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ORIGINAL ARTICLE

Occurrence of pathogens and nematodes in forest beetles from Curculionidae and Attelabidae in Bulgaria

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Abstract

Beetles from Curculionidae and Attelabidae collected from 14 localities (mainly in coniferous stands) in Bulgaria were investigated for the presence of pathogens and nematodes. A microsporidium belonging to genus *Nosema* in the fat body of *Pityogenes chalcographus* (prevalence: 0.9%) and the fungus *Beauveria bassiana* in the oak-leaf roller *Attelabus nitens* (prevalence: 62%) were detected for the first time. Morphological data and characteristics of *Nosema* sp. spores and conidia of *B. bassiana* are presented. Nematode species (*Cryptaphelenchus diversispicularis, Parasitorhabditis subelongati* and *Parasitylenchus dispar*), and specimens belonging to other ten nematode genera were found (*Bovianema, Bursaphelenchus, Cryptaphelenchus, Neoparasitylenchus, Parasitylenchus, Parasitaphelenchus, Panagrolaimus, Parasitorhabditis, Prothallonema* and *Sulphuretylenchus*). Their prevalences varied from 17.4% to 90%. Nine of the host beetles (*Dryocoetes autographus*, *Ips sexdentatus*, *I. acuminatus, Orthotomicus laricis, O. erosus, Pityogenes quadridens, P. conjunctus, Hylurgus ligniperda* and *Taphrorychus villifrons*) are reported as vectors of *Bursaphelenchus* sp.

Keywords Bark beetles · *Nosema* · *Beauveria bassiana* · *Attelabus nitens* · Nematodes · Bulgaria

Introduction

Studies of diversity and occurrence of insect pathogens are related to the development of environmentally friendly methods to control the pest mass outbreaks. Therefore their pathogen complex and parasites are intensively investigated.

Until now at least 16 microsporidian species were revealed in bark beetles (Takov et al. 2010, 2011; Holuša et al. 2016). Hosts of these pathogens were 35 bark beetle species, some of them with seriously economical importance. *Beauveria bassiana* is the most distributed pathogen causing mycoses in insects. It was found in about 700 arthropod species (Goettel et al. 1990) and is a common fungal pathogen in bark beetle species (Landa et al. 2001; Wegensteiner 1994, 2004; Polovinko et al. 2010). Nematodes of the orders Diplogasterida, Rhabditida and Tylenchida have been reported from bark beetles. Linstow (1890) was the first who observed and described the parasitic nematode *Contortylenchus diplogaster* (von Linstow, 1890) in

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Ips typographus from Germany. Data about occurrence and distribution of entomophilic nematodes of *Ips* spp. were reviewed and summarized by Grucmanová and Holuša (2013). They presented data about 11 nematode species associated with bark beetles (*Ips* spp.) and 12 endoparasitic nematode species found in the same genus in central Europe (Grucmanová and Holuša 2013).

In Bulgaria, Takov et al. (2006, 2007, 2011, 2012) and Nedelchev et al. (2008, 2011) investigated economically important bark beetle species – *Ips typographus, I. sexdentatus, I. acuminatus*, *Tomicus piniperda*, *Pityogenes chalcographus* etc. They detected viruses, fungi, protozoa, microsporidia and nematodes in these hosts. The parasitic nematodes *Contortylenchus diplogaster*, *Parasitylenchus dispar* and the associated nematode *Cryptaphelenchoides macrobulbosus* (Rühm, 1956) were observed in *I. typographus*. *Parasitorhabditis subelongati, Parasitaphelenchus sexdentati* Fuchs, 1937 and *Cryptaphelenchus diversispicularis* parasitize *I. sexdentatus*. The nematode *Rhodolaimus pini* Fuchs 1930 was found also in the same host. *Contortylenchus acuminati* Rühm, 1956 was detected in *I. acuminatus* (Nedelchev et al., 2008). Nedelchev et al. (2011) described a new nematode species *Prothallonema tomici*, parasitizing *T. piniperda*. However, the leaf-rolling weevils were not studied for the presence of pathogens or parasites in Bulgaria.

The aim of this study is to investigate the presence and the occurrence of pathogens and nematodes of scolytids and a leaf-rolling weevils in Bulgaria and also to obtain additional data on their species composition, distribution and localization in the hosts.

Materials and methods

Totally 1112 adult specimens of forest beetles belonging to 12 species of bark beetles and the weevil *Attelabus nitens* were collected from 14 localities in Bulgaria (Fig. 1).

Bark beetles were collected from bark which was peeled off from infested trees. In one location (Fig. 1; № 14) dead adults of *A. nitens* were collected from oak leafs. All collected beetles were transferred to the laboratory and kept refrigerated at 1–4 °C to reduce movement and prevent horizontal transmission of infection before investigation.

Bark beetles were dissected in vivo in a drop of water under a stereo microscope as described by Wegensteiner (1996) and were observed for presence of pathogens and nematodes under light microscopes Zeiss and Olympus BX60 DIC at magnifications 100× and 400×. Dead adults of *A. nitens* were also inspected microscopically. When symptoms of mycosis were found, small pieces of tissue were removed after surface sterilization and placed in a humid chamber to allow the sporulation of the fungal pathogens. Other pieces were placed on SDAY (Sabouraud dextrose agar with yeast extract) plates for isolation of fungi into pure cultures. Fungal conidia and conidiogenous cells from insect cadavers in the humid chamber and from pure cultures were used to prepare smears stained with methylen-blue or slide preparations with lactophenol and aniline blue closed by nail vanish (after Humber 1997). Fungal pathogens were identified according to their morphological characteristics (Samson et al. 1988; Humber 1997).

Nematodes found in bark beetles were isolated, heat-killed (65 °C), fixed in TAF (triethanolamin, destilled water and

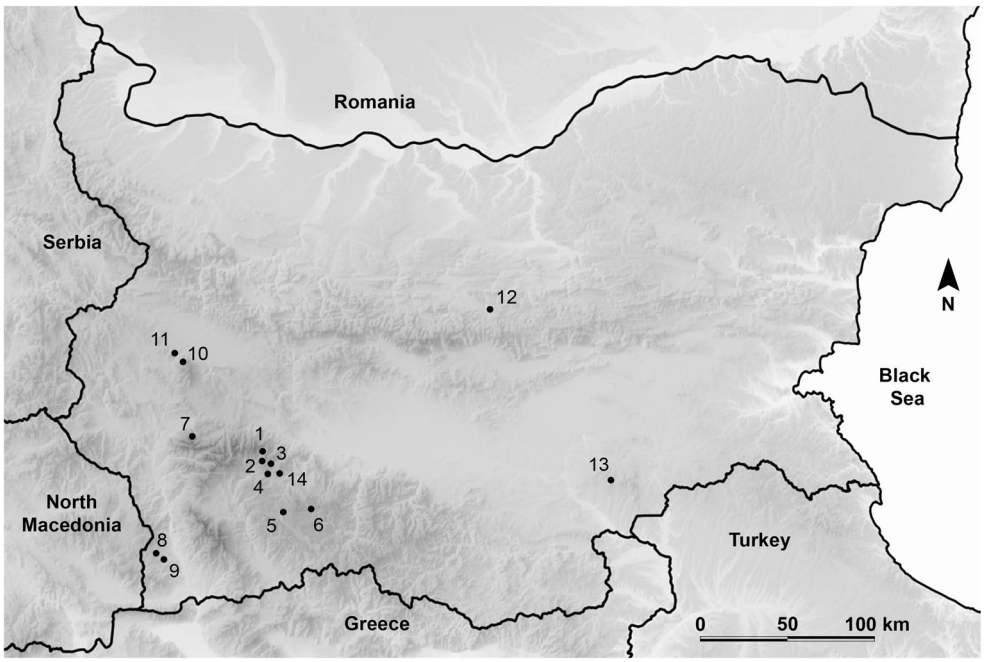

Fig. 1 Map of localities. 1. Rila Mt., Belmeken Dam, 1940 m a.s.l.; 2. Rila Mt., Kurtovo Place, 1760 m a.s.l.; 3. Rila Mt., Yundola Place, 1550 m a.s.l.; 4. Western Rodopes Mts., Pashovi skali Place, 1360 m a.s.l.; 5. Western Rodopes Mts., Kara tepe Hut, 1530 m a.s.l.; 6. Western Rodopes Mts., Beglika Place, 1560 m a.s.l.; 7. Rila Mt., Skakavitsa Hut, 1700 m a.s.l.; 8. Maleshevska planina Mt., W of Tsaparevo Vill., 940 m a.s.l.; 9. Maleshevska planina Mt., S of Tsaparevo Vill., 820 m a.s.l.; 10. Vitosha Mt., Zlatnite mostove Place, 1400 m a.s.l.; 11. Lyulin Mt., Bonsovi polyani Place, 900 m a.s.l.; 12. Balkan Range, near Gabrovo, 660 m a.s.l.; 13. Sakar Mt., Zvezdata Place, 500 m a.s.l.; 14. Western Rodopes Mts., W of Velingrad, 1000 m a.s.l

formalin), processed in glycerin (Seinhorst 1959) and studied in permanent mounts. In most of cases only larval stages of nematodes were observed.

For transmission electron microscopy (TEM) of the found microsporidium infected tissues were fixed in 2.5% glutaraldehyde in 1 M cacodylate buffer (pH 7.2) and postfixed for 2 h in 2% OsO_4. The tissues were then dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite or in Poly/Bed 812/Araldite 502 (Becnel 2012). Thick sections (1.0 mm), stained according to Richardson et al. (1960) were inspected using light microscopy to locate infected cells. Thin sections were cut on an Ultracut E. Reichert microtome, stained with uranyl acetate and lead citrate, and examined with a Philips EM 208 transmission electron microscope.

The infection levels were calculated as total prevalence of the parasites for each host species.

Results

A microsporidium belonging to genus *Nosema* Nägeli, 1857 was detected in the fat body of *P. chalcographus* with a prevalence of only 0.9% (Table 1). The microsporidian spores were oval and measured 2.4 μm (2.0–2.8) x 4.7 μm (3.7–5.6). The ultrastructural investigations of this microsporidium revealed internal structure typical for the genus *Nosema*, with binuclear sporogonial stages and spores. The nuclei were in diplokaryotic arrangement. The polaroplast was lamellar and the polar filament is isofilar with 15–16 coils, situated in one row (Figs. 2 and 3).

The microsporidium reported in this study is the first *Nosema* sp. found in *P. chalcographus*.

Most of the collected dead adults of *A. nitens* (23 of 37) showed symptoms of mycoses (Table 1). Beetles were covered with a dense whitish mycelium and some of them showed

Table 1 Host trees, found pathogens and parasites, their localization and prevalence in the insect hosts

Host species	Locality (Fig. 1)	Host tree	N *	Found pathogens and parasites	Localization (number of infected/ total prevalence)
Dryocoetes autographus (Ratzeburg, 1837)	7	*Picea abies* (L.) H.Karst.	40	*Parasitylenchus* sp. – larvae *Bursaphelenchus* sp. – larvae	gut lumen (24/ 60%)
Hylurgus ligniperda (Fabricius, 1787)	13	*Pinus sylvestris* L.	108	*Bursaphelenchus* sp. – larvae	haemocoel (93/ 86.1%)
Ips acuminatus (Gyllenhal, 1827)	3	*P. sylvestris*	68	*Bursaphelenchus* sp. – larvae *Parasitaphelenchus* sp. – larvae	haemocoel and gut lumen (24/ 35.2%)
I. sexdentatus (Börner, 1776)	13	*P. sylvestris*	10	*Bursaphelenchus* sp. – larvae *Parasitaphelenchus* sp. – larvae	haemocoel and gut lumen (9/ 90%)
I. typographus (Linnaeus, 1758)	2, 10	*P. abies*	30	*Parasitylenchus dispar* (Fuchs 1915) – adults	haemocoel (21/ 70%)
Orthotomicus erosus (Wollaston, 1857)	13	*P. sylvestris*	15	*Neoparasitylenchus* sp. – larvae *Bursaphelenchus* sp. – larvae *Cryptaphelenchus* sp. – larvae *Panagrolaimus* sp. – larvae	haemocoel and gut lumen (12/ 80%)
O. laricis (Fabricius, 1792)	12	*P. sylvestris*	9	*Bursaphelenchus* sp. – larvae *Sulphuretylenchus* sp. – larvae	haemocoel and gut lumen (5/ 55.5%)
Pityogenes chalcographus (Linnaeus, 1761)	4, 6	*P. abies*	552	*Cryptaphelenchus diversispicularis* Korenchenko, 1987 – adults *Parasitorhabditis subelongati* Slobodjanjuk, 1973 – adults *Nosema* sp.	haemocoel (254/ 46%) fat body (5/ 0.9)
P. conjunctus (Reitter, 1887)	1	*Pinus mugo* Turra	22	*Bovianema* sp. – larvae	haemocoel (8/ 36.4%)
P. quadridens (Hartig, 1834)	4	*P. sylvestris*	23	*Bursaphelenchus* sp. – larvae *Cryptaphelenchus* sp. – larvae	malpighian tubules and haemocoel (4/ 17.4%)
Taphrorychus villifrons (Dufour, 1843)	11	*Fagus sylvatica* L.	85	*Bursaphelenchus* sp. – larvae	haemocoel (21/ 24.7%)
Tomicus piniperda (Linnaeus, 1758)	5, 8, 9, 12	*P. sylvestris*	113	*Parasitorhabditis* sp. – larvae *Parasitaphelenchus* sp. – larvae *Prothallonema tomici* Nedelchev, Takov and Pilarska, 2011 – adults *Bursaphelenchus* sp. – larvae	haemocoel (39/ 34.5%)
Attelabus nitens (Scopoli, 1763)	14	*Quercus petraea* (Matt.)	37	*Beauveria bassiana* (Bals.- Criv.) Vuill. (1912)	insect body (23/ 62%)

N *- number of investigated specimens

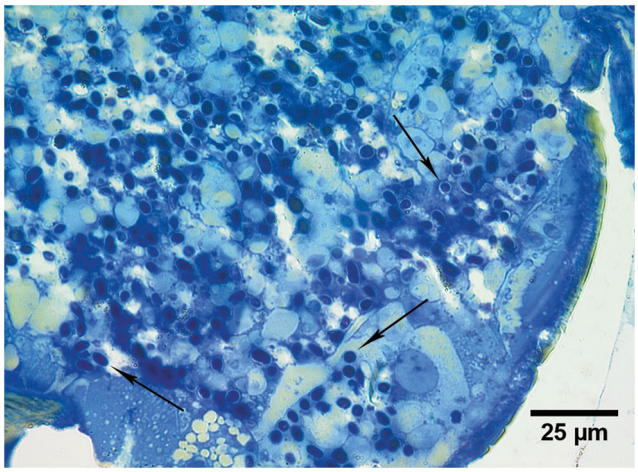

Fig. 2 Numerous *Nosema* sp. spores in fat body of *Pityogenes chalcographus* (semithin section, stained after Richardson et al. 1960)

colour changes of the elytra (Fig. 4). The isolates obtained from *A. nitens* were identified as *Beauveria bassiana* (Ascomycota anamorph form, Sordariomycetes: Hypocreales, Cordycipitaceae). Morphological characters of the examined fungal pathogen were typical for the species. Isolates from pure cultures on SDAY formed round raised colonies, most of them with powdery surface, with pigmentation from white to cream. Reverse sites of the colonies were with pale cream to pink-tan pigmentation (Fig. 5). Some of the isolates released pink pigment in the media which later paled and gradually disappeared. Conidiogenous cells were densely clustered in whorls, hyaline, smooth and short. They had a globose or elongated base terminated in a narrow extended denticulate apex with a distinctly zig-zag threadlike denticulate rachis with one conidium per denticle (Fig. 5). Conidia were one-celled, hyaline, thin-walled, hydrophobic, subglobose, with average dimensions 1.21 μm, 1.43 μm, 1.60 μm, 1.87 μm etc. for different *B. bassiana* isolates.

In this study nematodes were isolated from 12 beetle species, and we established 10 nematode genera. Three nematodes were determined to the species level. Infection levels varied from 17.4% to 90%. The nematodes were localized in the gut and haemocoel of the bark beetles.

Nematodes of the genus *Bursaphelenchus* Fuchs, 1937 were established in 9 of 12 investigated beetle species (Table 1): *Dryocoetes autographus*, *Ips sexdentatus*, *I. acuminatus, Orthotomicus laricis*, *Oerosus*, *Pityogenes quadridens*, *Hylurgus ligniperda, Tomicus piniperda* and *Taphrorychus villifrons* were identified as vectors for *Bursaphelenchus* in Bulgaria.

Parasitylenchus dispar was detected in *I. typographus* and *Parasitylenchus* sp. in *D. autographus*. *Cryptaphelenchus diversispicularis* was found in *P. chalcographus* and *Cryptaphelenchus* sp. in *P. quadridens* and *O. erosus*. Nematodes of genus *Parasitaphelenchus* Fuchs 1930 were found in the haemolymph of three bark beetle species – *I. acuminatus, I. sexdentatus* and *T. piniperda*. *Parasitorhabditis* sp. was detected in two hosts – *T. piniperda* and *P. chalcographus*. Nematodes of the genus *Sulphuretylenchus* Rühm, 1956 were found in one host – *O. laricis*. Another nematode genus, *Neoparasitylenchus* Nickle, 1967 was detected in *O. erosus*. *Panagrolaimus* sp. was found also in *O. erosus*.

Discussion

A literature review revealed that infections with microsporidia of the genus *Nosema* have been found in 14 curculionid beetle species todate (Table 2).

In most cases the target tissue of *Nosema* spp. was the fat body. Some *Nosema* spp. were also found in the gut tissue (mainly in midgut), Malpigian tubules and gonads. Two *Nosema* spp. were detected in bark beetles collected in Bulgaria – *H. ligniperda* (in the midgut) and *T. villifrons* (in the fat body and gonads) (Takov et al. 2007, 2011; Table 2). The size of the *Nosema* sp. found in this study was smaller than *Nosema* sp. from *T. villifrons* and from *H. ligniperda,* however, the size is similar to *Nosema calcarati*, *N. typographi* and

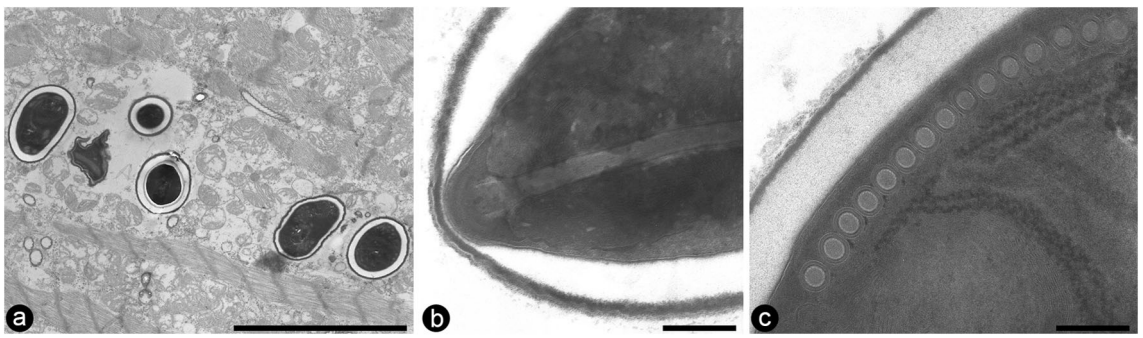

Fig. 3 TEM micrographs of *Nosema* sp. spores in *P. chalcographus*: **a** – total view, scale bar 10 μm; **b** – anterior spore part with anchoring disk, scale bar 0.5 μm; **c** – coils of the polar filament, scale bar 0.5 μm

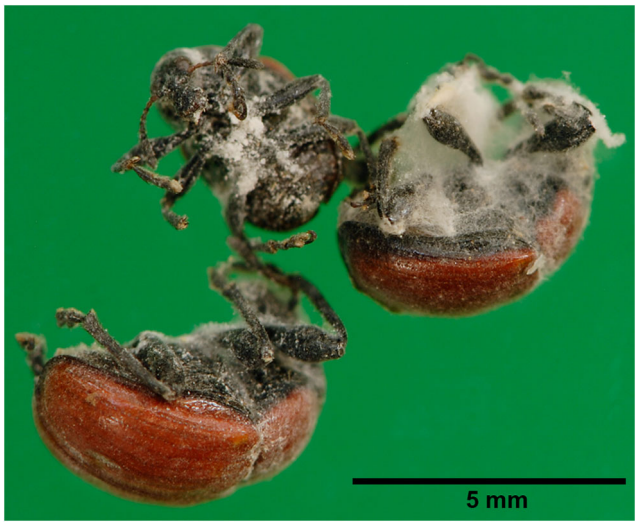

Fig. 4 Dead adults of *Attelabus nitens* with symptoms of mycosis caused by *Beauveria bassiana*

N. hylobii. *Nosema calcarati* and *N. typographi* possessed an isofilar polar filament with coils arranged in a single row and 12 coils (*N. calcarati)*, 16–17 coils (*N. typographi)*. *Nosema hylobii* has an anisofilar polar filament with 9–13 coils. The *Nosema* sp. in our study has an isofilar filament with 15–16 coils arranged in one row and thus shows similarity to *N. typographi*. The structure of the polaroplast was lamellar as described for *Nosema raphidae* Yaman, Radek, Tosun & Unal, 2009 by Yaman et al. (2009). However, for a precise species identification of the microspridium found in this study, molecular analysis is needed.

We also detected a mycosis caused by *B. bassiana* in the oak-leaf roller *A. nitens*. Unlike scolytids, *A. nitens* is not considered as a significant pest species. In Bulgaria its adults make specific and easily recognizable cylindrical rolls on leaves of *Quercus* spp. predominantly, and occasionaly on *Castanea sativa* Mill. According to Urbanová and Urban (2016) the young shoots of trees are prefered.

In Bulgaria, fungal infections caused by *B. bassiana* were detected in populations of different coleopteran forest pests by

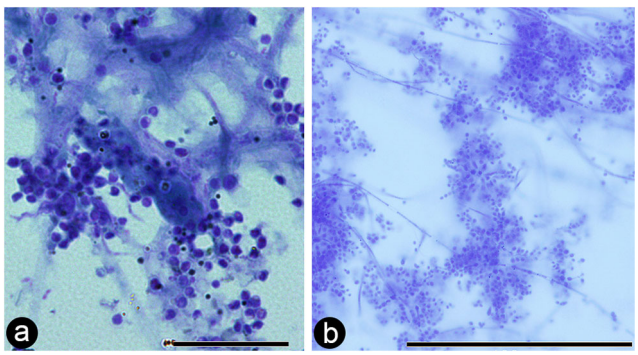

Fig. 5 Conidiophores and conidia of *B. bassiana* isolates from *A. nitens*: **a** 718Bb, scale bar 20 μm; **b** 720Bb, scale bar 100 μm

Markova (1992), Draganova et al. (2007, 2010) and Takov et al. (2011, 2012). However, so far no infection caused by *B. bassiana* was registerd in *A. nitens* and thus this leaf roller is a new host for the pathogen.

Nematodes of the genus *Bursaphelenchus* occur in coniferous forests in Europe, and 28 species (Braasch 2001) were found in more than 15 coniferous tree species in 20 countries of Europe. The list of vectors of this genus includes over 20 beetle species, 16 of which are bark beetles (Linit 1988; Kulinich and Orlinskii 1998). One beetle species can carry several *Bursaphelenchus* species, and one nematode species can use several beetle species as vectors. So far, only *Bursaphelenchus sexdentati* Rühm, 1960 has been found in *T. piniperda* from Bulgaria (Choleva, personal communication). Our study confirms *Bursaphelenchus* species in nine species of bark beetles. Nematodes of the genus *Bursaphelenchus* were also found by Burjanadze and Goginashvili (2009) in *I. typographus* from Georgia.

We recorded *Parasitylenchus dispar* in *I. typograhus*. This species was reported for the first time in Germany (Fuchs 1915) and later in Poland and former USSR (Filipjev and Schuurmans Stekhoven 1941), parasitizing *I. typographus*. In Bulgaria, Nedelchev et al. (2008) also reported this nematode species from *I. typographus* collected from spruce trees.

The impact of *Parasitylenchus* spp. on the host was investigated by different researchers (Fuchs 1915; Schvester 1950, 1957; Nickle 1971; Poinar Jr and Caylor 1974). They found changes in the structure of the galleries, shortened lifespan as well as a reduction of the fat body in the infected beetles. Bark beetles infected with *Parasitylenchus* spp. died shortly after the construction of short horizontal galleries (Nickle 1971; Schvester 1957). Rühm (1956) also investigated the impact of these nematodes and noted that 6% of the studied *Pityokteines curvidens* population were sterile and had reduced gonads.

Lieutier (1981) reported a delay in the reproduction of *Ips sexdentatus* after experimental infections with the nematodes *Contortylenchus diplogaster*, *Parasitaphelenchus sexdentati* and *Parasitaphelenchus* sp. in laboratory and field studies. Fat body and ovaries of bark beetles infected with these nematodes were less developed than in healthy beetles and reproduction and egg laying were delayed (Lieutier 1982, 1984).

We recorded *Parasitorhabditis* sp. in two bark beetle species – *P. chalcographus* and *T. piniperda*. Many of the representatives of the genus *Parasitorhabditis* Fuchs, 1937 are associated with bark beetles and only a few species can cause harm to their hosts. Larvae of *Parasitorhabditis* reduced the size of the midgut cells of *Ips confusus* (LeConte, 1876) (Nickle 1963). *Parasitorhabditis piniperdae* (Fuchs, 1937) may enter the hemocoel of *Tomicus piniperda* and *T. destruens* (Wollaston, 1865) (Rhum 1956; Laumond and Carle 1971). Laumond and Carle (1971) reported that *Parasitaphelenchus piniperdae* and *P. papillatus* Fuchs,

Table 2 *Nosema* species detected in other hosts

Nosema species	Curculionid hosts	Tissue*	Size of spores	Origin	Authors
Nosema typographi Weiser, 1955	*Ips typographus, Hylurgops palliatus* (Gyllenhal, 1813)	FB	3.6–5.3 × 2.0–3.5	CZ, DE, AU	Weiser (1955), Purrini (1978), Weiser et al. (1997)
Nosema dendroctoni (Weiser, 1970)	*Dendroctonus pseudotsugae* Hopkins, 1905	FB, MT	2.0–3.0 × 2.1	CA	Weiser (1970)
Nosema curvidentis Weiser, 1961	*Pityokteines curvidens* (Germar, 1824), *Scolytus scolytus* (Fabricius, 1775)	FB	2.5–3.6 × 1.5–2.0	CZ	Weiser (1961), Weiser (1968)
Nosema scolyti Lipa, 1968	*Scolytus ensifer* Eichhoff, 1881, *S. multistriatus* (Marsham, 1802), *S. pygmaeus* (Fabricius, 1787), *S. scolytus*	M, MT	3.6–6.2 × 2.0–3.3	PL, RU, DE	Lipa (1968)
Nosema dryocoetesi Purrini and Ormieres, 1981	*Dryocoetes autographus*	FB	2.5–3.0 × 1.2–1.5	DE	Purrini and Ormieres (1981)
Nosema calcarati Purrini and Halperin, 1982	*Pityogenes calcaratus* (Eichhoff, 1878)	G	3.5–5 × 2.5–3.0	DE	Purrini and Halperin (1982)
Nosema hylobii Purini, 1981	*Hylobius abietis* (Linnaeus, 1758)	M	4.5–6.0 × 1.5–3.0		Purrini (1981)
Nosema cf. *typographi*	*Ips amitinus* (Eichhoff, 1871)	FB	No data	CZ	Holuša et al. (2016)
Nosema sp.	*I. typographus*	FB	No data	GE	Burjanadze and Goginashvili (2009)
Nosema sp.	*H. palliatus*	FB, M	3.0–4.5 × 1.5–2.5	AU	Händel (2001), Händel and Wegensteiner (2003)
Nosema sp.	*Hylurgus ligniperda*	M	4.8–7.2 × 1.8–3.3	BG	Takov et al. (2007)
Nosema sp.	*Taphrorychus villifrons*	FB, G	2.4–3.9 × 1.2–3.0	BG	Takov et al. (2011)
Nosema sp.	*Pityogenes chalcographus*	FB	3.7–5.6 × 2.0–2.8	BG	Present study

*FB – fat body; MT – Malpighian tubules; G – gonads; M – midgut

AU – Austria, BG – Bulgaria, CA – Canada, CZ – Czech Republic, DE – Germany, GE – Georgia, PL – Poland, RU – Russia, TU – Turkey

1937 caused a reduction of fat body size and abnormal development of the gonads in adults of *T. destruens*.

Other studies confirm the high diversity of nematodes in bark beetles: Tomalak et al. (1989) investigated 31 species of bark beetles collected from nine host plants genera in Canada. Twenty one bark beetle species were infected with 56 nematode species. Among them 30 species were allantonematids, 14 rhabditid and 12 aphelenchoid species. Authors described three new species of *Sulphuretylenchus: S. pseudoundulatus* Tomalak, Welch & Galloway, 1989 parasitizing *Polygraphus rufipennis* (Kirby, 1837), *S. nopimingi* Tomalak, Welch & Galloway, 1989 in *Pityokteines sparsus* (LeConte, 1868) and *S. postuteri* Tomalak, Welch & Galloway, 1989 in *Ips perroti* Swaine, 1915.

According Ashraf and Berryman (1970) and Massey (1964) nematodes of genera *Sulphuretylenchus* and *Neoparasitylenchus* could cause sterility and decreased longevity of their hosts. Michalková et al. (2012) showed that *Neoparasitylenchus* sp. caused massive infections in *I. typographus* and was found in co-infections with protozoa *Gregarina typographi* Fuchs 1915.

In our study we found *Bovienema* sp. in *Pityogenes conjunctus*. Nematodes of genus *Bovienema* Nickle, 1963 were found for the first time in this host species. Bovien (1937) described *Bovienema tomicis* (Bovien, 1937) from the bark beetle *Pityogenes bidentatus* (Herbst, 1784) in Denmark. Nickle (1963) showed that 4% of *Pityogenes fossifrons* (LeConte, 1876) individuals collected from lodgepole pine in 1961 were found to harbor body cavity parasites similar to *B. tomicis*.

The genus *Panagrolaimus* was described by Fuchs (1930) who discovered the species *Panagrolaimus tigrodon* Fuchs 1930 in *T. destruens*. Rühm (1956) investigated *T. piniperda* and nematodes occurrence in its galleries including also *P. tigrodon*. Laumond and Carle (1971) found the same nematode species in *T. destruens* from France. Korentchenko (1992) investigated two bark beetles species, *Orthotomicus laricis* and *O. suturalis* (Gyllenhal, 1827), and described *Panagrolaimus orthotomici* Korentchenko, 1992.

Later Grucmanová et al. (2014) described in *Ips duplicatus* (Sahlberg, 1836) the nematode species *Contortylenchus diplogaster*, *Parasitylenchus* cf. *aculeatus* (Slankis, 1972), *Parasitorhabditis obtusa* (Fuchs, 1915), *Cryptaphelenchus* cf. *macrogaster* (Fuchs, 1937), *Micoletzkya buetschlii* (Fuchs, 1915) and *Parasitaphelenchus* sp., with a prevalence ranging from 1.5 to 74.3%. In *Ips cembrae* (Heer, 1836), Grucmanová et al. (2016) found *C. diplogaster*, *P. dispar*, *C.* cf. *macrogaster*, *P. obtusa*, *M.* cf. *buetschlii*, *Bursaphelenchus*

sp. and *Laimaphelenchus* sp. with a prevalence of 41.7 to 68.2%.

Holuša et al. (2017) studied the occurrence of insect pathogens and nematodes in *Orthotomicus laricis*, *O. erosus* and *O. nobilis* (Wollaston, 1862) in Central-South Europe and found nonspecific gut nematodes and nematodes in the haemolymph, identified as *Contortylenchus laricis* (Fuchs, 1929). Nematode prevalence in the haemolymph ranged from 3 to 25% in the three *Orthotomicus* species. Authors concluded that the incidence of pathogens and nematodes in the host species seemed unrelated to the beetle species, the deposition of eggs, the ability to outbreak, or the beetle distribution range.

Conclusion

In this study, we document the diversity of pathogens and nematodes in forest-related beetles in Bulgaria. This is the first report of microsporidiosis caused by *Nosema* species in the bark beetle *P. chalcographus* and of mycosis caused by the fungus *B. bassiana* in the oak-leaf roller *A. nitens*. The nematode genera *Sulphuretylenchus* and *Neoparasitylenchus* are also recorded for the first time in *O. laricis* and *O. erosus*, respectively. *Dryocoetes autographus*, *I. sexdentatus*, *I. acuminatus, O. laricis, O. erosus, P. quadridens, H. ligniperda, P. conjunctus* and *T. villifrons* are confirmed as vectors of *Bursaphelenchus* sp. in Bulgaria. The influence of the pathogens and nematodes and their effect on the hosts remain to be investigated, however other studies indicate a potential in the biological control of bark beetles. Therefore further laboratory investigations and field experiments about influence and effects of this pathogens and parasites are needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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