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ORIGINAL PAPER

Nosema ceranae an emergent pathogen of *Apis mellifera* in Chile

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Abstract The microsporidian *Nosema apis and Nosema ceranae* have been associated with colony disorders of *Apis mellifera* and *Apis cerana*, respectively. *N. apis* is endemic in South America. Recently, *N. ceranae* has been detected in Brazil, Uruguay and Argentina. No report of its presence, distribution and prevalence in Chile is available. Here, we present a real-time PCR-based method that was able to discriminate between *N. apis* and *N. ceranae.* The dynamic range of this assay was 100 to 100,000 spores per honeybee. False-negative results were avoided due to the use of *ACTIN* gene as internal standard. False-positive results were obtained neither in experimentally nor in naturally contaminated samples. Using this method, we screened 240 beehives from the Chilean region where 42% of the total country honey production take places (Región del Biobío). *Nosema* spp. were detected in the four provinces and in 20 of the 26 communes of the region. Among the samples analysed, 49% were positive for *N. ceranae*. Their infection level ranged from 200 to more than 100,000 spores per honeybee. *N. apis* was not detected in this region. Hence, our data show that in Chile *N. ceranae* is an emergent pathogen that is been replacing *N. apis*. Also, they support that *N. ceranae* maybe the actual responsible for nosemosis in *A. mellifera* in South America.

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Introduction

Microsporidia genus *Nosema* (Nosematidae) causes honeybee nosemosis (Giersch et al. 2009; Klee et al. 2007). *Nosema* spp. are parasitic fungi which colonise the epithelial of the ventriculus and the midgut of adult workers (de Graaf et al. 1994; Fries 1988). This results in digestive diseases leading to undernourishment and shortening of the honeybee life span (Calderón et al. 2008; Ritter 2001). Affected colonies diminish their honey production and experience a significant decrease in their population (Chauzat et al. 2007; Higes et al. 2008; Oldroyd 2007). Thus, nosemosis causes considerable economical losses to apicultures (Anderson Anderson and Giacon 1992; Fries et al. 1996; Fries 2010; Hornitzky 2008).

Nosema apis infects *Apis mellifera*, the Western honeybees (Zander 1909). *Nosema ceranae* infects *Apis cerana*, the Asian honeybees (Fries et al. 1996). Recently, it has been shown that this exclusivity is no longer true. For instance, *A. mellifera* can be infected by *N. apis* and *N. ceranae* separately or as mixed infection (Higes et al. 2006; Huang et al. 2008). Currently, there are conclusive evidences showing neither higher virulence (Forsgren and Fries 2010; Higes et al. 2007; Paxton et al. 2007) nor the factors that determine it (Martín-Hernández et al. 2011a) in any of these species. However, it has been proposed that worldwide *N. ceranae* is displacing *N. apis* (Klee et al. 2007; Martín-Hernández et al. 2011b; Paxton et al. 2007; Stevanovic et al. 2010; Tapaszti et al. 2009). Thus, *N. ceranae* was been detected in Europe (Chauzat et al. 2007; Higes et al. 2006; Klee et al. 2007; Stevanovic et al. 2010; Whitaker et al. 2010), North Africa (Higes et al. 2009), Asia (Chaimanee et al. 2011; Yoshiyama and Kimura 2011), Oceania (Giersch et al. 2009), North America (Chen et al. 2008; Traver and Fell 2011; Williams et al. 2008a) and

Central America (Calderón et al. 2008; Guzman-Novoa et al. 2011). In Chile, nosemosis has been declared an endemic disease. Up to now, *N. apis* is recognised as its unique causal agent (Hinojosa and González 2004; Huaiquil et al. 2009). Since *N. ceranae* have been progressively identified in South American countries, e.g. Brazil, Uruguay and Argentina (Invernizzi et al. 2009; Klee et al. 2007; Medici et al. 2011), our aim was to determine the presence, distribution and prevalence of *Nosema* spp. in Chile. First, we look for *N. apis* and *N. ceranae* in honeybees clinically diagnosed with nosemosis. As we found *A. mellifera* infected with *N. ceranae*, we decided to perform a screening of *Nosema* spp. in Región del Biobío, which concentrates 42% of the national production of honey. For this, we settled a real-time PCR-based method that was able to discriminate between *N. apis* and *N. ceranae*. Using this method, we analysed 240 beehives sampled, in the spring of 2010 and the summer of 2011, in 26 communes of the four provinces of Region del Biobío, Chile.

Materials and methods

Extraction and quantification of *Nosema* spp. spores

A. mellifera individuals were collected and stored in 70% ethanol at 4°C. At least 1 week later, the abdomens of 10 worker honeybees were chirurgical removed and crushed in 3 ml of sterile water. Homogenate was filtered through two layers of gauze and centrifuged at 800×*g*, for 6 min. Two millilitres of the supernatant was removed and the pellet was resuspended with the remaining supernatant. Spores present in the sample were analysed under light microscope and counted using an hemocytometer (Cantwell 1970).

To generate spore stocks, honeybees clinically diagnosed with nosemosis were used as start material. Extracted spores were diluted up to 1×10^7 spores/ml and stored at 4°C.

Isolation of DNA from *Nosema* spp. spores

The isolation of the DNA of *Nosema* spp. spores was performed with the UltraClean Soil DNA isolation kit (Mo Bio Laboratories Inc, California, USA). Genomic DNA was eluted with 40 μl of elution buffer, quantified spectrophotometrically and stored at −20°C.

Characterization of *16S rRNA* gene of field samples

Fifty nanograms of DNA isolated from spores extracted from *A. mellifera* clinically diagnosed with nosemosis was amplified in a final volume of 50 μl containing 0.2 μM of primers targeted to *Nosema* spp. *16S rRNA* gene (INTER-F/INTER-R, Higes et al. 2007), 3 mM $MgCl_2$, 0.2 mM dNTP, 0.2 mg/ml BSA, PCR buffer (200 mM Tris–HCl, pH 8, 500 mM KCl) and 1 U Taq polymerase. Amplification protocol was: one cycle at 94°C for 5 min; 25 cycles at 94°C for 45 s, at 56°C for 45 s and at 72°C for 2 min; one cycle at 72°C for 7 min. After been electrophoresed, amplicons (649 bp for *N. apis* and 659 bp for *N. ceranae*) were purified using the extraction kit Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Restriction analysis of amplicons was performed with endonucleases TaqI or AluI. Sequencing of the amplicons was done in the automated DNA sequencer ABIPRISM 3100 (Applied Biosystems, California, USA). Similarity search was performed using the Basic Local Alignment Search Tool (Altschul et al. 1990).

Generation of DNA positive controls

Reference DNAs from *N. apis* and *N. ceranae* were amplify using primers APIS-F/APIS-R and MITOC-F/MITOC-R, respectively (Martín-Hernández et al. 2007). Amplicons (321 and 218 bp, respectively) were cloned using the pGEM-T cloning kit (Promega, Madison, WI, USA). Recombinant plasmids thus generated, named pJNA-2 and pNCH-1, respectively, were propagated in *Escherichia coli* cultured in Luria–Bertani Broth medium supplemented with 100 μg/ml ampicillin, under aerobic atmosphere at 37°C (Sambrook and Russel 2001), purified using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI, USA), and quantified spectrophotometrically. Restriction analysis of recombinant plasmids was performed with endonucleases EcoRI and SalI. Plasmid stocks were stored at −20°C.

Detection of *N. apis and N. ceranae* by real-time PCR

Real-time PCR was performed in a final volume of 10 μl containing 1 μl of DNA, 0.5 uM primers, 1.6 mM $MgCl_2$ and 1× SYBR Green Mastermix (Roche, Germany). To amplify the *16S rRNA* gene of *N. apis* and *N. ceranae*, the primers used were APIS-F/APIS-R and MITOC-F/MITOC-R, respectively (Martín-Hernández et al. 2007). To amplify *ACTIN* (endogenous gene), the primers used were ACT-F/ACT-R (Soares et al. 2007). The amplification conditions were adjusted in order to perform real-time PCR in a LightCycler instrument (Roche, Germany). The program was: one cycle at 95°C for 10 min; 40 cycles at 95°C for 10 s, at 60°C for 10 s and at 72°C for 9 s; one cycle of melting temperature analysis: 0.1°C/s up to 95°C. Amplicon melting temperature (T_m) was determined using the LightCycler software version 3.5 (Roche, Germany). Amplicon size was estimated by electrophoresis in 1.2% agarose gel stained with ethidium bromide (Sambrook and Russel 2001).

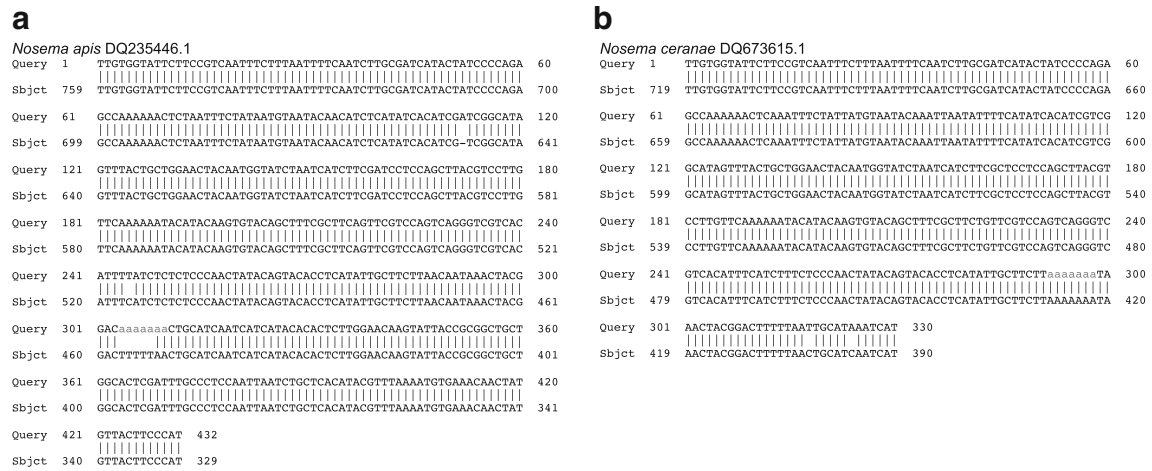


Fig. 1 Identification of *N. apis* and *N. ceranae* in Chilean *A. mellifera*. Amplicons obtained from DNA of spores extracted from honeybees clinically diagnosed with nosemosis using primers INTER-F/INTER-R were sequence and compared with the available sequence of *N. apis* gb. DQ235446.1 (**a**) and *N. ceranae* gb. DQ673615.1 (**b**). In both cases, identities were 99% (430/432 and 328/330, respectively)

Negative control corresponds to reaction settled without template DNA. Positive control corresponds to reaction settled with pJNA-2 for *N. apis* and pNCH-1 for *N. ceranae*.

Screening of *N. apis* and *N. ceranae* in field samples

A. mellifera individuals were collected from 240 beehives located in 26 communes of the four provinces of Región del Biobío, Chile, during the spring (October to November) of 2010 and the summer (January to April) of 2011. Each beekeeper that agreed to participate in the study randomly collected 30 honeybees from the three central frames of the brood chamber, irrespective of its clinical state. All samples were analysed using the real-time PCR-based method reported in this article.

Results

Identification of *N. ceranae* in Chilean honeybees

The amplicons of *16S rRNA* gene generated from spores extracted from *A. mellifera* clinically diagnosed with

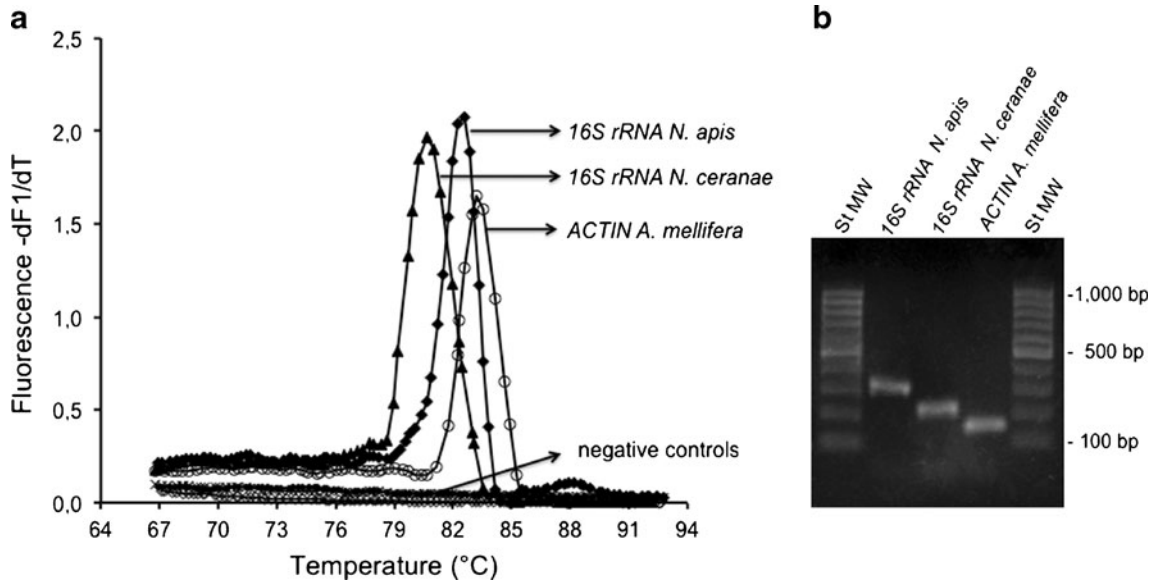


Fig. 2 Detection of *N. apis* and *N. ceranae* by real-time PCR. Amplicons obtained with *N. apis* DNA using primers APIS-F/APIS-R, *N. ceranae* DNA using primers MITOCF/MITOC-R and *A. mellifera* DNA using primers ACT-F/ACT-R were characterised according to their T_m (melting curves) (**a**) and size (agarose gel electrophoresis) (**b**). Theoretical T_m and size were 83.5°C/321 bp for 16S rRNA gene of *N. apis*; 80.4°C/218 bp for 16S rRNA gene of *N. ceranae* and 84.8°C/156 bp for ACTIN gene of *A. mellifera*

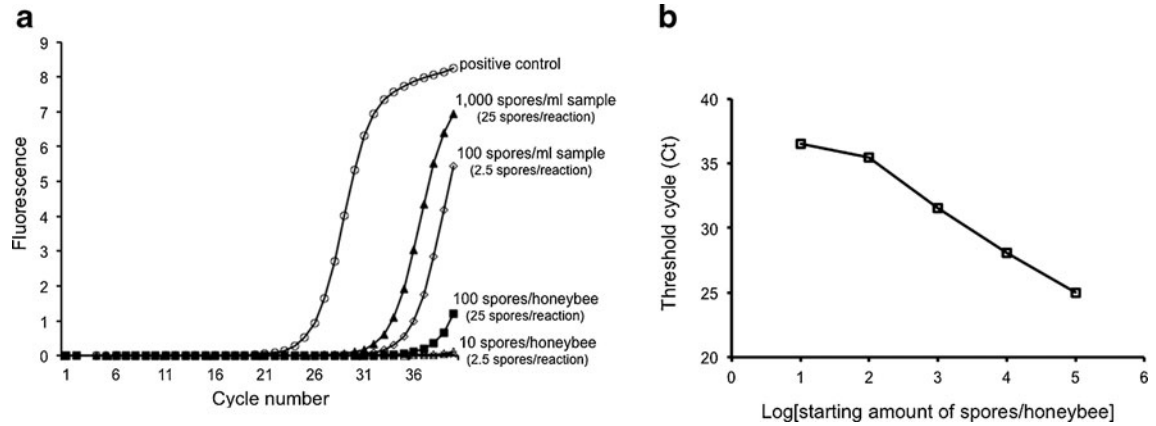


Fig. 3 Detection limit and dynamic range of real-time PCR settled for *N. ceranae*. Serial dilutions of extracted spores (*insert*) and experimentally contaminated honeybee were amplified using primers MITOC-F/MITOC-R (**a**). The relationship between spore number and cycle of amplification start was linear in the range 100 to 100,000 spores/honeybee (**b**)

nosemosis showed two different restriction patterns. Samples cut with TaqI resulted in fragments of 238, 209 and 201 bp, as expected for *N. apis*. Samples cut with AluI resulted in fragments of 379, 216, 38 and 25 bp, as expected for *N. ceranae*. Consistently, when amplicons were sequenced and compared with available sequence for *N. apis* (gb. DQ235446.1) and *N. ceranae* (gb. DQ673615.1), identities were 99% in both cases (Fig. 1). The detection of *N. apis* was expected. However, for the first time, we observed that *N. ceranae* is present in Chilean samples of *A. mellifera* with nosemosis.

Reliable and sensible detection of *N. apis and N. ceranae* by real-time PCR

Under optimum reaction and cycling conditions, the amplification of *N. apis* DNA (pJNA-2) using primers APIS-F/APIS-R resulted in a product with a T_m=82.5±0.3°C and over 300 bp (Fig. 2a, b). By another hand, the amplification of *N. ceranae* DNA (pNCH-1) using primers MITOC-F/MITOC-R resulted in a product with a T_m=80.6±0.6°C and over 200 bp. Finally, the amplification of honeybee DNA using primers ACT-F/ACT-R resulted in a product with a T_m=83.5±0.3°C and over 100 bp. Expected T_m and size were 83.5°C/321 bp for *16S rRNA* gene of *N. apis*, 80.4°C/218 bp for *16S rRNA* gene *of N. ceranae* and 84.8°C/156 bp for *ACTIN* gene of *A. mellifera*. The similarity between empirical and theoretical data for the three genes analysed was also observed in extracted spores, experimentally contaminated honeybees and field honeybees (Fig. 3a).

Regarding to the selectivity of our method, we observed no amplification when *N. apis* DNA were subjected to PCR using primers MITOC-F/MITOC-R or when *N. ceranae* DNA were subjected to PCR using primers APIS-F/APIS-R (data not shown). About false-positive and false-negative results, we detected no amplification when uncontaminated honeybees were assessed and amplification of *ACTIN* gene was mandatory for the analysis of field samples. Compared to light microscopy analysis, our real-time PCR-based method has 100% sensitivity and 100% specificity (Table 1). The detection limit of our method, when we used the following as template: (1) plasmid DNA was one copy of *16S rRNA* gene per reaction (data not shown), (2) spore DNA was 2.5 spores per reaction, equivalent to 100 spores per ml of spore extract (Fig. 3a), (3) DNA from honeybee artificially contaminated with spores was 25 spores per reaction, equivalent to 100 spores per honeybee (Fig. 3a). The dynamic range of our real-time PCR-based method was 100 to 100,000 spores per honeybee (Fig. 3b).

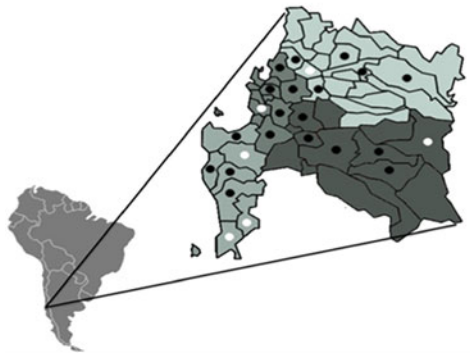
High prevalence of *N. ceranae* in honeybees from apiaries located in the South of Chile

Nosema spp. *16S rRNA* gene was detected in the four provinces and in 20 of the 26 communes of the Region del Biobío (Fig. 4a). *N. apis* was not detected (0/240) and *N. ceranae* was detected in 49% (117/240) of samples analysed (Fig. 4b). The infection level (spore load) of these samples ranged from 200 to more than 100,000 spores per honeybee.

Table 1 Detection of *N. ceranae* spores in field honeybees

Light microscopy	Real time-PCR	
	Negative	Positive
Negative	15	0
Positive	0	15

a



b

province	beehives total	positive	infected (%)
Arauco	54	17	31
Biobío	57	33	58
Concepción	78	42	54
Ñuble	51	25	49
TOTAL	240	117	49

Fig. 4 Screening of *Nosema* spp. in honeybees from apiaries located in Región del Biobío, Chile, South America. 2,400 honeybees collected from 240 beehives located in the four provinces (*different grey tones*) of Región del Biobío were screened. Samples from communes labelled with a *white dot* were negative for both *N. apis* and *N. ceranae*. Samples from communes labelled with a *black dot* were negative for *N. apis* and positive for *N. ceranae* (**a**). Data also was analysed from each provinces (**b**). All samples scored amplified for ACTIN gene

Discussion

We show for the first time that *N. ceranae* is present in Chilean apiaries. Furthermore, its genomic DNA is highly conserved compared with *N. ceranae* found in the rest of the world (Chen et al. 2009; Williams et al. 2008a).

In order to perform epizoological studies, we simplify the extraction of spore DNA using a commercial kit and adapted the conventional PCR described by Martín-Hernández et al. (2007) to real-time PCR. This improved method discriminates between *N. apis* and *N. cerenae*, has a detection limit of 100 spores per honeybee and lets us know the infection level (spore load) of samples. Similarly to other available molecular techniques, our real-time PCR-based method is as good as light microscopy analysis (Bourgeois et al. 2010; Chaimanee et al. 2011; Chen et al. 2008; Hamiduzzaman et al. 2010; Klee et al. 2007; Martín-Hernández et al. 2007; Ravikumar et al. 2011; Stevanovic et al. 2010; Traver and Fell 2011; Yoshiyama and Kimura 2011). Nevertheless, it does not rely on the expertise of the observer (Weiss et al. 1999) and allows the complete (*16S rRNA* of *N. apis*, *16S rRNA* of *N. ceranae* and *ACTIN* of *A. mellifera*) analysis of 10 samples in 3 h.

Using the method here reported, we analysed 2,400 honeybees collected from 240 beehives from the region where half of the Chilean apiary are located and in two different season of the year (Botías et al. 2011). Six of the 26 communes assessed were free of *Nosema* spp. Among the others communes, all samples infected with *Nosema* spp. were negative for *N. apis* and positive for *N. ceranae*. Results from samples collected between 2004 and 2006, analysed by light microscopy and published in 2007, show that *Nosema* spp. were present in Región del Biobío (www.agrarias.uach.cl/apicola/pdf/enfermedades_jul06_web.pdf). Their prevalence was 11–16% of adult honeybees and in all cases the microsporidia found was *N. apis*. In the absence of other reports, we cannot estimate when and how *N. ceranae* arrived to Chile. By another hand, as *N. apis* was detectable in 2004–2006 but not now (2010 and 2011), we hypothesise that *N. ceranae* displaced *N. apis* in Región del Biobío, as been proposed for other places in the world (Klee et al. 2007; Martín-Hernández et al. 2011a, b; Paxton et al. 2007; Tapaszti et al. 2009). Due to the fact that, compared with light microscopy, real-time PCR-based method has 100% sensitivity and 100% sensibility, we might conclude that the prevalence of *Nosema* spp. increases in Chile in the last 5 years (11–16% in 2004–2006 versus 49% in 2010–2011). This is not unexpected since *Nosema* spp. spores are massively expelled by diseased individuals and remain viable in field for more than a year (Fries 1993); there is no safety treatment available (Williams et al. 2008b) and alternative control strategy are under development (Porrini et al. 2010).

We envision that the method and the data presented in this article maybe useful for the screening and the follow-up of *Nosema* spp. infection. Also, will contribute to the understanding of the ecological behaviour of Nosematidae, in order to improve the control of nosemosis and related colony disorders worldwide.

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Ethical standards Experiments comply with current law of Chile.

Conflicts of interest Authors declare no conflict of interest.

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