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The first report of the prevalence of *Nosema ceranae* in Bulgaria

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Nosema apis and *Nosema ceranae* are the two main microsporidian parasites causing nosematosis in honey bee *Apis mellifera*. The object of the present study is to investigate the presence of *Nosema apis* and *Nosema ceranae* in the Bulgarian honey bee. The 16S (SSU) rDNA gene region was chosen for analysis. A duplex PCR assay was performed on 108 honey bee samples from three different parts of the country (South, North and West Bulgaria). The results showed that the samples from the northern part of the country were with the highest rate of invasion (77.2%) for *Nosema ceranae* while those from the mountainous parts (the Rodopa Mountains, South Bulgaria) were with the lowest rate (13.9%). Infection with *Nosema apis* alone and co-infection *N. apis/N. ceranae* were not detected in any samples. These findings suggest that *Nosema ceranae* is the dominant species in the Bulgarian honey bee. It is not known when the introduction of *Nosema ceranae* in Bulgaria has occurred, but like in the rest of the world, this species has become the dominant one in Bulgarian *Apis mellifera*. In conclusion, this is the first report for molecular detection of *Nosema* infection in Bulgarian honey bee that confirms the worldwide dissemination and prevalence of *Nosema ceranae*.

Title: The first report of the prevalence of *Nosema ceranae* in Bulgaria

Short Title: Colonization of the Bulgarian honey bee by *Nosema ceranae*

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Abstract

Nosema apis and *Nosema ceranae* are the two main microsporidian parasites causing nosematosis in honey bee *Apis mellifera*. The object of the present study is to investigate the presence of *Nosema apis* and *Nosema ceranae* in the Bulgarian honey bee. The 16S (SSU) rDNA gene region was chosen for analysis. A duplex PCR assay was performed on 108 honey bee samples from three different parts of the country (South, North and West Bulgaria). The results showed that the samples from the northern part of the country were with the highest rate of invasion (77.2%) for *Nosema ceranae* while those from the mountainous parts (the Rodopa Mountains, South Bulgaria) were with the lowest rate (13.9%). Infection with *Nosema apis* alone and co-infection *N. apis/N. ceranae* were not detected in any samples. These findings suggest that *Nosema ceranae* is the dominant species in the Bulgarian honey bee. It is not known when the introduction of *Nosema ceranae* in Bulgaria has occurred, but like in the rest of the world, this species has become the dominant one in Bulgarian *Apis mellifera*.

In conclusion, this is the first report for molecular detection of *Nosema* infection in Bulgarian honey bee that confirms the worldwide dissemination and prevalence of *Nosema ceranae*.

Key words: *Apis mellifera*, *Nosematosis*, PCR diagnosis, Bulgaria

Inroduction

Bulgaria has a long tradition in beekeeping. Historically, this occupation existed before the First Bulgarian Empire. Initially, local people gathered honey from feral bees, while later they started to raise them in apiaries. Gradually, beekeeping became a significant occupation and honor in human life. It is not surprising that this hard-working insect has been presented in songs, traditions and beliefs. From honey, the ancient people prepared mead – an alcoholic beverage created by fermenting honey with water. The first Bulgarian empire kept lively trade with Genoa, Venice, Dubrovnik, and the Eastern Roman Empire etc.

Honey bee colonies suffer from various specific pathogens. These include various bacteria, viruses, fungi and endo- and ecto-parasites. Some of them, microsporidians, are obligate intracellular parasites belonging to the kingdom Fungi (Keeling and McFadden, 1998; Hirt et al., 1999; Sina et al., 2005*). Nosema* is a microsporidian genus causing an infection called *Nosematosis* of adult honey bees. Until now two main species of *Nosema* parasites have been recognized – *Nosema apis* and *Nosema ceranae*. It is well known that *N. apis* is specific for the Western honey bee, *Apis mellifer*a L., whilst the Eastern honey bee *Apis cerana* harbours *Nosema ceranae* (Fries et al., 1996). However, many recent investigations have revealed that *N. ceranae* is not restricted only to *A. cerana*, but it transferred to *A. mellifera*, and even became a dominant species in many parts in the world (Klee et al., 2007; Paxton et al., 2007; Chen et al., 2008; Invernizzi et al., 2009; Tapaszti et al., 2009; Stevanovic et al., 2010; Gajger et al., 2010; Papini et al., 2017). The exact time and transmission route of transfer of *N. ceranae* from *A. cerana* to *A. mellifera* is not known worldwide. It is possible that during the last decades, the rapid, long-distance dissemination of *N. ceranae* is likely due to the transport of infected honey bees and/or by the increased mobility of people, goods and livestock.

There are two main techniques for identifying *Nosema* species – microscopic and molecular. The microscopic methods such as Light microscopy, Giemsa and Toluidine staining, and Transmission electron microscopy were introduced first (Fries et al., 2013), but they are still a valuable, relatively cheap and simple method for screening and identification of *Nosema* infection. Despite the fact that *N. apis* and *N. ceranae* spores are morphologically different, in case of low rate of invasion or presence of vegetative forms of *Nosema,* they are often not detected using microscopy techniques. This requires the search for methods that are more sensitive. Therefore, various molecular methods have been developed. Those include mainly PCR techniques (conventional or duplex PCR, PCR-RFLP, qPCR etc.) (Evans et al., 2013) involving usually a wide range of species-specific PCR primers (Martín-Hernández et al., 2007; Klee et al. 2007; Chen et al. 2008).

Until now, there has been no data regarding the distribution of *N. apis* and *N. ceranae* throughout Bulgaria as well as information if *N. ceranae* has become a dominant species, although Nosema infection for the surrounding Balkan countries is well studied (Stevanovic et al., 2010; Whitaker et al., 2010; Hatjina et al., 2011).

The main goal of the current study is to investigate and determine the presence and spreading of the two different *Nosema* spp. in the Bulgarian honey bee. Because this is the first report regarding the distribution of *Nosematosis* in Bulgaria, we believe that the results of this investigation will improve the overall picture not only for the Balkans, but also worldwide.

Materials and Methods

Sample collection. A total of 108 honey bee samples were collected from three different parts in the country: Rousse district (North Bulgaria, N=44), Sofia district (West Bulgaria, N=28) and

Smolyan district (South Bulgaria, N=36), (Table 1, Fig. 1) in April-May 2017. The first two regions are characterized by their low-lying and generally flat plains, while the last region is situated in the Rodop Mountains. Sampling was done according to the guidelines of the Office International des Epizooties (2008). None of the honey bee colonies were treated against *Nosema* infection for at least 6 months. In each hive, five adult worker honey bees were randomly selected at entrance of the hive or on frames away from the brood nest. The honey bees were placed in a falcon tube, put in a cooler bag and stored at – 20 °C prior to analysis.

DNA extraction. Briefly, prior to DNA extraction, the abdomen of single bee was cut off with scissors, mechanically homogenized with a cell lysis buffer and centrifuged for 1 min at 15 000 rpm. The pellet was resuspended in a cell lysis buffer; proteinase K was added and incubated overnight at 56 °C. Total DNA was isolated by using GeneMATRIX Tissue and Bacterial DNA purification Kit (Cat. No. E3551-01, EURx Ltd., Poland) according to the manufacturer instructions. The extracted DNA was resuspended in 50 µL of Tris:EDTA buffer (pH 8.0). The DNA concentration was determined spectrophotometrically and the quality of the DNA samples was examined on 1% agarose gel electrophoresis stained with Greensafe premium (Cat. No. MB13201, Nzytech, Portugal). The purified DNA was stored at –20 °C until PCR assay.

Gene selection and PCR amplification. The small subunit (16S) ribosomal RNA gene was chosen for molecular identification of *Nosema ceranae* and *Nosema apis*. A fragment of this gene was amplified in both *Nosema* species using primers designed by Martín-Hernández et al. (2007). 321APIS-FOR (5'-GGGGGCATGTCTTTGACGTACTATGTA-3'; 321APIS - REV (5'-GGGGGGCGTTTAAAATGGAAACAACTATG-3') for Nosema apis and 218MITOC FOR (5'-CGGCGACGATGTGATATGAAAATATTAA-3'); 218MITOC-REV: (5'-CCCGGTCATTCTCAAACAAAAAACCG- 3') for *N. ceranae*.

Both primer sets were used together for performing a duplex PCR for identification and discrimination of *Nosema* species.

The expected number of amplified bases in *N. ceranae* using the 218MITOC primers can be either 218 or 219 depending on the sequences for *N. ceranae* available in the GenBank database (http://www.ncbi.nlm.nih.gov/) (Martín-Hernández et al., 2007). In the case of *N. apis*, the expected size of the amplicon using the 321APIS primers was 321 bp. In addition, a negative control was included for all PCR reactions. The PCR mixtures contained 25 µL of NZYTaq 2× Colourless Master Mix (Cat. No. MB04002, Nzytech, Portugal), 0.4 µM of each species-specific primer (FOR/REV), 1 µL of template DNA PCR water (Cat. No. E0211-01, EURx Ltd., Poland) in a total volume of 50 µL. All PCR reactions were carried out using Little Genius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial denaturation at 94 ºC for 5 min; 30 cycles (denaturation at 94 ºC for 30 s; primer annealing at 50 ºC for 30 s; extension at 72 ºC for 1 min) and final extension at 72 ºC for 10 min. PCR products were visualized on 2 % agarose gel with Greensafe premium (Cat. No. MB13201, Nzytech, Portugal). The fragment size was determined using Gene-Ruler™ 100 bp Ladder Plus (Cat. No. SM0323, ThermoFisher Scientific Inc.).

Results

Duplex PCR with species-specific primers (321APIS-FOR/REV and 218MITOC-FOR/REV) produced PCR products in 57 samples out of 108 analyzed (52.8% successful amplifications), while 51 samples failed to produce a PCR product (47.2%). There were no PCR products in the negative controls.

The highest level of infection was observed in North Bulgaria. From all 44 investigated samples, 34 (77.2%) were *Nosema* positive (Table 1). In the west part of the country (Sofia district), *Nosema* positive samples were detected in 18 from all 28 studied samples (64.3%). These two regions are situated in flat parts of the country. The lowest level of infections was found in the honey bee samples from the mountainous part of the country (Smolyan district, the Rodopa Mountains). From all 36 investigated samples, only 5 were *Nosema* positive. Surprisingly, in all the studied samples from three different regions of the country only *Nosema ceranae* was found. The presence of *Nosema apis* as well as co-infections *N. apis/N. ceranae* were not detected (Table 1). Moreover, the honey bee samples from the flat part of the country (Sofia and Rousse districts) have demonstrated a higher level of invasion as compared with samples obtained from the mountain part (Smolyan district).

Discussion

Bulgaria has long-standing traditions in the production of honey and bee products, a precondition for which is the varied and rich vegetation of the Balkan Peninsula suitable for the production of honey and also the favorable natural, climatic and ecological conditions.

In the current study we have presented for the first time molecular identification of two *Nosema* spp. and their distribution in Bulgaria. The results indicate the presence of *N. ceranae* in 57 of 108 investigated samples, which suggests the dominance of *N. ceranae* in all investigated regions. The results of many studies from Balkan countries have indicated that *N. ceranae* displaces *N. apis* (Stevanovic et al., 2010; Whitaker et al., 2010; Hatjina et al., 2011; Gajger et al., 2010) (Fig. 1). One reasonable question is why this introduced parasite (*N. ceranae*) has become in a short time the dominant species worldwide? Concerning the virulence of the two *Nosema* spp., the results are contradictory. It is an interesting fact that in many European countries numerous studies report that *N. ceranae* is more virulent and thus possesses a competitive advantage compared to *N. apis* (Klee et al., 2007; Paxton et al., 2007; Forsgren and Fries, 2010). Contrary to this, more recent research, done mainly in the USA, does not support these observations (Huang et al., 2015; Milbrath et al., 2015). These studies suggest that the US honey bees may be less susceptible to *N. ceranae* infections than European bees or that the US isolates of the pathogen are less infective and less virulent than European isolates.

These findings are a suitable way to explain our results. We found that *N. ceranae* infection prevailed in honey bee colonies from the part of the country characterized by a more flat landscape (Rousse and Sofia district), while in the mountainous parts (Smolyan district, the Rodopa Mountains) the infection was the lowest (Table 1). Different subspecies of *Apis mellifera* are raised in flat and in mountainous regions. *A. m. macedonica* is considered to be a native honey bee for Bulgaria (Ruttner, (1988). More than three decades ago, *A. m. ligustica*, *A. m. carnica* and *A. m. caucasica* were

introduced and were bred in Bulgaria (Bouga et al., 2011). These subspecies are disseminated mainly in the flat regions of the country. On the other hand, in Bulgaria there exists a local honey bee subspecies called *A. m. rodopica*, geographically distributed only in the Rodopa Mountains massive (Petrov, 1995; Bouga et al., 2011; Ivanova et al., 2012; Nikolova and Ivanova, 2012). According to Petrov (2010), *A. m. rodopica* possesses a lot of advantages compared to the introduced subspecies – good adaptation to the specific local climatic conditions, resistance to diseases, etc. This may explain why *N. ceranae* infection is the lowest in the Rodopa Mountains where *A. m. rodopica* exists. Another fact which may explain the low rate of infection of *A. m. rodopica* is the long geographical isolation of this subspecies. Moreover, the beekeepers are encouraged to raise this local honey bee and even not to allow genetic introgression with other subspecies in Bulgaria.

Another fact which may explain the high rate of infestation with *N. ceranae* in honey colonies from the plain regions compared to the mountainous regions of the country is the different climatic conditions in these places. Many papers have discussed that warmer climatic conditions favored prevalence of *N. ceranae* (Tapaszty et al., 2009; Stevanovic et al., 2011) whereas *N. apis* remains more prevalent in colder climates (Budge et al., 2010; Gisder et al., 2010).

Conclusion

This is the first report of the distribution of *Nosematosis* from honey bee colonies in Bulgaria. We found that *N. ceranae* is the dominant species in the Bulgarian honey bee. Currently we cannot answer when and how *N. ceranae* started infecting the Bulgarian honey bees and even became the prevalent *Nosema* species due to the lack of bee samples from past decades. A local honey bee *A. m. rodopica* bred in the Rodopa Mountains seems to be more resistant compared to the introduced species. Because of this, local honey bees should be kept as a part of the genetic biodiversity and the related conservation activities.

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298

299 **Figure 1.** Map showing sampling locations in Bulgaria. *Nosema* species distribution are
300 represented in all Balkan countries.

Table 1(on next page)

Table 1

Differential diagnostic investigations of (Nosema) species in Apis mellifera from three regions in Bulgaria.

Table 1. Differential diagnostic investigations of *Nosema* species in *Apis mellifera* from three regions in Bulgaria.

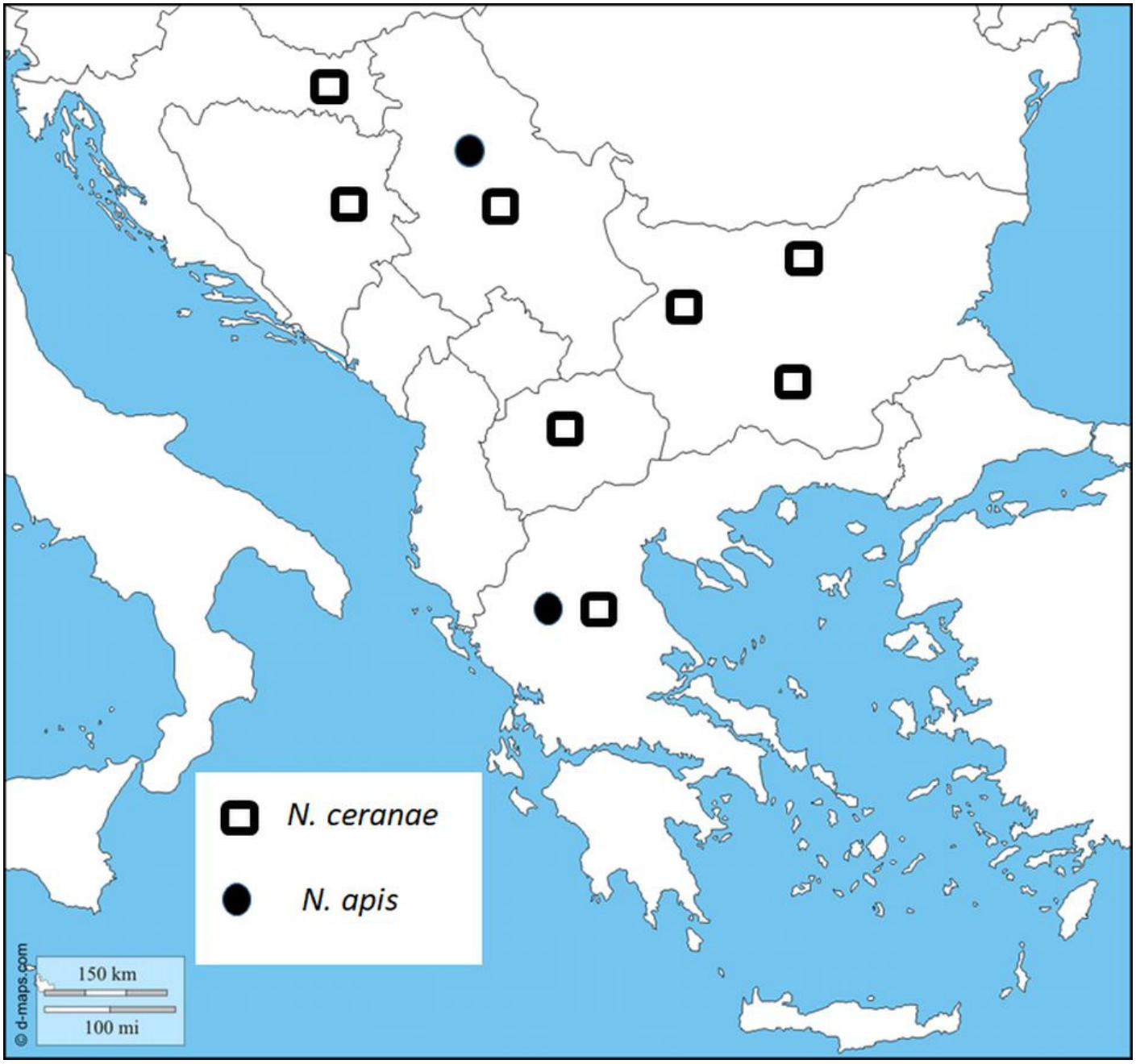
Region	**No. of collected samples**	**No. of *Nosema* positive samples**	**% of *Nosema* positive samples**	***N. ceranae***	***N. apis***	**Co-invasion**
Smolyan (SB)	36	5	13.9	5	-	-
Sofia (WB)	28	18	64.3	18	-	-
Russe (NB)	44	34	77.2	34	-	-
Total	**108**	**57**	**52.8**	**57**	**-**	**-**

Abbreviations: SB – South Bulgaria; WB – West Bulgaria; NB – North Bulgaria

Figure 1

Map

Map showing sampling locations in Bulgaria. Nosema species distribution are represented in all Balkan coutry.



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