Prevalence and distribution of *Nosema ceranae* in Croatian honeybee colonies

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ABSTRACT: Nosema disease of European honey bees afflicts bees worldwide. *Nosema ceranae* is a recently described microsporidian parasite of the honey bee (*Apis mellifera*) and its geographical distribution is not well known. The disease may have many negative effects on bee colonies and cause high losses for apiculture and consequently in agriculture. With this in mind, a total of 204 samples of dead bees from different localities in Croatia were selected and investigated for distribution, prevalence and diversity of *N. ceranae* infection, using light microscopic examination and multiplex PCR. Our results show that *N. ceranae* is the only nosema species found to infect honeybees in our geographically varied collection. The nucleotide sequences of amplicons from Nosema-infested honeybee samples were 100% identical with the *N. ceranae* sequence deposited in the GenBank database. *N. ceranae* infected bees were found in samples collected from each of 21 districts, and in all three climatic areas, i.e., mediterranean, mountain, and continental parts regions of Croatia.

Keywords: *Nosema ceranae*; *Nosema apis*; honey bees; distribution; colonization

Nosemosis is a parasitic disease of adult honey bees (*Apis mellifera*) caused by two species of microsporidia, *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries et al., 1996). The disease occurs throughout the world, including Croatia, and causes significant honey production and economic losses, such as reduced yields of honey and other bee products (Anderson and Giacon, 1992), and poor pollination resulting in lower quality and reduced yields in agriculture (Goodwin et al., 1990). Honey bees afflicted with nosemosis start to forage earlier (Fries, 1995), while pathological changes of their mid – gut epithelial cells, as well as digestive and metabolic disorders (Hassanein, 1951), cause malnutrition (Muresan et al., 1975) resulting in decreased populations in honey bee colonies (Malone et al., 1995), leading to premature colony deaths (Morse and Shimanuki, 1990). Affected honey bees tend to die away from the hive, and there are no obvious signs of disease, making it difficult to notice, hence it is often referred to as "the silent killer"(Hornitzky, 2005). Spores enter the digestive tract of bees via contaminated food or liquid honey during trophallaxis (Fries et al., 1996; Sulimanovic et al., 1995). Factors favouring the spread of the disease include the robbing of honey bee colonies and poor beekeeping practices, as well as sudden temperature fluctuations, poor foraging, and the disturbance, and frequent movement of honey bee colonies (Sulimanovic et al., 1995).

Previously, nosema infections in Europe were attributed just to *N. apis* (Ellis and Munn, 2005), but it appears that *N. ceranae* is an emerging pathogen that has increased its distribution to include European honey bees (Klee et al., 2007) and may be displacing *N. apis* in this area. *N. ceranae* was first detected in the European honey bee in Taiwan and Spain (Higes et al., 2006; Huang et al., 2007) and Klee et al. (2007) speculated that *N. ceranae* may be a long established and wide spread infection in European honey bees. *N. apis* is a parasite of the bee digestive tract and is considered not to be highly virulent and with a clear sesonality such that infections are least prevalent during the summer months.In contrast, *N. ceranae* is highly pathogenic when experimentally inoculated into European honey bees (Higes et al., 2007; Cornman et al., 2009). However, there are usually no symptoms of diarrhea or visible adult

bee deaths, and there is a total lack of seasonality in the diagnosis (Martin-Hernandez et al., 2007). A long asymptomatic incubation period may explain the absence of evident symptoms prior to colony death (Higes et al., 2008).

During the last few years, an increase in the number of nosema infected honeybees has been detected in Croatia (Kezic et al., 2009) and in some other European countries (Faucon, 2005), while increasing numbers of honey bee colony losses and low production in the same areas have been reported by beekeepers. Also, for the first time Higes et al. (2008) showed that natural infection with *N. ceranae* can cause the sudden collapse of bee colonies, establishing a direct correlation between infection with that parasite and the death of honey bee colonies under field conditions. In view of these observations the principal hypothesis that may explain these problems is the recent entry and dissemination of *N. ceranae* into new areas. Although a routine optical microscopy assessment can confirm infection with both *Nosema* species, it is not possible to distinguish between the species because of the absence of clear morphological characteristics for species recognition. Thus, it is necessary to use molecular diagnostic tools and identification methods (Fries et al., 2006; Higes et al., 2006). Because *N. ceranae* has been diagnosed in some neighboring countries and because of high percentages of nosema infections have been described during the summer months, we have suspected its presence in pure or mixed infections with *N. apis*. The aim of this research was to investigate and determine the presence of *N. ceranae*, its prevalence and distribution in all 21 districts of Croatia, using light microscopic examination and multiplex PCR (Anonymous, 2008).

MATERIAL AND METHODS

The diversity of nosema species was studied using 204 honeybee samples sent to the Department for Biology and Pathology of Fish and Bees from different districts (all 21) in Croatia by beekeepers, as shown in Figure 1. Bee samples originated mainly from bee colonies with different pathological problems like depopulation, weakness and high colony mortality. Samples were collected during the first spring inspection of honeybee colonies, in February and March 2009, and each common sample represented one location. All the bee samples were stored at –20 °C prior to examination.

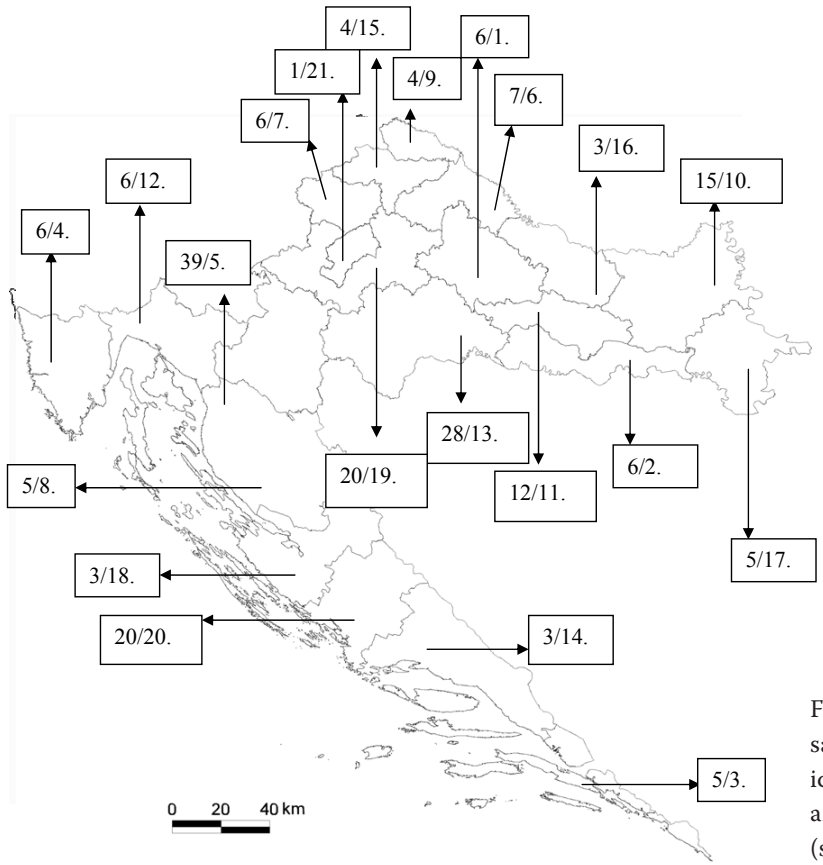

Figure 1. Total number of common bee samples (locations) from each geographical district of the Republic of Croatia and (/) the ordinal number of the district (see Table 1 for locality names)

Thirty adult bees were randomly selected from each sample and suspensions were created by adding 20 ml distilled water to the abdomens of bees from a single colony, which was crushed using mortar and pestle. Afterwards, the spore suspensions were filtered to remove coarse bee parts, centrifuged at 1000 g for 20 min and the supernatants removed. Pellets of isolated spores were resuspended in 1.5 ml of distilled water and transferred to a fresh tube. Each sample was microscopically examined at 400× magnification for the presence of *Nosema* sp. spores. Samples of spores were kept at 4 °C. Extraction of genomic DNA and further molecular analysis was performed as follows: for each of the selected suspensions of isolated *Nosema* spores, an aliquot of 50 µl was transferred to a fresh tube, boiled at 100 °C for 30 min and centrifuged at 14 000 g for 10 min. Thirty µl of the supernatant was removed and supplemented with 10× TE buffer to a final concentration of 10mM Tris and 5mM EDTA, pH8. This supernatant served as a source of template DNA and was stored at –20 °C, or, used immediately for multiplex PCR. Primers used for specific amplification of *N. apis* DNA were 321APIS-FOR (5'-GGGGGCATGTCTTTGACGTACTATGTA-3') and 321APIS-REV (5'GGGGGGCGTTTAAAATGTGAAACAACTATG-3') and the expected size of the amplicion was 321 bp. Primers for *N. ceranae* were 218MITOC-FOR (5'CGGCGACGATGTGATATGAAAATATTAA-3') and 218MITOC-REV (5'-CCCGGTCATTCTCAAACAAAA-AACCG-3') and amplicon size was expected to be 218–219 bp in size. Primers were designed so as to be specific to each of the two species, and to allow for simultaneous amplification and separation using agarose gel electrophoresis.

The PCR protocol was 2 min at 94 °C, followed by 10 cycles of 10 s at 94 °C, 15 s at 60 °C, and 30 s at 70 °C, and 25 cycles of 10 s at 94 °C, 15 s at 62 °C, and 30 s at 72 °C plus a two-second elongation cycle for each successive cycle and a final extension step at 72 °C for 7 min. The PCR conditions used followed the instructions of the manual of the manufacturer of Taq polymerase (Sigma, USA). In brief, the PCR reaction mix contained a final concentration of 200µM for each dNTP, 3mM MgCl, 0.5µM forward and reverse primer, and one unit Taq DNA Polymerase. The amount of template DNA varied and usually between 1 µl and 4 µl of the extracted samples was added to the reaction. The molecular size of PCR products were determined by electrophoresis in a 2% agarose TAE (Tris-acetate-ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with SYBR green, and visualized using the UviTec gel documentation system (Figure 2). The nucleic acid sequences of the PCR products were determined and compared to the nucleotide sequences deposited in GenBank using the BLAST program (www.ncbi.nlm.nih.gov/BLAST/). DNA extracted from *Nosema*-infected bees (provided by Dr. A. Gregorc from Agricultural Institute in Slovenia), that had been confirmed to be positive for both *N. ceranae* and *N. apis* by PCR, was used as a positive control. Negative controls (water instead of sample DNA) were included in

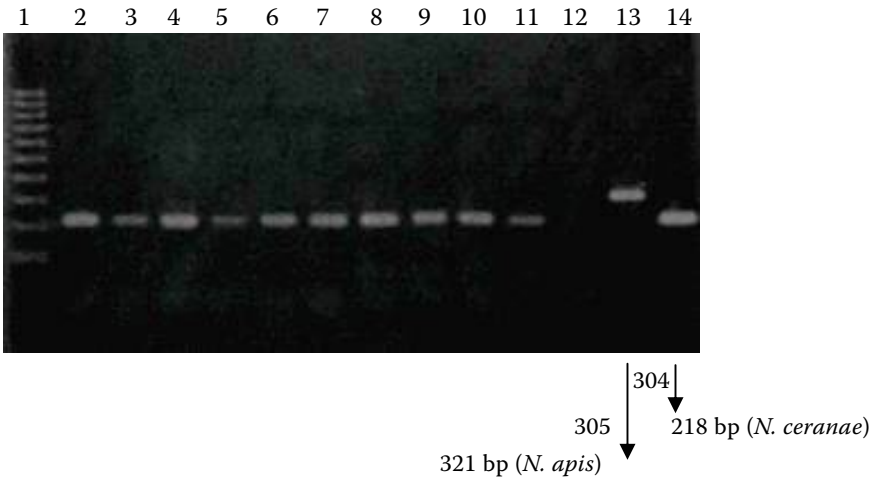

Figure 2. PCR amplification of representative bee samples infected with *N. ceranae*, negative control and reference DNA extracts of *N. apis* and *N. ceranae* (positive controls). Line 1 = DNA ladder (DNA molecular weight marker VI; Roche, Germany), line 2 = 11 PCR reactions of positive bee samples, line = 12 negative control – blank, line 13 = positive control for *N. apis*, line 14 = positive control for *N. ceranae*

each run of PCR amplification to exclude possible DNA contamination.

RESULTS

Results of the microscopic examination of nosema spores in samples are presented in Table 1. The highest percentages (100%) of nosema positive samples were from four districts of Croatia: Karlovacka district 39/39, Pozesko – Slavonska district 12/12, Varazdinska district 4/4 and Zagreb 1/1. All these regions are located in the continental part of Croatia. The lowest percentages (16.6%) of nosema spores were detected in Istarska district 1/6, and Primorsko – Goranska district 1/6, both located in the mountainous part of Croatia. After light microscopy, a total of 150 samples from different localities were selected (135 positive and 15 negative samples) and investigated by multiplex PCR for the presence of *N. apis* and *N. ceranae*. Our results showed that *N. ceranae* was the only nosema species found to infect honeybees from our widespread geographic collection in Croatia. *N. ceranae*-infected bees were found in positive samples collected from each of 21 districts and in all three climatic areas, i.e., mediterranean, mountain, and continental parts of Croatia. Eleven of the 15 "negative" samples were negative for both *Nosema* sp., and no mixed infections were detected. The results of PCR amplification with the generic nosema primer pair perfectly matched the results of amplification with the *N. ceranae*-specific primer pair. Also, the results show that all examined bees were negative for *N. apis* when amplified with the *N. apis*-specific primer pair. PCR amplifications of representative bee samples, negative and positive controls are presented in Figure 2. The nucleotide sequences of amplification products from the Nosema infested honeybee samples were 100% identical with the *N. ceranae* sequence deposited in the GenBank database.

Table 1. Results of light microscopy examination for *Nosema* sp. spores in common samples originating from each district of the Republic of Croatia

Name of district		Samples				
		total (*n*)	negative (*n*)	negative (%)	positive (*n*)	positive (%)
1	Bjelovarsko-Bilogorska	6	4	66.66	2	33.33
2	Brodsko-Posavska	6	4	66.66	2	33.33
3	Dubrovačko-Neretvanska	5	2	40.00	3	60.00
4	Istarska	6	5	83.33	1	16.66
5	Karlovacka	39	0	0	39	100.00
6	Koprivnicko-Krizevacka	7	3	42.85	4	57.14
7	Krapinsko-Zagorska	6	3	50.00	3	50.00
8	Licko-Senjska	5	2	40.00	3	60.00
9	Međimurska	4	3	75.00	1	25.00
10	Osjecko-Baranjska	15	2	13.33	13	86.66
11	Pozesko-Slavonska	12	0	0	12	100.00
12	Primorsko-Goranska	6	5	83.33	1	16.66
13	Sisacko-Moslavacka	28	2	7.14	26	92.85
14	Splitsko-Dalmatinska	3	2	86.66	1	33.33
15	Varazdinska	4	0	0	4	100.00
16	Viroviticko-Podravska	3	1	33.33	2	66.66
17	Vukovarsko-Srijemska	5	4	80.00	1	20.00
18	Zadarska	3	1	33.33	2	66.66
19	Zagrebacka	20	6	30.00	14	70.00
20	Sibensko-Kninska	20	3	15.00	17	85.00
21	Grad Zagreb	1	0	0	1	100.00
Total		204	52	25.49	152	74.50

DISCUSSION

Our results show that *N. ceranae* is the only nosema species found to infect honey bees in the geographic territory of Croatia. From the literature we can see that *N. ceranae* has been present in some European countries (Paxton et al., 2007), as well as in the United States (Chen et al., 2008) for the past decade. This indicates that *N. ceranae* is not a new emerging pathogen for European honey bees, and has presumably been transferred from its original host *Apis ceranae* to *Apis mellifera* (Klee et al., 2007) much earlier than previously recognized. The exact dates when *N. ceranae* appeared and started to parasitize Croatian bees is unknown, and it is impossible to investigate the historical incidence because of a lack of bee samples. Our presented data imply a strong need for epidemiological and pathogenic studies to identify the conditions that resulted in displacement of *N. apis* with *N. ceranae* in Croatia. Martin-Hernandez et al. (2007) report the absence of differences in the number of positive samples between months, showing evident lack of seasonality, and indicating a change in the clinical and epidemiological patterns of nosema disease. Also, little is known about the pathogenicity (Oldroyd, 2007) of *N. ceranae* upon infection of *A. mellifera*, and it may be possible that this parasite is a crucial factor contributing to high bee mortalities (Cornman et al., 2009). Higes et al. (2006, 2007) suggest that *N. ceranae* is a serious threat to the global beekeeping industry and natural biodiversity. Our results showed that eleven out of 15 microscopically negative samples examined with multiplex PCR were in fact positive, confirming the advantages of molecular diagnostic methods. The benefits of molecular methods include increased sensitivity, specificity, and perhaps more significantly, the ability to identify all developmental stages of the *Nosema* sp. (Martin-ernandez et al., 2007).

Serious problems arise from controlling nosemosis caused by *N. apis*, and especially *N. ceranae* because of their asymptomatic duration (Martin-Hernandez, 2007). Beekeepers devote insufficient attention or often neglect nosemosis, because of its lack of symptoms. However, eradication can be achieved by an interchange of frames with a brood in a disinfected hive and often the use of new wax (Sulimanovic, 1995). European Union and Croatian regulations prohibit the use of antibiotics and fumagillin in the treatment of nosemosis and other bee diseases because of the potential development of resistance and concerns about residues. Currently, beekeepers in other parts of the world are using Fumagillin, which is effective for *N. apis* infections. Fumagillin's impact on *N. ceranae* it is still under evaluation (Williams et al., 2008). Poor results using Fumagillin to control *Nosema bombi* in bumblebees (Whittington and Winston, 2003), raises the possibility that this antibiotic will not be useful for treating longterm *N. ceranae* infections. Herbal alternatives are being investigated and recent results with "Nozevit" demonstrated high effectiveness as a preventive measure and curative treatment for bees infected with *N. ceranae* (Tlak Gajger et al., 2009a,b).

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REFERENCES

Anderson DL, Giacon H (1992): Reduced pollen collection by honey bee (*Hymenoptera, Apidae*) colonies infected with *Nosema apis* and sacbrood virus. Journal of Economic Entomology 85, 47–51.

Anonymous (2008): Nosemosis of Honey Bees. OIE Terrestrial Manual, Chapter 2.2.4., 410–414.

Chen Y, Evans JD, Smith IB, Pettis JS, (2008): *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. Journal of Invertebrate Pathology 97, 186–188.

Cornman RS, Chen JP, Schatz MC, Street C, Zhao Y, Desany B, Egholm M, Hutchison S, Pettis JS, Lipkin W, Evans JD (2009): Genomic analyses of the microsporidian *Nosema ceranae*, an emergent pathogen of honey bees. PloS Pathogens 5, 6, e1000466. doi:10.1371/journal.ppat.1000466

Ellis JD, Munn PA (2005): The worldwide health status of honey bees. Bee World 86, 88–101.

Faucon JP (2005): La nosemose. Sante Abeille 209, 343–367.

Fries I (1995): *Nosema apis* – a parasite in the honey bee colony. Bee World 74, 5–19.

Fries I, Feng F, Da Silva A, Slemenda SB, Pieniazek NJ (1996): *Nosema ceranae* sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis ceranae* (Hymenoptera, Apidae). European Journal of Protistology 32, 356–365.

Fries I, Martin R, Meane A, Garcia-Palencia P, Higes M (2006): Natural infections of *Nosema ceranae* in European honey bees. Journal of Apicultural Research 45, 230–232.

Goodwin M, Ten Houten A, Perry J, Blackman R (1990): Cost benefit analysis of using fumagillin to treat *nosema*. New Zealand Beekeeper 208, 11–12.

Hassanein MH (1951): Studies on the effect on infection with *Nosema apis* on the physiology of the queen honey bee. Journal of Cell Science 92, 225–231.

Higes M, Martin R, Meana A (2006): *Nosema ceranae*, a new microsporidian parasite in honey bees in Europe. Journal of Invertebrate Pathology 92, 93–95.

Higes M, Garcia-Palencia P, Martin-Hernandez R, Meana A (2007): Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). Journal of Invertebrate Pathology 94, 211–217.

Higes M, Martin-Hernandez R, Botias C, Bailon EG, Gonzales-Porto V, Barrios L, Del Nozal J, Bernal JL, Jimenez JJ, Palencia PG, Meana A (2008): How natural infection by *Nosema ceranae* causes honey bee colony collapse. Environmental Microbiology, doi: 10.1111/j.1462-2920.2008.01687.x

Hornitzky M (2005): A report for the Rural Industries Research and Development Corporations, Kingston, Australia, 1–16. http:/www.rirdc.gov.au/reports/HBE/05-055.pdf.

Huang W-F, Jiang J-H, Chen Y-W, Wang C-H (2007): A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie 38, 30–37.

Kezic N, Drazic M, Tlak Gajger I, Tomljanovic Z (2009): Colony losses in Croatia during winter 2008/09. In: Proceedings of the 4th Coloss Conference, Zagreb, 12.

Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, Chinh TX, Puerta F, Ruz JM, Kryger P, Message D, Hatjina F, Korpela S, Fries I, Paxton RJ (2007): Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. Journal of Invertebrate Pathology 96, 1–10.

Malone LA, Giacon HA, Newton MR (1995): Comparison of the responses of some New Zealand and Australian honey bees (*Apis mellifera* L.) to *Nosema apis* Z. Apidologie 2, 495–502.

Martin-Harnandez R, Prieto L, Martinez Salvador A, Garrido-Bailon E, Higes M (2007): Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Applied Environmental Microbiology 73, 6331–6338.

Morse RA, Shimanuki H (1990): Summary of control methods. In: Morse RA, Nowogrodzki R (eds.): Honey Bee Pests, Predators and Diseases. 2nd ed. Cornell University Press, Ithica and London, 341–354.

Muresan E, Duca C, Papay Z (1975): The study of some histochemical indicies of the mid – gut, healty and infected with *Nosema apis* Z., of the *Apis mellifica carpatica* bee. In: Proceeding of XXVth International Congres, Apimondia. Public House, Grenoble, 384–385.

Oldroyd BP (2007): What s killing American honey bees? PloS Biology 5, 1195–1199.

Paxton RJ, Klee J, Korpela S, Fries I (2007): *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie 38, 558–565.

Sulimanovic D, Zeba Lj, Markovic J (1995): Recognition and Eradication of Honeybee Diseases (in Croatian). PIP, Zagreb. 88–100.

Tlak Gajger I, Petrinec Z, Pinter L, Kozaric Z (2009a): Experimental treatment of nosema disease with "Nozevit" phyto-pharmacological preparation. American Bee Journal 149, 485–490.

Tlak Gajger I, Vugrek O, Pinter L, Petrinec Z (2009b): "Nozevit patties" treatment of honeybees (*Apis mellifera*) for the control of *Nosema ceranae* disease. American Bee Journal 149, 11, 1053–1056.

Whittington R, Winston ML (2003): Effects of *Nosema bombi* and its treatment fumagillin on bumble bee (*Bombus occidentalis*) colonies. Journal of Invertebrate Pathology 84, 54–58.

Williams GR, Sampson MA, Shutler D, Rogers REL (2008): Does Fumagillin control the recently detected invasive parasite *Nosema ceranae* in western honey bees (*Apis mellifera*)? Journal of Invertebrate Pathology 99, 342–344.

Zander E (1909): Tierische Parasiten als Krankheitserreger bei der Biene. Munchener Bienenzeitung 31, 196–204.

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