Apidologie 40 (2009) 117–123
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DOI: 10.1051/apido/2008065

Available online at:
www.apidologie.org

Original article

Nosema ceranae infects honey bees (*Apis mellifera*) and contaminates honey in Australia*

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Received 3 July 2008 – Revised 1 October 2008 – Accepted 14 October 2008

Abstract – Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and microscopy were used to test 307 adult bee and 37 honey samples collected in Australia for the presence of two microsporidia, *Nosema ceranae* and *Nosema apis*. *N. ceranae* was detected in samples from 4 states (Queensland, New South Wales, Victoria and South Australia) and was most commonly found in samples from Queensland where 28 (33.7%) of 83 samples were positive. New South Wales had the second highest prevalence with 15 (15.8%) of 95 samples positive. South Australia and Victoria had 4 (16%) of 25 and 2 (4.5%) of 44 samples positive respectively. *N. ceranae* was not detected in samples from Western Australia and Tasmania. *N. apis* was detected in samples from all states. Three honey samples (8.1%) were PCR positive for *N. ceranae.* These positive honey samples originated from beekeepers in Queensland. Six imported honey samples tested were negative for both *Nosema* spp.

Nosema ceranae / *Nosema apis* / nosemosis / *Apis mellifera* / PCR / RFLP

1. INTRODUCTION

Nosemosis is the most widespread of adult bee diseases and causes significant economic losses to beekeepers worldwide. This disease was originally thought to be caused by a single *Nosema* sp., *Nosema apis* Zander, a microsporidian which has a range of effects on honey bee colonies and adult bees. The effects of *N. apis* on the colony include spring dwindling of adult bee populations, decreased honey production, decreased brood production and in severe cases nosemosis may kill colonies (Langridge, 1961; Bailey and Ball, 1991; Fries, 1995). *N. apis* reduces the lifespan of infected bees by about half in colonies in spring and summer (Kang et al., 1976). Infected bees do not fully develop their hypopharyngeal glands, resulting in up to 15% of eggs in severely infected colonies failing to produce mature larvae in early summer (Wang and Moeller, 1969). Infected queens are generally superseded within a few weeks (Bailey and Ball, 1991; Fries, 1995).

In 1994, a microsporidian similar to *N. apis* was described in Asian honey bees (*Apis cerana* Fabricius) from China (Fries et al., 1996). This parasite, called *Nosema ceranae* was subsequently detected in European honey bees (*Apis mellifera* L.) in Taiwan (Huang et al., 2005). More recently it has been found in Brazil, USA, Vietnam, and much of Europe (Klee et al., 2007).

Whilst *N. apis* infection causes a fast acting, short duration syndrome, this has not been the case for *N. ceranae*, which instead has been observed in association with non-specific symptoms, such as a gradual depopulation, higher autumn/winter colony deaths or low honey production (Fries et al., 2006). It has also recently been shown that *N. ceranae* does not display the seasonality that is seen with

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* Manuscript editor: David Tarpy

N. apis. In a study of bee samples collected in Spain from 1999 to 2005 (Martin-Hernandez et al., 2007), the typical *Nosema* seasonality was observed between 1999 and 2002, as characterised by an increase in infection levels especially in spring. However, from 2003 to 2005, this seasonality diminished and consistently high numbers of samples infected with *N. ceranae* were detected throughout 2005 (Martin-Hernandez et al., 2007). *N. ceranae* has also been demonstrated to cause significantly higher mortalities in laboratory experiments indicating that it may be more virulent than *N. apis* (Paxton et al., 2007).

It is now clear that *N. ceranae* is not a new parasite of the European honey bee. *N. ceranae* was detected in bee samples collected in the USA in 1996 and France in 2002 (Chen et al., 2008; Chauzat et al., 2007). The delay in recognising *N. ceranae* is attributable to the routine use of microscopy as a diagnostic technique for the detection of *Nosema*-like spores. However, the spores of *N. ceranae* and *N. apis* are similar in size, which has resulted in their being diagnosed as the latter. Although, on average, *N. ceranae* spores are slightly smaller (Fries et al., 2006), microscopy cannot be used to reliably discriminate between the two species. Molecular techniques such as PCR have not been commonly used to diagnose nosemosis, but these are necessary to identify each of the two species (Chen et al., 2008; Martin-Hernandez et al., 2007). The advent of molecular assays for *Nosema* spp. has driven the detection of *N. ceranae* worldwide.

During a metagenomic study of microbes in honey bee colony collapse disorder (CCD) *N. ceranae* was identified in a single sample of apparently healthy bees imported from Australia while this study was being undertaken. Cox-Foster et al. (2007) detected *N. ceranae* in 30 (100%) of samples from colonies with CCD and 17 (80.9%) of non-CCD samples, suggesting a potential role for *N. ceranae* in CCD. Beyond the detection of *N. ceranae* in the single bee sample that had originated in Australia, nothing more is known about the presence of this microsporidian in Australia. This study was undertaken to determine whether bees in Australia are infected with *N. ceranae*.

Australia is an island continent with restrictions on the importation of beekeeping equipment. Honey, however, can be imported without restriction into all states except Western Australia and may be a reservoir for infection although *N. ceranae* has not been reported to be found in honey. This study also aimed to determine whether *N. ceranae* could be detected in honey.

2. MATERIALS AND METHODS

2.1. Collection and processing of adult bee samples

Adult bee samples were collected by beekeepers and apiary inspectors in New South Wales, Queensland, Victoria, South Australia, Western Australia and Tasmania. Submitters were asked to collect bees from the top lids of bee hives or from around the outside of the brood nest. Bee samples were stored in 70% (v/v) ethanol at room temperature prior to testing. These samples were collected from August 2007 to May 2008.

Fifteen adult bees from each sample were macerated in 15 mL of distilled water with a mortar and pestle. The suspension was decanted to remove coarse material and examined by dark-field microscopy for the presence of *Nosema* spores. The homogenates were used directly for DNA extraction or stored at –20 °C until further use.

2.2. Collection and processing of honey samples

Honey samples were submitted by beekeepers and a honey packing plant. Six imported honey samples (Argentina, Spain, Korea [2] and Brazil [2]) were also examined for *N. ceranae*.

Honey samples (50 mL) were mixed with an equal volume of phosphate buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.3 g/L KH_2PO_4) and centrifuged for 45 min at 3000 × *g*. The supernatant was decanted, the pellet resuspended in 0.5 mL PBS. A 3 μL sub-aliquot was examined for *Nosema* spores and the remainder was subjected to DNA extraction.

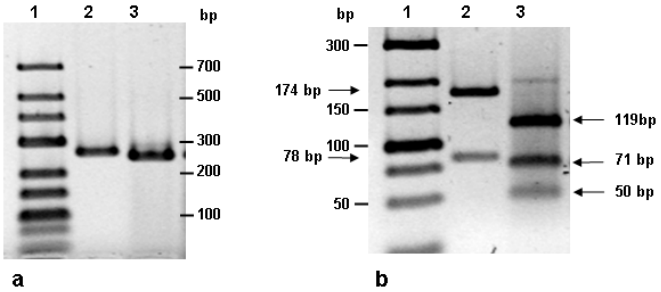

Figure 1. (a) PCR products of *N. apis* and *N. ceranae*: Lane 1: O'Generuler DNA ladder; lane 2: *N. ceranae* (252 bp); lane 3: *N. apis* (240 bp). **(b)** Lane 1, O'Generuler low range DNA ladder; lane 2, *N. ceranae* digested with *Dpn*II (174, 78 bp); lane 3, *N. apis* digested with *Dpn*II (119, 71, 50 bp).

2.3. Extraction of DNA from adult bees and honey

Bee homogenate (1 mL) or resuspended honey pellet (0.5 mL) was centrifuged for 15 min at 18 000 × *g*. The supernatant was discarded and DNA was extracted from the pellet with a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The DNA was eluted in a final volume of 50 μL and the extracts were stored at –20 °C until used as template in PCR.

2.4. PCR

The primers used in this study: NOS-FOR: 5'-TGCCGACGACGATGTGATATG**AG**-3'/ NOS-REV: 5'-CACAGCATCCATTGAAAACG-3' (Higes et al., 2006) bind to the 16S rRNA gene of *Nosema* spp. to amplify products of 240 bp from *N. apis* and 252 bp from *N. ceranae*. Primer NOS-REV has 100% homology to both *N. apis* and *N. ceranae*, whereas NOS-FOR is mismatched at 2 positions to *N. ceranae* (GenBank entry U26533, and all other available sequences); the 3' AG in *N. apis* (shown in bold) corresponds to GA in *N. ceranae*.

PCR was performed using a Mastercycler (Eppendorf) in a reaction volume of 20 μL containing 2 μL of template DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each forward and reverse primer and 0.7 U Taq polymerase (Roche). Given the mismatches between NOS-FOR and *N. ceranae*, the PCR annealing temperature was reduced from 62 °C to 55 °C to allow reliable amplification from both *N. apis* and *N. ceranae* templates. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 20 s at 94 °C, 20 s at 55 °C and 30 s at 72 °C, and a final extension step at 72 °C for 5 min. PCR products were analysed on 2% (w/v) agarose gels. The O'Generuler low range DNA ladder (Fermentas) was co-electrophoresed as a size marker. Gels were stained with ethidium bromide and visualised using UV illumination.

2.5. RFLP

Since the size difference of the PCR amplicons did not readily provide unambiguous discrimination between the species *N. apis* and *N. ceranae* (Fig. 1a), the sequences of *N. apis* (GenBank entries U26534, U97105, X73894, DQ235446), and *N. ceranae* (Accession No. U26533) were aligned using Sequencher (V4.7, Genecodes Corp.) and examined for discriminating restriction endonuclease sites. The presence of a unique *Dpn*II restriction site in all known *N. apis* sequences provided characteristic RFLP patterns for *N. apis* and *N. ceranae.*

The PCR amplicons were digested in a final volume of 22.5 μL containing 2.0 U of *Dpn*II (New England Biolabs) in 1 × *Dpn*II buffer , with incubation at 37 °C for 18 h. Restriction fragments were separated in 3% (w/v) agarose gels and analysed as described above.

Analysis of the *N. apis* and *N. ceranae* 16 S rDNA genes suggested that the PCR products amplified using the NOS-FOR/NOS-REV primers could be distinguished by digestion with restriction endonuclease *Dpn*II. The *Dpn*II recognition sequence, GATC, occurred twice in the *N. apis* amplicon and only once in the *N. ceranae* amplicon. Digestion of the 240 bp *N. apis* amplicon was demonstrated to generate fragments of 119, 71 and

Table I. Prevalence of *N. ceranae* and *N. apis* in adult bee samples collected in Australia.

State of Origin	*N. ceranae*	*N. apis*	Negative	Total
Queensland	28	4	51	83
New South Wales	15*	48	34	95
Victoria	2	40	2	44
Western Australia	0	23	1	24
South Australia	4	4	17	25
Tasmania	0	23	13	36
TOTAL	49	142	118	307

* Two samples contained both *N. ceranae* and *N. apis*.

Table II. PCR results in relation to *Nosema* spore presence in adult bee samples

Microscopic analysis for *Nosema* – like spores	PCR results			
	N. ceranae	*N. apis*	Negative	Total
Negative	8	30	111	149
Positive	41*	112	7	158
TOTAL	49	142	118	307

* Two samples contained both *N. ceranae* and *N. apis*.

50 bp, whereas *Dpn*II restriction of the 252 bp *N. ceranae* amplicon yielded fragments of 174 and 78 bp.

3. RESULTS

3.1. Adult bee samples

Three hundred and seven adult bee samples were submitted for examination (Tab. I). These samples were primarily from individual beekeepers from widely dispersed geographical regions within each state, except for 50 samples from Queensland which were from one beekeeper and 16 samples which were from a single beekeeper from Tasmania.

PCR was demonstrated to be a more sensitive assay than microscopy for the detection of *Nosema* (Tab. II). Of the 144 bee samples that were negative for *Nosema*-like spores when analysed by microscopy, 38 adult bee samples (26%), were found to be positive for *Nosema* spp when subjected to PCR-RFLP analysis (8 samples containing *N. ceranae* and 30 samples containing *N. apis*). Of the 163 samples in which microscopy did detect *Nosema*-like spores, 7 samples (4%) were negative by PCR (Tab. II). Using microscopy *Nosema*-like spores were not detected in the honey samples that tested positive for *N. ceranae* by PCR.

N. ceranae was detected in samples from 4 states (Queensland, New South Wales, Victoria and South Australia) and was most commonly found in samples from Queensland where 28 (33.7%) of 83 samples were positive (10 of the 50 samples submitted by one single beekeeper from Queensland were *N. ceranae* positive). New South Wales had the second highest prevalence with 15 (15.8%) of 95 samples positive. South Australia and Victoria had 4 (16%) and 2 (4.5%) of samples positive (Tab. I). *N. ceranae* was not detected in any samples from Western Australia and Tasmania.

A better assessment of the prevalence of *N. ceranae* in each state and the relative prevalence of *N. apis* can be provided by presenting the number of *N. ceranae*-positive samples as a percentage of the number of *Nosema* positive samples.Among the 32 bee samples from Queensland that were positive for *N. ceranae* or *N. apis*, 28 (87.5%) were found to carry *N. ceranae*. For New South Wales, South Australia and Victoria the percentage of *N. ceranae* positive samples were 23.8% (15 samples), 50% (4) and 4.8% (2) respectively.

3.2. Honey samples

Thirty seven honey samples were tested during this study. Eight (21.6%) were PCR positive for *N. apis* and 3 (8.1%) were PCR positive for *N. ceranae*. The *N. ceranae* positive honey samples originated from beekeepers in Queensland. All 6 of the imported honey samples were negative for both *Nosema* spp.

4. DISCUSSION

The combination of PCR and RFLP used in this study was demonstrated to be an effective method of detecting *N. ceranae* and *N. apis.* This methodology detected *N. apis* or *N. ceranae* in 38 samples which were found to be negative using microscopy. The 7 bee samples which tested negative using PCR-RFLP, but were positive using microscopy, contained very few *Nosema*-like spores. The detection of *Nosema* using PCR is also not restricted to the detection of DNA in *Nosema* spores. Hence, bees infected with DNA which may be contained in the polar filament extruded from spores or contained in cells in which spores have not yet developed may also be PCR positive.

This study has demonstrated that in Australia *N. ceranae* infects bees in Queensland, New South Wales, South Australia and Victoria. However, there is a striking difference in the percentage of *N. ceranae* positive samples among the *Nosema* positive samples between states. Of the *Nosema* positive samples from Queensland 87.5% were *N. ceranae* positive but only 4.8% were positive from Victoria.

The four South Australian samples found to contain *N. ceranae* consisted of queen bees and escorts (worker bees caged with the queen) which had been imported from New South Wales. This indicates that queens and their escorts may be a source of infection although no other detections of *N. ceranae* were made from the remaining 21 samples in that state. These four samples should be considered as samples from New South Wales rather than South Australia.

The high percentage of *N. ceranae*-positive samples from Queensland compared with those detected from New South Wales and Victoria may be due to two reasons. *N. ceranae* may have been first introduced into Australia in Queensland and thus been established longest in that state. Most commercial beekeepers in Australia are migratory, often moving from state to state to take advantage of honey flows and suitable conditions for their bees. In doing so, *N. ceranae* may have spread to other colonies through movement of infected bees into and out of Queensland, or the sale of *N. ceranae*-contaminated equipment. Alternatively, *N. ceranae* may have been introduced elsewhere in Australia, but be better adapted to multiply and disseminate in tropical climates such those occurring in Queensland. Although *N. ceranae* has been reported in much of Europe, the impact of *N. ceranae* in Spain seems to be greater than in other countries with more temperate conditions in Europe (Martin-Hernandez et al., 2007). The same may hold true for the more temperate climates of New South Wales and Victoria.

N. ceranae was detected far more commonly than *N. apis* in samples from Queensland than in samples from other states. Of the *Nosema* positive samples from Queensland 28/32 (87.5%) contained *N. ceranae* and 4/32 (12.5%) contained *N. apis* (Tab. I) indicating that *N. ceranae* may be replacing *N. apis*. The replacement of *N. apis* with *N. ceranae* has previously been described by Paxton et al. (2007), Klee et al. (2007) and Chen et al. (2008).

Little is known about the epidemiology of *N. ceranae.* Higes et al. (2008) demonstrated that *N. ceranae* can contaminate both honey bee pollen baskets and pollen collected from commercial apiarists. However, there are no reports of honey contaminated with *N. ceranae.* In Australia honey is commonly moved from state to state, with the exception of honey destined for Western Australia. In this study, 37 honey samples sourced from Australia and overseas were tested for both *N. ceranae* and *N. apis.* Three samples were positive for *N. ceranae* and eight samples were positive for *N. apis.* The three honey samples that were positive for *N. ceranae* originated from Queensland where the prevalence of *N. ceranae* in bees was the highest. The absence of

detectable spores in these honey samples supports the higher sensitivity of PCR compared to microscopy, but could also be an indication that there was non-spore associated *Nosema* DNA in honey.

Following the first detection of European foulbrood in Australia in 1977 (Tham, 1978), Western Australia implemented restrictions on the importation of bees and bee products. Whilst European foulbrood has spread among the remaining states, Western Australia remains free of this disease. It seems likely that importation restrictions may have prevented *N. ceranae* from entering Western Australia, given that all 24 adult bee samples from Western Australia were *N. ceranae* negative when analysed by PCR-RFLP during this study. Nevertheless, a more comprehensive testing regime is required to unequivocally confirm that *N. ceranae* does not occur in Western Australia.

Tasmania is the state with the least number of beekeepers and colonies in Australia. It is also separated from mainland Australia by a large body of water. None of the 36 samples submitted from this state contained *N. ceranae*. Nevertheless, beekeepers in Tasmania source queen bees from mainland Australia, so the importation of infected queens and escorts poses a risk for the introduction and establishment of *N. ceranae* in Tasmania.

Martin-Hernandez et al. (2007) carried out a study of bee samples collected in Spain from 1999 to 2005. Data from the first period 1999 to 2000 showed a consistency with the seasonal pattern usually associated with nosemosis, ie. an infection peak in spring (Langridge, 1961). However, in 2004 and 2005 the *Nosema* seasonality diminished to the point where, in 2005, consistently high numbers of *Nosema*-positive samples were detected throughout the year. In this study, 7 samples collected in March-April (Autumn), a time when *Nosema* is not commonly found, were confirmed to be *N. ceranae*-positive, indicating that a similar situation may be emerging in Australia.

This study has confirmed that bees in Australia are infected with *N. ceranae* and that the prevalence of infection varies between states. Tasmania and Western Australia appear to be *N. ceranae* free, but more intensive studies are required to establish their freedom from this parasite. It was recently shown that pollen can be contaminated with *N. ceranae* (Higes et al., 2008), and this study has demonstrated that both *N. ceranae* and *N. apis* can contaminate honey. As *N. apis* spores can stay viable in honey for several months depending on the honey type (Malone et al., 2001) it is likely that *N. ceranae* was introduced into Australia through imports of either contaminated honey or pollen. The extent of infected samples detected in multiple states indicates that *N. ceranae* has been in Australia for some time and that any attempts at eradication would be unlikely to be successful.

ACKNOWLEDGEMENTS

We thank the Rural Industries Research & Development Corporation for funding. We thank Professor Ingemar Fries from the Swedish University of Agricultural Sciences, Uppsala, Sweden for providing a sample of *Nosema ceranae* spores which was used as the positive control for the molecular studies. We also thank the beekeepers, apiary officers and Capilano Honey for submitting adult bee and honey samples.

***Nosema ceranae* infecte les abeilles domestiques (*Apis mellifera*) et contamine le miel en Australie.**

***Nosema ceranae* / *Nosema apis* / nosémose / parasite / *Apis mellifera* / détection**

Zusammenfassung – ***Nosema ceranae* infiziert Honigbienen (*Apis mellifera*) und kontaminiert Honig in Australien.** Nosemosis ist die am weitesten verbreitete Krankheit der adulten Bienen und verursacht bei den Imkern weltweit erhebliche wirtschaftliche Schäden. Im Jahr 1994 wurde bei der östlichen Honigbiene (*Apis cerana*) in China ein ähnliches Mikrosporidium wie *N. apis* beschrieben (Fries et al., 1996). Dieser Parasit, *Nosema ceranae*, wurde anschließend in europäischen Honigbienen (*Apis mellifera*) in Taiwan gefunden (Huang et al., 2005). In jüngerer Zeit wurde er in Brasilien, den USA, Vietnam und in mehreren europäischen Staaten nachgewiesen (Klee et al., 2007). Während bei *N. apis*-Infektionen die Syndrome meist rasch auftreten und von begrenzter Dauer sind, verursacht *N. ceranae* eher unspezifische Krankheitssymptome wie einen sukzessiven Populationsrückgang, höhere Winterverluste oder eine geringere Honigproduktion (Fries et al., 2006). Inzwischen ist klar, dass *N.*

ceranae kein neuer Parasit bei europäischen Honigbienen ist. So wurde *N. ceranae* in Bienenproben aus dem Jahr 1996 in den USA und 2002 in Frankreich nachgewiesen (Chen et al., 2008; Chauzat et al., 2007). Die weit verbreiteten Nachweise von *N. ceranae* sind eine Folge der molekularen Nachweismethoden, mit denen im Gegensatz zur mikroskopischen Analyse eine klare Unterscheidung zwischen *N. ceranae* und *N. apis* möglich ist. Da es bisher keine Informationen bezüglich des Vorkommens von *N. ceranae* in Australien gibt, sollte mit dieser Studie geklärt werden, ob Honigbienen in Australien mit *N. ceranae* infiziert sind.
307 adulte Bienen und 37 Honigproben aus Australien wurden mit PCR, RFLP (Restriction Fragment Length Polymorphism) und über mikroskopische Analysen auf das Vorkommen von *N. ceranae* und *N. apis* hin untersucht. *N. ceranae* wurde in Proben aus 4 Staaten nachgewiesen: Queensland, New South Wales, Victoria und South Australia. Nicht nachgewiesen wurde *N. ceranae* in Proben aus Western Australia und Tasmania. *N. apis* wurde dagegen in Proben aller Staaten gefunden. Drei Honigproben von Imkern in Queensland waren für *N. ceranae* positiv. Sechs Proben von Importhonigen waren für beiden Nosema-Arten negative. Diese Studie hat bestätigt, dass auch Bienen in Australien mit *N. ceranae* infiziert sind und dass die Verbreitung der Infektion in den einzelnen Staaten unterschiedlich ist. Tasmania und Western Australia scheinen noch frei von *N. ceranae* zu sein, doch müsste dies mit umfangreicheren Analysen abgesichert werden. Der Umfang der positiven Nachweise in mehreren Staaten weist darauf hin, dass *N. ceranae* bereits seit einiger Zeit in Australien vorkommt und dass Versuche zur Ausrottung des Erregers kaum Aussicht auf Erfolg haben.

***Nosema ceranae* / *Nosema apis* / Nosemosis / *Apis mellifera* / PCR / RFLP**

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