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

M. Calin¹, P. Vieira², C. Costache¹, H. Braasch³*, J. Gu⁴, J. Wang⁴ and M. Mota²

¹Central Phytosanitary Laboratory, 11 Voluntari, Voluntari, 077 190, Romania; e-mail: calin.monica@fcf.ro
³Federal Biological Research Centre for Agriculture and Forestry, Messeweg 11, Braunschweig, D-38104, Germany
⁴Technical 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. hofmannii*, *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 coniferous 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) (Doniș 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% Nonident 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'-TTTCACTCCGCTTACTAAGG-3' (Vrain, 1993). The PCR mixture (total volume 25 µL) contained 1x buffer enzyme with MgCl₂, 0.5 mM MgCl₂, 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 *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

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

<table>
<thead>
<tr>
<th><em>Bursaphelenchus</em> sp.</th>
<th>Location</th>
<th>Region</th>
<th>Host</th>
<th>Year of detection</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. hofmanni</em></td>
<td>Curtea de Arges</td>
<td>Arges</td>
<td><em>Picea</em> sp.</td>
<td>2010</td>
<td>Sawdust from conifers wood-processing industry</td>
</tr>
<tr>
<td></td>
<td>Onesti</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Musetesti</td>
<td>Gorj</td>
<td><em>Pinus</em> sp.</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dobra</td>
<td>Hunedoara</td>
<td></td>
<td>2010</td>
<td>Sawdust from conifers wood-processing industry</td>
</tr>
<tr>
<td></td>
<td>Ciurea</td>
<td>Iasi</td>
<td></td>
<td>2010</td>
<td>Sawdust from conifers wood-processing industry</td>
</tr>
<tr>
<td></td>
<td>Osica de Sus</td>
<td>Olt</td>
<td><em>Pinus</em> sp.</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Andreiasu de Jos</td>
<td>Vrancea</td>
<td><em>Pinus</em> sp.</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td><em>B. poligraphi</em></td>
<td>Musetesti</td>
<td>Gorj</td>
<td><em>Pinus</em> sp.</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td><em>B. vallesianus</em></td>
<td>Malin</td>
<td>Suceava</td>
<td><em>Picea abies</em></td>
<td>2008</td>
<td>First detection of the genus</td>
</tr>
<tr>
<td><em>B. willibaldi</em></td>
<td>Ocol silvic Ploiesti</td>
<td>Prahova</td>
<td><em>Abies</em> sp.</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td><em>Bursaphelenchus</em> sp. NR512</td>
<td>Sita Buzaului</td>
<td>Covasna</td>
<td><em>Picea</em> sp.</td>
<td>2012</td>
<td>Sawdust from conifers wood-processing industry</td>
</tr>
</tbody>
</table>
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. The spicules are not strongly curved and have a prominent rostrum. The lateral field shows three lines. The length of the spicules is not strongly curved and have 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.

*B. 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

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

The ITS–RFLP fragments for B. vallesianus (AM160662), B. sexdentati (AM269914) and B. pinophilus (AM160664) were calculated from the sequences available on NCBI.
Enquête sur le genre *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoïdidae) en Roumanie


**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.

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**Включение обнаружения видов рода *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoïdidae) 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 сосной ствольной 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

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

References


