

A comparison of the effectiveness of the microscopic method and the multiplex PCR method in identifying and discriminating the species of *Nosema spp.* spores in worker bees (*Apis mellifera*) from winter hive debris

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Abstract

The objective of this study was to compare the effectiveness of the multiplex PCR method and traditional light microscopy in identifying and discriminating the species of *Nosema spp.* spores in worker bees from winter hive debris in the Province of Warmia and Mazury (NE Poland). A total of 1000 beesdead after from the bottom of the hive from bee colonies were analyzed. Spores were identified with the use of a light microscope (400-600x magnification). Spores were assigned to species by the multiplex PCR method. The microscopic evaluation revealed the presence of *Nosema spp.* spores in 803 samples (80.3%). *Nosema ceranae* spores were observed in 353 positive samples (43.96%), *Nosema apis* spores were found in 300 samples (37.35%), while 150 samples (19.67%) showed signs of a mixed infection. A multiplex PCR analysis revealed that 806 samples were infested with *Nosema spp.*, of which 206 were affected only by *Nosema ceranae*, 600 showed signs of mixed invasion, while no samples were infected solely by *Nosema apis* parasites. In two cases, the presence of spores detected under a light microscope was not confirmed by the PCR analysis.

The results of the study indicate that *Nosema ceranae* is the predominant parasitic species found in post-winter worker bees from the bottom of the hive in the region of Warmia and Mazury.

Key words: *Apis mellifera*, *Nosema ceranae*, *Nosema apis*, multiplex PCR, 16S rRNA

Introduction

Nosemosis (*nosemosis apium*), a parasitic disease affecting honey bees, is caused by *Nosema spp.* parasites of class *Microsporea* (*Fungi*). At present, *Nosema*

ceranae spores pose the greatest threat to honey bees (*Apis mellifera*) in Europe (Forsgren et al. 2010). This parasite has been found to be more pathogenic than *Nosema apis*, for which the European honey bees act as the definitive host (Gajda 2010). The first honey

bee infestation with *Nosema ceranae* was observed in Spain in 2005, while in Poland the disease was first noted in 2006 in bee specimens forwarded for testing to Spain (Topolska et al. 2007). Today, nosemosis is a common parasitic disease affecting bees around the world. Worker bees are infested *per os* via food and water contaminated with spores. The physical and chemical environment in a worker bee's midgut supports spore germination. Spores proliferate and mature in the midgut epithelial cells, hypopharyngeal salivary glands, Malpighian tubules and the fat body (Chen et al. 2009a). Newly formed spores damage epithelial cells to reach the lumen of the intestine, and they are excreted with feces and undigested food. The bee colony affected by the spores quickly loses its vitality, and the damaged intestinal epithelium becomes a gateway for viral infections. The progression of nosemosis caused by *Nosema ceranae* may vary. In some cases, the disease produces mild initial symptoms, it does not cause great losses, and diarrhea is not observed. The affected bee colonies do not winter well, they do not use up the accumulated food reserves, and the reason for large numbers of dead bees found on the hive floor in the spring is difficult to determine (Higes et al. 2008).

The standard practice of determining *Nosema spp.* spores under a light microscope does not yield highly accurate results. The species is difficult to identify owing to similarities in the spore size of both analyzed parasites. The morphological structure of *Nosema apis* and *Nosema ceranae* spores is also relatively similar. The main differences are noted in respect of the length of the polar filament, and they can be detected only under an electron microscope (Fries 2010).

Nosema spp. spores are routinely determined with the use of a light microscope. Analyses are generally performed in early spring on samples of dead bees collected from the bottom of the hive after winter (mostly worker bees) (Topolska et al. 2005). The presence of spores is determined in microscopic preparations of crushed abdomen samples. Owing to minor differences in the structure of *Nosema apis* and *Nosema ceranae* spores, the pathogen infecting a bee colony is difficult to identify under a light microscope. For this reason, the spore species is additionally verified using PCR assays with the use of primers designed for the small subunit 16S rRNA (Higes et al. 2006, Kasprzak et al. 2007).

The objective of this study was to compare the effectiveness of the multiplex PCR method and traditional light microscopy in identifying and discriminating the species of *Nosema spp.* spores in dead bees samples collected after winter in the Province of Warmia and Mazury (NE Poland).

Materials and Methods

Materials

The experimental material comprised 1000 dead bees collected from the bottom of the hive from bee colonies in the region of Warmia and Mazury in the early spring of 2010. The collected hive debris samples were stored at a temperature of -18°C .

Microscopic method

Nosema spp. spore invasions were determined by the Kirkor method modified by Hartwig and Topolska (Topolska et al. 2005). Each test was performed on a random sample of 10 worker bee abdomens collected from hive debris. The abdomens were ground in a mortar with the addition of 10 ml of water. A drop of the resulting suspension was placed on a microscope slide and covered with a coverslip. The preparation was studied under a light microscope in five fields of view at 400-600x magnification. The presence of a single spore in five fields of view was regarded as a positive result. Preliminary attempts were made to determine the pathogen's species by analyzing the spore's size and measuring its length and width under a light microscope, using a program for image acquisition and visualization (QuickPHOTO CAMERA 2.3). The studied samples were classified into: group I – *Nosema ceranae* (4.4 μm in length, 2.2 μm in width), group II – *Nosema apis* (6.0 μm in length, 3.0 μm in width), group III – mixed invasion, and group IV – uninfected samples (Fig. 1).

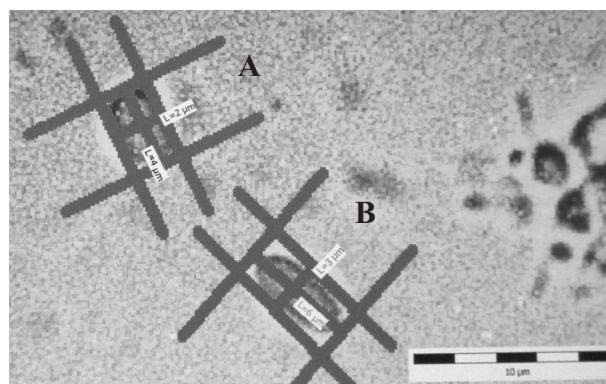


Fig. 1. Spores of *Nosema ceranae* (A) and *Nosema apis* (B).

DNA extraction

To identify the observed spores to species, all samples were analyzed using multiplex PCR methods after the DNA extraction of *Nosema spp.*, using the filtered and centrifuged (800 g for 6 minutes) suspen-

sion from the microscopic test. Genomic DNA was isolated using the Genomic Mini A&A Biotechnology kit (Gdynia, Poland) for DNA extraction by relying on genomic DNA's ability to bind to silica in the presence of high concentrations of chaotropic salts. The isolation process was carried out in accordance with the manufacturer's guidelines, and purified DNA was stored for further analyses in test tubes at a temperature of -20°C.

Multiplex PCR kits and techniques

The multiplex PCR assay involved the amplification of small subunit rRNA sequences (16S rRNA) of *Nosema apis* and *Nosema ceranae*. The sequence of DNA primers developed by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences was obtained from the OIE Terrestrial Manual. Primer sequences for the studied reaction are presented in Table 1.

Table 1. Sequences of the primers used in multiplex PCR.

	Sequence of the primers	Product volume (bp)	source
<i>Nosema ceranae</i>	5'-CGGCGACGATGTGATATGAAAATATTA-3' 5'-CCCGGTCAATTCTCAAACAAAAACCG-3'	218	OIE
<i>Nosema apis</i>	5'-GGGGGCATGTCTTTGACGTACTATGTA-3' 5'-GGGGGCGTTTTAAAATGTGAAACAATATG-3'	321	OIE

The multiplex PCR analysis was carried out using the HotStarTaq *Plus* Polymerase (Qiagen) and the HotStarTaq *Plus* Master Mix Kit (Qiagen). The reaction mix of 20 µl comprised around 120 ng of isolated DNA (from 1 to 3 µl), 10 µl HotStarTaq *Plus* Master Mix 2x, 2 µl CoralLoad Concentrate 10x and 0.1 µl of each primer (with a final concentration of 0.5 µM), supplemented with RNase-Free Water to 20 µl. Every reaction involved three controls: two positive controls with the DNA of *Nosema apis* and *Nosema ceranae* (Centro *Nosema* Apicola Regional, Dirección General de la Producción Agropecuaria, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, Spain) and one negative control where DNA was replaced with water. The reaction was carried out in the Eppendorf Mastercycler thermocycler. PCR commenced with initial denaturation for 5 minutes at 95°C. The reaction mixture was then processed for 35 cycles consisting of the following steps: denaturation at 94°C for 45 seconds, primer annealing at 55°C for 45 seconds and extension at 72°C for 1 minute. The last cycle was followed by final chain synthesis stage at 72°C for 10 minutes. The products of the multiplex PCR reaction were separated by electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bro-

midide for visualizing the resulting DNA fragments of *Nosema spp.* The size of the obtained products was evaluated by comparison with the GeneRuler™ 100bp 36 Ladder Plus (Fermentas) molecular size marker. Electrophoresis results were archived using the GelDoc (Bio-Rad) gel documentation system.

Results

A total of 1000 microscopic specimens were prepared for the needs of this study. *Nosema spp.* spores were determined in 803 samples (80.3%), and 197 (19.7%) samples were found to be free of the investigated pathogen (Fig. 2). Under a light microscope, *Nosema ceranae* was observed in 353 (43.96%) samples (presence of spores), *Nosema apis* was found in 300 samples (37.35%), while a mixed invasion (*Nosema apis* and *Nosema ceranae*) was noted in 150 samples (19.67%)

Multiplex PCR assays confirmed the presence of *Nosema spp.* DNA in 806 (80.6%) samples, while 194 (19.4%) hive debris samples were free of the pathogen. *Nosema spp.* was observed in all positive samples from the microscopic examination as well as in three samples classified as negative (Fig. 2). An analysis of positive samples revealed the presence of *Nosema ceranae* in 206 samples (25.55%). *Nosema apis* spores were not detected, and a mixed invasion was noted in 600 samples (74.45%). In 353 samples where *Nosema ceranae* had been identified in a microscopic examination, the genetic material of those spores was observed in multiplex PCR, and 163 of those samples were also marked by the presence of *Nosema apis*, which was indicative of a mixed invasion (Fig. 3).

In the group of samples where *Nosema apis* was identified under a light microscope, the analyzed spore's genetic material was reported in 209 samples together with *Nosema ceranae* (mixed invasion), while the sole presence of *Nosema ceranae* was observed in 10 samples (Fig. 4).

In microscopic samples indicative of a mixed invasion, the noted results were positively confirmed in 97% of samples by the PCR method. In the group of 197 negative microscopic samples, the presence of

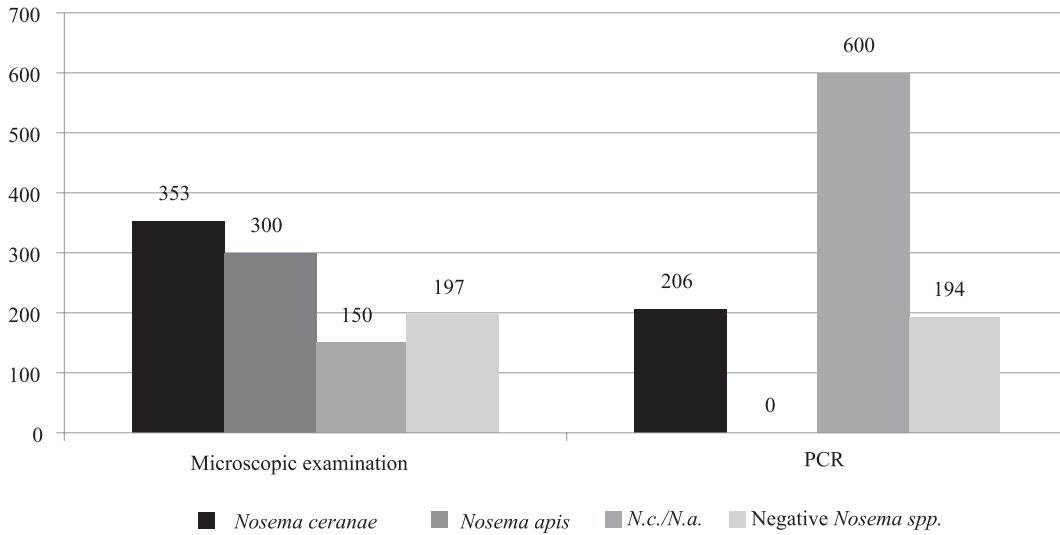


Fig. 2. Species assignment of *Nosema* spp. in microscopic examination and PCR

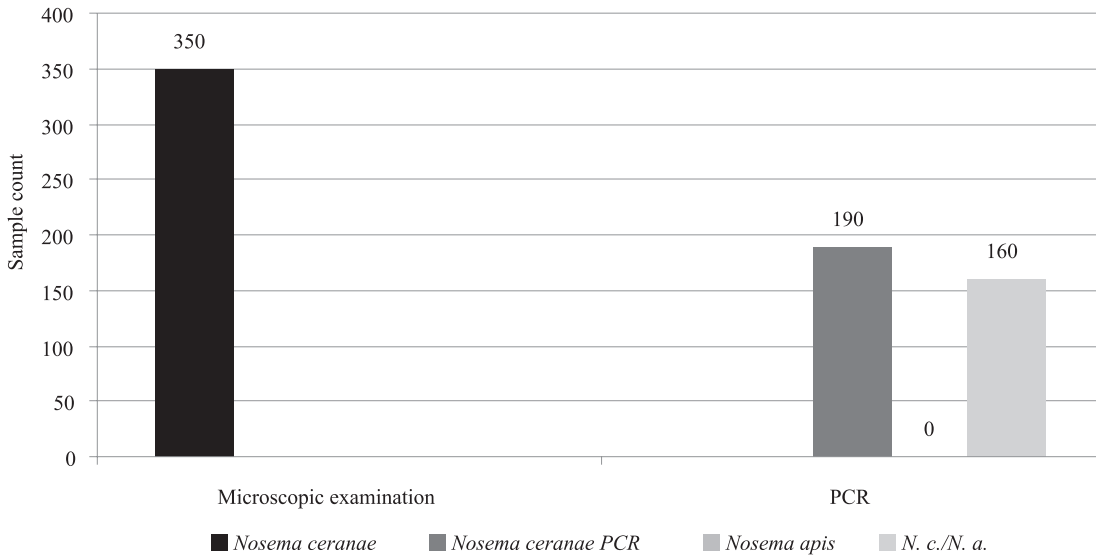


Fig. 3. Difference in species determination between microscopic examination (in the group I) and PCR.

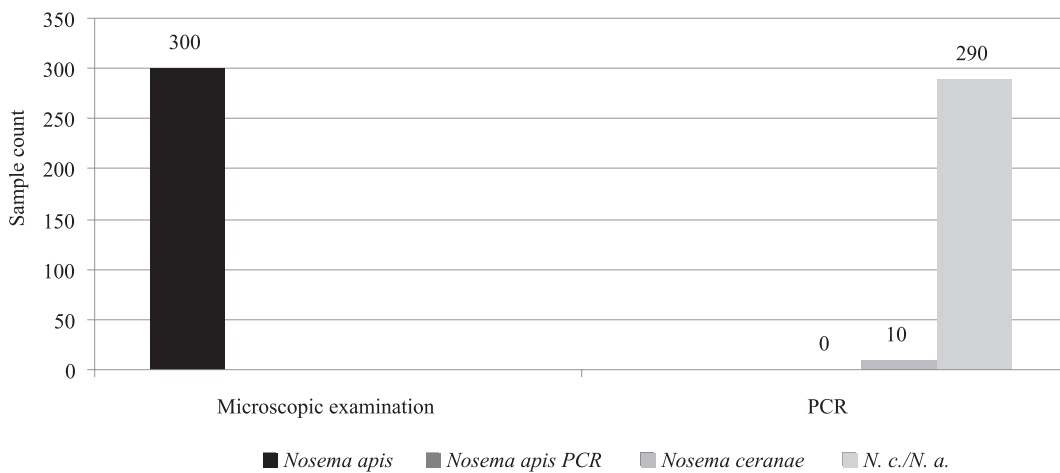


Fig. 4. Difference in species determination between microscopic examination (in the group II) and PCR.

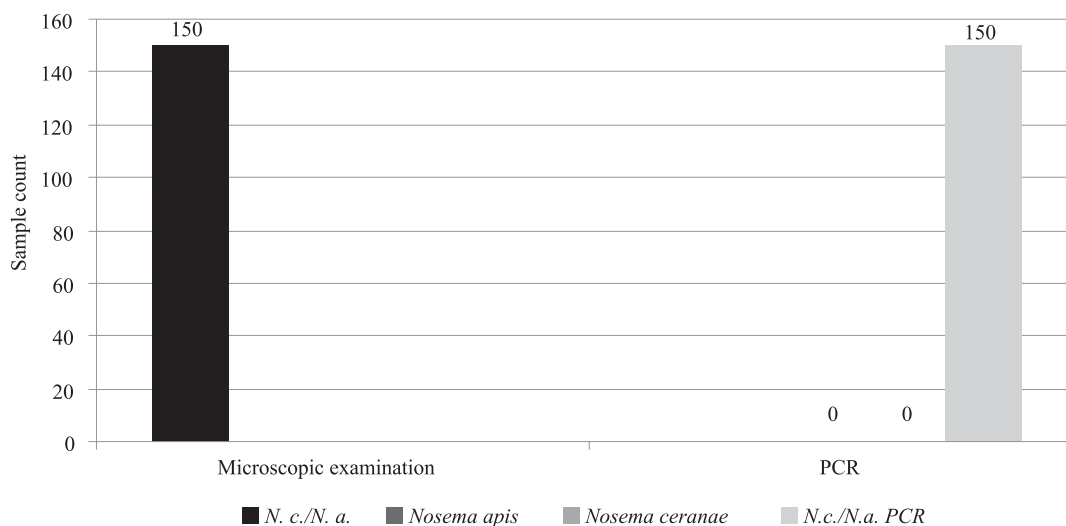


Fig. 5. Difference in species determination between microscopic examination (in the group III) and PCR.

Nosema ceranae DNA was determined in three samples using the PCR technique. The above suggests the presence of infrequent spores that were not detected by the microscopic examination (Fig. 5).

Discussion

Today *Nosema spp.* is identified by the PCR technique, as recommended, among others, by OIE (OIE Terrestrial Manual 2008). In 2005, Spanish researchers relied on the PCR method to determine *Nosema ceranae* spores in European worker bees. This was the first documented discovery of *Nosema ceranae* in the European honey bee (*Apis mellifera*). *Nosema ceranae* is a spore species that is commonly found in the Eastern honey bee (*Apis ceranae*). The researchers relied on primers designed for the small subunit 16S rRNA based on a gene bank sequence (U26533) (Kasprzak et al. 2007).

Marin-Hernández et al. analyzed 87 bee samples for the presence of *Nosema spp.* using two different diagnostic methods (light microscopy and PCR). Positive results were reported for 81.6% samples analyzed with the PCR method and 53.3% samples examined under a microscope. The presence of *Nosema apis* was determined in only five of the 87 studied samples, while *Nosema ceranae* was identified in the remaining samples. The results noted by the above authors prove the high effectiveness of the PCR technique, and they point to the expansion of the analyzed parasite in the territory of Spain (Martin-Hernández et al. 2006). In a study on 15 dead post-winter bees from the bottom of the hive Topolska and Kasprzak found *Nosema spp.* spores in 10 samples. Based on an analysis of their morphological properties under a light microscope, the above authors determined the combined presence of *Nosema apis* and *Nosema ceranae* spores in eight

samples, while two samples contained only *Nosema ceranae* spores. In a PCR analysis, *Nosema ceranae* was observed in all samples, while the presence of *Nosema apis* spores was additionally determined in three samples (Topolska et al. 2007).

The results of this study also suggest that the PCR technique supports the discrimination of spore species with a much higher level of accuracy than a light microscope (see the Figures). The noted results have confirmed the assumption that *Nosema ceranae* is the predominant parasitic species affecting bee colonies in the region of Warmia and Mazury (PCR assay). These findings are of great significance for the further handling of bee colonies invaded by the studied parasite. The observed results indicate that in the region of Warmia and Mazury, *Nosema ceranae* is becoming dominant over *Nosema apis* spores which, until recently, were identified solely in microscopic examinations.

The effectiveness of molecular biology methods has been validated by numerous researchers. In a PCR-based study of bee colonies from different regions of Hungary, Tapaszki et al. (2009) observed that *Nosema ceranae* produces less acute infection symptoms than *Nosema apis*. In the group of 38 analyzed samples, *Nosema apis* spores were found in only one sample, while the remaining 37 samples showed the presence of *Nosema ceranae*. The above authors have concluded that *Nosema ceranae* has become the predominant pathogenic species affecting Hungarian bee colonies (Tapaszki et al. 2009). Chen et al. (2008) investigated bee specimens infected with *Nosema spp.*, collected in 1995-2007 in 12 states of the USA. The above authors have suggested that *Nosema ceranae* had adapted to *Apis mellifera* much earlier than it is commonly believed, and that the presence of *Nosema ceranae* had not been detected because light microscopy was the generally applied method of investiga-

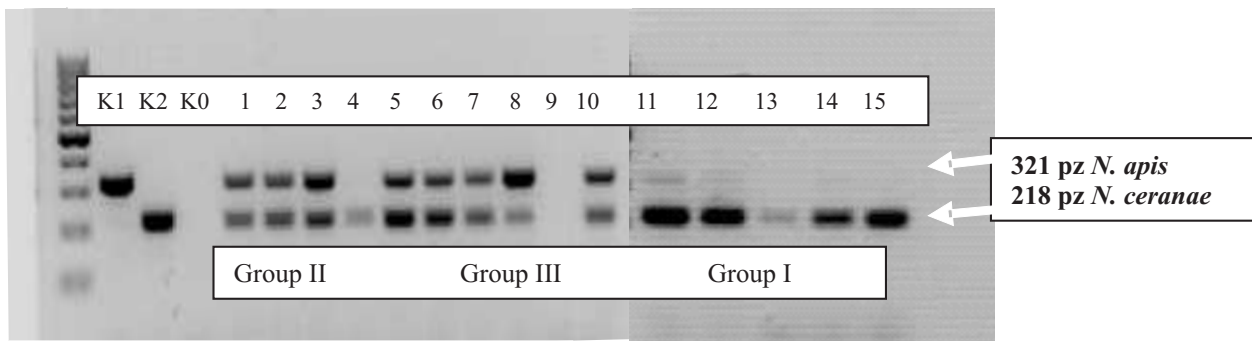


Fig. 6. Electrophoretic separation of amplification products (small-subunit rRNA *Nosema ceranae* i *Nosema apis*), obtained from a honey bee in multiplex PCR in 2% agarose gel.

M – GeneRuler™ 100bp DNA Ladder Plus volume marker (Fermentas)

K¹⁺ – positive control, includes DNA isolated from *Nosema apis* (Centro Apicola Regional Spain)

K²⁺ – positive control, includes DNA isolated from *Nosema ceranae* (Centro Apicola Regional Spain)

K0 – “zero” control, does not includes DNA isolated from *Nosema apis* and *Nosema ceranae*.

- Path 1- 5 DNA isolated from self sample in light microscopy considered as *Nosema apis*: 1- 3 i 5 – mixed invasion, 4 – invasion of *Nosema ceranae*,
- Path 6-10 – mixed invasion (light microscopy): 9 – DNA *Nosema apis* and *Nosema ceranae* were not detected,
- Path 11-15 – *Nosema ceranae* (light microscopy) 11, 12 – mixed invasion, the rest *Nosema ceranae*.

tion. They examined a total of 180 bee specimens invaded by *Nosema spp.*, of which 16% (28 samples) were infected with *Nosema ceranae*. According to the above authors, the exact place and time when a specific barrier had been broken and when *Nosema ceranae* moved to a different host remain unknown (Chen et al. 2008). According to Chen et al., *Nosema ceranae* produced higher infestation rates than *Nosema apis* in bee colonies infected with the colony collapse disorder (CCD). *Nosema ceranae* spores were detected in 31% samples with the use of the PCR method. The analyzed samples were collected in Taiwan, Japan and China. The above authors investigated 78 Eastern honey bee specimens to conclude that 71% samples were invaded by *Nosema ceranae*, 19% samples were affected by a mixed infection, and 31% samples – by *Nosema apis* spores (Chen et al. 2009b). Webster et al. (2004) studied worker bees in late autumn to determine their ability to transfer parasitic spores to queen bees. Although *Nosema apis* spores were observed under a light microscope in specimens infected with noseiosis as well as in the intestines of worker bees and queen bees, they were not found in DNA analyses. The cited authors have suggested that the above resulted from the absence of sporulation and spore deposition in the intestine for long periods of time. Undigested spores have flexible walls, and they are resistant to DNA extraction (Webster et al. 2004). The results of this study support the above theory. Spores were observed under a light microscope in two cases, but their presence was not confirmed by PCR tests, as demonstrated in Fig. 6, lane 9.

Paxton et al. (2007), Martin-Hernández et al. (2007) have demonstrated that multiplex PCR is a highly effective method for discriminating *Nosema spp.* (Martin-Hernández et al. 2007, Paxton et al. 2007). In an experiment investigating material collected in the previous years, Paxton et al. concluded that *Nosema ceranae* had been present in Finland for a long time, but it was not detected due to the unavailability of PCR techniques at the time (Martin-Hernández et al. 2007).

This study analyzed post winter dead bees from the bottom of the hive whose volume (number of dead bees) is an indicator of noseiosis intensity. The presence of *Nosema apis* and *Nosema ceranae* was determined in samples analyzed under a light microscope and with the involvement of PCR assays (Fig. 1). The obtained results demonstrate that PCR delivers a much higher level of accuracy than light microscopy. PCR altered the species identification of parasitic spores in comparison with that determined by the microscopic evaluation. The results of this study have confirmed the presence of *Nosema ceranae* spores in bee colonies as well as the dominant character of this parasitic species. This study delivers vital information about the incidence of noseiosis in bee colonies in the region of Warmia and Mazury.

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