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COMMUNICATION

Identification of *Nosema ceranae* in the Valparaíso District, Chile[#]

Identificación de *Nosema ceranae* en la Región de Valparaíso, Chile

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RESUMEN

El presente estudio demostró por primera vez la presencia de *Nosema ceranae* en la V Región de Valparaíso. Las muestras analizadas fueron obtenidas en la Región de Valparaíso, pertenecientes a colmenas ubicadas en las localidades de San Antonio, Limache y La Ligua. Las muestras obtenidas de grupos de 60 abejas fueron maceradas y analizadas por microscopia de contraste de fases amplificada a 40x y posteriormente confirmadas por la técnica molecular PCR múltiple. Los resultados obtenidos indicaron que las muestras provenientes de apiarios chilenos fueron positivas a *N. ceranae,* mostrando ausencia de *Nosema apis.* En el período de estudio (2011-2012) se produjo la muerte de 2.915 colmenas en la Región de Valparaíso que cuenta con 6.167 colmenas, cifra que corresponde al 47,3% del total. La muerte de colmenas observada en este periodo en la V Región de Chile podría deberse a la presencia de *N. ceranae,* pudiendo estar asociada a la falta de capacitación en el manejo y control sanitario de las colmenas.

Palabras clave: *Nosema ceranae, Apis mellifera, Nosema apis,* Nosemosis.

SUMMARY

This study aimed to demonstrate for the first time the presence of *Nosema ceranae* in the Valparaíso District. The analised bee samples were obtained in the Valparaíso Region, between 2011 and 2012, from beehives located in San Antonio, Limache and La Ligua. Samples obtained from groups of 60 bees were macerated and analysed by phase contrast microscopy at 40x. Then, DNA was extracted and the gene that encodes for 16SrRNA of *Nosema apis* and *N. ceranae* was amplified by multiplex PCR. 100% of the samples were infected by *N. ceranae*, while *N. apis* was not detected. During the period of study 2,915 colonies died in the Valparaíso Region which is 47.3% of the total hives of the Region. The loss of beehives during this period in the V Region of Chile could be related to either the lack of training in management and health monitoring of the colonies or the presence of *N. ceranae.*

Key words: *Nosema ceranae, Apis mellifera, Nosema apis,* Nosemosis.

INTRODUCTION

Fungal diseases affecting the honey bee *Apis mellifera* are caused by parasitic fungi such as *Ascosphaera apis* which is the etiologic agent of the honey bee larval disease known as chalkbrood, *Aspergillus flavus* which causes mummification of the brood of a honey bee, while *Nosema apis* and *Nosema ceranae* are intracellular microsporidian parasites infecting the midgut epithelial cells of adult honey bees. *N. apis* was the only Microsporidia that had been found in *A. mellifera* until *N. ceranae* was identified in this host in 2005, in Europe (Higes *et al* 2006) and Taiwan (Huang *et al* 2007). Among fungal diseases, *N. ceranae* has a greater relevance, both national and internationally, because it is an emerging pathogen distributed in all countries where beekeeping exists; also it is highly contagious and easily spread, causing economic damages of great importance (Higes *et al* 2010).

From an economic point of view, nosemosis has proven to be a growing issue for beekeepers worldwide (Botias *et al* 2009). Among its harmful effects on production are the loss of adult bees (especially at the end of winter and early spring) which decreases production of honey by 25%, and since the consumption of honey increases during the winter up to 50% and there is no production of royal jelly, high quality queens cannot be produced, neither healthy larvae (Peldoza 2002).

Fries *et al* (1996) reported that the disease affecting European honey bees (*A. mellifera*) was called nosemosis and it was caused by the microsporidian *N. apis*, whereas

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in East Asia *N. ceranae* was considered restrictive of *Apis cerana*. However, later studies confirmed that *N. ceranae* can also infect *A. mellifera* (Fries *et al* 2006, Higes *et al* 2006, Huang *et al* 2007, Klee *et al* 2007) and could be responsible as well for the increased number of incidents related to the disease observed in some European countries (Martín-Hernández *et al* 2007, Fries *et al* 2006, Paxton *et al* 2007)

Later studies also showed that infections caused by *N. ceranae* can promote other bee diseases, such as RNA virus (Higes *et al* 2008) and chalkbrood (Hedtke *et al* 2011).

N. ceranae has been progressively identified in South American countries, including Brazil, Uruguay and Argentina (Invernizzi *et al* 2009, Klee *et al* 2007, Medici *et al* 2011). In Chile, *N. apis* was first detected in 1978, through the analyses carried out in the laboratories of the Agriculture and Livestock Service (SAG). Hinojosa and González (2004) after researching six communities from the O'Higgins District, found *N. apis* with a prevalence of 60.0 ± 6.9% in the spring of 1999, 78.3 ± 5.8% in the spring of 2000, 14.3 ± 5% in the summer of 2000, and 23.0 ± 6% in the summer of 2001. These results indicate that in this area the highest incidence occurs in the spring, due to high fecal contamination existing within the colony in late winter.

To diagnose the presence of the etiologic agent *N. ceranae* in Chile, in 2009 the SAG sent samples to the Regional Apicultural Research Center of Marchamalo, Spain, to confirm the existence of this pathogen. Moreover, Martínez *et al* (2012) identified the presence of *N. ceranae* in Chile only in the Bío-Bío District, which covers 42.0% of the country's honey production. The study evaluated four provinces of the Valparaíso District, obtaining information from a total of 26 communities and 49.0% of them showed the presence of *N. ceranae*. Rodriguez *et al* (2012) reported the presence and coexistence of viruses and *N. ceranae* in the same apiaries from the Maule District.

The aim of this study was to demonstrate for the first time the presence of *N. ceranae* in the Valparaíso District. This objective was based on the report of INDAP-Agroapilculture 2011-2012 Ltda., which reported the death of 2,915 out of 6,167 colonies in the Valparaíso District, 47.3% of the total colonies in the District.

MATERIAL AND METHODS

SAMPLES

The analysed samples were taken from colonies of the Valparaíso District that presented low population with a large numbers of dead bees, and a reduced number of worker bees inside of them, acording to information provided by INDAP-Agro Apiculture Ltda. Live adult bees were collected from 12 apiaries (pooled samples were 60 bees from affected colonies), refrigerated, transported to the laboratory and stored at 60 ºC until analysis. These sample apiaries came from San Antonio (32°42'00"S, 71°23'00"W), Limache (33°01'00"S, 71°16'00"W) and La Ligua (32°27'09"S, 71°13'52"W), collection date is shown in table 2. Samples were processed by macerating the abdomens of 30 bees in 5 mL of distilled water. The suspension obtained was filtered to remove traces of the exoskeleton of the bee and centrifuged at 1200 rpm for 10 min, afterwards the obtained pellet was resuspended in 1 mL of distilled water and analysed by optical microscopy amplified at 40x.

SPORE DETECTION AND EXTRACTION OF DNA

To determine the presence or absence of spores belonging to *Nosema spp,* the pellet was analysed by phase contrast microscopy, amplified at 40x in an optical microscope Nikon Eclipse 80i DIC (Differential Interference Contrast) to verify the presence of spores (OIE, 2008); the spores were then counted in a Neubauer chamber (Cantwell 1970). An aliquot of the pellet (400 µL) was shaken together with 0.1 g of glass beads (2 mm diameter) 30 times per s for 4 minutes on TissueLyser (Qiagen) shaker to break the wall of the possible spores present. Subsequently, the extraction of the DNA was done (in triplicate), previously incubating 150 µL of each of the samples processed at 56 °C with 20 µL of proteinase K and 30 µL of ATL buffer (Qiagen) overnight. After incubation the DNA extraction was conducted using BioSprint DNA Blood Kit ™ 96 (384) (Qiagen, Cat No. 940057) in a Biosprint 96 (Qiagen) robot using the program BS96_DNA_Tissue.

MULTIPLEX PCR

After obtaining the extracts, these were analysed for the presence of *N. ceranae* and *N. apis* using a multiplex polymerase chain reaction (PCR) that allows to detect both *Nosema* species in a single reaction (Martín-Hernández *et al* 2007) and also amplifies a fragment of subunit I of the gene cytochrome c oxidase I (COI) of *Apis mellifera*, which serves as an internal control of the reaction (Martín-Hernández e*t al* 2012).

The PCR reactions were held in a thermocycler Mastercycler ep gradient S (Eppendorf ®) in a total reaction volume of 25 µL containing 2.5 µL of DNA extract, 2.5 µL of H_2O of PCR and 20 µL of the PCR mix, which contains the polymerase FastStart PCR Master 12.5 µL (Roche 04710452001), 0.2 mg/mL of bovine serum albumin, 0.1% Triton X-100, 0.4 µM of 218MITOC and 321APIS and 0.03 µM of the primers COI (see table 1). Along with the samples, the negative controls (distilled H_2O) were processed in parallel to detect possible contamination in the phase of the PCR and the positive control corresponding to patterns of *Nosema apis* and *Nosema ceranae* (Martín-Hernández *et al* 2007).

Table 1. Primers selected to detect the presence of *N. ceranae*, *N. apis* and *Apis mellifera*.
Partidores seleccionados para detectar la presencia de *N. ceranae*, *N. apis* y *Apis mellifera*.

First	Sequence[a]	Product size of PCR (pb)	Especificity
218MITOC-FOR	5´-CGGCGACGATGTGATATGAAAATATTAA-3´	218-219[b]	*N. ceranae*
218MITOC-REV	5´-CCCGGTCATTCTCAAACAAAAAACCG-3´		
321APIS-FOR	5´-GGGGGCATGTCTTTGACGTACTATGTA-3´	321	*N. apis*
321APIS-REV	5-GGGGGGCGTTTAAAATGTGAAACAACTATG-3´		
COI-FOR	5´-GGGTCCAAGACCAGGAACTGGAT-3´	118	*Apis mellifera*
COI-REV	5´-GCGCGGAAATTCCTGATATATGAAGAGAAAA-3´		

[a]CG queues added to the primers are underlined; [b]There is 1 bp (base pair) of difference in the size of the applications obtained from *N. ceranae* depending from different sequences on this species available in GenBank (www.ncbi.nlm.nih.gov).

Table 2. Analysis of the presence of *N. apis* and *N. ceranae* in bees from Chile. + Positive; - Negative; according to Martín-Hernández (2007).
Análisis de la presencia de *N. apis* y *N. ceranae* en abejas de Chile. + Positivo; - Negativo; de acuerdo con Martín-Hernández (2007).

Sample	Location	Date	*N. apis*	*N.ceranae*
1	San Antonio	Summer 2012	–	+
2	San Antonio	Summer 2012	–	+
3	San Antonio	Summer 2012	–	+
4	Limache	Fall 2012	–	+
5	Limache	Fall 2012	–	+
6	Limache	Fall 2012	–	+
7	La Ligua	Fall 2012	–	+
8	La Ligua	Fall 2012	–	+
9	La Ligua	Fall 2012	–	+
10	San Antonio	Fall 2012	–	+
11	San Antonio	Fall 2012	–	+
12	San Antonio	Fall 2012	–	+

RESULTS AND DISCUSSION

Microscopic analysis of the samples coming from colonies that presented low populations and death in the Valparaíso District in three areas (San Antonio, Limache and the Ligua), showed the presence of *Nosema ceranae* spores by phase contrast microscopy (figure 1a) and confirmed by PCR showing the presence of *N. ceranae.* Conversely, *N. apis* (figure 1b) was not detected in the samples under study (table 2).

Therefore, this research is the first report of the presence of *Nosema ceranae* in the Valparaíso District of Chile.

Nosemosis does not present obvious symptoms, which makes it often difficult to detect (Huang *et al* 2007). However, the affected colonies exhibit a significantly lower production of honey and a reduction in their pollination capability (Anderson and Giacon 1992). Consequently, a field diagnosis must be confirmed by a laboratory analysis done by an optical microscopy visualization of *Nosema spp.* from macerated preparations of bee intestines or feces collected from the colony (Peldoza 2002). The spores of *N. apis* and *N. ceranae* are very similar and can hardly be distinguished by light microscopy, so molecular diagnostic techniques such as PCR are very effective tools for differential diagnosis of both pathogens. Additionally, it allows the detection in low levels of infestation (Bourgeois *et al* 2010). This is why in this study, the samples were analysed by molecular technique, to report for the first time in the Valparaíso District the presence of *N. ceranae.* This indicates a significant change in the condition of colonies, in contrast with the results presented by Neira (2006), who did not detect samples with the presence of nosemosis.

During the study it was observed a decline in the average of honey production per colony from 14.23 kg to 8.2 kg, falling by 42.4% (report data INDAP- Agro Apiculture 2011-2012) which might have been the cause by *N. ceranae*. This situation may occur for the same reasons reported in Europe during previous years, caused by the lack of training in management and health monitoring of the colonies. An epidemiological study conducted in Spain in the spring

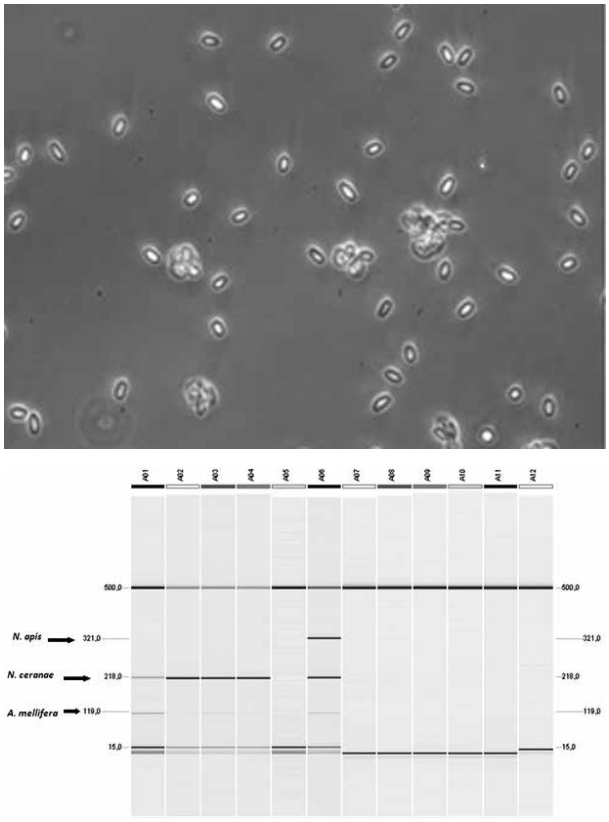

Figure 1. a) *Nosema ceranae* spores in a sample from Chilean apiaries observed in an optical microscope Nikon Eclipse 80i DIC (Differential Interference Contrast). b) Electrophoresis of the PCR product in nucleic acid analyzer QIAxcel (QIAGEN). The samples A1-A4 from Chilean apiaries of the Valparaíso Region (San Antonio A1-A2; A3 Limache and A4 La Ligua), A5 negative control and A6 positive control corresponding to patterns of *Nosema apis* (321pb) and *Nosema ceranae* (218pb) and internal control COI *Apis mellifera* (119pb).

a) Esporas de *Nosema ceranae* en una muestra de apiarios chilenos observado mediante microscopio óptico Nikon Eclipse 80i DIC (Differential Interference Contrast). b) Electroforesis de producto de PCR en analizador de ácido nucleico QIAxcel (QIAGEN). Las muestras A1-A4 de apiarios chilenos de la Región de Valparaíso (San Antonio A1-A2; A3 Limache y A4 La Ligua), control negativo A5 y control positivo A6 correspondiente a patrones de *Nosema apis* (321pb) y *Nosema ceranae* (218pb) y control interno COI *Apis mellifera* (119pb).

of 2006 showed a prevalence of over 50% of nosemosis. These data indicate that *N. ceranae* currently represents a health risk of the first-rate order for Spanish apiaries due to its high prevalence and its high pathogenicity, which could cause the death of colonies, as it has been shown in experimental colonies naturally parasitised by *N. ceranae* (Higes *et al* 2006, Higes *et al* 2010).

The results of our research demonstrate the need to develop training and surveillance policies for beekeepers regarding colonies management and disease control, since appropriate monitoring would control the development of this pathogen.

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