Dominance of Nosema ceranae in honey bees in the Balkan countries in the absence of symptoms of colony collapse disorder

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Original article

Dominance of *Nosema ceranae* in honey bees in the Balkan countries in the absence of symptoms of colony collapse disorder*

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Abstract – *Nosema* species were determined in honey bees from Balkan countries. A total of 273 *Nosema*-positive samples were analysed. Duplex PCR and PCR-RFLP with newly designed primers, nos-16S-fw/rv, were used to differentiate between *N. apis* and *N. ceranae*. *N. apis* was detected in only one sample (collected in 2008 in Serbia) and *N. ceranae* in all the others (N = 272) including 35 older samples from Serbia collected between 2000 and 2005. No co-infection was detected. The results suggest (1) the dominance of *N. ceranae* infection in all Balkan countries monitored throughout the last three years; (2) the presence of *N. ceranae* in Serbia at least since 2000, which means that *N. ceranae* has not recently displaced *N. apis*; (3) the higher efficacy of PCR-RFLP with newly designed primers, nos-16S-fw/rv, in comparison with duplex PCR (100%:82%, respectively). The prevalence of *N. ceranae* in Balkan countries was not associated with an increase in nosemosis or colony losses resembling Colony Collapse Disorder (CCD).

***Nosema ceranae* / PCR-RFLP / duplex PCR / Balkan countries**

1. INTRODUCTION

The European honey bee *Apis mellifera* was formerly considered parasitized with a single microsporidian species, *Nosema apis* Zander 1909. However, many recent reports show that another species of the same genus, *N. ceranae* Fries (Fries et al., 1996), infects *A. mellifera* almost worldwide (Huang et al., 2005, 2007; Higes et al., 2006; Fries et al., 2006; Klee et al., 2007; Paxton et al., 2007; Chauzat et al., 2007; Chen et al., 2008; Williams et al., 2008a; Invernizzi et al., 2009; Tapaszti et al., 2009; Giersch et al., 2009). Those reports also confirm that *N. ceranae* is not restricted to *A. cerana* in East Asia as it was originally considered (Fries et al., 1996; Fries, 1997). Recently, the presence of *N. ceranae* (Microsporidia) was detected even in North African honey bees (*A. mellifera intermissa*) (Higes et al., 2009a).

According to Klee et al. (2007), *N. ceranae* is an emergent pathogen of the European honey bee that most likely jumped from its host, the Asian honey bee *A. cerana,* to *A. mellifera*. Although it is still impossible to determine the exact time of the jump and transmission route of *N. ceranae* from *A. cerana* to *A. mellifera*, Klee et al. (2007) suggested that the process occurred most likely within the last decade, based on pure *N. apis* infection revealed in older (pre-2003) samples of *A. mellifera*. The same authors proposed that *A. mellifera* was formerly parasitized by *N.*

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apis alone and not *N. ceranae* and that *N. ceranae*, as an exotic parasite of *A. mellifera*, is displacing *N. apis* in *A. mellifera* populations. This view is supported by the results of Paxton et al. (2007) who found that *N. ceranae* has been present in Europe (Finland) since at least 1998, and that all *Nosema* infections in 2006 involved *N. ceranae*, either alone or with *N. apis*, meaning that *N. ceranae* has increased in frequency across this time period relative to *N. apis*. In the USA, Chen et al. (2008) found *N. ceranae* in *A. mellifera* as early as in 1995 and concluded that *N. ceranae* was not a new emerging pathogen of *A. mellifera*, but had transferred from *A. cerana* to *A. mellifera* earlier than Klee et al. (2007) previously recognized. In addition, there are indications of the presence of *N. ceranae* in Uruguay before 1990 (Invernizzi et al., 2009).

Several years ago, when incomprehensible colony losses occurred, *N. ceranae* was thought to be associated with the honey bee depopulation syndrome, lower honey production and increased mortality in colonies (symptoms resembling colony collapse disorder, CCD) detected throughout the year in central and southern Europe (Higes et al., 2005, 2006; Imdorf et al., 2006). It was shown that *N. ceranae* was highly virulent when experimentally inoculated into *A. mellifera* (Higes et al., 2007), but this study lacked any controls with *N. apis*. When the virulence of *N. apis* and *N. ceranae* was directly compared in cage experiment performed in 1994, the latter induced significantly higher mortality in comparison to the former (Paxton et al., 2007). According to Martín-Hernández et al. (2007), a significant causative association between the presence of *N. ceranae* and hive depopulation indicates that the colonization of *A. mellifera* by *N. ceranae* is related to bee losses accompanied by symptoms of CCD. Moreover, Higes et al. (2008, 2009b) reported sudden collapses of bee colonies in field conditions as a result of *N. ceranae* infection. On the other hand, studies of Cox-Foster et al. (2007); vanEngelsdorp et al. (2009) and Johnson et al. (2009) did not confirm *N. ceranae* as the causal agent of CCD.

In Serbia and surrounding Balkan countries, *N. apis* was considered the only *Nosema* species that infects *A. mellifera* colonies, until Klee et al. (2007) revealed *N. ceranae* in honey bees in south Serbia in 2006. However, the prevalence and distribution of this species throughout Serbia is still obscure, and no data about *Nosema* species in honey bees are available for surrounding countries, Bosnia and Herzegovina, Montenegro and Former Yugoslav Republic of Macedonia (FYROM). According to the records of Veterinary Directorate, Ministry of Agriculture, Forestry and Water Management, Republic of Serbia (MAFWMRS) there were no significant changes in the incidence of nosemosis in Serbia in the last decade. However, lack of seasonality, a characteristic of nosemosis caused by *N. ceranae* (Martín-Hernández et al., 2007), was reported by beekeepers in the last three-year time.

Today, molecular techniques are available that enable accurate differentiation between *N. apis* and *N. ceranae*: sequencing of the 16S SSU rRNA gene (Fries et al., 1996), PCR–RFLP of partial SSU rRNA (Klee et al., 2007), and duplex PCR using species-specific primer pairs (Martín-Hernández et al., 2007). The two latter methods were used in this study to reveal the species of *Nosema* prevalent in honey bees in Serbia, Bosnia and Herzegovina, Montenegro and FYROM in last three-year time (2006–2009) and to demonstrate whether or not *N. ceranae* displaced *N. apis* in these countries in the recent past. Furthermore, older samples collected between 2000 and 2005 from Serbia were analysed to reveal if *N. ceranae* is an emerging parasite that became prevalent in *A. mellifera* recently, or if *A. mellifera* has been harbouring *N. ceranae* for some time but was overlooked in routine microscopical diagnosis due to its morphological similarity with *N. apis*.

2. MATERIALS AND METHODS

2.1. Samples, spore detection and DNA extraction

A total of 325 honey bee samples were collected from *Apis mellifera* colonies located in four Balkan countries: Serbia (N = 215), Bosnia and Herzegovina (N = 45), Montenegro (N = 28) and FYROM

(N = 37) in the years 2006–2009. Samplings were done in accordance with the guidelines of the Office International des Epizooties (OIE, 2008) by official person (Ivan Pavlovic, details given in Acknowledgements). Each sample was comprised of bees from the same hive (at least 60 adult forager bees collected from the hive entrance). Three to six hives were sampled per apiary/location. Sampling locations were picked depending on their geographic position with the aim of getting samples throughout the chosen Balkan countries. Bees were sampled in spring and summer (March–July). Hives were sampled irrespective of nosemosis and/or signs of other diseases; for each sampled colony it was recorded if the characteristics of CCD (sudden disappearance of adult bees prior to colony death, unattended brood, colony weakness, no dead or trembling bees around the hives) were observed at the time of sampling and in the years afterwards. In the final re-evaluation, updated CCD characteristics defined by vanEngelsdorp et al. (2009) were taken into consideration. Among the samples from Serbia 35 were older, collected between 2000 and 2005 (provided by veterinary institutes). These were collected in spring; the colonies from which these samples originated were categorised in the institutes' protocols as *Nosema*-positive with medium infection. No CCD symptoms were recognized after reviewing the records of the bee owners.

The samples were examined microscopically at 400× magnification for the presence of *Nosema* spores according to the guidelines of OIE (OIE, 2008). Only *Nosema*-positive samples (N = 273) were used for DNA extraction (178 from Serbia, 38 from Bosnia and Herzegovina, 22 from Montenegro and 35 from FYROM). From each colony, abdomens of 60 adult bees were homogenized in a mortar with 60 mL of ddH_2O. In case of *Nosema*-positive samples, homogenates were filtered to remove coarse bee parts and obtained spore suspensions stored at –20 °C until used for DNA extraction.

For DNA extraction, 1 mL of spore suspension was centrifuged (5 min, 16 100 *g*) and the supernatant discarded. Pellets were frozen in liquid nitrogen and crushed using sterile pellet pestles. DNA was extracted applying DNeasy Plant Mini Extraction Kit (Qiagen, Cat. No. 69104). The extracts were kept at –20 °C until needed as DNA template in PCRs. Reference DNA extracts of *N. apis* and *N. ceranae* (kindly provided by Eva Forsgren, SLU-Uppsala, Sweden) confirmed to be positive by sequencing were used as the template for the positive controls. Double-distilled water (ddH_2O) was the template for the negative control.

2.1.1. *Identification of Nosema species with duplex PCR*

In duplex PCR, species-specific primers designed by Martín-Hernández et al. (2007) were used for differential diagnosis of *N. apis* and *N. ceranae* (321APIS-FOR/REV primers for detection of *N. apis* and 218MITOC-FOR/REV primers for detection of *N. ceranae*).

PCR conditions given by Martín-Hernández et al. (2007) were followed, with slight modifications. All PCRs were performed using a Mastercycler Personal (Eppendorf) in 25 μL volumes containing 1× PCR-buffer A (Kapa Biosystems), 3 mM $MgCl_2$ (Kapa Biosystems), 0.4 mM of each deoxynucleoside triphosphate (Qiagen), 0.4 μM of each primer (321APIS-FOR, 321APIS-REV, 218MITOC-FOR, 218MITOC-REV, made by Invitrogen, Carlsbad, CA), 0.2 mg/mL bovine serum albumin (New England Biolabs), 0.5 U of *Taq* polymerase (Kapa Biosystems) and 5 μL of template DNA.

The thermocycler program consisted of 94 °C for 4 min, followed by 10 cycles of 15 s at 94 °C, 30 s at 61.8 °C, and 45 s at 72 °C, 20 cycles of 15 s at 94 °C, 30 s at 61.8 °C, and 50 s at 72 °C plus an additional 5 s of elongation for each successive cycle, and a final extension step at 72 °C for 7 min. For each PCR, positive controls (reference *N. apis* and *N. ceranae* DNA extracts as template) and negative controls (ddH_2O as template) were run along with DNA extracts of isolates as template. PCR products (4 μL of amplified DNA) were electrophoresed on 2% agarose gels (1× TBE), stained with ethidium bromide, and visualised under UV light. A commercial O'RangeRuler™ 50 bp DNA Ladder (Fermentas) was used as a size marker.

2.1.2. *Identification of Nosema species with PCR-RFLP*

A region of the 16S rRNA gene which is conserved for *N. apis* and *N. ceranae* (Klee et al., 2007) was selected for primer design using MacVector 6.5 (Oxford Molecular Ltd., Oxford, UK). Primers nos-16S-fw (5′- CGTAGACGCTATTCCCTAAGATT -3′, positions 422 to 444 in U97150; Gatehouse and Malone, 1998) and

nos-16S-rv (5′– CTCCCAACTATACAGTACAC-CTCATA -3′, positions 884 to 909 in U97150, Gatehouse and Malone, 1998) were used to amplify ca. 488 bp of partial 16S rRNA gene. All PCRs were performed using a Mastercycler Personal (Eppendorf) in 25 μL volumes containing 1× PCR-buffer A (Kapa Biosystems), 2.5 mM $MgCl_2$ (Kapa Biosystems), 0.2 mM of each deoxynucleoside triphosphate (Qiagen), 0.5 μM of each primer (nos-16S-fw, nos-16S-rv, made by Operon Biotechnologies, Inc., Huntsville, Al), 0.5 U of *Taq* polymerase (Kapa Biosystems) and 5 μL of template DNA.

The PCR parameters for amplification were: initial DNA denaturation of 4 min at 95 °C followed by 45 cycles of 1 min at 95 °C, 1 min at 53 °C and 1 min at 72 °C, and terminated with a final extension step at 72 °C for 4 min. For each PCR, positive controls (reference *N. apis* and *N. ceranae* DNA extracts as template) and negative controls (ddH_2O as template) were run along with DNA extracts of isolates as template. PCR products (4 μL of amplified DNA) were electrophoresed on 1.4% agarose gels (1× TBE), stained with ethidium bromide, and visualised under UV light. A commercial O'RangeRuler™ 50 bp DNA Ladder (Fermentas) was used as a size marker.

To differentiate between the species *N. apis* and *N. ceranae*, discriminating restriction endonuclease sites present in the PCR amplicon were used (Klee et al., 2007). The restriction endonuclease *Pac* I provides one unique digestion site for *N. ceranae* whilst the enzyme *Nde* I only digests *N. apis*. *Msp* I digests *N. apis* and *N. ceranae* and is used as a control for successful restriction digestion of PCR products. The predicted restriction fragments produced from digestion of the PCR amplicons are 97 bp, 118 bp, and 269 bp for *N. ceranae* and, 91 b, 131 bp and 266 bp for *N. apis* (Fig. 1). PCR amplicons were digested with *Msp* I/*Pac* I and with *Msp* I/*Nde* I (New England Biolabs, NEB) in two reactions at 37 °C for 3 h to analyze and confirm the presence of each *Nosema* species in each sample. Digests were performed in 12.5 μL volume with 10 μL of the amplified DNA and 1.5 Units of each enzyme. The 1× NEBuffer 2 (provided by NEB with *Msp* I) was used as buffer for the reactions. *Msp* I and *Nde* I show 100% activity with this buffer whilst *Pac* I performs to 75% in this buffer. Fragments were separated in a 3% NuSieve agarose gel (Cambrex Bio Science) in 1× TBE buffer with a 20 bp ladder as size marker at 110 V for 1 h 30 min. Gels were stained with ethidium bromide and visualised under UV light.

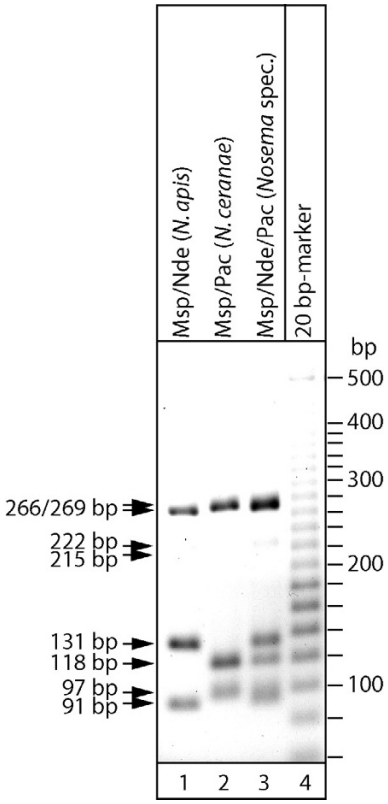

Figure 1. Restriction fragment length polymorphism (RFLP) of a 16S rRNA gene region conserved for *N. apis* and *N. ceranae*. A conserved region of the 16S rRNA gene of *N. apis* and *N. ceranae* was amplified by PCR. Subsequently, discriminating restriction endonuclease sites within this region were used to differentiate between the two Nosema species. Digestion by *Msp* I/*Nde* I results in three bands (91 bp, 131 bp, 266 bp) for *N. apis* (lane 1). Digestion by *Msp* I/*Pac* I results in three bands (97 bp, 118 bp, 269 bp) for *N. ceranae* (lane 2). Weak bands of 222 bp and 215 bp represent incomplete digests by *Nde* I for *N. apis* and *Pac* I for *N. ceranae*, respectively. Mixed infections can also be identified using this method (lane 3).

3. RESULTS

By microscopic examination of collected bee samples (N = 325) 273 were found positive for *Nosema* sp. (84.0%). In samples from Serbia, 178 (including all 35 older samples collected between 2000 and 2005) out of 215 examined, were *Nosema*-positive

Table I. The results of differential diagnostic investigations of *Nosema* species in *Apis mellifera* throughout Serbia, Bosnia and Herzegovina, Montenegro and FYROM[a].

Country	No. of collected samples	No. of *Nosema*-positive samples	% of *Nosema*-positive samples	*N. ceranae*	*N. apis*	Co-infections
Serbia	215*	178*	82.8%	177*	1	/
Bosnia and Herzegovina	45	38	84.4%	38	/	/
Montenegro	28	22	78.6%	22	/	/
FYROM	37	35	94.6%	35	/	/
Overall	325	273	84.0%	272	1	/

[a] FYROM, Former Yugoslav Republic of Macedonia. * Including 35 historical samples.

(82.8%). The percentage of *Nosema*-positive samples was 84.4% (38 infected out of 45 examined) among those from Bosnia and Herzegovina, 78.6% (22 infected out of 28) from Montenegro and 94.6% (35 infected out of 37) from FYROM (Tab. I). *Nosema*-positive samples originated from apiaries scattered across the monitored countries (Fig. 2). All positive samples (N = 273) underwent further analysis to identify the species of *Nosema*.

Duplex PCR with species-specific primers (321APIS-FOR/REV and 218MITOC-FOR/REV) enabled differentiation between *N. apis* and *N. ceranae* in 224 samples out of 273 analysed (82% successful amplifications), while 49 samples failed to produce a PCR product (18%).

As expected, the PCR product of the reference *N. apis* sample with the primer pair 321APIS-FOR/REV was 321 bp, and the PCR product of reference *N. ceranae* sample with primer pair 218MITOC-FOR/REV was 218–219 bp. None of the two fragment lengths were present in the negative controls. Moreover, no co-infections were found. Among successfully amplified samples, 223 amplicons exibit a length of 218–219 bp which corresponds to *N. ceranae*, and only one sample (collected in the northernmost of Serbia in 2008) produced amplicon of 321 bp in length corresponding to *N. apis* (Fig. 3).

All 273 samples (100%) were successfully amplified with primers nos-16S-fw/rv and produced amplicons approximately 488 bp in size (Fig. 4a). After digestion with the enzymes *Pac*I, *Nde*I and *Msp*I, RFLP patterns of 272 samples corresponded to that of *N. ceranae* control, and one sample matched the pattern of *N. apis* control (the same sample found to be *N. apis* in duplex PCR). No co-infections were found (Fig. 4b). The results of this study are summarized in Table I.

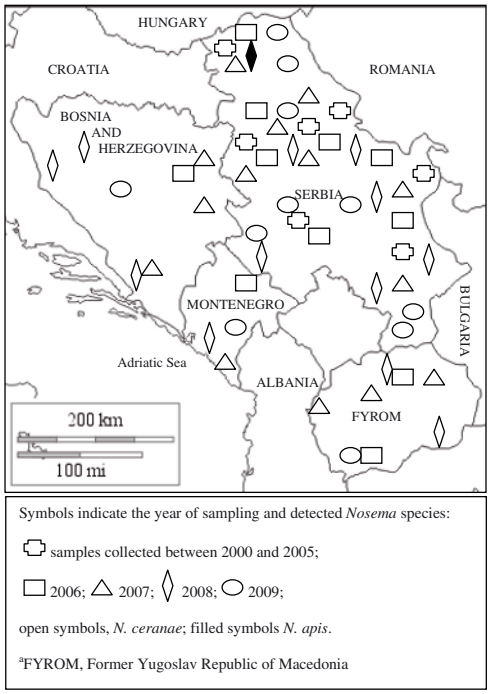

Figure 2. Map indicating sampling locations in Serbia, Bosnia and Herzegovina, Montenegro and FYROM[a], and *Nosema* species distribution.

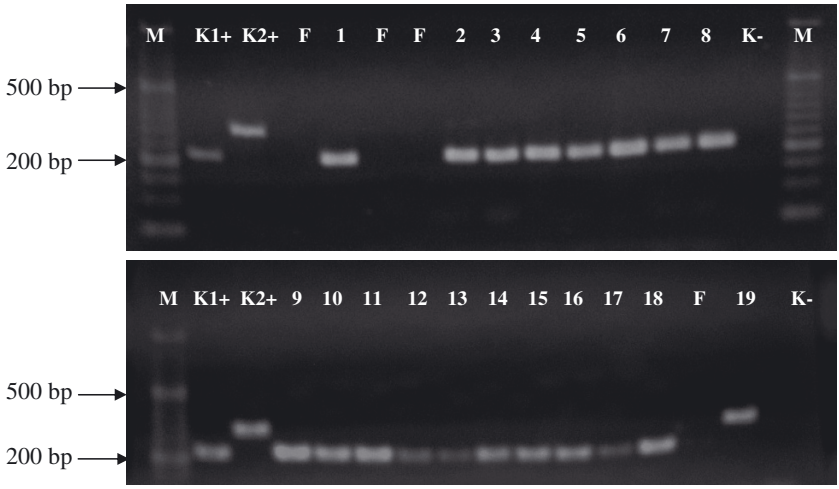

Figure 3. Ethidium bromide stained agarose gels showing the results of duplex PCR using primers 321APIS-FOR/REV and 218MITOC-FOR/REV. Lane M, 50 bp ladder; K1+, positive *N. ceranae* control; K2+, positive *N. apis* control; F, failed PCR; K-, negative control; 1–18, PCR products of samples that correspond to *N. ceranae*; 19, PCR product of a single sample that correspond to *N. apis*.

Furthermore, no characteristics of CCD were observed in the colonies sampled in this study, either at the moment of sampling or at subsequent re-evaluation steps.

4. DISCUSSION

As in other geographic regions of Europe, *N. apis* was suspected as the single cause of nosemosis in *A. mellifera* colonies in Serbia and surrounding Balkan countries (Bosnia and Herzegovina, Montenegro and FYROM). The first report of *N. ceranae* in south Serbia (Klee et al., 2007) and the lack of data from surrounding Balkan countries indicated the need for differential diagnostic investigations of *Nosema* species in honey bees throughout Serbia, Bosnia and Herzegovina, Montenegro and FYROM. In addition, the recent reports of causative association between the presence of *N. ceranae*, bee losses (Martín-Hernández et al. 2007) and the sudden colony collapse of western honey bees due to *N. ceranae* (Higes et al., 2008, 2009b) justify such investigations.

The results of this study demonstrated *N. ceranae* in 272 samples and *N. apis* in only one sample which suggest the dominance of *N. ceranae* infection in all Balkan countries monitored throughout the last three years (2006–2009).

In this study, the presence and dominance of *N. ceranae* in *A. mellifera* in the territory of Bosnia and Herzegovina, Montenegro and FYROM in the period from 2006–2009 is first reported. No *N. apis* or mixed *N. apis*/*N. ceranae*-infections were detected in samples from these countries. These results indicate that *N. ceranae* did not displace *N. apis* in these countries in the recent past, but any discussion about the time when the replacement took place is a matter of speculation due to the absence of samples taken prior to 2006 originating from these Balkan countries.

The detection solely of *N. ceranae* in older samples collected between 2000 and 2005 from Serbia indicates that this species has been present in Serbia since at least 2000, but remained undetected since molecular techniques that enable differential diagnosis of *N. apis* and *N. ceranae* were not available at that time. The finding of *N. ceranae* in older samples (from 2000 onwards) revealed that this parasite may have caused the infection in honey bees in Serbia earlier than it had been previously recognized by Klee et al. (2007). *N. apis* was

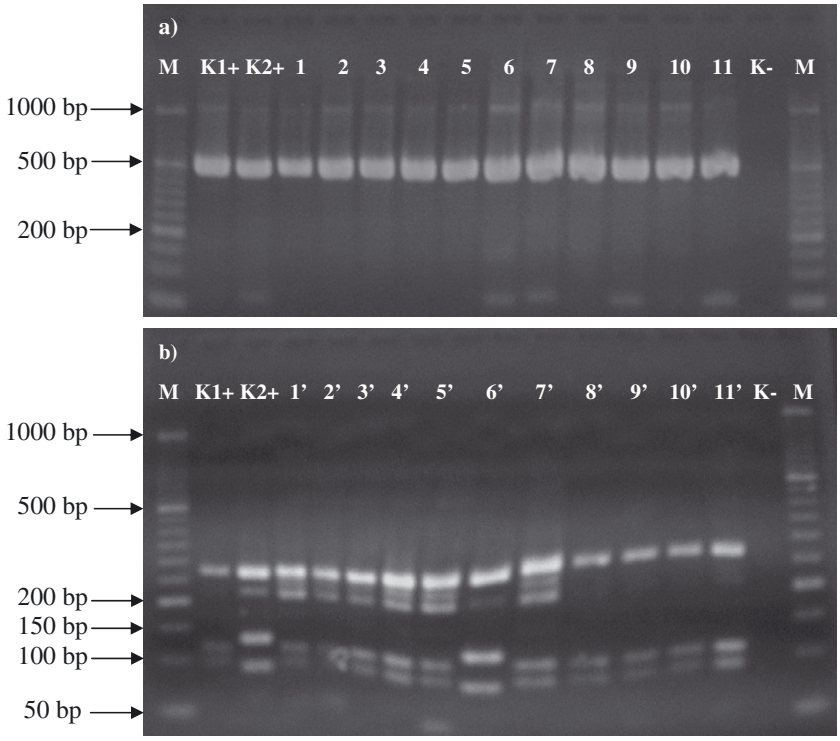

Figure 4. Ethidium bromide stained agarose gels showing the results of PCR-RFLP. a) 1–11, PCR products using primers nos-16S-fw/rv; Lane M, 50 bp ladder; K1+, positive *N. ceranae* control; K2+, positive *N. apis* control; K-, negative control. b) RFLP products after digestion with the enzymes *Msp*I, *Pac*I and *Nde*I. Lane M, 50 bp ladder; K1+, positive *N. ceranae* control; K2+, positive *N. apis* control; K-, negative control; 1′–5′ and 7′–11′, RFLP of samples that correspond to *N. ceranae*; 6′, RFLP of a single sample that correspond to *N. apis*.

not found in any of older samples collected between 2000 and 2005. In the samples collected in the period 2006–2009, *N. apis* was detected in only one sample (collected from the northernmost of Serbia in 2008). Although most honey bee colonies and individual bees may be co-infected with both microsporidian species, *N. apis* and *N. ceranae* (Klee et al., 2007; Paxton et al., 2007), no co-infections were found in our study, similarly to the findings of Huang et al. (2008) in Taiwan and Martinique. Such results question whether or not *N. apis* was ever prevalent in Balkan countries. The impressive dominance of *N. ceranae* confirmed by analysing a considerable number of samples in this study, including older samples collected between 2000 and 2005, as well as the absence of any molecular evidence of *N. apis* as a predominant honey bee microsporidian in Serbia, may indicate that *N. ceranae* is not an emergent pathogen of honey bees in Serbia, at least it was not in the last 10 years. This is congruent with the data of MAFWMRS that state that the incidence of nosemosis has not changed throughout the last 10 years, although lack of seasonality, characteristic for *N. ceranae* (Martín-Hernández et al., 2007), was reported by beekeepers in the last three years. As the symptoms of CCD were not observed in the apiaries sampled in this study, the association between *N. ceranae* and CCD cannot be affirmed, which coincides with the most recent findings of vanEngelsdorp et al. (2009) and Johnson et al. (2009).

In all the Balkan countries monitored, the usage of fumagillin for the control of

nosemosis has been common and extensive, despite the fact that if not used properly it can have harmful effects on human health (Stanimirovic et al., 2006, 2007; Stevanovic et al., 2006, 2008). However, future studies are necessary to reveal if fumagillin could have played a role in the displacement of *N. apis* with *N. ceranae*, since its efficacy against both parasite species has been proven, but without simultaneous comparative investigations (Williams et al., 2008b).

The identification of *Nosema* species in this study was done using two methods: duplex PCR according to Martín-Hernández et al. (2007) and PCR-RFLP deploying the primers nos-16S-fw/rv. The efficacy in achieving the final results was 82% over 100% in favour of PCR-RFLP. Although PCR-RFLP is more time-consuming and more expensive than duplex-PCR detection, PCR-RFLP should be the method of choice if 100% accurate results are needed.

The subspecies of *A. mellifera* and the duration of exposure to the pathogen were suggested as factors that may affect the virulence of *N. ceranae* (Invernizzi et al., 2009). In the present study there were two southeastern European subspecies, *A. m. carnica* and *A. m. macedonica*, which both belong to the C2D and C2E mtDNA haplotype (Susnik et al., 2004; Kozmus et al., 2007) of the C Mediterranean lineage (Garnery et al., 1992; Franck et al., 2000; Meixner et al., 2007). Indigenous non-hybridized *A. m. carnica* populations in the region (Stanimirovic et al., 2005; Sušnik et al., 2004; Kozmus et al., 2007), an important genetic resource for future bee breeding programs (Stanimirovic et al., 2005; De la Rua, 2009), may be more resistant to *N. ceranae*. Nevertheless, the influence of bee subspecies on their susceptibility could be the subject of further investigation. Finally, there may be distinctions in the virulence of the haplotypes of *N. ceranae* (Williams et al., 2008a, b) similarly to the genotype-specific ones of *Paenibacillus larvae* (Genersch et al., 2005, 2006; Ashiralieva and Genersch, 2006). Thus, if the bees from two distant European regions harbor different *N. ceranae* haplotypes, a different outcome of *N. ceranae* infection can be easily comprehensible.

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Dominance *de Nosema ceranae* chez les abeilles des pays balkaniques en l'absence de symptômes du syndrome d'effondrement des colonies.

***Nosema ceranae* / PCR-RFLP / PCR duplex / pays des Balkans / abeilles**

Zusammenfassung – ***Nosema ceranae* dominiert in den Honigbienenvölkern auf dem Balkan in Abwesenheit von Symptomen von ‚colony collapse disorder' (CCD).** Honigbienenvölker (*Apis mellifera*) wurden in Serbien, Bosnien-Herzegowina, Montenegro und der Ehemaligen jugoslawischen Republik Mazedonien (Former Yugoslav Republic of Macedonia, FYROM) zwischen 2006 und 2009 beprobt, um zu untersuchen, ob und mit welcher *Nosema*-Spezies diese Völker in den letzten drei Jahren infiziert waren. Zusätzlich wurden Nosema-positive Bienenproben aus Serbien aus dem Zeitraum 2000–2005 untersucht, um die Frage zu beantworten, ob *N. ceranae* entweder ein neuer Krankheitserreger ist, der erst seit kurzem *A. mellifera* infiziert, oder schon seit längerer Zeit in den untersuchten Ländern bei *A. mellifera* vorkommt. Zur Differenzierung von *N. apis* und *N. ceranae* wurden zwei verschiedene molekulare Methoden eingesetzt: Duplex-PCR-Analysen unter der Verwendung Spezies-spezifischer Primer sowie PCR-RFLP-Analysen, die mit hier erstmals veröffentlichten Primern (nos-16S-fw/rv; Abb. 1)

durchgeführt wurden. Insgesamt wurden 273 Nosema-positive Proben (N = 178, 38, 22 und 35 jeweils aus Serbien, Bosnien-Herzegowina, Montenegro und Mazedonien) untersucht. In nur einer Probe wurde *N. apis* nachgewiesen, während in allen anderen Proben (N = 272) einschließlich der älteren Proben aus Serbien, die aus dem Zeitraum 2000–2005 stammten, nur *N. ceranae* nachgewiesen werden konnte (Tab. I, Abb. 2–4). Mischinfektionen wurden nicht gefunden. Diese Ergebnisse zeigen, (1) dass *N. ceranae*-Infektionen in den genannten Balkanstaaten in den letzten drei Jahren klar dominierten, und (2) dass *N. ceranae* in Serbien mindestens seit dem Jahr 2000 vorkommt und hier nicht erst kürzlich *N. apis* in der Honigbienenpopulation verdrängt hat. Darüber hinaus zeigte der Methodenvergleich, dass die PCR-RFLP-Analysen mit den neuen Primern nos-16S-fw/rv im Vergleich zur Duplex-PCR genauere Ergebnisse liefern (100 % gegenüber 82% korrekt positive Ergebnisse). Ein wichtiges Ergebnis dieser Studie ist auch, dass das Vorkommen von *N. ceranae* in den untersuchten Ländern nicht einherging mit einem Anstieg an Nosemosis oder mit Völkerverlusten, die von „colony collapse disorder"-ähnlichen (CCD-ähnlichen) Symptomen begleitet waren.

***Nosema ceranae* / PCR-RFLP / Duplex-PCR / Balkanstaaten**

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