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

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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% hardwood; 18% oaks; and 20% other broadleaf trees (15% hardwood, 5% softwood) (Doniță 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'-TTTCACTCGCGTTACTAAGG-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 *Alu*, *HaeIII*, *HinII*, *MspI* and *RsaI* 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

<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>Bacau</td>
<td>–</td>
<td>2010</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>Andreiaus 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>
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.

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**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

**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.
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 (Doniță et al., 1990; Borlea et al., 2006).

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Detailed information about the molecular phylogenetic status of *Bursaphelenchus* sp. NR512 is provided. The phylogenetic tree was generated using 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**


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

**References**


