apis* (Fig. 1), and were supported by bootstrap values $\geqslant 50\%$ (although most bootstrap values at the species level were >96%). When *V. imperfecta* was excluded from the ML analysis, the split between *N. ceranae* and *N. apis* was supported by a bootstrap value of 100%. This suggests that the low bootstrap support for the ML analysis was influenced by the relatively large degree of divergence between the genera *Nosema* and *Vairimorpha*.

Phylogenetic clustering of sequence data was further supported by genetic distances calculated using Tamura-Nei's model; average within-group distance for *N. ceranae* and *N. apis* was 0.2% and 0% (standard deviations 0.2% and 0%), respectively, and the average distance between groups was 6.4% (standard deviation 1.7%). In addition, the sequence of our North American *N. ceranae* SS rRNA differs from those reported from China (Fries et al., 1996) and Spain (Higes et al., 2006) by the insertion of either an adenine or guanine at the 19th bp position and by a transition mutation at the 21st bp position; however, the North American haplotype is shared with those reported from Austria (i.e., EF458655-58).

The detection of *N. ceranae* in European honey bees from the Maritime Provinces of Canada and Minnesota, USA expands the known distribution of this emerging parasite. As in other geographic regions of North America, *N. apis* was suspected as the single cause of nosemosis in European honey bee colonies, although Klee et al. (2007) just reported that *N. ceranae* was detected in eastern USA (District of Columbia) in 2004. Thus, *N. ceranae* is likely a relatively recent arrival to Canada compared to other regions of the world, such as southern and eastern Europe, Asia, and the rest of the Americas, where the more virulent *N. ceranae* has possibly displaced *N. apis* (Klee et al., 2007). Further work is needed to determine the temporal pattern of *N. ceranae* range expansion into beekeeping operations that were previously thought to contain only *N. apis*.

The presence of only a single *N. ceranae* 16 S rRNA haplotype in North America suggests that parasites here originated from a single source, likely Europe. Different haplotypes of *N. bombi* may vary in virulence in bumblebees (Tay et al., 2005). Thus, virulence of these different *N. ceranae* haplotypes in European honey bees should be examined, as a more virulent haplotype may infect honey bees in North America.

Although we did not detect *N. ceranae* in New Brunswick, it is likely present (but perhaps at a low prevalence) because it was found in neighbouring Nova Scotia and Prince Edward Island (the latter is only ~13 km offshore). Moreover, New Brunswick is the only Maritime Province with an effectively open border to inter-province honey bee importation, and it has a history of being the first in Canada to receive exotic bee parasites, such as the mite *Varroa destructor* in 1989 (Clay, 1996). New Brunswick also borders Maine, which accepts thousands of honey bee colonies each spring from all parts of the USA for low-bush blueberry (*Vaccinium angustifolium*) pollination. This could potentially expose Canadian colonies to infected bees on the American side of the border, and vice versa. Intensive surveys are needed to determine the distribution and prevalence of *N. ceranae* in North America.

Recent survey and experimental work suggests that *N. ceranae* is a serious threat to the global beekeeping industry (Higes et al., 2006, 2007). Little is known about the pathogenicity of this parasite to European honey bees, and it is possible that this parasite is one of many factors contributing to high bee mortalities recently being reported (Oldroyd, 2007). Currently, Fumagilin-B® (Medivet Pharmaceuticals Ltd.) is the only registered product in Canada available to beekeepers for the control of *N. apis*; it was ineffective against a closely related species, *Nosema bombi*, in the bumble bee *Bombus occidentalis* (Whittington and Winston, 2003). Studies are needed to determine the efficacy of Fumagilin-B® against *N. ceranae*.

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