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 aim of the present study is to investigate the presence of *Nosema apis* and *Nosema ceranae* in the area of Bulgaria. 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 prevalence (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 of honey bee in Bulgaria. The results showed that *N. ceranae* is the main *Nosema* species in Bulgaria.

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 aim of the present study is to investigate the presence of *Nosema apis* and *Nosema ceranae* in the area of Bulgaria. 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 prevalence (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 of honey bee in Bulgaria. The results showed that *N. ceranae* is the main *Nosema* species in Bulgaria.

Inroduction

The Western honey bee (*Apis mellifera* L., Hymenoptera: Apidae) is species of crucial economic, agricultural and environmental importance. The biological significance of bees is rooted in the fact that they are main pollinators in the natural environment. About 80% of the pollination of entomophilous plants is carried out by *Apis mellifera*. In all crops, active pollination significantly increases their yields. Honey bees are a valuable economic asset due to the ensemble of their products which are includes honey, bee pollen, propolis, royal jelly, and bee venom, used by humans for food and treatment.

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.

Honey bee colonies suffer from numerous 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 Nosemosis of adult honey bees (Klee et al., 2007). Only two the main species of *Nosema* causing infection in *Apis mellifera* have been recognized – *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries et al., 1996). 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; Ansari et al., 2017; 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. Recently, a so called "Colony Collapse Disorder" (CCD) disease has been described in the United States (Chen et al., 2008) and Europe (Topolska et al., 2008). The *Nosema ceranae* was suspected to one of the contributor to this illness, especially winter colony loses (Klee et al., 2007).

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.According to Ptaszyńska et al. (2014), *N. ceranae* spores seem to be more sculptured with deeper ornamentation than those of *N. apis*. Despite the fact that *N. apis* and *N. ceranae* spores are morphologically different, in case of low rate of infection or presence of vegetative forms of *Nosema,* differentiation between spores of *N. apis* and *N. ceranae* is very difficult 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) (Fries 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 distribution of the two different *Nosema* spp. in the Bulgarian honey bee.

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. There is no bias concerning the obtained honey bee samples. 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 the 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 a single bee was cut off with scissors, mechanically homogenized with a cell lysis buffer and centrifuged for 1 min at 15 000 rpm. 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. Shortly, the pellet was resuspended in a cell lysis buffer (a component of DNA purification kit); proteinase K was added and incubated overnight at 56 °C. The extracted DNA was resuspended in 50 µL of elution buffer. The DNA concentration was determined spectrophotometrically and the quality of the DNA samples was examined on 1% agarose gel electrophoresis stained with Greensafe

106 premium (Cat. No. MB13201, Nzytech, Portugal). The purified DNA was stored at –20 °C until
107 PCR assay.

108 **Gene selection and PCR amplification**. The small subunit (16S) ribosomal RNA gene was
109 chosen for molecular identification of *Nosema ceranae* and *Nosema apis*. A fragment of this gene
110 was amplified in both *Nosema* species using primers designed by Martín-Hernández et al. (2007).
111 321APIS-FOR (5'-GGGGGCATGTCTTTGACGTACTATGTA-3'; 321APIS - REV (5'-
112 GGGGGGCGTTTAAAATGGAAACAACTATG-3') for Nosema apis and 218MITOC FOR (5'-
113 CGGCGACGATGTGATATGAAAATATTAA-3'); 218MITOC-REV: (5'-
114 CCCGGTCATTCTCAAACAAAAAACCG- 3') for *N. ceranae*.

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

117 The expected number of amplified bases in *N. ceranae* using the 218MITOC primers can be
118 either 218 or 219 depending on the sequences for *N. ceranae* available in the GenBank database
119 (http://www.ncbi.nlm.nih.gov/) (Martín-Hernández et al., 2007). In the case of *N. apis*, the
120 expected size of the amplicon using the 321APIS primers was 321 bp. In addition, a negative
121 control was included for all PCR reactions. As a positive control, cytochrome c-oxidase gene
122 (*CoI*2) of *Apis mellifera* was used in all studied samples. The sequence of primers used for positive
123 control was CoI2-F (5'-CCTGATATAGCATTTCCTCG-3') and CoI2-R (5'-
124 TGTGAATGATCTAAAGGTGG-3') designed on the base of the known mitochondrial genome
125 of *A. m. ligustica* (Acc. No. L06178, Crozier and Crozier, 1993). The PCR mixtures contained 25
126 µL of NZYTaq 2× Colourless Master Mix (Cat. No. MB04002, Nzytech, Portugal), 0.4 µM of
127 each species-specific primer (FOR/REV), 1 µL of template DNA PCR water (Cat. No. E0211-01,
128 EURx Ltd., Poland) in a total volume of 50 µL. All PCR reactions were carried out using a Little

129 Genius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial
130 denaturation at 94 ºC for 5 min; 30 cycles (denaturation at 94 ºC for 30 s; primer annealing at 50
131 ºC for 30 s; extension at 72 ºC for 1 min) and final extension at 72 ºC for 10 min. PCR products
132 were visualized on a 2 % agarose gel with Greensafe premium (Cat. No. MB13201, Nzytech,
133 Portugal). The fragment size was determined using Gene-Ruler™ 100 bp Ladder Plus (Cat. No.
134 SM0323, ThermoFisher Scientific Inc.).

135 **Sequence analysis**

136 The successfully amplified products for *Nosema* (20 samples) were purified by a PCR
137 purification kit (Gene Matrix, PCR clean-up kit, EURx, Poland) and sequenced in both directions
138 by a PlateSeq kit (Eurofins Genomics Ebersberg, Germany).

139 **Results**

140 Duplex PCR with species-specific primers (321APIS-FOR/REV and 218MITOC-FOR/REV)
141 produced PCR products in 57 samples out of 108 analyzed (52.8% successful amplifications),
142 while 51 samples failed to produce a PCR product (47.2%). There were no PCR products in the
143 negative controls. The results from the obtained sequences support identity only to *Nosema*
144 *ceranae* species.

145 From all investigated samples only *Nosema ceranae* infection was detected. The highest level
146 of infection was observed in North Bulgaria. From all 44 investigated samples, 34 (77.2%) were
147 *Nosema* positive (Table 1). In the west part of the country (Sofia district), *Nosema* positive samples
148 were detected in 18 from all 28 studied samples (64.3%). The lowest level of infections was found
149 in the honey bee samples from the mountainous part of the country (Smolyan district, the Rodopa
150 Mountains). From all 36 investigated samples, only 5 (13.9%) were *Nosema* positive. Surprisingly,
151 in all the studied samples from three different regions of the country only *Nosema ceranae* was
152 found. The presence of *Nosema apis* as well as co-infections *N. apis/N. ceranae* were not detected
153 (Table 1). Moreover, the honey bee samples from the flat part of the country (Sofia and Rousse
154 districts) had a higher prevalence of *N. ceranae* infection as compared with samples obtained from
155 the mountain part (Smolyan district).

157 **Discussion**

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

172 These findings are a suitable way to explain our results. We found that *N. ceranae* infection
173 prevailed in honey bee colonies from the part of the country characterized by a more flat landscape
174 (Rousse and Sofia district), while in the mountainous parts (Smolyan district, the Rodopa Mountains)
175 the prevalence was the lowest (Table 1). Different subspecies of *Apis mellifera* are raised in flat and in
176 mountainous regions. *A. m. macedonica* is considered to be a native honey bee for Bulgaria (Ruttner,
177 1988). More than three decades ago, *A. m. ligustica*, *A. m. carnica* and *A. m. caucasica* were introduced
178 and were bred in Bulgaria (Bouga et al., 2011). These subspecies are disseminated mainly in the flat
179 regions of the country. On the other hand, in Bulgaria there exists a local honey bee subspecies called
180 *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). Our previous investigation on mitochondrial heredity have revealed that *A. m. rodopica* is distinct concerning malfunction COI protein in compared to other honey bee subspecies reread in Bulgaria (Radoslavov et al., 2017).

According to Petrov (2010), *A. m. rodopica* possesses a lot of advantages in compared to the introduced subspecies – good adaptation to the specific local climatic conditions, resistance to diseases. These findings might explain the differences in the prevalence of *N. ceranae* in the different areas since different subspecies of bees are reared in each area. 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 bee colonies from the plain regions in compared to the mountainous regions of the country is the different climatic conditions in these places. The Rodopa Mountains climate is rather colder then the other two investigated regions. This determine the later development of honey bee colonies (May-August). Moreover, malfumction of COI protein in *A. m. rodopica* may associated with lower mitochondrial respiration metabolism, which may determinate and lower activity of honey bees. 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; Natsopoulou et al., 2015).

Conclusion

This is the first report of the distribution of *N. ceranae* of honey bee colonies in Bulgaria. We found that *N. ceranae* is the dominant species in the Bulgarian honey bee. A local honey bee *A. m. rodopica*

bred in the Rodopa Mountains seems to be more resistant in 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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Table 1(on next page)

Table 1

Distribution of N. ceranae in three different regions in Bulgaria.

Table 1. Distribution of *N. ceranae* in three different regions in Bulgaria.

Region	No. of collected samples	No. of *Nosema* positive samples	% of *Nosema* positive samples	*N. ceranae*	*N. apis*	Co-infection
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

Figure 1

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