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Comparative study of *Nosema ceranae* (Microsporidia) isolates from two different geographic origins

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ABSTRACT

The intestinal honey bee parasite *Nosema ceranae* (Microsporidia) is at the root of colony losses in some regions while in others its presence causes no direct mortality. This is the case for Spain and France, respectively. It is hypothesized that differences in honey bee responses to *N. ceranae* infection could be due to the degree of virulence of *N. ceranae* strains from different geographic origins. To test this hypothesis, we first performed a study to compare the genetic variability of an rDNA fragment that could reveal differences between two *N. ceranae* isolates, one from Spain and one from France. Then we compared the infection capacity of both isolates in *Apis mellifera iberiensis*, based on the anatomopathological lesions due to *N. ceranae* development in the honey bee midgut, *N. ceranae* spore-load in the midgut and the honey bee survival rate. Our results suggest that there is no specific genetic background of the two *N. ceranae* isolates, from Spain or France, used in this study. These results agree with the infection development, honey bee survival and spore-loads that were similar between honey bees infected with both *N. ceranae* isolates. Probably, differences in honey bee response to infection are more related to the degree of tolerance of honey bee subspecies or local hybrids to *N. ceranae*, or experimental conditions in the case of laboratory trials, than to differences between *N. ceranae* isolates. Further studies should be done to estimate the contribution of each of these factors on the response of the honey bees to infection.

1. Introduction

Nosema spp. are obligate unicellular parasites that belong to the phylum Microspora. They are characterized by the production of a resistant spore that contains a polar filament which serves to transmit the genetic material to the host cell (Wittner and Weiss, 1999). Microsporidiosis of adult honey bees caused by *Nosema apis* and *Nosema ceranae* is a common worldwide disease with both, a direct negative impact on colony strength and productivity (Fries, 1988; Higes et al., 2010a; Mayack and Naug, 2009) and an indirect effect by interacting with other environmental stressors weakening colony health (Alaux et al., 2010b; Pettis et al., 2012; vanEngelsdorp et al., 2009). While it has been known for a century that *N. apis* infects the European honey bee, *N. ceranae* was first isolated in 1996 in the Asian honey bee *Apis ceranae* (Fries et al., 1996) and recently detected in the European honey bee *Apis mellifera* in 2005 (Higes et al., 2006; Huang et al., 2007). Among the factors related to *Nosema* pathology, the existence of different *N. ceranae* isolates from distant geographic areas (Chen et al., 2009) that exhibit different degrees of virulence may explain the differences in the response of the honey bee to infection (Genersch, 2010). Contradictory results on *N.*

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ceranae virulence have been obtained in a large number of laboratory experiments studying *N. ceranae* effects (Forsgren and Fries, 2010; Higes et al., 2007; Martín-Hernández et al., 2009; Suwannapong et al., 2010) as well as in field surveys of commercial apiaries (vanEngelsdorp et al., 2009; Higes et al., 2010b).

More precisely, there are apparent differences in honey bee mortality due to *Nosema* spp. between Spain and France. A large study carried out between 2002 and 2006 showed the presence of *Nosema* spp. in France in 50–79% of the surveyed apiaries from different regions (Chauzat et al., 2010a,b). *N. ceranae* was found to be predominant over *N. apis* (Chauzat et al., 2007). In all surveyed years, the presence of the parasite was not significantly related to any acute mortality (Chauzat et al., 2010a,b), with the exception of apiaries from a region in France not considered in the previous study where *N. ceranae* appeared to have a central role in massive losses during 2005–2006 (Borneck et al., 2010). In contrast, a study of the epidemiological factors involved in colony losses in different regions of Spain suggested that *N. ceranae* is a key factor in colony depopulation (Higes et al., 2010b). *N. ceranae* was much more common than *N. apis* and it was present in 95% of the samples from depopulated colonies, compared to 5% of asymptomatic beehives (Higes et al., 2010b). Laboratory experiments also showed a similar tendency. In France, experimental infections resulted in 10% and 50% bee mortality, 10 and 20 days post-infection respectively (Alaux et al., 2010b; Vidau et al., 2011); whereas in Spain, higher mortality has been observed over shorter study periods, the most dramatic being 100% of bee mortality 8 days post-infection (Higes et al., 2007).

In consequence, we tested the hypothesis that different degrees of virulence of *N. ceranae* isolates from each country could explain this differential effect of *N. ceranae*. We compared the effects on the honey bee of two *N. ceranae* isolates, originating from the National Institute of Agricultural Research (INRA) of Avignon, South of France, and the Regional Apicultural Centre (CAR) in Central Spain. In order to avoid the confounding effects of methodology we used one honey bee subspecies (*Apis mellifera iberiensis*) and a single laboratory protocol. We carried out a genetic study of a variable fragment of rDNA, looking for similar sequences or characteristic fragments of both *N. ceranae* isolates that could define strains from two different geographic origins; at the same time, we performed cage experiments to compare the development of infection of both *N. ceranae* isolates in the honey bee through the observation of histopathological lesions in the midgut and the measurement of daily mortality and spore counts.

2. Materials and methods

In October 2010, naturally-infected forager honey bees from two colonies were collected from Central Spain (Regional Apicultural Center, CAR, at Marchamalo), and the South of France (National Institute of Agronomic Research, INRA, from Avignon), to obtain fresh *N. ceranae* spores to be used in experimental infections. *N. ceranae* from Spain are known to cause rapid mortality of honey bees (Higes et al., 2008, 2010b), while *N. ceranae* from France have not been related to mass colony depopulation (Chauzat et al., 2010a,b).

Both sets of bee samples were collected from the entrance of the hive on the same day at the two different geographic locations and maintained at room temperature, whether in Spain, in France or during the transport of the French samples to the CAR laboratory in Spain where the infections were carried out.

2.1. Obtaining *N. ceranae* spores for experimental infection

Bee samples from both geographic locations obtained as described above, were processed in parallel, in exactly the same way, to obtain fresh mature spores as described in Botías et al. (2011). Briefly, the abdomens of all bees were homogenized in 25 ml H_2O PCR grade for 2 min at high speed in a Stomacher® 80 Biomaster (Seward, West Sussex, UK) using strainer bags (BA6040/STR, Seward). The homogenate was recovered in a tube and 15 ml of H_2O PCR grade was again added to the strainer bag to repeat the homogenization under the same conditions. Honey bee homogenates were centrifuged (6 min at 800 *g*), the supernatant was discarded as the spores remained in the sediment. The pellet was resuspended in 1 ml of distilled water. To confirm the *Nosema* species of the spores, an aliquot of each homogenate was analyzed by PCR as previously described (Martín-Hernández et al., 2011b) using 218MITOC FOR/218MITOC REV and 321APIS FOR/321APIS REV primers specific for *N. ceranae* or *N. apis* respectively and COI-F/COI-R primers for *A. mellifera* COI, as internal control of each reaction. All the PCRs reactions were carried out in a Mastercycler® ep gradient S (Eppendorf®, Hamburg, Germany). Each PCR product was analyzed in a QIAxcel System (Qiagen, Hilden, Germany), using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002) to detect positive and negative reactions. Negative controls were analyzed in parallel to detect possible contaminations in all phases of this technique. Once the *N. ceranae* species were confirmed, the spores were purified with Percoll® to obtain fresh pure spores suspension for artificial infection. During extraction and before infection, spores were kept at room temperature. The spore number was counted using a hemocytometer chamber and a phase contrast microscope.

2.2. Genetic variability of an rDNA fragment from two *N. ceranae* isolates

Up until now no reliable genetic markers have been found to be suitable to distinguish between strains of *N. ceranae*. In fact, two genetic markers can produce different phylogenetic trees of the same sample, leading to incongruous results (Ironside, 2007). Because of this, we performed a study on the variability of an rDNA fragment between samples as in Sagastume et al. (2011), through the amplification by PCR and cloning of the product, to look for a decrease in variability that consequently occurs because of geographic isolation.

Total DNA was previously extracted from honey bee samples naturally infected by *N. ceranae* from Central Spain and South of France (see above). PCR was performed