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NOTES AND COMMENTS

Presence of *Nosema ceranae* in Africanized honey bee colonies in Costa Rica.

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Nosemosis is a severe disease caused by the microsporidian *Nosema* sp. affecting adult honey bees (*Apis mellifera*) (Bailey and Ball, 1991). *Nosema* spores infect the epithelial layer of the ventriculus and midgut of adult bees, causing digestive disorders and shortening the life span of bees, with a resulting decrease in bee population (Ritter, 2001). So far only two microsporidian parasites have been described in honey bees: *N. apis* and *N. ceranae*. *Nosema apis* was the first described microsporidian in honey bees (Zander, 1909). *Nosema* ceranae was first described in colonies of the Asian hive bee (*A. cerana*) (Fries *et al*., 1996), but recently it was found in *A. mellifera* colonies in both Taiwan and Europe (Higes *et al*., 2006). Very few data are available about the spread of *N. ceranae* in Africanized honey bees (AHB) (*A. mellifera scutellata*) (Klee *et al*., 2007). *N. apis* is known to be distributed in honey bees worldwide (Matheson, 1993).

Spores produced by the two *Nosema* species are quite similar and very difficult to distinguish using traditional light microscopic analysis. Molecular tools have been developed to improve accurate diagnosis of *Nosema* disease in the laboratory (Higes *et al*., 2006; Klee *et al*., 2007; Paxton *et al*., 2007). Here we used PCR techniques in order to characterize infections or co-infections by these two pathogens in AHB colonies in Costa Rica. Prior to PCR analysis, spores were visually detected in adult bee samples using light microscopy.

In our study, conducted between August and December 2006, 262 AHB colonies from different areas of Costa Rica were randomly sampled for *Nosema* spp. Most samples were taken from colonies not suffering from symptoms of the disease. Interestingly, reduced honey production had been reported by beekeepers in some of the sampled areas over the previous two years. Adult bees were sampled from frames. The abdomens of thirty adult bees from each colony were macerated in 30 ml of distilled water and examined for the presence of *Nosema* spp. *Nosema* spores were identified and counted under the cover slip using a light microscopy (400x). Once the presence of microsporidian spores was confirmed, 22 samples from across Costa Rica were selected from the 73 positive ones for molecular diagnosis and were stored in 95 % ethanol and kept under refrigeration. All bee samples belonged to AHB colonies naturally parasitized by *Nosema*, showing diverse levels of infection.

Nosema DNA extractions were performed as reported by Klee *et al*. (2007). Five abdomens of adult bees from each sample were homogenized in 2 ml ddH_2O. The homogenates were filtered and washed 1-2 times with ddH_2O by centrifugation (5 min, 14000 rpm). After centrifugation the supernatants were discarded and the pellets frozen using liquid nitrogen and mashed with a sterile sealed pipette tip, in order to disrupt the *Nosema* spore cell walls. A DNeasy® Plant Mini Extraction Kit (Qiagen) was used to extract DNA following the manufacturer's recommendations. Extracted DNA was stored at -20°C until PCR amplification.

The primers used in this study were those reported by Chen *et al*. (2008). They aligned the complete sequences of the ribosomal RNA gene of both *N. apis* (GenBank Accession No: U97150) and *N. ceranae* (GenBank Accession No: DQ486027) to design a specific pair of primers for each *Nosema* species (Table 1). These primers discriminate between both *Nosema* species. Firstly, they produce species-specific PCR amplification and secondly, their PCR products differ in length, 401 and 250 bp for *N. apis* and *N. ceranae*, respectively.

Amplification by PCR was performed using HotStarTaq® (Qiagen). 10x PCR-buffer, 5x Q-solution, dNTPs (final concentration of 20 µM), Taq (0.625 U), forward and reverse primers (final concentration of 0.5 µM each) and 5 µl of DNA template were mixed in a total PCR volume of 25 µl. Amplification took place in a MJ Research DYAD thermal cycler using the following parameters: initial DNA denaturation of 15 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C, and ended with a final extension step of 72°C for 4 min. PCR products (5 µl) were resolved on 1.2 % agarose gel (1× TBE) stained with ethidium bromide by 30 min and pictured under UV light. A 100 bp ladder was used as a size marker.

Based on our molecular data, the microsporidia isolated from Costa Rican samples of AHB are indeed *N. ceranae* and it has been present in Costa Rica since at least 2006. The origin of the bee samples showed that the microsporidian parasite is distributed throughout the country. Using the genetic technique, all the samples were found to be infected with *N. ceranae*. Almost 28.0 % of the samples were infected with *N. apis* and *N. ceranae* together (Fig. 1). Until now it has been considered that AHB colonies in Costa Rica were infected by one microsporidian, *N. apis* (Matheson, 1993). Some earlier observations of microsporidian infections in AHB may in fact have been observations of *N. ceranae*. How the parasite *N. ceranae* was introduced into Costa Rica is unknown, but it is most likely through the transport of honey bee queens, which beekeepers import from different countries. The pathological consequences of *N. ceranae* in AHB colonies are not known, but because of recent reports of colony losses of honey bees in Europe connected to microsporidian infections (Higes *et al*., 2006), where *N. ceranae* is reported to be highly virulent (Paxton *et al*., 2007), the virulence of *N. ceranae* in African races of *A. mellifera* needs to be investigated.

Table 1. Specific primers for *N. apis* and *N. ceranae* detection.

Primer/ specificity	**Sequence 5' ⟶ 3'**
N. apis forward	CCA TTG CCG GAT AAG AGA GT
N. apis reverse	CAC GCA TTG CTG CAT CAT TGA C
N. ceranae forward	CGG ATA AAA GAG TCC GTT ACC
N. ceranae reverse	TGA GCA GGG TTC TAG GGA T

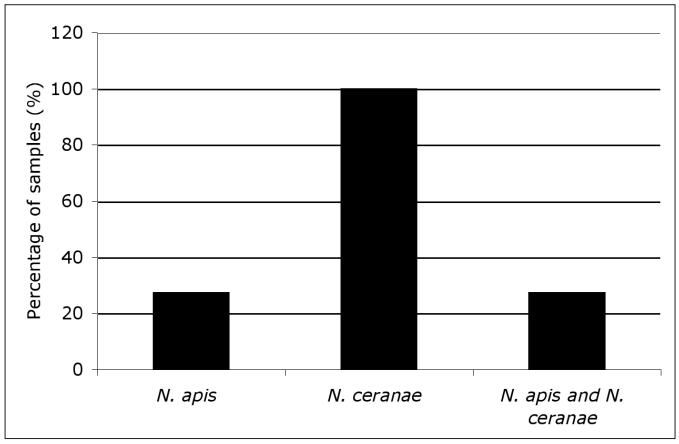

Fig. 1. Percentage of adult bee samples containing only *N. apis*, only *N. ceranae* or *N. apis* and *N. ceranae* together.

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