Forest Pathology
BLACKWELL
For. Path.
doi: 10.1111/efp.12031

On the genus *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchinae) associated with wood and insects from declining forest trees in the Czech Republic

V. Čermák[1], P. Vieira[2], V. Gaar[1], M. Čudejková[1], J. Foit[3], M. Zouhar[4], O. Douda[5] and M. Mota[2]

[1]Division of Diagnostics, State Phytosanitary Administration, Šlechtitelů 773/23, Olomouc, 779 00, Czech Republic; [2]Lab. Nematologia/ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7002-554 Évora, Portugal; [3]Department of Forest Protection and Game Management, Mendel University in Brno, Zemědělská 3, Brno, 613 00, Czech Republic; [4]Department of Plant Protection, Czech University of Agriculture, Kamýcká 129, Praha-Suchdol, 165 00, Czech Republic; [5]Department of Entomology, Research Institute of Crop Production, Drnovská 507/73, Praha-Ruzyně, 161 06, Czech Republic; [6]Email: vaclav.cermak@srs.cz (for correspondence)

Summary

An overview of the genus *Bursaphelenchus* in the Czech Republic is presented, based on a recent survey for monitoring the presence of the pinewood nematode, *Bursaphelenchus xylophilus*, as well as on previous reports of this genus in the country. In addition, we provide a morphological and molecular characterization of four *Bursaphelenchus* species (*B. eremus*, *B. pinophilus*, *B. vallesianus* and *B. borealis*) found during the monitoring programme for forest pests, conducted during 2006–2010, within the Moravian and Bohemian regions. Nematodes were extracted from over 1917 insects and 1493 wood samples collected from deciduous and coniferous trees exhibiting wilting and declining symptoms. *Bursaphelenchus* species were found only in 0.73% of insects and 0.47% of the total number of wood samples. *Bursaphelenchus borealis* and *B. pinophilus dauer* juveniles were found associated with the insect vectors *Dryocetes autographus* and *Pityogenes bidentatus*, respectively. While a total of seven *Bursaphelenchus* species are now reported from the Czech Republic, the status of *B. xylophilus* remains as absent.

1 Introduction

The detection of the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, in mainland Portugal and Madeira Island (Mota et al. 1999; Fonseca et al. 2012) marks one of the most recent introductions of an invasive species into pine forest ecosystems in Europe. More recently, three incursions have been reported from Spain as well (Abelleira et al. 2011; Robertson et al. 2011). Following the detection of the PWN in one of the member states of the European Union, all other members have been conducting specific programmes for the detection of this quarantine species in their own forests and strategic areas where the nematode could enter as a result of human activities (Anonymous 2007).

In the Czech Republic, coniferous forests are of high importance, occupying approximately 74% of the total forest area. Norway spruce (*Picea abies* L.) occupies 52.2% of the total forest area, followed by Scots pine (*Pinus sylvestris* L.) representing 17% of that area (Anonymous 2009). The other coniferous areas together represent 5.1% of the total area, distributed among larch (*Larix decidua* Miller, 3.9%), fir (*Abies alba* Miller, 1%) and other pine species that together represent no more than 0.2% (*Pinus mugo* Turra, *P. nigra* Arnold and *P. strobus* L.). The remaining area (25%) is occupied mostly by oak, beech, birch, hornbeam and alder species (Anonymous 2009). The potential introduction of the PWN in the Czech Republic could create a high impact on the country's forest resources, as the major coniferous species (*P. abies* and *P. sylvestris*) are hosts of the PWN (Evans et al. 1996).

Following EPPO recommendations, medium- and long-term monitoring for quarantine species are fairly well established in the country. To detect a hypothetical introduction of the PWN, several surveys have been conducted in the past years. Up to now, the PWN has not been detected in Czech forests (Běhalová 2006). During 2006–2011, a new survey was performed in several areas of Moravia and Bohemia, for monitoring the presence of *Bursaphelenchus* species in these regions. During this survey, several species were identified. Currently, the information about the genus *Bursaphelenchus* in the Czech Republic is very scarce based on single reports without any detailed morphological or molecular characterization of the species identified. Herein, we present an updated distribution of all *Bursaphelenchus* species reported for the country, including a morphological and molecular characterization of the *Bursaphelenchus* species found during our survey. Furthermore, a list of the most common insect species found associated with wilting or declining symptomatic trees is also provided, including the insect-associated nematodes.

2 Materials and methods

2.1 Wood sampling and nematode extraction

During 2006–2011, a survey was conducted in the Moravian and Bohemian regions of the Czech Republic as part of a general survey for forest quarantine species, including *B. xylophilus*. A total of 1493 wood samples were collected from decidu-

Received: 6.1.2012; accepted: 29.1.2013; editor: R. N. Sturrock

Table 1. Occurrence of *Bursaphelenchus* species in the Czech Republic.

Bursaphelenchus sp.	Location	Elevation (m a.s.l.)	Region	Host	Insect vector	Publication
B. borealis	N49 06.574 E13 30.340	857	Svojše (Bohemia)	*Picea abies*	*Dryocetes autographus*	This study
B. eremus	–	190	Bačov (Bohemia)	*Quercus robur*	*Scolytus intricatus*	Kubátová et al. 2000;
	N50 37.859, E15 05.269	329	Sychrov (Bohemia)	*Quercus* spp.	–	This study
B. fungivorus	–	–	–	Coniferous bark	–	Braasch et al. 2002;
B. hofmanni	–	–	–	Imported coniferous wood	–	(Tomiczek & Braasch, unpublished), in Braasch 2001;
B. mucronatus	–	–	–	*Pinus sylvestris*	–	(Tomiczek, *pers. comm.*), in Braasch 2001;
B. pinophilus	N48 53.873, E17 12.360	192	Rohatec (Moravia)	*Pinus sylvestris*	*Pythiogenes bidentatus*	This study
	N48 53.871, E17 12.652	167	Bzenec (Moravia)	*Pinus sylvestris*		This study
B. vallesianus	N49 58.995, E 13 49.349	490	Karlova ves (Bohemia)	*Pinus sylvestris*	–	This study
	N48 53.879, E17 11.127	200	Rohatec (Moravia)	*Pinus sylvestris*	–	Gaar et al. 2006

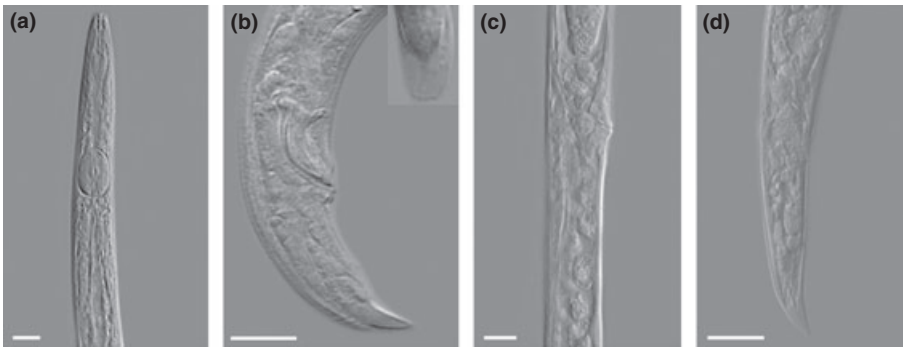

Fig. 1. Light micrograph of *Bursaphelenchus eremus* collected from wood. (a) Anterior region. (b) Male tail and bursa (ventral view). (c) Vulva region. (d) Female tail. Scale bars = 10 μm.

ous and coniferous trees (20–100 years old) exhibiting wilting and decline symptoms (pine, 1222; spruce, 129; larch, 6; oak, 33; orchard trees, 39; other deciduous trees, 64). Samples (approximately 50 g each) were collected from the trunk and branches of all sizes, displaying signs of the presence of insects (insect holes, galleries). Samples were collected after tree debarking and obtained using a drill or with the use of a chisel. On average, wood samples were incubated for 10 days at 27°C, within sealed plastic bags to avoid moisture loss. Nematodes were extracted using the 'tray method' technique over 48 h at room temperature (Whitehead and Hemming 1965). The extracted nematodes were killed and fixed in 4% formalin and transferred to pure glycerine according to Seinhorst (1959). Using a light microscope (Olympus, Bx50), morphological and morphometric analyses were performed from specimens directly collected from wood samples. Nematode identification was based on observations of the main morphological features for the genus *Bursaphelenchus*, particularly spicule shape, number and position of caudal papillae, number of incisures in the lateral field, presence of a vulval flap and female tail shape (Ryss et al. 2005; Braasch et al. 2009). For each species of *Bursaphelenchus* found, 10–20 specimens collected directly from wood samples were used to generate the respective morphological and morphometric data. Wood samples containing a sufficient number of *Bursaphelenchus* specimens were transferred and maintained in cultures of *Botrytis cinerea* Pers. ex Ft., growing in 5% malt extract agar (MEA).

2.2 Insect sampling and associated nematode identification

Within the same period, 1917 bark- and wood-boring insects were collected from the same declining or symptomatic trees and screened for the presence of *Bursaphelenchus* species. Bark and longhorn adult beetles were captured with soft entomological forceps after debarking parts of the trunk displaying symptoms of insect attack and kept in plastic tubes at 7°C until dissection. In addition, intact trunk sections cut from affected trees that contained potential longhorn beetle larvae were also

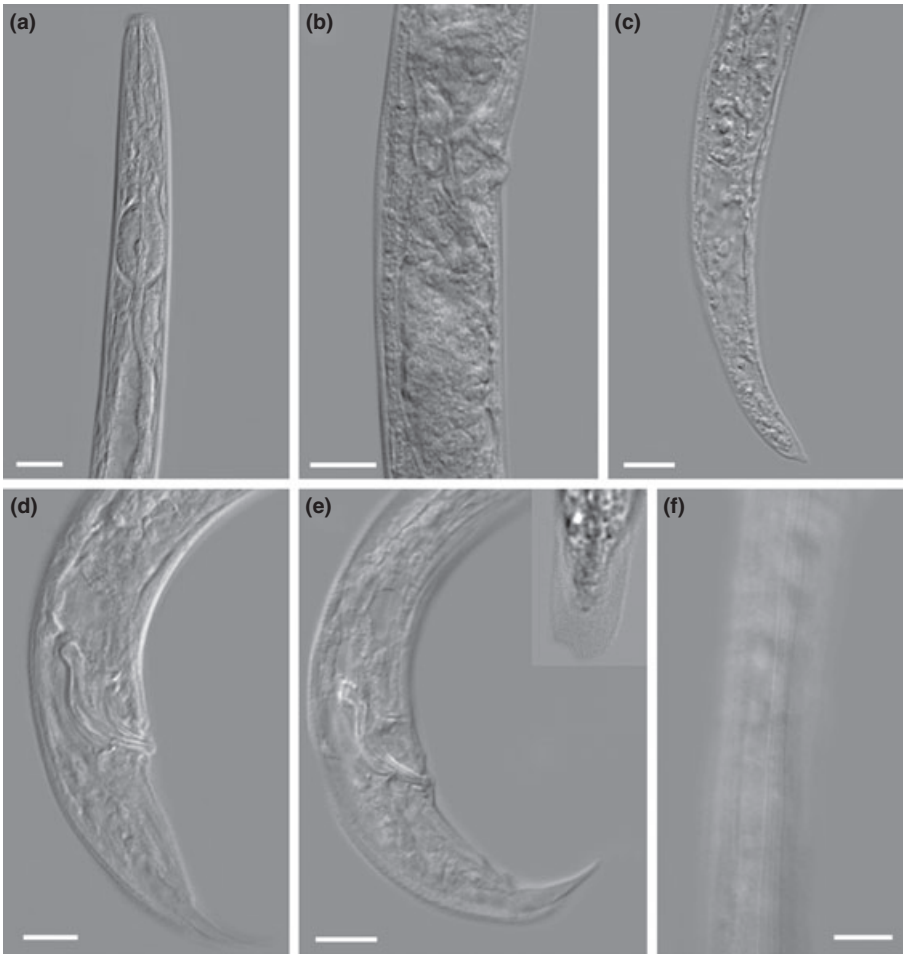

Fig. 2. Light micrograph of *Bursaphelenchus pinophilus* collected from wood. (a) Anterior region. (b) Vulva region. (c) Female tail. (d-e) Male tail, with ventral view of the bursa in (e). (f) Lateral field with four incisures. Scale bars = 10 μm.

collected. These sections were kept in the laboratory at room temperature until emergence of the insects. All adult insects were identified to species level. Each adult insect was individually dissected in water and examined for the presence of nematodes. All nematodes resembling *dauer* juveniles of the genus *Bursaphelenchus* were collected and identified using ITS-RFLP analysis, as described later. All other nematodes were mounted in temporary slides and identified up to the genus level.

2.3 Molecular analysis

Nematodes (1–15) were collected into a drop of demineralized water in a 1.5-ml Eppendorf tube and stored at −20°C for subsequent molecular characterization. Genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol for isolation of nucleic acids from mammalian tissues. DNA was eluted in 50 μl of pre-heated elution buffer. The target region containing the ITS1, 5.8S and ITS2 of ribosomal DNA was amplified by PCR using the forward primer 5′-CGTAACAAGGTAGCTGTAG-3′ (Ferris et al. 1993) and reverse primer 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain 1993). Each PCR was performed in a total volume of 50 μl, containing 1× PCR buffer (75 mM Tris–HCl pH 9,0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 4 mM $MgCl_2$, 400 μM dNTPs, 600 nM of each primer, 2 U of Biotools DNA polymerase (Biotools, Madrid, Spain) and 10 μl of template DNA. DNA was amplified in a Mastercycler Gradient thermocycler (Eppendorf) or Thermal XP Cycler (Bioer Technology Co., LTD, Hangzhou, China) using an initial denaturation step for 5 min at 94°C, followed by 40 reaction cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and extension for 2 min at 72°C, with a final extension for 10 min at 72°C. Ten microlitres of PCR product was analysed by electrophoresis using ethidium bromide stained 1.5% agarose gel in TBE buffer, and size of the products was compared with 100-bp DNA ladder (Promega Corporation, Madison, WI, USA). Six microlitres of the amplified DNA was digested for at least 2 h at 37°C, using 2.5 U of each of the five enzymes (*Alu*I, *Hae*III, *Hinf*I, *Msp*I and *Rsa*I) in the total volume of 10 μl. These five restriction enzymes are known to generate species-specific ITS-RFLP profiles for *Bursaphelenchus* species (Burgermeister et al. 2009). The restriction fragments were resolved by electrophoresis in a 3% (w/v) agarose polyacrylamide gel in TBE buffer and stained with ethidium bromide. The size of the fragments was estimated by comparing with 100-bp DNA ladder (Promega Corporation).

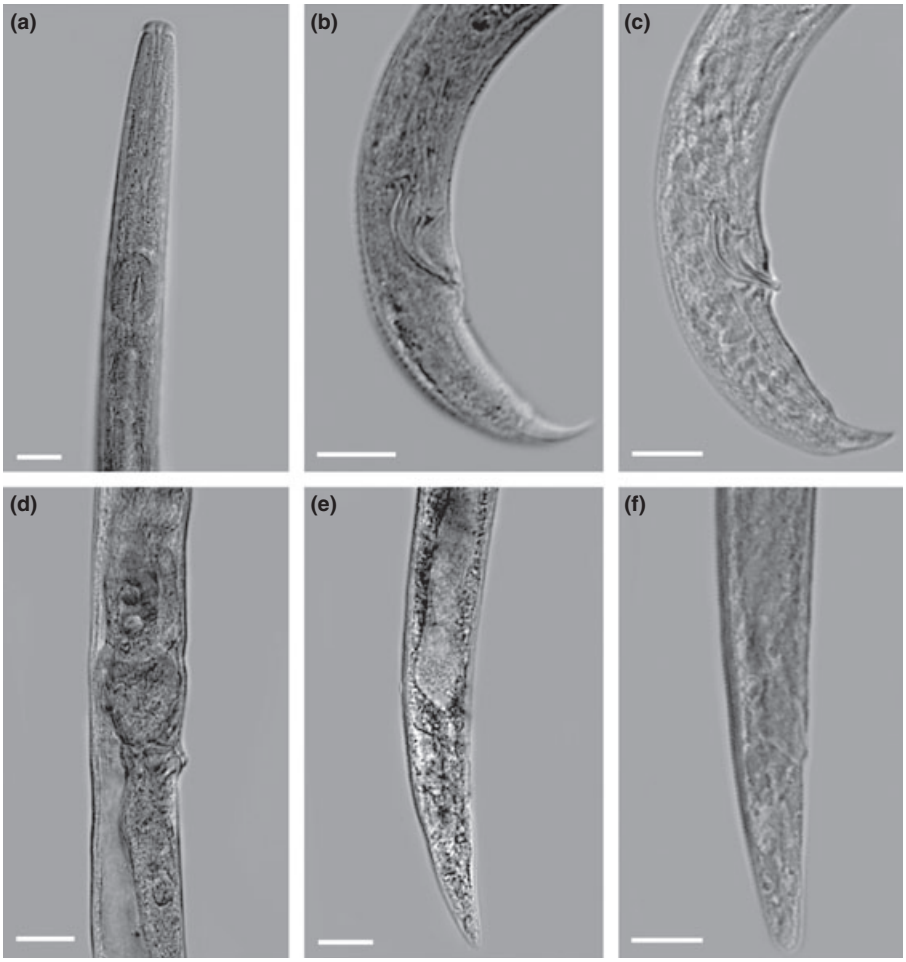

Fig. 3. Light micrograph of *Bursaphelenchus vallesianus* collected from wood. (a) Anterior region. (b–c) Male tail. (d) Vulva region. (e–f) Female tail. Scale bars = 10 μm.

3 Results

The geographical distribution of the *Bursaphelenchus* species found during this survey, as well as the species previously reported from the Czech Republic is summarized in Table 1. From the 1493 wood samples collected from the Moravia and Bohemia regions, only five samples contained *Bursaphelenchus* species (Table 1).

3.1 *Bursaphelenchus* spp. – Tree host association

A total of three different *Bursaphelenchus* species were identified from the 1493 wood samples collected from trees. These were *B. eremus* Rühm, 1956 (Fig. 1), *B. pinophilus* Brzeski and Baujard 1997 (Fig. 2), and *B. vallesianus* Braasch, Shönfeld, Polomski & Burgermeister, 2004 (Fig. 3). The morphological (Figs. 1–3) and morphometric data (Table 2) determined were generally in agreement with the respective original descriptions. *B. eremus* is characterized by short spicules (Fig. 1b), the presence of seven caudal papillae on males, three lateral incisures, females with no vulval flap (Fig. 1c), conical and gradually tapering female tail (Fig. 1d). Both *B. pinophilus* and *B. vallesianus* had stout and curved spicules, with prominent rostrum and condylus (Figs 2d, e and 3b, c, respectively), the presence of seven caudal papillae, four lateral incisures (Fig. 2f) and females with a small vulval flap (Figs 2b and 3d, respectively). Whereas *B. pinophilus* females have a more pointed and conoid tail (Fig. 2c), sometimes mucronate, *B. vallesianus* females present a conical, gradually tapering tail (Fig. 3e, f).

3.2 Insect–Nematode association

The 1917 insects collected from the declining trees were distributed among 13 different species, belonging to the families Buprestidae, Cerambycidae and Curculionidae. Nematodes were only detected in insects belonging to the subfamily Scolytinae (Curculionidae, 0.73% of the total number of insects collected), including two species of the genus *Bursaphelenchus* (Table 3), one of which was also detected in our wood samples. *Bursaphelenchus borealis* was isolated from several

Table 2. Morphometrics of three *Bursaphelenchus* species associated with declining conifer trees in the Czech Republic. All measurements are in micrometre and in the format: mean ± SD (range).

	Bursaphelenchus eremus (Sychrov population)		*Bursaphelenchus pinophilus* (Bzenec population)		*Bursaphelenchus vallesianus* (Rohatec population)	
	♀♀ n = 20	♂♂ n = 20	♀♀ n = 12	♂♂ n = 13	♀♀ n = 10	♂♂ n = 10
L	883.9 ± 109.8 (731–1200)	815.1 ± 49.3 (709–895)	777.9 ± 75.0 (706–983)	743 ± 62.2 (620–829)	1000.3 ± 73.4 (866–1116)	967.3 ± 96.0 (752–1107)
a	39.4 ± 5.0 (29.7–50.1)	43.9 ± 3.4 (38.0–51.3)	31.5 ± 2.0 (26.9–35.9)	45.8 ± 6.4 (35.3–54.3)	53.7 ± 4.8 (47.3–64.1)	61.0 ± 5.8 (47.0–68.9)
b	12.2 ± 1.4 (10.2–16.1)	11.2 ± 0.7 (9.9–12.8)	11.3 ± 0.3 (10.7–11.8)	11.7 ± 0.9 (10.6–13.5)	12.4 ± 0.3 (12.1–12.8)	12.9 ± 0.2 (12.4–13.1)
b′	6.2 ± 0.8 (5.0–7.9)	5.6 ± 0.3 (5.0–6.2)	5.6 ± 0.4 (5.2–6.5)	5.7 ± 0.9 (4.5–7.7)	6.7 ± 0.3 (6.1–7.1)	6.9 ± 0.6 (6.6–8.1)
c	25.6 ± 3.3 (20.4–35.7)	26.6 ± 1.7 (23.6–29.4)	22.2 ± 2.4 (20.1–27.5)	21.4 ± 0.9 (19.4–22.8)	38.8 ± 4.6 (29.4–43.6)	33.5 ± 5.8 (25.9–38.2)
c′	3.3 ± 0.4 (2.9–4.5)	2.3 ± 0.2 (1.9–2.5)	3.0 ± 0.3 (2.8–3.9)	2.8 ± 0.3 (2.4–3.4)	2.7 ± 0.3 (2.5–3.1)	2.5 ± 0.2 (2.2–2.7)
V [%]	74.1 ± 1.0 (71.9–75.8)	–	71.8 ± 1.2 (69.6–73.3)	–	73.2 ± 1.0 (72.0–75.1)	–
Post-uterine sac length/vulva to anus [%]	65.2 ± 7.2 (46.7–76.3)	–	60.4 ± 16.0 (34.7–88.3)	–	–	–
Stylet length	13.9 ± 0.5 (12.9–14.9)	13.9 ± 0.5 (12.9–14.9)	13.4 ± 0.2 (13.1–13.8)	13.3 ± 0.3 (13.2–14.1)	13.0 ± 0.9 (11.0–14.0)	12.9 ± 0.7 (12.0–14.0)
Excretory pore position	57.4 ± 3.2 (51.2–62.3)	57.6 ± 5.7 (43.66–65.2)	44.3 ± 4.4 (40.2–56.2)	54.6 ± 5.5 (41.0–60.3)	57.0 ± 2.9 (54.0–61.0)	57.5 ± 1.9 (55.0–60.0)
Max. body diam.	22.7 ± 3.4 (16.8–29.9)	18.7 ± 1.8 (15.8–22.1)	24.7 ± 2.0 (22.4–30.3)	16.6 ± 2.7 (12.5–21.8)	18.7 ± 1.2 (17.0–21.0)	15.9 ± 1.1 (15.0–18.0)
Anal body diam.	10.5 ± 0.8 (8.3–11.4)	13.6 ± 0.6 (12.5–14.9)	11.9 ± 1.1 (9.8–13.4)	12.6 ± 1.2 (10.6–14.6)	10.5 ± 0.5 (10.0–11.0)	11.1 ± 0.0 (11.0–11.5)
Tail length	34.7 ± 2.9 (30.5–41.2)	30.8 ± 2.0 (24.4–34.2)	35.2 ± 2.9 (32.0–40.5)	34.7 ± 3.0 (28.3–38.6)	26.0 ± 2.6 (23.0–33.0)	29.0 ± 2.2 (26.0–35.0)
Post-uterine sac length	125.9 ± 19.4 (88.2–178.0)	–	111.5 ± 33.5 (60.0–167.0)	–	130.1 ± 17.3 (11.0–155.0)	–
Spicule length (curved median line)	–	13.4 ± 0.5 (12.5–14.8)	–	14.2 ± 1.4 (12.3–17.4)	–	15.6 ± 2.3 (13.0–21.0)

Table 3. Bark- and wood-boring insects collected from declining and symptomatic trees from Bohemia and Moravia regions and respective associated nematodes.

Insect species	Examined specimens	Specimens infested with nematodes	Associated nematodes
Buprestidae			
Chrysobothris affinis	1	0	–
Phaenops cyanea	4	0	–
Cerambycidae			
Monochamus galloprovincialis	8	0	–
Plagionotus arcuatus	1	0	–
Spondylis buprestoides	3	0	–
Curculionidae			
Molytinae			
Pisodes piniphilus	22	0	–
Scolytinae			
Dryocetes autographus	6	5	*Bursaphelenchus borealis* *Rhabditis*
Hylurgus ligniperda	18	2	*Aphelenchoides*
Ips acuminatus	80	23	*Contorthylenchus* *Cryptaphelenchus* *Ektaphelenchus* *Parasitaphelenchus* *Parasitorhabditis* *Parasitylenchus*
Ips duplicatus	930	186	*Cryptaphelenchus* *Ektaphelenchus* Unidentified nematodes
Orthotomicus proximus	62	37	*Cryptaphelenchus* *Contorthylenchus.* *Parasitaphelenchus* *Parasitorhabditis*
Pityogenes bidentatus	37	12	*Bursaphelenchus pinophilus*
Pityogenes chalcographus	569	85	*Cryptaphelenchus* Unidentified nematodes
Pityophthorus pityographus	34	5	Unidentified nematodes
Tomicus piniperda	74	9	*Contorthylenchus* *Parasitorhabditis*
Tomicus minor	68	12	*Parasitorhabditis*
Total	1917	376	

specimens of the bark beetle, *Dryocetes autographus* Ratzerburg, collected from symptomatic *Picea abies* trees. The *dauer* juveniles (Fig. 4) displayed three lateral incisures (Fig. 4a), slender body (Fig. 4b), dome-shaped head and a degenerated stylet (not clearly visible), oval metacorpus (Fig. 4e), with a variable conical tail (Fig. 4c, d). Our molecular analyses confirmed the identification of these specimens as *B. borealis* Korentchenko 1980 (Fig. 6).

Dauer juveniles of *B. pinophilus* were found associated with *Pityogenes bidentatus* (Herbst) (Čermák et al. 2012), collected from declining *P. sylvestris*. The *dauer* juveniles (Fig. 5a) were morphologically very similar, displaying the typical dome-shaped head, indistinct stylet, and elongated conoid to digitate tail (Fig. 5b, d). The morphobiometric data for the *dauer* juveniles of both species are presented in Table 4.

In the case of *Dryocetes autographus*, *Ips acuminatus* (Gyllenhal), *Ips duplicatus* (Sahlberg), *Orthotomicus proximus* Eich., *Pityogenes chalcographus* (L.) and *Tomicus piniperda* (L.), more than one nematode genus were carried by these insects (Table 3).

3.3 Molecular characterization

For each species of *Bursaphelenchus* that was collected in this study, a molecular characterization was performed using the ITS1, 5.8S and ITS2 region of the ribosomal DNA. The ITS-RFLP patterns obtained for all *Bursaphelenchus* species extracted from the declining trees or insect vectors, showed the same reference patterns established by Burgermeister et al. (2009) and therefore validated our morphological and morphometric characterization (Fig. 6).

4 Discussion

Information regarding the diversity of the genus *Bursaphelenchus* in the Czech Republic is scarce and occurs in a variety of brief reports or abstracts without any morphological or morphometric characterization of the different species reported. The objective of our survey was to see whether *B. xylophilus* occurred within the regions of Bohemia and Moravia. Four *Bursaphelenchus* species – *B. eremus*, *B. pinophilus*, *B. vallesianus*, and *B. borealis* – were isolated and characterized. Based on our findings and other available data, we discuss the present knowledge and distribution of the genus within the country.

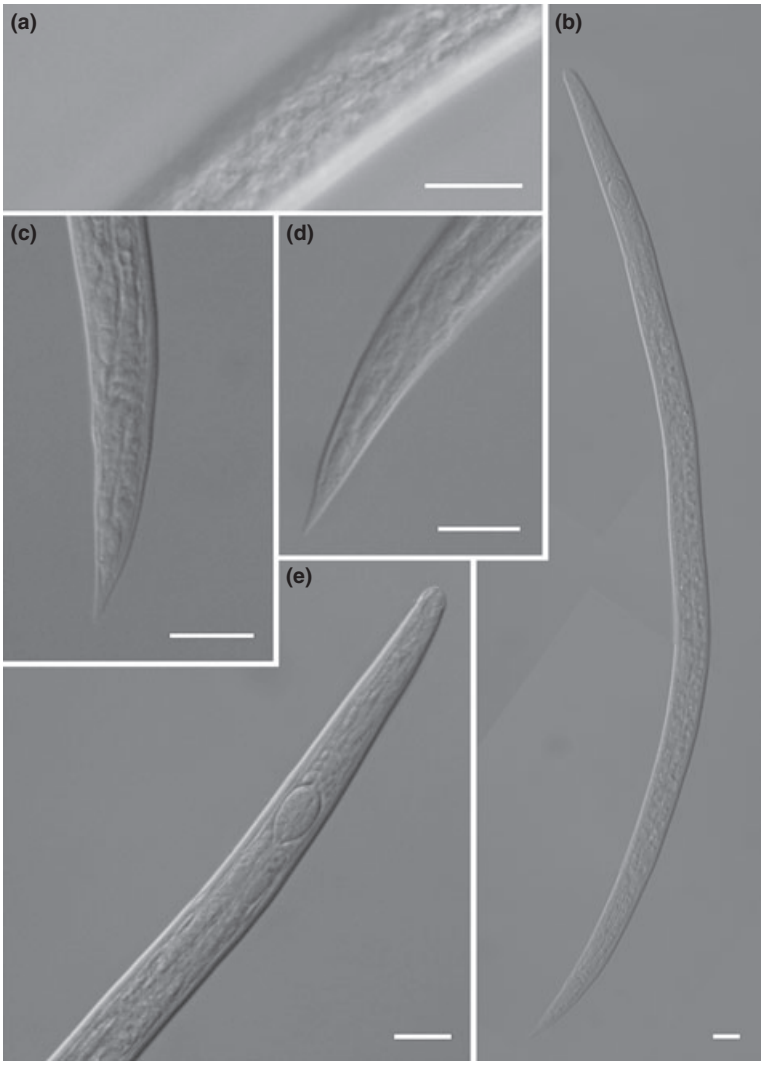

Fig. 4. Light micrograph of *dauer* juveniles of *Bursaphelenchus borealis*. (a) Lateral lines. (b) Whole *dauer* body. (c–d) Variation of *dauer* tails. (e) Anterior region. Scale bars = 10 μm.

The first report of the genus *Bursaphelenchus* for the Czech Republic was by Kubátová et al. (2000), who reported the association of *B. eremus* with the hyphomycetous microfungus *Esteya vermicola* and the bark beetle *Scolytus intricatus* (Ratzeburg), collected from several *Quercus robur*, in central Bohemia. During our survey, adults and juveniles of *B. eremus* were found within the trunk of declining *Quercus* spp., in the Sychrov castle park (near Mohelka River) in Bohemia. As confirmed by our morphological and molecular analyses, the presence of *B. eremus* seems to be widespread within country. The cerambycid *Plagionotus arcuatus* (L.) and the buprestid *Chrysobothris affinis* (Fabricius) collected from the same declining *Quercus* trees (Čermák et al. 2009) did not carry *Bursaphelenchus*.

Following the first report of *B. eremus*, three other species were recorded in the Czech Republic, that is, *B. fungivorus* Franklin & Hooper, 1962 (Braasch et al. 2002), *B. mucronatus* Mamiya & Enda, 1979 (unpublished data, in Braasch 2001) and *B. hofmanni* Braasch, 1998, which were all intercepted in imported coniferous wood (unpublished data, in Braasch 2001). However, no morphological description and no morphometric or molecular characterization was included in these reports. Due to the lack of information regarding the origin of the collected wood material, we were unable to trace their geographical location within the Czech Republic.

Bursaphelenchus vallesianus was found associated with declining *P. sylvestris* in both Moravia and Bohemia. This species had previously been reported to occur in the Moravia region (Gaar et al. 2006; Zouhar et al. 2006). Although *B. vallesianus* has repeatedly been associated with *P. sylvestris* in central Europe (CABI 2010), it has recently been reported from different regions of Eurasia, such as Turkey (Akbulut et al. 2008) and China (CABI 2010). The potential involvement of *B. vallesianus* on *P. sylvestris* decline in Valais (Switzerland) has been suggested (Polomski et al. 2008). Furthermore, some pathogenic potential of *B. vallesianus* was also demonstrated experimentally when 3-year-old *P. sylvestris* trees, under a drought stress situation, were inoculated with species (Polomski and Rigling 2010). Whether this species can be considered pathogenic

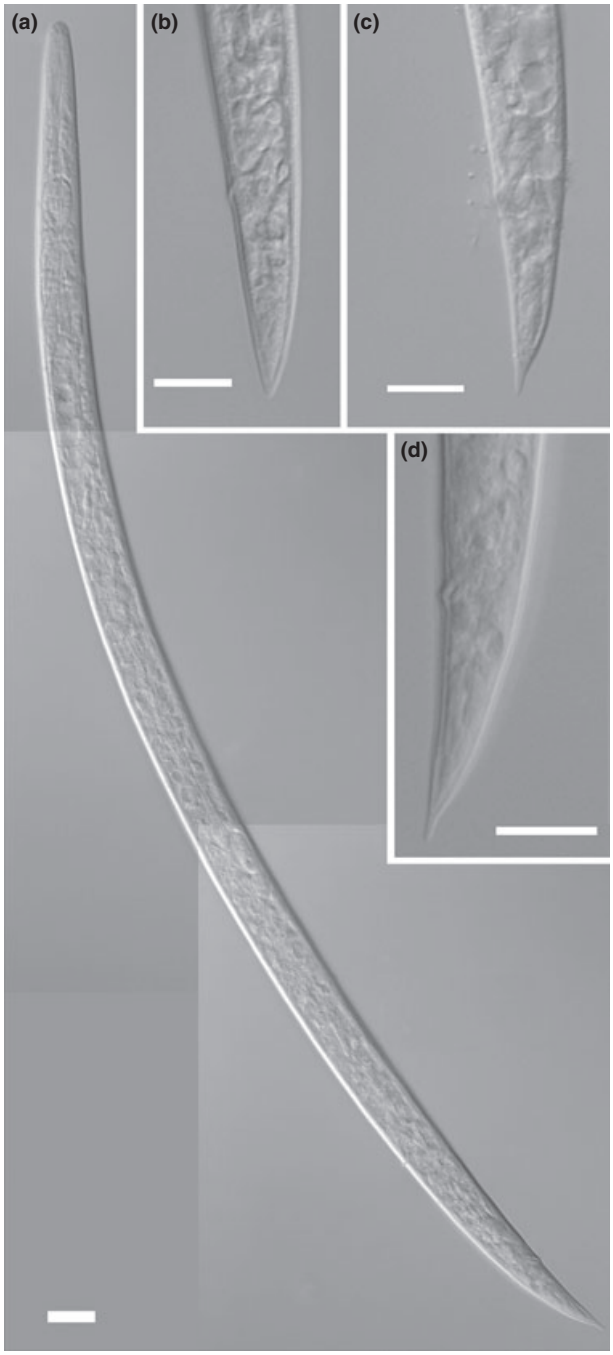

Fig. 5. Light micrograph of *dauer* juveniles of *Bursaphelenchus pinophilus*. (a) Whole *dauer* body. (b–d) Variation of *dauer* tails. Scale bars = 10 μm.

and harmful to pine forests remains unclear. Although all trees surveyed during this study showed symptoms of decline, *B. vallesianus* occurred in only two trees. Due to the low number of individuals found on each tree, and occasional distribution, no correlation could be drawn between the declining pine trees and the presence of this species.

During our survey, *B. pinophilus* was found for the first time in the Czech Republic and it was associated with declining *P. sylvestris*, in two different areas of Bohemia. This species has been reported from Poland (Brzeski and Baujard 1997), Germany (unpublished data, in Braasch 2001), Portugal (Penas et al. 2007) and recently from Korea, where it was associated with dead *Pinus koraiensis* (Han et al. 2009). The *sexdentati* group of species has been showing a tight correlation with insects belonging to the Scolytinae (Ryss et al. 2005; Braasch et al. 2009). Our previous findings showed for the first time an association of *B. pinophilus* with its insect vector, the bark beetle *P. bidentatus* (Čermák et al. 2012).

Our survey results also enable us to report for the first time in the Czech Republic, *B. borealis* in association with the bark beetle *D. autographus*, which occurs under the bark of declining *P. abies*. *D. autographus*, which is considered a

Table 4. Morphometrics of *dauer* juveniles of *Bursaphelenchus borealis* and *B. pinophilus* isolated from the bark beetles *Dryocetes autographus* and *Pityogenes bidentatus*, respectively. All measurements are in micrometre and in the format: mean ± SD (range).

	Bursaphelenchus borealis (*dauer* juveniles isolated from *D. autographus*) n = 5	*Bursaphelenchus pinophilus* (*dauer* juveniles isolated from *P. bidentatus*) n = 8
L	406.4 ± 42.0 (346–466)	374.4 ± 11.9 (363–394)
a	31.7 ± 2.1 (29.8–35.2)	25.6 ± 1.1 (23.8–28.0)
c	15.9 ± 1.5 (20.1–27.5)	14.3 ± 0.6 (13.8–15.0)
c´	3.4 ± 0.2 (3.1–3.7)	2.9 ± 0.1 (2.9–3.1)
Head - MB	49.2 ± 3.5 (46.2–54.7)	46.6 ± 1.2 (45.0–48.5)
Max. body diam.	12.8 ± 1.1 (11.6–14.3)	14.7 ± 0.5 (14.3–15.5)
Anal body diam.	7.5 ± 0.4 (7.1–8.2)	8.9 ± 0.4 (8.2–9.4)
Tail length	25.7 ± 2.4 (22.5–28.9)	26.2 ± 0.7 (25.0–26.7)
MB width	6.5 ± 0.2 (6.2–6.8)	7.4 ± 0.5 (6.5–7.8)
MB length	11.3 ± 0.2 (11.0–11.6)	13.5 ± 1.1 (11.3–14.7)
Genital primordium (GP) length	15.0 ± 2.1 (12.7–19.0)	19.1 ± 4.3 (12.0–23.6)
Head – GP	257.7 ± 36.7 (204.4–314.0)	247.0 ± 25.1 (217.0–297.0)

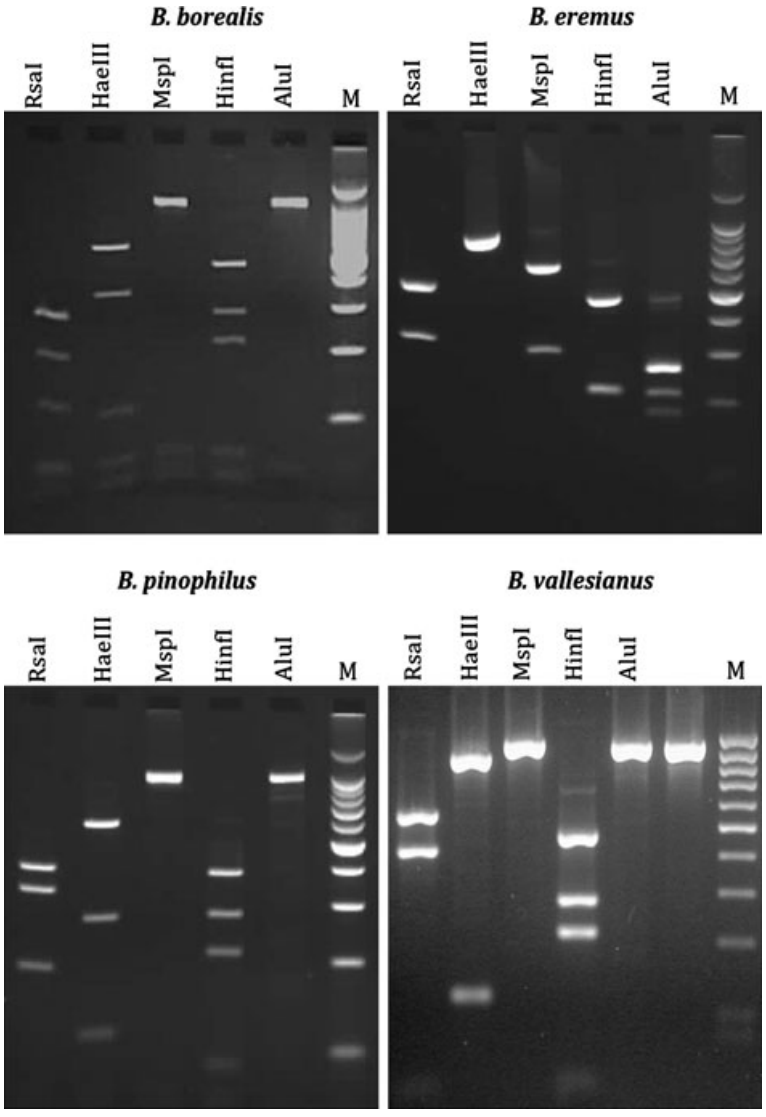

Fig. 6. ITS-RFLP profiles of *B. borealis, B. eremus*, *B. pinophilus* and *B. vallesianus*. The enzymes used for the restriction digest are indicated above the corresponding line. M: DNA size marker (100 bp).

Palearctic species occurring abundantly from low to high altitudes of central Europe, has been reported before as the insect vector of *B. borealis* (Braasch et al. 1999). This insect species develops mainly under the bark of spruces (*Picea* spp.), and occasionally in some other conifers, feeding on heavily weakened, dying or felled trees that were previously attacked by more aggressive bark beetles (Postner 1974). Its preferential occurrence with the base or stumps of the tree, due to the favourable moist conditions (Pfeffer 1955), could also reflect the preferable habitat for *B. borealis*. After its original description from Russia (Magadan territory), in association with the vector *Ips subelongatus* Motschulsky (Korentchenko 1980), *B. borealis* has been occasionally reported for Germany (Braasch et al. 1999), in intercepted wood from Russia (Braasch et al. 2001) and associated with *Pinus brutia* Tenore in Cyprus (Braasch and Philis 2002).

During our survey of dying or symptomatic trees, special attention was also paid to the collected insects that are also known to vector *Bursaphelenchus* species. Most of the insects we collected belong to the subfamily Scolytinae, suggesting a strong association between these species and decaying trees. The number of associated nematodes with different species within the Scolytinae demonstrates a broader association of these insects with a different set of nematode genera, including two species of *Bursaphelenchus.* Within the Cerambycidae, only eight specimens of *Monochamus galloprovincialis* (Olivier), the insect vector of *B. xylophilus* in Portugal (Sousa et al. 2001), were captured, without any associated nematodes. Although *Monochamus* spp. are considered secondary forest pests, mainly associated with weakened or dying host trees (Evans et al. 2004), they assume great importance as they are the major insect carriers of *B. xylophilus* worldwide (Akbulut and Stamps 2012). In the Czech Republic, five species of the genus *Monochamus* have been reported, namely *M. galloprovincialis*, *M. saltuarius* (Gebler), *M. sartor* Fabricius, *M. sutor* (L.) and *M. urussovi* (Fischer), with both *M. galloprovincialis* and *M. sutor* representing the most widely distributed species (Sláma 1998). The wide distribution of species of the genus *Monochamus* within the Czech Republic reinforces the idea that a continuous plan for monitoring the main forests could be advantageous, because all the conditions for potential introduction and propagation of *B. xylophilus* are met in certain areas of the country. Up to know, and based upon the analysis of several hundreds of samples, the status of *B. xylophilus* in the country remains as being absent.

To summarize, to date seven *Bursaphelenchus* species have been reported to occur in the Czech Republic. Future surveys within other regions of the Czech Republic will generate a better knowledge of the full diversity and distribution of the genus *Bursaphelenchus* in the Czech forests.

Acknowledgements

This research was partly supported by an ERASMUS, Portugal–Czech Republic Cultural Agreement Programme (2007–2010), Czech Ministry of Agriculture, project number QF4156, the Ministry of Education of the Czech Republic, number MSM6046070901 and MSM 6215648902. P. Vieira and M. Mota were partially supported by the EC 7th Framework project REPHRAME (KBBE.2010.1.4-09, 'Analysis of the potential of the pine wood nematode (*Bursaphelenchus xylophilus*) to spread, survive and cause pine wilt in European coniferous forests in support of EU plant health policy'). The authors kindly thank to Kateřina Široká and Hana Orságová from the Division of Diagnostics Olomouc, State Phytosanitary Administration, for technical assistance.

References

Abelleira, A.; Picoaga, A.; Mansilla, J. P.; Aguin, O., 2011: Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in Northwestern Spain. Plant Dis. **95**, 776.

Akbulut, S.; Stamps, W. T., 2012: Insect vectors of the pinewood nematode: a review of the biology and ecology of *Monochamus* species. For. Path. **42**, 89–99.

Akbulut, S.; Elekcioglu, I. H.; Keten, A., 2008: First record of *Bursaphelenchus vallesianus* Braasch, Schonfeld, Polomski, and Burgermeister in Turkey. Turk. J. Agric. For. **32**, 273–279.

Anonymous, 2007: EPPO standards: national regulatory control systems. EPPO Bull. **37**, 219–220.

Anonymous, 2009: Report on forestry of the Czech Republic 2009. Prague: Ministry of Agriculture of the Czech Republic, pp. 113.

Běhalová, M., 2006: Recent surveys for *Bursaphelenchus xylophilus* in the Czech Republic. EPPO Bull. **36**, 467–469.

Braasch, H., 2001: *Bursaphelenchus* species in conifers in Europe: distribution and morphological relationships. EPPO Bull. **31**, 127–142.

Braasch, H.; Philis, J., 2002: New records of *Bursaphelenchus abietinus* sp. in Cyprus. Nematol. Medit. **30**, 55–57.

Braasch, H.; Metge, K.; Burgermeister, W., 1999: *Bursaphelenchus*-Arten (Nematoda, Parasitaphelenchidae) in Nadelgehölzen in Deutschland und ihre ITS-RFLP-Muster. Nachricht. Deutsch. Pflanzenschutz. **51**, 312–320.

Braasch, H.; Tomiczek, C. H.; Metge, K.; Hoyer, U.; Burgermeister, W.; Wulfert, I.; Schönfeld, U., 2001: Records of *Bursaphelenchus* spp. (Nematoda, Parasitaphelenchidae) in coniferous timber imported from the Asian part of Russia. Forest Pathol. **31**, 129–140.

Braasch, H.; Bennewitz, A.; Hantusch, W., 2002: *Bursaphelenchus fungivorus* - ein Nematode aus der Gruppe der Holznematoden im Pflanzsubstrat eines Gewächshauses und in Holz-und Rindenimport. Nachricht. Deutsch. Pflanzenschutz. **54**, 1–4.

Braasch, H.; Burgermeister, W.; Gu, J., 2009: Revised intra-generic grouping of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae). J. Nematode Morphol. System. **12**, 65–88.

Brzeski, M. W.; Baujard, P., 1997: Morphology and morphometrics of *Bursaphelenchus* (Nematoda: Aphelenchoididae) species from pinewood of Poland. Ann. Zool. **47**, 305–319.

Burgermeister, W.; Braasch, H.; Metge, K.; Gu, J.; Schröder, T.; Woldt, E., 2009: ITS-RFLP analysis, an efficient tool for differentiation of *Bursaphelenchus* species. Nematology **11**, 649–668.

CABI, 2010: *Bursaphelenchus vallesianus*. Distribution Maps of Plant Diseases (October): Map 1084 (Edition 1081).

Čermák, V.; Gaar, V.; Orságová, H.; Foit, J.; Široká, K.; Tománková, T., 2009: The re-discovering of *Bursaphelenchus eremus* Rühm, 1956 (Nematodea, Parasitaphelenchidae) in the Czech Republic in 2008. In: Abstract of Šafránková, I.,Šefrová, H., Sborník abstraktů XVIII. České a Slovenské konference o ochraně rostlin. 1 vydání Brno: MZLU v Brně, 2009. Czech Republic, 2009, pp. 140 (abstract).

Čermák, V.; Vieira, P.; Gaar, V.; Čudejková, M.; Foit, J.; Široká, Mota, M., 2012: Bursaphelenchus pinophilus Brzeski & Baujard, 1997 (Nematoda: Parasitaphelenchinae) associated with nematangia on Pityogenes bidentatus (Herbst, 1783) (Coleoptera: Scolytinae), from the Czech Republic. Nematology **14**, 385–387.

Evans, H. F.; McNamara, D. G.; Braasch, H.; Chadouef, J.; Magnusson, C., 1996: Pest risk analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monochamus*. EPPO Bull. **26**, 199–249.

Evans, H.; Moraal, L. G.; Pajares, J. A., 2004: Buprestidae and Cerambycidae. In: Bark and wood boring insects in living trees in Europe, a synthesis. Ed. by Lieutier, F.; Day, K. R.; Battista, A.; Gregoire, J. C.; Evans, H. F. Dordrecht: Kluwer, pp. 447–474.

Ferris, V. R.; Ferris, J. M.; Faghihi, J., 1993: Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. Fund. Appl. Nematol. **16**, 177–184.

Fonseca, L.; Cardoso, J. M. S.; Lopes, A.; Pestana, M.; Abreu, F.; Nunes, N.; Mota, M.; Abrantes, I., 2012: The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. Helminthologia **49**, 96–103.

Gaar, V.; Zouhar, M.; Douda, O.; Marek, M.; Nováková, E.; Rysánek, P., 2006: First occurrence of *Bursaphelenchus vallesianus* in the Czech republic. In: Abstracts of Pine wilt disease: a worldwide threat to forest ecosystems. International Symposium, 10-14 July, 2006. Lisbon, Portugal, 2006, pp. 62 (abstract).

Han, H.; Chung, Y. J.; Shin, S. C., 2009: First report of *Bursaphelenchus pinophilus* on Korean pine (*Pinus koraiensis*). Plant Dis. **93**, 1.

Korentchenko, E. A., 1980: New species of nematode from the family Aphelenchoididae, parasites of stem pests of the Dahuran Larch. Zoologicheskii Zhurnal **59**, 1768–1780.

Kubátová, A.; Novotný, D.; Prášil, K.; Mráček, Z., 2000: The nematophagous hyphomycete *Esteya vermicola* found in the Czech Republic. Czech Mycol. **52**, 227–235.

Mota, M.; Braasch, H.; Bravo, M. A.; Penas, A. C.; Burgermeister, W.; Metge, K.; Sousa, E., 1999: First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology **1**, 727–734.

Penas, A. C.; Bravo, M. A.; Valadas, V.; Mota, M., 2007: Detailed morphobiometric studies of *Bursaphelenchus xylophilus* and characterization of other *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) associated with *Pinus pinaster* in Portugal. J. Nematode Morphol. System. **10**, 137–163.

Pfeffer, A., 1955: Kůrovci – Scolytidae. Fauna ČSR. Svazek 6. Praha: ČSAV, pp. 324.

Polomski, J.; Rigling, D., 2010: Effect of Watering Regime on Disease Development in *Pinus sylvestris* Seedlings Inoculated with *Bursaphelenchus vallesianus* and *B. mucronatus*. Plant Dis. **94**, 1055–1061.

Polomski, J.; Rigling, D.; Schweingruber, F., 2008: Development of external and internal symptoms in pine seedlings (*Pinus sylvestris*) due to inoculation with *Bursaphelenchus vallesianus*. In: Pine Wilt Disease: A Worldwide Threat to Forest Ecosystems. Ed. by Mota, M.; Vieira, P. Netherlands: Springer Science + Business Media B.V., pp. 335–344.

Postner, M., 1974: Scolytidae (= Ipidae), Borkenkäfer. S. In: Die Forstschädlinge Europas. Bd. II. Ed. by Schwenke, W. Paul Parey: Berlin, pp. 334–482.

Robertson, L.; Cobacho Arcos, S.; Escuer, M.; Santiago Merinos, R.; Esparrago, G.; Abelleira, A.; Navas, A., 2011: Incidence of the pinewood nematode *Bursaphelenchus xylophilus* Steiner & Buhrer, 1934 (Nickle, 1970) in Spain. Nematology **13**, 755–757.

Ryss, A.; Vieira, P.; Mota, M.; Kulinich, O., 2005: A synopsis of the genus *Bursaphelenchus* Fuchs (Nematoda: Parasitaphelenchidae) with a key to species. Nematology **7**, 393–458.

Seinhorst, J. W., 1959: A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. Nematologica **4**, 67–69.

Sláma, M. E. F., 1998. [Long-horned beetles (Cerambycidae) of the Czech Republic and the Slovak Republic (Beetles-Coleoptera).], Krhanice: Milan Sláma, pp. 383.

Sousa, E.; Bravo, M. A.; Pires, J.; Naves, P.; Penas, A. C.; Bonifácio, L.; Mota, M., 2001: *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal. Nematology **3**, 89–91.

Vrain, T. C., 1993: Restriction fragment length polymorphism separates species of the *Xiphinema americanum* group. J. Nematol. **25**, 361–364.

Whitehead, A. G.; Hemming, J. R., 1965: A comparison of some quantitative methods of extracting small vermiform nematodes from soil. Annals Appl. Biol. **55**, 25–38.

Zouhar, M.; Gaar, V.; Douda, O.; Marek, M.; Nováková E., ; Ryšánek, P., 2006: First occurrence of *Bursaphelenchus vallesianus* in the Czech Republic. In: Abstracts of the Proceedingsof XVII. Czech and Slovak Plant Protection Conference, 12-14 September, 2006. Czech Agricultural University, Prague, Czech Republic, pp. 426-428 (abstract).