Microscopic and Molecular Detection of *Nosema ceranae* in Honeybee *Apis mellifera* L. from Romania

Status on pathogen worldwide distribution

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Until now, in Romania, a country in southeastern Europe located on the Balkans Peninsula, information regarding the status on A. mellifera honey bee infection by Nosema spp. has not been published. The aims of the present study were to survey the occurrence and identification of Nosema spp. in A. mellifera honeybee colonies from 37 apiaries located in Arad, Caras-Severin and Timis Counties, Romania. Also, based on published literature an update on the distribution of N. ceranae infection among A. mellifera colonies worldwide was performed. Overall, a frequency of 55.1% (389/690) of Nosema infection was observed in the analyzed regions (ranging from 52% to 66%), by light microscopy. By PCR and DNA sequencing, N. ceranae was the only microsporidia identified. Nosema ceranae is clearly a novel, emergent pathogen of A.mellifera with potentially very serious effects on the individual and honeybee's colonies in Romania. Data obtained provide new and important information on N. ceranae geographic prevalence and distribution, and on its impact at colony level and/or its role in colony losses. The present study intends to contribute to highlight the importance of implementing prevention, treatment and control measures of honeybee nosemosis, in Romania.

Keywords: Apis mellifera honeybee; Microsporidia; Nosemosis; Nosema ceranae; Molecular characterization

For centuries, the importance of honeybees to honey and wax production and to the pollination of most crops is well recognized. Honey remains an important international good with global production estimated at 1,07 million metric ton in 2007 (FAO, 2009), and almost fifty percent of leading global food commodities depend on honey bee pollination for either fruit or seed set (Klein *et al.*, 2007). This insect is the most efficient pollinator for most crop monocultures around the world (McGregor et al,1976; Delaney *et al.*, 2009). The western honeybee, *Apis mellifera*L., one of the most economically important species of the genus *Apis*, has been transported worldwide for beekeeping purposes from its native range in Europe, Africa and the Near East (Ruttner, 1988).

During the last decade multiple factors like pathogens, pesticides, and abiotic stressors have been identified associated with unusually high and inexplicable honey bee colony losses (Genersch *et al.*, 2010; Ratnieks and Carreck, 2010; Cornman *et al.*, 2012; Pettis *et al.*, 2013). Among the pathogens characterized and discussed in this context are two microsporidian species from genus *Nosema*, (Cox-Foster *et al.*, 2007; Higes *et al.*, 2008; Genersch, 2010) which infect adult honeybees (Bailey L, 1955), *Nosema apis* and *Nosema ceranae*. Both species are intracellular pathogens that are thought to represent very primitive, but highly specialized spore-forming fungi. Initially, nosemosis in *A.mellifera* was thought to be caused by a single species, *Nosema apis* Zander. However, in 1994, a microsporidium like *N. apis*, called *N. ceranae* was described in Eastern honey bee (*Apis cerana* Fabricius) from China (Fries *et al.*, 1996). Transmission of *Nosema* in honeybee colonies is mainly via the fecal-oral route. The infection process starts with the ingestion of infective spores by adult honeybees when they are eating contaminated food or when they are cleaning up fecal material from infected bees. The spores germinate in the insect midgut by extruding the polar tube and releasing their sporoplasm into midgut epithelial cells where they generate more spores and leave the body of infected host by defecation. Once in the gut, they invade the ventricular cells causing disease, but the clinical and epidemiological characteristics of the parasitization by either species are different; the infection by *N.apis* (type A nosemosis) does not usually cause the death of the colonies and is characterized by dysentery, general weakening of the adults, locomotion impairment and crawling (OIE, 2014). These symptoms are not present in *N. ceranae* infections (type C nosemosis) (Higes *et al.*, 2010), which produce alterations in the temporal polyethism, foraging activity and life span of infected bees (Goblirsch *et al.*, 2013; Dussaubat *et al.*, 2013; Alaux *et al.*, 2014**).** A large-scale depopulation phenomenon, named colony collapse disorder (CCD), has been reported in the United States of America (USA) (Chen *et al.*, 2008) and Europe (Topolska, Gajda & Hartwig, 2008). *Nosema ceranae* was suspected to be one of the contributors to this illness, particularly winter colony loses (Klee *et al.*, 2007). However, although some studies implicated

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N.ceranae infection in the colony collapse of bees in the Mediterranean, particularly in Spain (Higes *et al.*, 2008; Martín-Hernández *et al.*, 2007), their relationship is still an open question. Probably, the delay to detect the first natural infections of *A. mellifera* colonies by *N. ceranae* (Higes *et al.*, 2006; Huang *et al.*, 2007) are associated with the use of 3 light microscopy as a routine diagnostic tool for the detection of *Nosema*-like spores. Since both species have spores with similar size, an incorrect result can easily occur only by using this methodology. Instead, *N. ceranae* could be clearly separated from its congener *N. apis* based on 16S small subunit (SSU) rRNA gene sequences and ultrastructural features (Fries *et al.*, 1996). Several PCR based molecular techniques for the diagnosis and identification of *N. apis* and *N. ceranae*, such as conventional or duplex PCR, PCR–RFLP, qPCR, multiplex PCR (Higes *et al.*, 2006; Klee *et al.*, 2007; Martin-Hernandez *et al.*, 2007; Chen *et al.*, 2008; Fries *et al.*, 2013) have been described. During the last decade, the arising of molecular techniques capable of distinguish *Nosema* spp. has led to an increased and even surprising high detection of *N. ceranae* in worldwide *A. mellifera* populations. Furthermore, it was even found to be the predominant *Nosema* species in many regions of the globe. Several of the major studies reported worldwide on *Nosema* spp. honeybee infection are summarized in table 1. These epidemiological evidences point towards the hypothesis that *N. ceranae* has been replacing *N. apis* in the honey bee populations throughout the world (Klee *et al.*, 2007; Chen *et al.*, 2008; Williams *et al.*, 2008; Invernizzi *et al.*, 2009; Chen and Huang, 2010; Yoshiyama and Kimura, 2011; Copley *et al.*, 2012) In Romania, a country in southeastern Europe located on the Balkans Peninsula, *N. apis* (F. Begnescu) and *N. ceranae* (I.D.S.A. Bucharest) were described for the first time in 1934 and 2008, respectively. Based on few scientific data presented in workshops and/or national periodics, is thought that *A. mellifera* nosemosis by these two microsporidian species, is a frequently spread infection in this country (Agripina *et al.*, 2017). However, to date there is a lack of publications on this subject in international peer-reviewed journals.

The aims of the present study were to survey the occurrence and identification of *Nosema* spp. in *A. mellifera* honeybee colonies from apiaries located in Arad, Caras-Severin and Timis Counties**,** Romania.

Experimental part

Materials and Methods

Bee sampling collection

Adult bee samples were collected during 2015 by beekeepers and apiary inspectors in Arad, Caras-Severin and Timis, Counties localized in Southwestern region of Romania, near the border with Hungary, and/or Serbia. Submitters were asked to collect bees from the top lids of bee hives or from around the outside of the brood nest. Most bee samples were collected during the latter part of winter and early spring. All the bee samples were stored in 70% (v/v) ethanol at room temperature prior to testing.

Microscopic analysis

Diagnosis of *Nosema* spp. infections was performed by light microscopy examination at 400× magnification, of homogenized adult worker honeybees abdomens according to the *Manual of Standards for Diagnostics and Vaccines* published by the Office International des Epizooties (OIE), the World Organization for Animal Health (OIE, 2013). A total of 690 adult worker honeybees were examined for the presence of *Nosema* spores.

Spore counting was estimated in some randomly selected positive samples (n = 60) by hemocytometer (Fries *et al.*, 2013). Infection levels were classified as low (<5.0 million spores per bee), medium (P≥5.0–<10.0 million spores per bee), and high (P≥10.0 million spores per bee) according to Yücel and Gogaroglu (2005). As morphological characteristics of *N. ceranae* and *N. apis* spores are very similar and can hardly be distinguished by light microscopy, all samples were also screened by PCR assay based on 16S rRNA-gene-targeted primers, and DNA sequencing to distinguish these two species. Bee homogenates were filtered to remove coarse bee parts and the obtained suspensions were stored at -20°C until used for DNA extraction.

Molecular analysis

Genomic DNA was extracted from the bee homogenates suspensions, using the Fast DNA SPIN kit for soil (Lobo *et al.*, 2006a). A nested PCR protocol, previously described (Lobo *et al.*, 2006b) was used to amplify a fragment of the 16S region of the rRNA gene of *Nosema* group. The DNA extracted from the homogenates was analyzed at least three times by PCR. The products obtained were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining.

The secondary PCR products were purified using a Jetquick kit (Genomed, Lohne, Germany) and sequenced in both directions. The accuracy of the nucleotide sequence was confirmed by sequencing three separate PCR products from the same homogenate. The sequences obtained were analyzed together with the reference sequences from the GenBank database using the BLASTN (www.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997 in papini 2017).and ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) programs.

Results and discussions

Six hundred and ninety adult bee samples obtained from 37 apiaries from three geographical regions in Romania were submitted for examination of *Nosema* spp. spores, by light microscopy. *Nosema* spores appear as oval corpuscles with a size of 2.5–3.0 μm in width and 4.3-5.0μm in length (n = 60), refractory and with highlighted sporal membrane (fig. 1).

The spore counts by light microscopy indicate the average of 5.5 x 10^6 spores/bee, without distinction between *Nosema* spp. species. One hundred and fifty-two

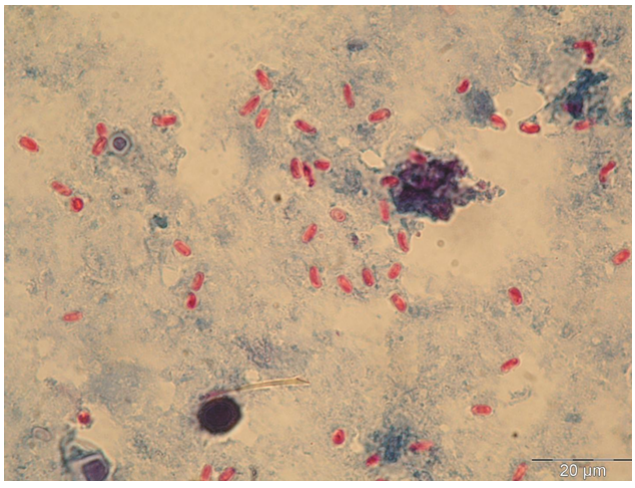

Fig. 1. Light micrograph showing the appearance of *Nosema ceranae* free spores in smears stained by the Gram-chromotrope method. Magnification= x1000; scale bar= 20 µm.

out of 292 (52%) samples of bees collected from 16 apiaries located in Arad County, 178 out of 323 (55%) samples of bees collected from 13 apiaries of Timis County and 50 out of 75 (66%) samples of bees collected from eight apiaries in Caras-Severin County were diagnosed with nosemosis, by light microscopy. By PCR, *N. ceranae* was the only *Nosema* species identified in samples from the three counties. Additionally, selected PCR products from the *N. ceranae* assays were also sequenced and this species was confirmed as the one present in our samples using reference sequences deposited in GenBank. The sequences obtained from the *Nosema*- infested Romanian honeybees were 100% identical with the *N. ceranae* sequence, and 92% like *N. apis* from GenBank database.

Prevalence data reported worldwide for *N. apis* and *N. ceranae* showed that the latter one became the main species in the global *A. mellifera* honey bee populations and it was suggested that *N. ceranae* has replaced or is about to replace its congener globally (Chen *et al.*, 2012; Martin-Hernandez *et al.*, 2012). In the European honeybee *N. ceranae* was first detected in Spain (2006), and this microsporidian has been pointed as the main cause of large-scale colony losses. In Europe, a South to North gradient was observed with *N. ceranae* being dominant in Southern European countries (such as Italy and Greece)while*N.apis* was still dominant in some countries from the Northern/Western part of Europe (Ireland, Sweden, Norway, and Germany) (table 1) (Klee *et al.*, 2007). This difference may be a consequence of distinct influence of climatic factors in *N. ceranae* spread and assertiveness (Fenoy *et al.*, 2009; Martin-Hernandez *et al.*, 2009; Gisder *et al.*, 2010; Chen *et al.*, 2012; Natsopoulou *et al.*, 2015). These data add strong evidence for an advantage of *N. ceranae* over *N. apis* in warmer climates. On the other hand, the cold-sensitivity of *N. ceranae* spores might slow down the replacement process in colder climates (Gisder *et al.*, 2010).The precise momentum in time and the transmission pathway of *N. ceranae* from *A. cerana* to *A. mellifera* are not known. It is hypothesized that throughout the last decades, the rapid, long-distance dissemination of *N. ceranae* is possible due to the transport of infected honey bees and/or by the increased mobility of people, goods and livestock.

Romania is a country included in the Balkans region, and is one of the most important honey producers from Europe and has many melliferous zones of acacia, lime and sun flower plantations all over the country. In 2008, there were registered more than eight hundred thousand bee colonies of *A. mellifera*. During the last two years, an increase in infection by microsporidian parasites in honey bees has been detected in different regions of Romania, while apparently healthy and strong colonies become weak and finally die. In 2007-2008 the number of *Nosema* outbreaks increased, compared with 2004-2006. The presence of *N. ceranae* was confirmed in Romania (2008) using molecular biology techniques in samples that were first analyzed by microscopy methods (Chioveanu G *et al.*, unpublished). Until now, information regarding the status on *A. mellifera* honey bee infection by *Nosema* spp. in this country has not been published. The present study has demonstrated that in Romania *Nosema* spp. infects bees in the three Western Country Counties of Arad, frequency of 55.1% of *Nosema* infection was observed in the regions analyzed (table 1). In addition, *N. ceranae*, was the only species detected by PCR and DNA sequencing. An unpublished study (Chioveanu G *et al.*, unpublished) reports 45.8% (60/131) of *Nosema* infection based on light microscopy, in bee samples collected from several Counties (including Arad County) of Romania (March 2007-September 2008). In that study, *N. ceranae* was the only species detected by PCR-RFLP, as it was observed in the present study.

Some data on nosemosis prevalence in bee colonies from Romania bordering countries, and others from Balkan Peninsula are available (table 1). However, comparative study of prevalence rates should be treated with caution, as frequently the results reported cannot be compared because studies used distinct methodologies in the diagnosis of nosemosis that could bias the results and conclusions. For instance, between 2008 and 2012, Stevanovic *et al.* (2013) found a higher prevalence (73–98%) of *N. ceranae* by PCR-RFLP combined technique, in about 200 Serbian *A. mellifera* honey bee colonies (Stevanovic *et al.*, 2011, 2013) (table 1), then the prevalence obtained in our study. A recent retrospective study (2007 to 2015) demonstrated that *N. ceranae* was ubiquitous in that country. An overall frequency of 95.7% (table 1),ranging annually from 83.3% to 100% occurred, according the 57 locations and season of the year monitored (Stevanovic *et al.*, 2016). This microsporidian species has been present in Serbia since at least 2000 and is not considered anymore as an emergent pathogen in this region. Due to the absence of any molecular data there is no confirmation that *N. ceranae* has displaced *N. apis* within Serbian honey bees (Stevanovic *et al.*, 2011). This is in contrast to honey bee microsporidia populations from other regions of Europe where both species have been described, usually with predominance of *N. ceranae* over *N. apis* (Klee *et al.*, 2007; Martín-Hernandez *et al.*,2007) or less frequently predominance of *N. apis* over *N. ceranae* (Forsgren and Fries, 2013). In Hungary, from 38 *Nosema*-infested bee samples (2006-2007) from geographically distant bee colonies representing all regions of the country, only one sample contained *N. apis*, and in the remaining samples *N. ceranae* was detected, which also indicates the dominance of *N. ceranae* in Hungarian apiaries. More recently, Shumkova *et al.*, 2018, reported *A. mellifera* infection by *N. ceranae* with an overall prevalence rate of 52.8% (13.9-77.2%) in three different regions of Bulgaria. As observed in the present study and in Serbia apiaries, *N. apis* species was not detected in Bulgaria. In the Northern border of Romania, a study carried on Ukraine (Odnosum HV, 2017) reported the occurrence of 38.9, 25.5 and 35.6% of *N. ceranae*, *N. apis* and co-infection by both species, respectively. These data add evidence to the predominance of *N. ceranae* in all these monitored countries from the Balkans region and Eastern Europe.

The occurrence of a high prevalence of *Nosema* infection observed in all the apiaries studied, even without the presence of clinical symptomatology, may also constitute an increased risk for the honeybee colonies of being affected concomitantly by other pathogens. It is known, that the infection of honeybees with one parasite may affect their susceptibility to infection by another parasite, either by increasing or decreasing host susceptibility to co-infection (reviewed in Evans and Schwarz [2011] and Schwarz *et al.* [2015b]). Thus, it is important to monitor these apiaries for other common bee pathogens.

Whilst *N. apis* infection seems to cause a fast acting, short duration syndrome, this has not been the case for *N. ceranae*, which instead has been observed in association with non-specific symptoms, resembling colony collapse disorder (CCD), such as a gradual depopulation, higher autumn/winter mortality in colonies or low honey production (Fries *et al.*, 2006). It has also

recently been shown that *N. ceranae* does not display the seasonality that is seen with *N. apis* (Giersch *et al.*, 2009). When the virulence of *N. apis* and *N. ceranae* was evaluated in experimentally inoculated *A. mellifera* assays the latter seemed to induce significantly higher mortality in comparison to *N. apis* (Higes *et al.*, 2007; Paxton *et al.*, 2007). Furthermore, in several European countries some studies describe *N. ceranae* as the more virulent species and thus may have a competitive advantage in comparison to *N. apis* (Klee *et al.*, 2007; Paxton *et al.*, 2007; Forsgren & Fries, 2010). Dissimilar data, reported mainly in the USA, do not support these observations (Huang *et al.*, 2015; Milbrath *et al.*, 2015). Accordingly, to their results, it is suggested that the North American honeybees 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. (Shumkova *et al.* (2018). Based on the available information, the effect of *N. ceranae* at the colony level and its role in colony losses diverge in different geographic regions. This difference is attributed to the presence of multiple factors, such as the genetic background of the pathogen and the host, climatic factors or other environmental conditions, and colony management.

During the present study, the health of honeybee colonies from the apiaries monitored for *Nosema* spp. infection was evaluated. About 54% of the total (52%) samples diagnosed with nosemosis from Arad County (region from wester Romania bordering with Southeast Hungary) were collected from asymptomatic bee colonies, most of which were observed during the warm season. Thirty percent of the samples infected with *Nosema* came from dead bee colonies in the latter half of winter. In most cases, over 90% of colonies of bees which began wintering as powerful colonies, died. All the bees were fallen from the honeycombs in which some of the stuffed nourishment can be seen. Some of the honey reserves were inappropriate, uncooked, fermented and moldy, which made it possible to trigger acute nosemosis manifested by diarrhea and mortality. At all these bee colonies, *Nosema* spores were identified at very high levels. At the end of winter and early spring, a small number of colonies (9%) presented CCD.

These bee colonies were introduced at strong wintering, according to the beekeepers' declarations.

In this study, the depopulated and missing colonies were observed in spring and autumn. In most cases, the colonies of dead bees diagnosed with nosemosis were observed in the second month of the year and less in the third month. In one situation, cases of dead bee colonies of nosemosis were diagnosed during autumn. The death of bees in the second half of winter is often caused by nosemosis (Higes *et al.*, 2013; Chen and Huang, 2010; Fries I, 2010, Paxton RJ, 2010), which can also be observed in the present study. It can be noticed that mortality can be associated with the diagnosis of nosemosis, but not always clinically manifested, since only a small part of the dead colonies also presented symptoms of diarrhea. These observations can point out to the diagnosis of C-type nosemosis (infection by *N. ceranae*) in which the evolution is acute and the colony of bees dies before the visible symptoms of the disease arise [Higes M. *et al.* 2010, Cox-Foster, D.L. *et al.*, 2007, Fries, I., 2007]. Bee colonies diagnosed with nosemosis associated with diarrhea symptoms were only recorded in the second half of the winter, January to February. During this period, *Nosema* infection may evolve into clinical manifestations that are probably linked to a set of factors that potentiate the intensity of the infection: the growth of the juvenile, the increase of the temperature in the hive, the intensification of the activity in the wintering pad, the increase of the consumption of energy-protein reserves, the effects of fecal accumulation inside the hive associated with the almost exclusive indoor activity of bees. Concerning Timis County (region below Arad County and bordering with Northeast Servia), about 69% of *Nosema*-positive samples determined for this county (55%) in the present study were originated from asymptomatic bee colonies. Most of dead bee's colonies diagnosed with *Nosema* infection were also found in the second half of winter and early spring, February to March. However, on these bees colonies, were detected symptoms of diarrhea concomitantly with very high levels of *Nosema* infection. The colonies of dead bees diagnosed with *Nosema* infection were also found during the autumn, but they did not show visible symptoms of disease. The colonies infected with *Nosema* can be found throughout the year and have an important epidemiological role. They maintain the risk of a clinical episode in the apiary, especially during winter. Infected bee colonies are dangerous not only for the apiary, but also represent a serious risk of contamination to other apiaries and can spread over hundreds of kilometers by practicing the pastoral. Bees infected with *Nosema* have less longevity than healthy bees, which explain depopulation in bee's colonies with nosemosis. In massive winter depopulation, *Nosema* has an important role in reducing the longevity of working bees due to lesions and energy stress, and due to the reduction of the queen eggs (Mayack & Naug, 2009). In Caras-Severin County (region Southwest Romania and bordering Servia), 38% of bee samples with nosemosis monitored in this area (66%) come from colonies of depopulated bees. About 28% of the samples diagnosed with *Nosema* infection were collected from dead bee colonies in the latter half of winter. While 20% of bee colonies with *Nosema* infection do not display symptoms, about 14% of the colonies presented diarrhea. During the study, in Caraº-Severin County, colonies of bees with CCD syndrome was not identified. Overall, health evaluation of bee colonies from the apiaries of the three counties monitored in the present study highlighted the presence of a high number of bee colonies without symptomatology but carriers of *Nosema* spp. And a lesser number of bee colonies with manifested disease. Also, it was observed bee colonies with depopulation, morbidity and positive testing for *Nosema* spp. In the present study, most of the dead bees infected with *Nosema* spp. were observed in the second/latter half of the winter, as reported for *N. ceranae* by other authors (Chen and Huang, 2010; Paxton RJ, 2010; OEI, 2014).

Some studies in Romania bordering countries and other countries from Balkan Peninsula present several results like those reported in this study, namely the predominance of *N. ceranae* over *N. apis* (table I), and some of the typical pattern of infection associated with *N. ceranae* nosemosis (Chen and Huang, 2010; Fries I, 2010; Stevanovic *et al.*, 2013; Odnosum HV, 2017). Based on the literature, *N. ceranae* infections have not reported typical signs of *Nosema* infection. For example, dysentery and crawling bees may well be absent, and many times infected bees are asymptomatic (Chen and Huang, 2010; Fries I, 2010, Paxton RJ, 2010; Higes *et al.*, 2013). Some discrepancies in data colony-level pathology, characteristics and outcome of *N. ceranae* infection, observed in the three Romanian counties monitored, have also been described in other studies (Alaux *et al.* 2010; Fries 2010; Higes *et al.* 2010a; Paxton RJ, 2010; Gisder *et*

Table 1

REVIEW OF THE MAIN STUDIES ON DETECTION AND DISTRIBUTION OF NOSEMA SPP. INFECTION IN HONEY BEE POPULATIONS WORLDWIDE REPORTED IN SCIENTIFIC PUBLICATIONS.

Host	Year	Region and/or country	N	*Nosema* species	Frequency of infection [a] % (n)	Methodology	Ref.
		Asia					
A. cerenae	1996	China	300I	*N. ceranae*	3 (9)	OM, PCR, Cloning,TEM	Fries *et al.*, 1996
A. cerenae	1992, 2007-2008	China,Japan, Taiwan	78S	*N. ceranae*	71 [b]	PCR/ DNA-Seq	Chen et al, 2009
				N. apis	31 [b]		
				N. apis + *N. ceranae*	19 [b]		
A. mellifera	2009	Japan	112C	*N. ceranae*	4.5 (5)	PCR/ DNA-Seq	Yoshiyama & Kimura et al, 2011
			336I		2.1 (7)		
A. mellifera	NA	Jordan	46A	*N. ceranae*	23.9 (11)	PCR/ DNA-Seq	Haddad JL, 2014
A. mellifera	2017	Saudi Arabia	50A	*Nosema* spp.	20.59 (10)	OM	Ansari *et al.*, 2017
			50A	*N. ceranae*	58 (29)	M-PCR	
A. cerenae	2006	Vietnam	4C	*N. ceranae*	100 (4)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2006	Vietnam	2C	*N. ceranae*	100 (2)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2005	Taiwan					
A. cerenae	2008-2009	Thailand	27C	*N. ceranae*	22.2 (6)	M-PCR	Chaimanee *et al.*, 2010
A. mellifera	2008-2009	Thailand	80C	*N. ceranae*	57.5 (46)	OM	Chaimanee *et al.*, 2010
					77.5 (62)	M-PCR	
		Eurasia					
A. mellifera	2005-2006	Turkey	84S	*N. apis*	4.8 (4)	PCR/ DNA-Seq	Whitaker *et al.*, 2011
				N. ceranae	3.6 (3)		
A. mellifera	2007-2009	Turkey	85C+11S	*N. ceranae*	84-89 [b]	OM and/or PCR/DNA-Seq	Muz *et al.*, 2010
				N. apis	11-16 [b]		
A. mellifera	2011-2012	Turkey	99A, 101C	*N. ceranae*	0-100 (0-12)	OM and/or PCR/ DNA-Seq	Tunca *et al.*, 2016
		Northern Europe					
A. mellifera	1988-2006	Denmark	9C	*N. apis*	11.2 (1)	PCR-RFLP	Klee *et al.*, 2007
				N. ceranae	44.4 (4)		
				N. apis + *N. ceranae*	44.4 (4)		
A. mellifera	1986-1995	Finland	6C	*N. apis*	100 (6)	PCR-RFLP	Paxton *et al.*, 2007
A. mellifera	1998	Finland	4C	*N. apis*	75 (3)	PCR-RFLP	Paxton *et al.*, 2007
				N. apis + *N. ceranae*	25 (1)	PCR-RFLP	Paxton *et al.*, 2007
A. mellifera	2002	Finland	8C	*N. apis*	87.5 (7)	PCR-RFLP	Paxton *et al.*, 2007
				N. apis + *N. ceranae*	12.5 (1)	PCR-RFLP	Paxton *et al.*, 2007
A. mellifera	2006	Finland	10C	*N. apis* + *N. ceranae*	50 (5)	PCR-RFLP	Paxton *et al.*, 2007
				N. ceranae	50 (5)	PCR-RFLP	Paxton *et al.*, 2007
A. mellifera	2006	Finland	2C	*N. ceranae*	100 (2)	PCR-RFLP	Klee *et al.*, 2007

A. mellifera	1998, 2003, 2005	Sweden	5C	*N. apis*	100 (5)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2006	Sweden	2C	*N. apis*	50 (1)	PCR-RFLP	Klee *et al.*, 2007
				N. apis + *N. ceranae*	50 (1)		
A. mellifera	NA	Sweden	NA	*Nosema spp.*	319	OM	Fries and Forsgren, 2008 (in Fries, 2010)
			319S	*N. apis*	26.02 (83)	PCR	
				N. apis + *N. ceranae*	5.33 (17)		
A.	2011-2014	Lithuania	347C	*Nosema*	4 (153)	PCR/ DNA-Seq	Blažytė-Čereškienė *et al.*, 2016
		colonies					
			153C	*N. ceranae*	38.6 (59)		
				N. apis	37.2 (57)		
				N. apis + *N. ceranae*	24.2 (37)		
		Eastern Europe					
A. mellifera	2014-2016	Ukraine	784S	*N. apis*	25.5 (200)	OM and/or PCR/ DNA-Seq	Odnosum HV, 2017
				N. ceranae	38.9 (305)		
				N. apis + *N. ceranae*	35.6 (279)		
		Central Europe					
A. mellifera	2003,2005-2006	Germany	69S	*N. ceranae*	78.3 (54)	M-PCR	Martin-Hernandez *et al.*, 2007
				N. apis	7.2 (5)		
				N. apis + *N. ceranae*	2.9 (2)		
A. mellifera	2006	Germany	34C	*N. ceranae*	50.0 (17)	PCR-RFLP	Klee *et al.*, 2007
				N. apis	23.5 (8)		
				N. apis + *N. ceranae*	26.5 (9)		
A. mellifera	2005-2016	Germany	5600C	*N. apis*	1.5-18.7 (4-43) [c]	OM and/or PCR-RFLP	Gisder *et al.*, 2017
				N. ceranae	1.2-14.9 (3-34) [c]		
				N. apis + *N. ceranae*	0.0-10.1 (0-21) [c]		
A. mellifera	1994	Hungary	1C	*N. apis*	100 (1)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2006-2007	Hungary	38S	*N. ceranae*	97.4 (37)	PCR-RFLP	Tapaszti *et al.*, 2009
				N. apis	2.6 (1)		
A. mellifera	2017	Hungary	225C	*Nosema spp.*	47.8 (112)	OM	Agripina *et al.*, 2017
A. mellifera	2006	Switzerland	36S	*N. ceranae*	63.9 (23)	M-PCR	Martin-Hernandez *et al.*, 2007
				N. apis	2.8 (1)		
A. mellifera	2011	Switzerland	408I	*N. ceranae*	23.5 (96)	PCR/ DNA-Seq	Tritscheler *et al.*, 2016
		Western					
		Europe					

A. mellifera	2002-2005	France	61S	*N. ceranae*	59 (36)	PCR/ DNA-Seq	Chauzat *et al.*, 2007
				N. apis	1.6 (1)		
				N. apis + *N. ceranae*	6.6 (4)		
A. mellifera	2006	France	36S	*N. ceranae*	75 (27)	M-PCR	Martin-Hernandez *et al.*, 2007
				N. apis + *N. ceranae*	16.7 (6)		
A. mellifera	2005	Ireland	1C	*N. apis*	100 (1)	PCR-RFLP	Klee *et al.*, 2007
	2007-NA	England	309 S	*N. apis*	10 (31)	RT qPCR	Budge *et al.*, 2008
				N. ceranae	4.5 (14)		
				N. apis + *N. ceranae*	1 (3)		
A. mellifera	2001, 2005, 2006	UK (Northern Ireland)	3C	*N. apis*	100 (3)	PCR-RFLP	Klee *et al.*, 2007
		Southeastern					
		Europe					
A. mellifera	2006-2009	FYROM	37S	*N. ceranae*	94.6 (35)	OM, PCR and/or PCR-RFLP	Stevanovic *et al.*, 2011
A. mellifera	2006-2009	Bosnia and Herzegovina	45S	*N. ceranae*	84.4 (38)	OM, PCR and/or PCR-RFLP	Stevanovic *et al.*, 2011
A. mellifera	2017	Bulgaria	108S; 36S	*N. ceranae*	52.8 (57) [d]; 13.9 (5)	PCR/ DNA-Seq	Shumkova *et al.*, 2018
			28S		64.3 (18)		
			44S		77.2 (34)		
A. mellifera	2006-2009	Montenegro	28S	*N. ceranae*	78.6 (22)	OM, PCR and/or PCR-RFLP	Stevanovic *et al.*, 2011
A. mellifera	2015	Romania	690	*N. ceranae*	55.1 (380)	OM, PCR/ DNA-Seq	In the present study
A. mellifera	2006	Serbia	4C	*N. ceranae*	100 (4)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2000-2005, 2006-2009	Serbia	215S	*N. ceranae*	82.3 (178)	OM, PCR and/or PCR-RFLP	Stevanovic *et al.*, 2011
				N. apis	0.5 (1)		
A. mellifera	2008-2012	Serbia	200C	*N. ceranae*	73-98 [b]	OM, PCR and/or PCR-RFLP	Stevanovic *et al.*, 2013
A. mellifera	2007-2015	Serbia	162C	*N. ceranae*	95.7 (155)	PCR and/or PCR-RFLP	Stevanovic *et al.*, 2016
		Southern					
		Europe					
A. mellifera	2005-2006	Greece	3C	*N. ceranae*	100 (3)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	1993,1998, 2010	Italy	6C	*N. ceranae*	50 (3)		Ferroglio *et al.*, 2012
				N. apis	16.7 (1)		
				N. apis + *N. ceranae*	16.7 (1)		
A. mellifera	1994	Italy	1C	*N. apis*	100 (1)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2005-2006	Italy	26C	*N. ceranae*	100 (26)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2014-2015	Italy	38A	*N. ceranae*	63.2 (24)	OM and M-PCR	Papini *et al.*, 2017

A. mellifera	2004-2005	Spain	12S	*N. apis*	8.3 (1)	PCR	Higes *et al.*, 2006
				N. ceranae	91.7 (11)		
A. mellifera	2004-2005	Spain	10C	*N. ceranae*	100 (10)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2005-2006	Spain	149S	*N. ceranae*	34.9 (52)	M-PCR	Martín-Hernandez *et al.*, 2007
				N. apis	14.1 (21)		
				N. apis + *N. ceranae*	7.4 (11)		
		North America					
A. mellifera	2008	Canada	169C	*N. ceranae*	51 (86)	Triplex PCR	Emsen *et al.*, 2016
				N. apis	34 (58)		
				N. apis + *N. ceranae*	15 (25)		
A. mellifera	2010	Canada	76C	*N. ceranae*	41 (31)	Triplex PCR	Emsen *et al.*, 2016
				N. apis	34 (26)		
				N. apis + *N. ceranae*	25 (19)		
A. mellifera	2012	Canada	181C	*N. ceranae*	91 (165)	Triplex PCR	Emsen *et al.*, 2016
				N. apis	4 (7)		
				N. apis + *N. ceranae*	5 (9)		
A. mellifera	2006-2007	Maritime Provinces of Canada and Minnesota	56C	*Nosema spp.*	23.2 (13)	OM	Williams *et al.*, 2008
			8S [c]	*N. ceranae*	100 (8)	PCR	
A. mellifera	1995-2007	USA	180I	*N. ceranae*	16 (28)	PCR/ DNA-Seq	Chen *et al.*, 2008
A. mellifera	2004	USA	1C	*N. ceranae*	100 (1)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2009	USA	293C	*Nosema spp.*	37.5 (110)	OM	Traver & Fell, 2011
			293C	*Nosema spp.*	69.3 (203) [f]	qPCR	
			293C	*N. ceranae*	34.8 (102) [f]		
				N. apis + *N. ceranae*	2.7 (8) [f]		
		South America					
A. mellifera	2006	Brazil	3C	*N. ceranae*	100 (3)	PCR-RFLP	Klee *et al.*, 2007
A. mellifera	2009-2012	Brazil	637S	*Nosema spp.*	79.9 (509)	OM	Teixeira *et al.*, 2013
			509S	*N. ceranae*	98.82 (503)	PCR/ DNA-Seq	
				N. apis + *N. ceranae*	0.79 (4)		
				N. apis	0.39 (2)		
A. mellifera	2010-2011	Chile	240C	*N. ceranae*	49 (117)	RT-PCR	Martinez *et al.*, 2012
A. mellifera	1995-1996	Mexico	10C	*N. ceranae*	100 (10)	PCR	Guerrero-Molina *et al.*, 2016
A. mellifera	~1990, 2004, 2007-2008	Uruguay samples	29S	*N. ceranae*	100 (29)	PCR-RFLP and DNA-Seq	Invernizzi et al, 2009

		Africa					
A. mellifera	2014-2015	Uganda	380C	*N. ceranae*	1.6 (6)	OM and/or RT PCR	Chemurot *et al.*, 2017
				N. apis	1.6 (6)		
				Nosema neumanni n. sp.	27.9 (106)		
		Oceania					
A. mellifera	2007-2008	Australia	307I	*N. apis*	45.6 (140)	OM and/or PCR-RFLP	Giersh *et al.*, 2009
				N. ceranae	15.3 (47)		
				N. apis + *N. ceranae*	0.7 (2)		
A. mellifera	2006	New Zeland	115C	*N. ceranae*	66.1 (76)	PCR-RFLP	Klee *et al.*, 2007
				N. apis	21.7 (25)		
				N. apis + *N. ceranae*	12.2 (14)		

al. 2010). Thus, further investigations on *N. ceranae* seasonality patterns and impact on individual/colony health are still necessary.

Romania has a climate that is temperate and continental. However, there are some regional differences. For instance, in the western parts, such as the region of Romanian Banat, where the three counties analyzed in this study are located, the climate is milder and has Mediterranean influences. Among other factors, the climatic conditions of the studied area may justify some of the similarities of prevalence, predominance and clinical impact of *N. ceranae* in colonies of *A. mellifera* bees that are characteristic of nosemosis reported in Mediterranean countries (Fries I, 2010; Gisder *et al.*, 2017).

Another potential factor involved in the *N. ceranae* impact on individual and/or colony level is the genetic background of the host. *Apis mellifera* subspecies differentiation strongly correlates with the distinct temperature zones of Romania. *Apis mellifera carnica* is more abundant in regions with the mean average temperature below 9 °C, whereas *A. m. macedonica* honeybees are more frequent in regions with mean temperatures above 9 °C. Coroian *et al.* (2014) reported *A. m. macedonica* as the more frequent endemic subspecies compared with *A. m. carnica*, in these southern regions of Romania. However, these two subspecies are reported to share the same habitat at Arad and Timis Counties. Currently, it is suggested the importance of the genetic background of bee colonies in the susceptibility process to *Nosema* spp. infection (Invernizzi *et al.*, 2009). For instance, some specific *A. mellifera* subspecies seem to be less susceptible to *N. ceranae* infection and thus could explain some differences on prevalence and effect of *N. ceranae* infection observed in distinct subspecies of *A. mellifera* bee colonies (Shunkova *et al.*, 2018). No information about the impact of *Nosema* spp., in the different endemic *A. mellifera* subspecies colonies are available for Romania. Thus, epidemiological studies evaluating host-parasite interactions throughout all Romanian regions are still necessary.

Molecular methods are necessary to distinguish *N. ceranae* from *N. apis* as the morphology of their spores is impossible to distinguish with 100% certainty by conventional microscopy. The rRNA gene sequence seems to be an excellent DNA barcodes (sensu Valentini *et al.*, 2009) to differentiate among these and other microsporidian species (Klee *et al.*, 2006), but not for intraspecific characterization of variants (O'Mahony *et al.*, 2007). Several studies suggested that the distinct effect of *N.ceranae* on individual and/or bee colony level reported in different geographic regions is strongly associated with intraspecific variability of *N. ceranae* (Higes *et al.*, 2006, 2013; Maside *et al.*, 2015; Branchiccela *et al.* 2016). The development and implementation of accurate molecular tools capable of characterizing *Nosema* genetic variants are still necessary to clarify this hypothesis. Recently, some authors provided new insights in this research context. Branchiccela *et al.* (2016) confirmed the existence of genetic variance in the *N. ceranae* genome, by inter-sequence single repetition (ISSR), allowing the identification of a conserved genetic variant circulating in different countries. In addition, these authors also showed that the *A. mellifera* bee immune response triggered against different *N. ceranae* genetic variants might be different. Genetic variability of *Nosema* isolates from *A. mellifera* largely remains unknown worldwide, including in Romania. However, considering that in the present study was observed similarities in the effect of *N. ceranae* infection in the monitored bee colonies, we would not be expecting to find great variability among the isolates obtained.

Conclusions

In conclusion, the combination of conventional microscopy and PCR/DNA sequencing demonstrated to be an effective method for *Nosema* spp. detection in *A. mellifera* honeybee, and evaluation of the prevalence of this pathogen in apiaries from three Southwestern counties of Romania. In addition, the exclusive presence of *N.ceranae* in all the monitored apiaries suggests the dominance of this species and its capacity to replace *N. apis* throughout time, in this region. *Nosema ceranae* is clearly a novel, emergent pathogen of *A. mellifera* with potentially very serious effects on the individual and honeybees colonies in Romania. Data obtained provide important information on *N. ceranae* geographic prevalence and distribution, and on its impact at colony level and/or its role in colony losses**.** The present study intends to contribute to highlight the importance of implementing prevention, treatment and control measures of honeybee nosemosis, in Romania. Additional epidemiological studies mostly based on specific-molecular tools, are needed to carry out a larger and detailed survey, involving the status of *Nosema* spp. and its honeybee host in apiaries of all the different regions of Romanian Territory.

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