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NOTES AND COMMENTS

Nosema ceranae has parasitized Africanized honey bees in Mexico since at least 2004

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Nosema apis, a spore-forming fungus of the Phylum Microsporidia was formerly thought to be the only *Nosema* species to infect the midgut epithelial cells of adult honey bees (*Apis mellifera* L.), and to cause Nosemosis in these insects (Matheson, 1993). It was, however, recently discovered that another microsporidian parasite, *N. ceranae*, also infects western honey bees in different continents (Fries *et al.*, 2006; Higes *et al.*, 2006; Huang *et al.*, 2007; Klee *et al.*, 2007). Surveys have been conducted in many countries to find out where and how widespread this parasite is. *N. ceranae* has been linked to the collapse of thousands of colonies in parts of Europe (Higes *et al.*, 2006; 2008; 2010; Martin-Hernandez *et al.*, 2007), although still no conclusive evidence exists to affirm that this parasite is an important factor in the recent extensive honey bee colony losses that have occurred in North America and several European countries (Forsgren and Fries, 2010; Guzman-Novoa *et al.*, 2010; Paxton, 2010).

Because at least two *Nosema* species are now known to infect western honey bees, surveys require correct identification of both microsporidia to determine their geographic distribution. Nosemosis has been known to affect honey bees in Mexico since the 1960s, and *Nosema* spp. spores were visually detected and first reported from samples collected in the Yucatan peninsula in 1980 (Guzman-Novoa, 1981). Here we present the first report of the presence of *N. ceranae* infecting Africanized honey bees (descendents of *A. m. scutellata*) in Mexico, using a newly developed PCR-assay.

Samples of honey bees that were collected in 2004 to study the spread of what was then thought to be exclusively *N. apis* in central Mexico were used in this study. The samples were obtained from hives located in the states of Morelos, Hidalgo, Mexico and Distrito Federal. In each hive, samples were collected by moving an open jar down the face of the comb and allowing worker bees to fall directly into 70% ethanol. The samples were stored at -20ºC until analyzed. A total of 99 samples belonging to an equal number of colonies located in the same number of apiaries were initially analyzed by microscopy. Each sample of 35 adult bee abdomens was macerated in 30 ml of double distilled water (dd H_2O) and examined for presence of *Nosema* spp. spores under a light microscope (Olympus BX41; Olympus; Markham, Ontario, Canada) at 400x (Shimanuki and Knox, 2000). Out of 99 samples analyzed under the microscope, 68 were *Nosema*-positive and 50% of them were randomly chosen for further molecular diagnosis. All items that were used for macerating bee abdomens or extracting DNA were thoroughly washed and then autoclaved prior to these procedures.

DNA extractions and PCR procedures performed on the 34 selected samples were as per Hamiduzzaman *et al.* (2010). Briefly, each sample of 10 adult bee abdomens was homogenized in extraction buffer (prepared as per Hunt, 1997) and Proteinase K solution. The homogenate was then filtered and incubated at 60ºC for 3 h and DNA was extracted with a two-step phenol-chloroform procedure (as per Hamiduzzaman *et al.*, 2010). The sample was centrifuged and washed with 95% ethanol and the pellet dried. The extracted DNA was stored at -20ºC until PCR amplification. Three sets of specific primers were used in a triplex PCR involving co-amplifying the *N. apis* and *N. ceranae* 16S rRNA gene with the ribosomal protein *RpS5* gene from the honey bee as control in the same reaction. PCR reactions were done with a Mastercycler (Eppendorf; Mississauga, Ontario, Canada). Each reaction contained 1.5 µl 10x PCR buffer (New England BioLabs; Pickering, Ontario, Canada), 0.5 µl 10 mM dNTPs (Bio Basic Inc.; Markham, Ontario, Canada), 1 µl 10 µM for each primer (Laboratory Services, University of Guelph, Canada), 0.2 µl 5U/µl *Taq* polymerase (New England BioLabs; Pickering, Ontario, Canada), 2 µl DNA sample (10 ng total in 2 µl), and 8.8 µl dd H_2O. The thermocycler was programmed to run at 94ºC for 2.5 min, followed by 10 cycles of 15 s at 94ºC, 30 s at 61.8ºC and 45 s at 72ºC, and 20 cycles of 15 s at 94ºC, 30 s at 61.8ºC and 50 s at 72ºC, and a final extension step at 72ºC for 7 min,

Table 1. Number of honey bee samples infected (+) with *N. apis* and *N. ceranae* from four different states of Central Mexico.

State	**N**	***N. apis* +**	***N. ceranae* +**
Mexico	19	2	17
Distrito Federal	4	0	4
Morelos	6	0	6
Hidalgo	5	0	5
Total	34	2	32

holding reactions at 4ºC for the rest of the time. DNA obtained from newly emerged, *Nosema*-negative honey bees was used as negative control in the experiments.

Primers for the *Nosema* 16S rRNA gene were MITOC-F (5' CGGCGACGATGTGATATGAAAATATTAA) and MITOC-R (5' CCCGGTCATTCTCAAACAAAAAACCG) to yield a 218 bp PCR product specific for *N. ceranae* and APIS-F (5' GGGGGCATGTCTTTGACGTAC-TATGTA) and APIS-R (5' GGGGGGCGTTTAAAATGTGAAACAACTATG) to yield a 321 bp PCR product specific for *N. apis* (Martin-Hernandez *et al*., 2007). Additionally, a honey bee housekeeping gene, the ribosomal protein S5 (*RpS5*) was used as a reference. Primer pairs RpS5-F (5' AATTATTTGGTCGCTGGAATTG) and RpS5-R (5' TAACGTCCAGCA-GAATGTGGTA) with 115 bp length product were used in the reactions (Thompson *et al*., 2007). PCR products were separated by electrophoresis in 1.1% agarose gels, stained with ethidium bromide and photo-graphed under UV light.

Based on the PCR products obtained, 32 of the samples analyzed were *N. ceranae* and only two were *N. apis* (Table 1). *Nosema ceranae* was detected in bees from each of the four states sampled in 2004, and 94% of the samples were positive for the "new" species *N. ceranae*, showing that this parasite has in fact been long established and wide-spread in Mexico's high plateau. Moreover, the fact that *Nosema* spores had been detected in over 68% of the samples shows that these microsporidia are common in Mexico. It may be that *N. ceranae* has been infecting bees in Mexico (and in many other countries) for a long time, but went undetected because the molecular markers to identify this microsporidian parasite are very recent. *N. ceranae* has been present in the Americas (samples from the USA) since at least 1995 (Chen *et al*., 2008) and in Europe since at least 1998 (Paxton *et al*., 2007). It could be that the parasite now identified as *N. ceranae* is in fact the historical cause of Nosemosis in Mexico and in many other countries across the world.

This is also the report of the oldest Africanized honey bee samples infected with *N. ceranae*. Calderón *et al*. (2008) found that Africanized bees collected in 2006 in Costa Rica were parasitized with *N. ceranae*, and Klee *et al*. (2007) found this parasite infecting honey bees collected in Brazil in 2006. The length of time that *N. ceranae* has been present in Mexico, whether or not infections of this parasite are associated with colony collapse, or whether Africanized bees are more resistant to this fungus than European bees, remains to be investigated.

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