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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*.

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

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

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23

**Abstract**

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

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

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

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## Introduction

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

49 Honey bee colonies suffer from various specific pathogens. These include various bacteria,  
50 viruses, fungi and endo- and ecto-parasites. Some of them, microsporidians, are obligate  
51 intracellular parasites belonging to the kingdom Fungi (Keeling and McFadden, 1998; Hirt et al.,  
52 1999; Sina et al., 2005). *Nosema* is a microsporidian genus causing an infection called  
53 *Nosematosis* of adult honey bees. Until now two main species of *Nosema* parasites have been  
54 recognized – *Nosema apis* and *Nosema ceranae*. It is well known that *N. apis* is specific for the  
55 Western honey bee, *Apis mellifera* L., whilst the Eastern honey bee *Apis cerana* harbours  
56 *Nosema ceranae* (Fries et al., 1996). However, many recent investigations have revealed that *N.*  
57 *ceranae* is not restricted only to *A. cerana*, but it transferred to *A. mellifera*, and even became a  
58 dominant species in many parts in the world (Klee et al., 2007; Paxton et al., 2007; Chen et al.,  
59 2008; Invernizzi et al., 2009; Tapasztó et al., 2009; Stevanovic et al., 2010; Gajger et al., 2010;  
60 Papini et al., 2017). The exact time and transmission route of transfer of *N. ceranae* from *A.*  
61 *cerana* to *A. mellifera* is not known worldwide. It is possible that during the last decades, the  
62 rapid, long-distance dissemination of *N. ceranae* is likely due to the transport of infected honey  
63 bees and/or by the increased mobility of people, goods and livestock.

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

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

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

83

84

## Materials and Methods

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

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

94 **DNA extraction.** Briefly, prior to DNA extraction, the abdomen of single bee was cut off  
95 with scissors, mechanically homogenized with a cell lysis buffer and centrifuged for 1 min at 15  
96 000 rpm. The pellet was resuspended in a cell lysis buffer; proteinase K was added and incubated  
97 overnight at  $56^{\circ}\text{C}$ . Total DNA was isolated by using GeneMATRIX Tissue and Bacterial DNA  
98 purification Kit (Cat. No. E3551-01, EURx Ltd., Poland) according to the manufacturer  
99 instructions. The extracted DNA was resuspended in  $50\ \mu\text{L}$  of Tris:EDTA buffer (pH 8.0). The  
100 DNA concentration was determined spectrophotometrically and the quality of the DNA samples  
101 was examined on 1% agarose gel electrophoresis stained with Greensafe premium (Cat. No.  
102 MB13201, Nzytech, Portugal). The purified DNA was stored at  $-20^{\circ}\text{C}$  until PCR assay.

103 **Gene selection and PCR amplification.** The small subunit (16S) ribosomal RNA gene was  
104 chosen for molecular identification of *Nosema ceranae* and *Nosema apis*. A fragment of this  
105 gene was amplified in both *Nosema* species using primers designed by Martín-Hernández et al.  
106 (2007). 321APIS-FOR (5'-GGGGGCATGTCTTTGACGTACTATGTA-3'; 321APIS - REV  
107 (5'-GGGGGGCGTTTAAAATGGAAACAACACTATG-3') for *Nosema apis* and 218MITOC FOR  
108 (5'-CGGCGACGATGTGATATGAAAATATTAA-3'); 218MITOC-REV: (5'-  
109 CCCGGTCATTCTCAAACAAAAACCG- 3') for *N. ceranae*.

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

112 The expected number of amplified bases in *N. ceranae* using the 218MITOC primers can be  
113 either 218 or 219 depending on the sequences for *N. ceranae* available in the GenBank database  
114 (<http://www.ncbi.nlm.nih.gov/>) (Martín-Hernández et al., 2007). In the case of *N. apis*, the  
115 expected size of the amplicon using the 321APIS primers was 321 bp. In addition, a negative  
116 control was included for all PCR reactions. The PCR mixtures contained 25 µL of NZYTaQ 2×  
117 Colourless Master Mix (Cat. No. MB04002, Nzytech, Portugal), 0.4 µM of each species-specific  
118 primer (FOR/REV), 1 µL of template DNA PCR water (Cat. No. E0211-01, EURx Ltd., Poland)  
119 in a total volume of 50 µL. All PCR reactions were carried out using Little Genius thermocycler  
120 (BIOER Technology Co., Ltd) under the following conditions: initial denaturation at 94 °C for 5  
121 min; 30 cycles (denaturation at 94 °C for 30 s; primer annealing at 50 °C for 30 s; extension at 72  
122 °C for 1 min) and final extension at 72 °C for 10 min. PCR products were visualized on 2 %  
123 agarose gel with Greensafe premium (Cat. No. MB13201, Nzytech, Portugal). The fragment size  
124 was determined using Gene-Ruler™ 100 bp Ladder Plus (Cat. No. SM0323, ThermoFisher  
125 Scientific Inc.).



127

## Results

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

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

144

**Discussion**

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

148 In the current study we have presented for the first time molecular identification of two  
149 *Nosema* spp. and their distribution in Bulgaria. The results indicate the presence of *N. ceranae* in  
150 57 of 108 investigated samples, which suggests the dominance of *N. ceranae* in all investigated  
151 regions. The results of many studies from Balkan countries have indicated that *N. ceranae*  
152 displaces *N. apis* (Stevanovic et al., 2010; Whitaker et al., 2010; Hatjina et al., 2011; Gajger et  
153 al., 2010) (Fig. 1). One reasonable question is why this introduced parasite (*N. ceranae*) has become  
154 in a short time the dominant species worldwide? Concerning the virulence of the two *Nosema* spp.,  
155 the results are contradictory. It is an interesting fact that in many European countries numerous  
156 studies report that *N. ceranae* is more virulent and thus possesses a competitive advantage compared  
157 to *N. apis* (Klee et al., 2007; Paxton et al., 2007; Forsgren and Fries, 2010). Contrary to this, more  
158 recent research, done mainly in the USA, does not support these observations (Huang et al., 2015;  
159 Milbrath et al., 2015). These studies suggest that the US honey bees may be less susceptible to *N.*  
160 *ceranae* infections than European bees or that the US isolates of the pathogen are less infective and  
161 less virulent than European isolates.

162 These findings are a suitable way to explain our results. We found that *N. ceranae* infection  
163 prevailed in honey bee colonies from the part of the country characterized by a more flat landscape  
164 (Rousse and Sofia district), while in the mountainous parts (Smolyan district, the Rodopa Mountains)  
165 the infection was the lowest (Table 1). Different subspecies of *Apis mellifera* are raised in flat and in  
166 mountainous regions. *A. m. macedonica* is considered to be a native honey bee for Bulgaria (Ruttner,  
167 (1988). More than three decades ago, *A. m. ligustica*, *A. m. carnica* and *A. m. caucasica* were

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

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

183

184

### Conclusion

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

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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.

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

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

- 3  
 4 **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 country.

