Bulletin OEPP/EPPO Bulletin (2013) **43** (1), 144–151 ISSN 0250-8052. DOI: 10.1111/epp.12011

Survey of the genus *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae) in Romania

M. Calin[1], P. Vieira[2], C. Costache[1], H. Braasch[3]*, J. Gu[4], J. Wang[4] and M. Mota[2]

[1]*Central Phytosanitary Laboratory, 11 Voluntari, Voluntari, 077 190, Romania; e-mail: calin.monica@lccf.ro*

[2]*Lab. Nematologia/ ICAAM – Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Núcleo da Mitra, Ap. 94, Évora, 7002-554, Portugal*

[3]*Federal Biological Research Centre for Agriculture and Forestry, Messeweg 11, Braunschweig, D-38104, Germany*

[4]*Technical Centre, Ningbo Entry-exit Inspection and Quarantine Bureau, 9 Mayuan Road, 315012, Ningbo, Zhejiang, China*

**Present address: Kantstrasse 5, Postdam, D-14471, Germany*

An overview of the occurrence of species of the genus *Bursaphelenchus* in Romania is presented. The data is based on recent surveys conducted for the first time throughout the country, to monitor and evaluate the potential entry of the pine wood nematode, *Bursaphelenchus xylophilus*. Wood samples were collected from declining trees, wood-processing companies and imported wood packaging material. Of the 895 wood samples examined, 11 contained *Bursaphelenchus* specimens. Morphological and molecular analyses were carried out to characterize the species detected. With respect to the possible presence of *B. xylophilus*, all samples were negative, confirming the absence of this quarantine pest in Romania. Nevertheless, five *Bursaphelenchus* species were found: *B. hofmanni*, *B. poligraphi*, *B. vallesianus*, *B. willibaldi,* and one putative new species belonging to the *sexdentati* group, classified here as *Bursaphelenchus* sp. NR512. These results constitute the first report of the genus *Bursaphelenchus* in Romania.

Introduction

In forest ecosystems, the pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, is regarded as one of the most important pests worldwide. *B. xylophilus* is an EU quarantine pest and on the EPPO A2 List of pests recommended for regulation as quarantine pests. The causal agent of pine wilt disease, *B. xylophilus* causes devastating damage to pine forests (Mota & Vieira, 2008). The detection of *B. xylophilus* in continental Portugal in 1999 (Mota *et al.*, 1999), and more recently in Madeira island (Fonseca *et al.*, 2012) and in Spain (Abelleira *et al.*, 2011; Robertson *et al.*, 2011), has triggered specific measures to control the potential spread and new outbreaks of this pest in other European countries. Due to the potential of *B. xylophilus* to have severe effects on pine forest ecosystems in Europe, and the implications for quarantine restrictions on wood exports from infested areas, each member state of the European Union has proceeded with national surveys and monitoring programmes to avoid the introduction of this pest into national forests. Following Council Directive 2000/29/EC, and also before the integration of Romania into the European Union in 2007, a national plan had been established for the continuous surveillance of natural conifer forests and potential entry areas such as wood processing industries or points of entry for wood imports.

The total forest area in Romania consists of over 6.3 million ha, corresponding to 27% of the total land area of the country. Romanian forests are diverse, consisting of 31% conifers (23% spruce, 5% fir trees, 3% other conifers); 31% beech; 18% oaks; and 20% other broadleaf trees (15% hardwood, 5% softwood) (Donița *et al.*, 1990; Borlea *et al.*, 2006).

Prior to this study, it was not known if *Bursaphelenchus* species were present in Romania. In the context of monitoring the potential introduction of *B. xylophilus* in Romania's forest resources and studying the distribution of other *Bursaphelenchus* species, national surveys were carried out under the Ministry of Agriculture and Rural Development authority since 2006. This study aimed to determine the occurrence and distribution of species belonging to the genus *Bursaphelenchus* in Romania.

Material and methods

Sampling and nematode extraction

From 2006 to early 2012, wood samples were collected in coniferous forests from declining or symptomatic trees (symptoms included needle discoloration or wilting), wood-processing industries (sawdust) in Romania, and wood packaging of commodities originating from other countries. Five core samples per tree/packaging material were taken up to a total of 150 g using a low-speed drill (20 mm). Wood samples were placed in polythene bags and sent to the Central Phytosanitary Laboratory in Voluntari.

Occasionally, bark and small wood pieces were also included within the different samples collected. Prior to nematode extraction, wood samples were incubated at approximately 25°C for 14 days. Nematodes were then extracted using a modified Baermann funnel method, and collected after 48 h in distilled water. The nematodes were observed using an Olympus SZ60 binocular stereoscope, and individuals were isolated for detailed characterization and identification under the microscope. Specimens belonging to the genus *Bursaphelenchus* were picked, killed and fixed in hot formalin (4%), and subsequently mounted on temporary slides. Nematodes were studied using a Leica DMLB light microscope fitted with a Leica DC300 camera, and the Leica DFC 295 image-processing software.

Molecular characterization

DNA extraction was carried out using one to three nematodes collected directly from wood samples. Nematodes were crushed between a glass slide and the cover slip by gentle pressure. The extract was recovered with 20 μL of lysis buffer (10 mM Tris pH = 8.8, 1 mM EDTA, 1% Nonidet P40, 100 μg mL^{-1} proteinase K) incubated at 60°C for 1 h, then at 95°C for 10 min (Ibrahim *et al.*, 1994). The ITS regions of rDNA were amplified using the forward primer 5′-CGTAACAAGGTAGCTGTAG-3′ (Ferris *et al.*, 1993) and reverse primer 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain, 1993). The PCR mixture (total volume 25 μL) contained 1× buffer enzyme with $MgCl_2$, 0.5 mM $MgCl_2$, 0.6 μM of each primer, 2 units Taq DNA polymerase (Qbiogene), 0.1 mM dNTPs (Qbiogene) and 4 μL DNA extract. A Techne-FlexiGene thermocycler was used for amplification, and the reaction consisted of a denaturation step at 94°C for 2.5 min followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension step of 5 min 72°C. Following PCR, 5 μL of the amplified product was analysed by electrophoresis in a 1% agarose gel. Amplified DNA was digested with *Alu*I, *Hae*III, *Hin*fI, *Msp*I and *Rsa*I restriction endonucleases (Promega and Fermentas) using an aliquot of 8.5 μL of the PCR product and 10 U of each enzyme, according to the manufacturer's instructions. Species-specific ITS–RFLP profiles for *Bursaphelenchus* were generated using these five restriction enzymes (Burgermeister *et al.*, 2009). Fragments were resolved by electrophoresis in 2% agarose gel. Data analysis was performed using GENi (Syngene) and 100 bp DNA Ladder (GeneRuler, Fermentas) as a molecular size marker.

For sequencing, a sample of the PCR product, together with both primers, was sent to Invitrogen (China). The ITS1/2 sequences were analysed and aligned using the software CLUSTAL X ver. 2 (Larkin *et al.*, 2007). The tree topology was obtained with the neighbour-joining (NJ) analysis with 1000 bootstrap replications using CLUSTAL X. The phylogenetic tree was visualized and annotated using the program FigTree (http://tree.bio.ed.ac.uk/software/figtree/). The ITS–RFLP profiles presented for the species of the *sexdentati* group were calculated from the ITS1/2 sequences available at the National Center for Biotechnology Information (NCBI), using the software EnzymeX 3 (http://www.mekentosj.com/science/enzymex).

Results

A total of 895 samples were collected from declining conifer trees, wood-processing industries and from wood packaging of imported goods throughout the country. Only 11 samples (approximately 1.1%) of the total number of wood samples contained *Bursaphelenchus* specimens (Table 1). In addition, 57 wood samples were randomly collected from wooden packaging material from other countries (including Brazil, China, Egypt, Indonesia, Libya, Portugal, Russia, South Africa, Turkey, Ukraine, USA); however, no nematodes were recovered from these samples.

The geographical distribution of *Bursaphelenchus* species found in Romania is shown in Table 1 and Fig. 1. Although several nematode species were present in numerous samples, only a few included *Bursaphelenchus* specimens. In all cases, nematode identification was based on observations of the main morphological and morphometric features for *Bursaphelenchus* (Ryss *et al.*, 2005; Braasch

Table 1 Distribution, localization and hosts of *Bursaphelenchus* species in Romania

Bursaphelenchus sp.	Location	Region	Host	Year of detection	Notes
B. hofmanni	Curtea de Arges	Arges	*Picea* sp.	2010	
	Onesti	Bacau	–	2010	Sawdust from conifers wood-processing industry
	Musetesti	Gorj	*Pinus* sp.	2011	
	Dobra	Hunedoara	–	2010	Sawdust from conifers wood-processing industry
	Ciurea	Iasi	–	2010	Sawdust from conifers wood-processing industry
	Osica de Sus	Olt	*Pinus* sp.	2010	
	Andreiasu de Jos	Vrancea	*Pinus* sp.	2011	
B. poligraphi	Musetesti	Gorj	*Pinus* sp.	2011	
B. vallesianus	Malin	Suceava	*Picea abies*	2008	First detection of the genus
B. willibaldi	Ocol silvic Ploiesti	Prahova	*Abies* sp.	2011	
Bursaphelenchus sp. NR512	Sita Buzaului	Covasna	*Picea* sp.	2012	Sawdust from conifers wood-processing industry

et al., 2009), particularly spicule shape and size, number and position of caudal papillae, number of incisures in the lateral field, and presence of a vulval flap. Four species were identified: *B. hofmanni* Braasch, 1998; *B. poligraphi* Fuchs, 1937; *B. vallesianus* Braasch, Schönfeld, Polomski and Burgermeister, 2004; and *B. willibaldi* Schönfeld, Braasch and Burgermeister, 2006.

B. hofmanni (Fig. 2) is around 600 μm long and has a delicate stylet of 13 μm (average) and spicules of the same length. The spicules are not strongly curved and have a prominent rostrum. The lateral field shows three lines. The female tail is conoid and has a finely rounded terminus. *Bursaphelenchus poligraphi* (Fig. 3) belongs to the species of the *sexdentati* group *sensu* Braasch *et al.* (2009), which have, in contrast to *B. hofmanni*, four lateral lines, stout and curved spicules, a small vulval flap and a long post-uterine branch. The stylet length of *B. poligraphi* is similar to that of *B. hofmanni*, but the spicules are slightly longer (15–18 μm). *Bursaphelenchus vallesianus* (Fig. 4) belongs likewise to the *sexdentati* group, has a small stylet (approx. 13 μm), a conical female tail with variable terminus, and males with spicules (on average 16 μm long) bearing a small cucullus at their distal end, a more or less pointed rostrum and a slightly dorsally bent condylus. *Bursaphelenchus willibaldi* (Fig. 5) belongs to the *fungivorus* group *sensu* Braasch *et al.* (2009) and is distinct from the other three species in lacking a vulval flap, but having slightly protruding vulval lips, a slim attenuated female tail, and 15–18 μm long spicules with blunt rostrum and slightly dorsally bent condylus and without cucullus. The *fungivorus* group species have also four lateral lines. All recorded *Bursaphelenchus* species differ significantly from *B. xylophilus* in their morphology, for instance in the shape of the female tail (rounded in *B. xylophilus*), lack of a large vulval flap, and completely different spicules, which are long, slender, semicircular, with distinct cucullus and a flattened condylus in *B. xylophilus* (Ryss *et al.*, 2005; Braasch *et al.*, 2009; EPPO, 2009).

ITS–RFLP analyses were performed to confirm the morphological identification. Three species have shown the same species-specific patterns (Fig. 6) as established previously by Burgermeister *et al.* (2009) for these species. In the case of *B. vallesianus,* only a morphological characterization was performed due to the restricted number of individuals found in the sample.

An additional species belonging to the *sexdentati* group *sensu* Braasch *et al.* (2009) (herein classified as *Bursaphelenchus* sp. NR512) was detected in wood sawdust collected from a wood-processing factory in the Covasna region. Amplification of the ITS1/2 region (including a partial region of both 18S and 28S rRNA genes) of this species resulted in a PCR product of 980 bp. Based on the full-length ITS1/2 sequence, this species is closest to *B. vallesianus*, forming a well supported clade with the other species belonging to the *sexdentati* group (Fig. 7). The position of this species in the *sexdentati* group cluster, as well as the differences of the ITS–RFLP patterns obtained (Table 2), suggests the occurrence of a new putative species for this group. However, due to a lack of sufficient specimens at present, a detailed morphological and molecular characterization of this species will follow later.

During the monitoring programme, a survey of vectors belonging to the genus *Monochamus* Dejean, 1821 (Coleoptera: Cerambycidae) was also conducted in the sampled forest areas. The only *Monochamus* specimens found during the entire 2010 survey were two specimens of *M. sutor* L. and two immature individuals identified as belonging to the *Monochamus* genus. However, no nematodes were found associated with these insects.

Discussion

Due to the huge potential economic and environmental damage that could be caused by the introduction of *B. xylophilus* into new non-endemic areas (as observed in

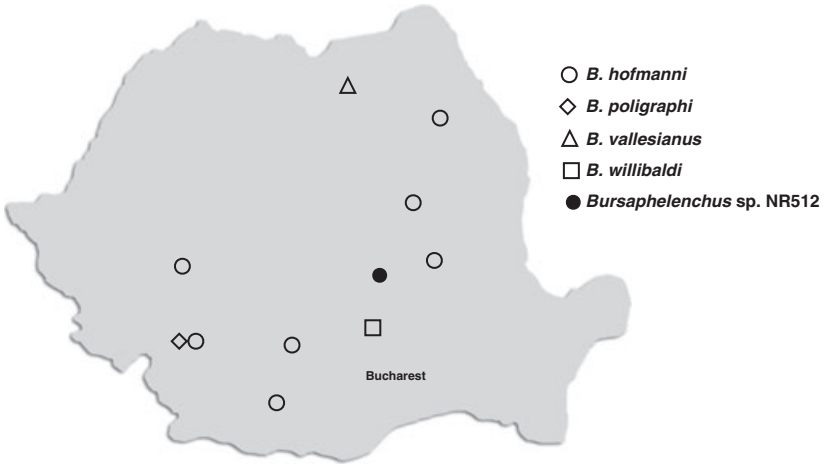

Fig. 1 Distribution of *Bursaphelenchus* species in Romania (see Table 1 for details of regions and locations).

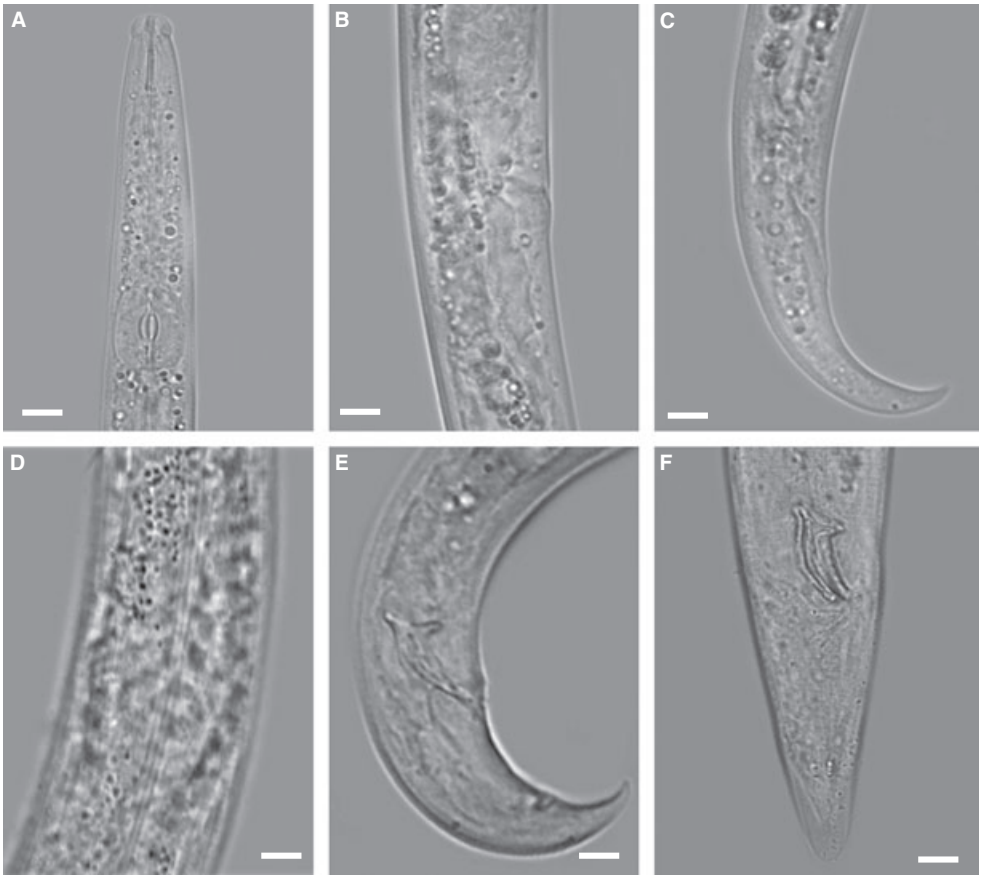

Fig. 2 Light microscope observations of *Bursaphelenchus hofmanni*, collected from Iasi county. A: anterior region; B: vulva region; C: female tail; D: lateral lines; E: male tail; F: ventral view of male tail and bursa. Scale bars: 10 μm.

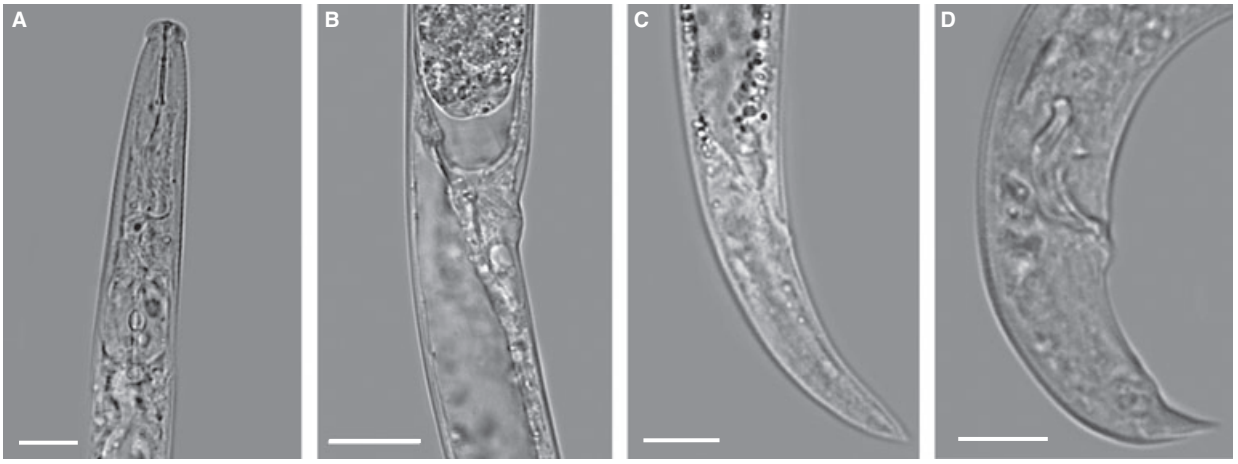

Fig. 3 Light microscope observations of *Bursaphelenchus poligraphi*, collected from Gorj county. A: anterior region; B: vulva region; C: female tail; D: male tail. Scale bars: 10 μm.

Portugal and Spain), it is imperative to conduct monitoring surveys related to the potential establishment of this species in forest areas of the European Union. The present study reflects the results obtained during the monitoring programme for *Bursaphelenchus* species in Romania from 2006 to early 2012, and reports the occurrence of *Bursaphelenchus* species for the first time in this country.

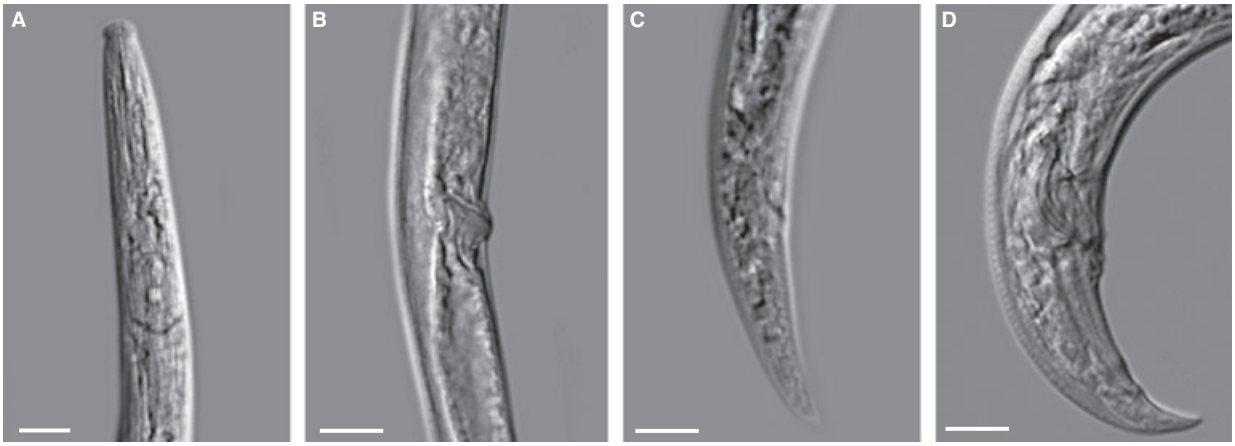

Fig. 4 Light microscope observations of *Bursaphelenchus vallesianus*, collected from Suceava county. A: anterior region; B: vulva region; C: female tail; D: male tail. Scale bars: 10 μm.

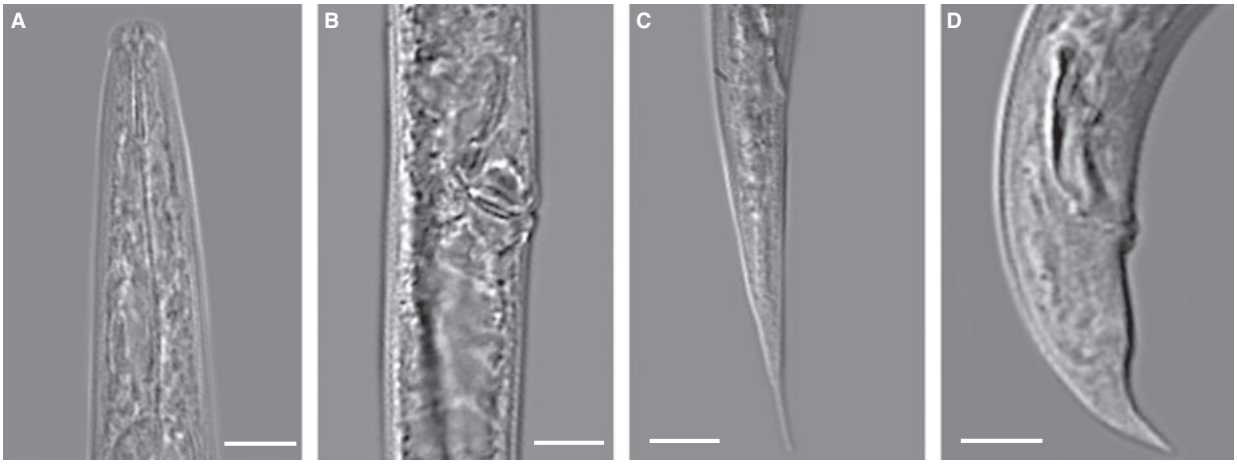

Fig. 5 Light microscope observations of *Bursaphelenchus willibaldi*, collected from Prahova county. A: anterior region; B: vulva region; C: female tail; D: male tail. Scale bars: 10 μm.

During the phytosanitary monitoring programme, special attention was given to conifer forest stands presenting declining/symptomatic trees in different areas, as well as to potentially high-risk areas such as wood-processing industries close to conifer forests. Wood samples were collected mainly from trees displaying needle discoloration or wilting that have shown any degree of susceptibility to *B. xylophilus* in nature or in laboratory trials, such as *Pinus*, *Picea* and *Abies* species (Evans *et al.,* 1996). In addition, samples collected from specific points of entry of imported wood from different countries, including countries where *B. xylophilus* occurs naturally, such as the USA, or from countries where it has become established, such as China and Portugal, were also considered for analyses during this monitoring programme. In each case, all samples examined were negative for *B. xylophilus*.

The main vectors of *B. xylophilus* are longhorn beetles belonging to the genus *Monochamus* (Akbulut & Stamps, 2012). In mainland Portugal, the only known insect vector of *B. xylophilus* is *M. galloprovincialis* Olivier (Sousa *et al.*, 2001, 2002), although in Spain and on the island of Madeira the specific insect vector is not yet known (Vicente *et al.*, 2012). In Romania, several *Monochamus* species have been reported, including *M. galloprovincialis*, *M. sartor* (Fabricius), *M. saltuarius* Gebler and *M. sutor* L. (Ruicănescu, 2007). Although special attention was paid to species of this genus, only a few specimens were found associated with declining/symptomatic trees during this monitoring programme; however, no association of these beetles with any nematodes was observed.

Despite the absence of *B. xylophilus*, according to the results of the monitoring programme the genus *Bursaphelenchus* is present, with several species occurring in Romanian conifer forests. The most common species found was *B. hofmanni*, showing a wide distribution range within the country, in association with different

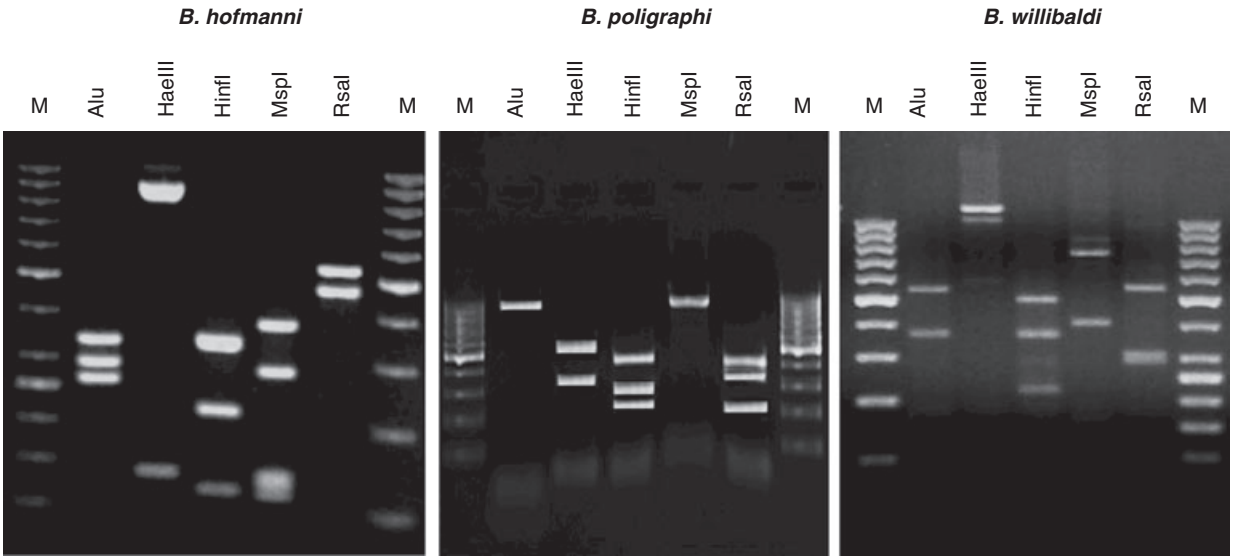

Fig. 6 ITS-RFLP patterns of *Bursaphelenchus* isolates found in Romania. The enzymes used for the restriction digest are indicated above the corresponding line. M: DNA size marker (100 bp).

Table 2 Size of DNA fragments obtained by ITS–RFLP analysis from sequencing results of the ITS1/2 region of *Bursaphelenchus* species belonging to the *sexdentati* group, closest to *Bursaphelenchus* sp. NR512

Bursaphelenchus sp.	PCR product (bp)	Restriction fragments (bp)				
		*Rsa*I	*Hae*III	*Msp*I	*Hin*fI	*Alu*I
Bursaphelenchus sp. NR512	980	543 415 22	477 385 118	980	464 209 159 70 53 25	967 13
B. vallesianus	981	543 416 22	862 119	981	465 280 212 24	967 14
B. sexdentati	981	543 416 22	585 277 119	981	465 280 212 24	967 14
B. pinophilus	983	416 346 199 22	586 278 119	983	381 280 212 86 24	983

The ITS–RFLP fragments for *B. vallesianus* (AM160662), *B. sexdentati* (AM269914) and *B. pinophilus* (AM160664) were calculated from the sequences available on NCBI.

host trees as demonstrated previously for other regions where this species was reported (Braasch, 1998; Braasch *et al.*, 2001; Braasch & Burgermeister, 2007). To date, the other four species have shown a more restricted distribution as they all were identified from a single specific area. Although the percentage of samples containing *Bursaphelenchus* species was very low (1.1%) in terms of the total number of samples, several samples collected from sawdust in wood-processing companies revealed the presence of *Bursaphelenchus* species, including a putative new species for the genus. These results reinforce the idea that a continuous monitoring programme, including wood samples from those industries, could be advantageous for studying not only the diversity of the genus in certain areas, but also the early detection of potentially infected trees containing *B. xylophilus*.

In conclusion, several species of *Bursaphelenchus* were found throughout Romania. To date, no single species belonging to the *xylophilus* group was detected in the several hundred wood samples processed. A continuous and more exhaustive sampling programme should support a better understanding of the diversity of the genus within the country, as Romania is rich in native tree species and natural forest ecosystems (Doniţă *et al.*, 1990; Borlea *et al.*, 2006).

Acknowledgements

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'). J. Gu and J. Wang were partially supported by the National Science and Technology Support Program (2012BAK11B03).

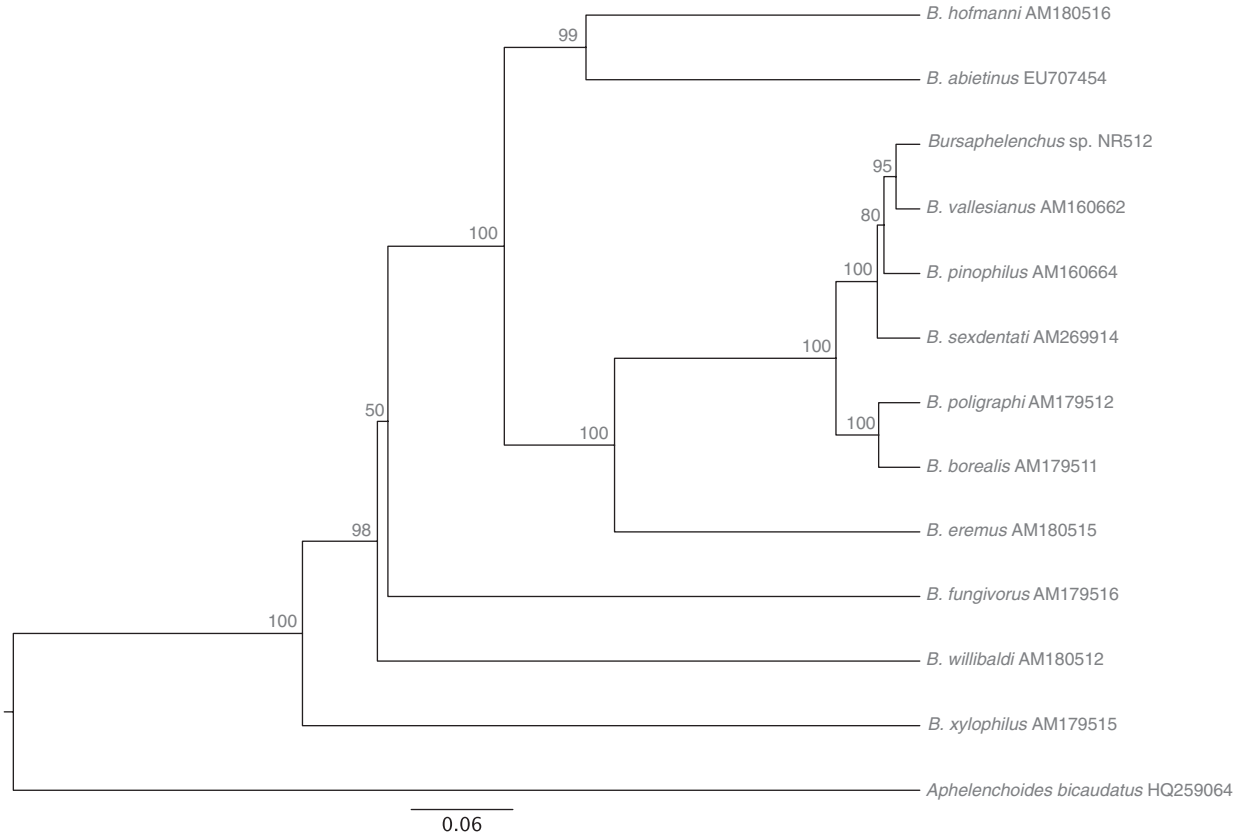

Fig. 7 Molecular phylogenetic status of *Bursaphelenchus* sp. NR512 based on the full ITS1/2 region sequence. The phylogenetic tree was generated by neighbour-joining analysis with 1000 bootstrap replications. The GenBank accession number follows the species names.

Enquête sur le genre *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae) en Roumanie

Une vue d'ensemble de la présence des espèces appartenant au genre *Bursaphelenchus* en Roumanie est présentée. Les informations sont basées sur des enquêtes récentes conduites pour la première fois sur l'ensemble du pays, afin de suivre et d'évaluer l'entrée potentielle du nématode du pin, *Bursaphelenchus xylophylus.* Des échantillons de bois ont été collectés sur des arbres affaiblis, chez des transformateurs et sur du bois d'emballage importé. Sur les 895 échantillons de bois examinés, 11 échantillons contenaient des spécimens de *Bursaphelenchus*. Des analyses moléculaires et morphologiques ont été réalisées pour caractériser les espèces détectées. Les échantillons étaient tous négatifs en ce qui concerne la présence possible de *B. xylophilus*, confirmant l'absence de cet organisme de quarantaine en Roumanie. Néanmoins, cinq espèces de *Bursaphelenchus* furent identifiées, à savoir *B. hofmanni*, *B. poligraphi*, *B. vallesianus*, *B. willibaldi,* et une nouvelle espèce appartenant provisoirement au groupe *sexdentati,* ici classifiée comme *Bursaphelenchus* sp. NR512. Ces résultats représentent le premier signalement de la présence de *Bursaphelenchus* en Roumanie.

Обследование на выявление видов рода *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae), проводившееся в Румынии

В статье дается обзор видов рода *Bursaphelenchus,* присутствующих в Румынии. Представленные в статье данные основаны на недавних обследованиях, впервые проведенных в масштабах страны, позволяющих оценить возможность проникновения сосновой стволовой нематоды *Bursaphelenchus xylophilus*. Пробы древесины были собраны не только с усыхающих деревьев, но и на предприятиях деревообрабатывающей промышленности, а также с древесных упаковочных материалов, завозившихся из других стран. Из общего количества 895 обследовавшихся проб древесины в 11-ти содержались особи рода *Bursaphelenchus*. Были проведены морфологические и молекулярные анализы, позволяющие охарактеризовать выявленные виды. В отношении возможного присутствия *B. xylophilus* все образцы оказались отрицательными, тем самым подтверждая отсутствие этого карантинного вредного организма в Румынии. При этом, однако, было обнаружено пять видов *Bursaphelenchus: B. hofmanni, B. poligraphi, B. vallesianus, B. willibaldi*, а также один

предполагаемый новый вид, принадлежащий группе *sexdentati*, классифицированный авторами как *Bursaphelenchus* NR512. Эти результаты представляют собой первое сообщение о присутствии рода *Bursaphelenchus* в Румынии.

References

Abelleira A, Picoaga A, Mansilla JP & Aguin O (2011) Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in Northwestern Spain. *Plant Disease* **95**, 776.

Akbulut S & Stamps WT (2012) Insect vectors of the pine-wood nematode: a review of the biology and ecology of *Monochamus* species. *Forest Pathology* **42**, 89–99.

Borlea GF, Radu S & Stana D (2006) Forest biodiversity preservation in Romania. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **34**, 21–27.

Braasch H (1998) *Bursaphelenchus hofmanni* sp. n. (Nematoda, Aphelenchoididae) from spruce wood in Germany. *Nematologica* **44**, 615–621.

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

Braasch H & Burgermeister W (2007) Bursaphelenchus hofmanni Braasch, 1998 in einem Weidenbaum in Sachsen, Deutschland. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* **59**, 183–185.

Braasch H, Burgermeister W & Gu J (2009) Revised intra-generic grouping of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae). *Journal of Nematode Morphology and Systematics* **12**, 65–88.

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.

Doniţă N, Chirita C & Stanescu V (1990). *Tipuri de ecosisteme forestiere din Romania*. ICAS, seria II, Bucuresti.

EPPO (2009) Diagnostics: *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **39**, 344–353.

Evans HF, McNamara DG, 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. *Bulletin OEPP/EPPO Bulletin* **26**, 199–249.

Ferris VR, Ferris JM & Faghihi J (1993) Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* **16**, 177–184.

Fonseca L, Cardoso JMS, Lopes A, Pestana M, Abreu F, Nunes N, Mota M & Abrantes I (2012) The pine wood nematode, *Bursaphelenchus xylophilus*, in Madeira island. *Helminthologia* **49**, 96–103.

Ibrahim SK, Perry RN, Burrows PR & Hooper DJ (1994) Differentiation of species and populations of *Aphelenchoides* and of *Ditylenchus angustus* using a fragment of ribosomal DNA. *Journal of Nematology* **26**, 412–421.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ & Higgins DG (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.

Mota M, Braasch H, Bravo MA, Penas AC, Burgermeister W, Metge K & Sousa E (1999) First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* **1**, 727–734.

Mota M & Vieira P (2008) *Pine wilt disease: a worldwide threat to forest ecosystems*. Dordrecht: Springer. 428 pp.

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.

Ruicañescu A (2007). Familia Cerambycidae. In *Checklist of Romania Fauna (terrestrial and freshwater species)*, (eds. Moldovan OT, Cîmpean M, Birda D, Iepure S & Ilie V), pp. 134–137. Institutul de Speologie Emil Racovita, Casa Cărţii de Ştiinţă.

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.

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

Sousa E, Naves P, Bonifácio L, Bravo MA, Penas AC, Pires J & Serrão M (2002) Preliminary survey for insects associated with *Bursaphelenchus xylophilus* in Portugal. *Bulletin OEPP/EPPO Bulletin* **32**, 499–502.

Vicente C, Espada M, Vieira P & Mota M (2012) Pine Wilt Disease: a threat to European forestry. *European Journal of Plant Pathology* **133**, 89–99.

Vrain TC (1993) Restriction fragment length polymorphism separates species of the *Xiphinema americanum* group. *Journal of Nematology* **25**, 361–364.