Morphological and molecular characterisation of
*Bursaphelenchus andrassyi* sp. n. (Nematoda: Aphelenchoididae)
from Romania and Turkey

Mehmet DAYI1, Monica CALIN2, Süleyman AKBULUT1,*, Jianfeng GU3, Thomas SCHRÖDER4, Paulo VIEIRA5 and Helen BRAASCH6,**

1 Düzce University, Faculty of Forestry, Konuralp Campus, 81620 Düzce, Turkey
2 Central Phytosanitary Laboratory, 11 Voluntari, 077 190 Voluntari, Romania
3 Technical Centre, Ningbo Entry-Exit Inspection and Quarantine Bureau, 9 Mayuan Road, Ningbo 315012, Zhejiang, P.R. China
4 Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for National and International Plant Health, Messeweg 11-12, D-38104 Braunschweig, Germany
6 Kantstrasse 5, D-14471 Postdam, Germany

Received: 17 April 2013; revised: 23 August 2013; accepted for publication: 23 August 2013; available online: 11 October 2013

Summary – *Bursaphelenchus andrassyi* sp. n., found in conifer wood samples from Romania and Turkey, is characterised morphologically and genetically. *Bursaphelenchus andrassyi* sp. n. clearly belongs to the *sexdentati* group, having a terminal bursa, four lateral lines, a very small female ‘vulval flap’, strongly arcuate spicules and the typical position of caudal papillae of males. It is morphologically most similar to *B. vallesianus* and *B. sexdentati*. It can be differentiated from *B. vallesianus* by the usually subcylindrical female tail with rounded or wedge-shaped terminus vs conical female tail with a more or less rounded terminus, slightly different shape of spicules (low square condylus, lacking a distinct cucullus, pointed rostrum) and from *B. sexdentati* by lacking a distinct post-vulval constriction, shorter stylet and shorter spicules. The species status is supported by ITS-RFLP patterns and sequencing results of both partial 18S and 28S rDNA regions.

Keywords – *Abies cilicica*, conifers, description, distribution, morphometrics, new species, *Picea, Pinus brutia*, *sexdentati* group, taxonomy.

After the detection of the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 in Portugal in 1999 (Mota et al., 1999), Member States of the European Union are required to carry out annual surveys for the possible presence of *B. xylophilus* in coniferous forests, according to the emergency measures implemented by the European Union Commission. During intensive annual surveys for the detection of PWN under the Ministry of Agriculture and Rural Development Authority in Romania, a putative new *Bursaphelenchus* species of the *sexdentati* group, previously named *Bursaphelenchus* sp. NR512 was found in the Covasna county in 2012 (Calin et al., 2013). Only a few specimens of this species were found in sawdust of *Picea* spp. in a wood processing factory. The molecular phylogenetic status of the specimens found, using the full ITS1/2 sequence region, suggested the presence of a new *Bursaphelenchus* species (Calin et al., 2013). Due to the small number of specimens found, which could not be preserved on slides, a detailed description of the new species was not possible at this time. However, this changed when the same species was found in two samples in Turkey in 2012.

*Corresponding author, e-mail: suleymanakbulut@duzce.edu.tr
**Formerly at: Federal Biological Research Centre for Agriculture and Forestry, Messeweg 11, D-38104 Braunschweig, Germany

© Koninklijke Brill NV, Leiden, 2014
DOI:10.1163/15685411-00002759
Turkey follows the phytosanitary regulations of EPPO for quarantine organisms and is located in a transitional area between Europe and Asia, thereby requiring special attention for the possible introduction of quarantine organisms. A survey monitoring for the presence of B. xylophilus was started in 2003. So far, several Bursaphelenchus species, but not B. xylophilus, have been found (Akbulut et al., 2006, 2007, 2008a, b). In 2012, a new project was started to determine the insect vectors of Bursaphelenchus species. Wood samples were collected from trap logs prepared for catching possible insect vectors at various locations. Two samples with an undescribed Bursaphelenchus species were isolated from Cilician fir, Abies cilicica (Antoine & Kotschy) Carrière, and Turkish red pine (Pinus brutia Ten.) in Bucak-Burdur (Mediterranean region of Turkey). Morphological and molecular studies revealed it to be the same species as found in Romania. The two isolates from Turkey were cultivated, preserved and the new species is described herein.

**Materials and methods**

**Nematode culturing and morphological observations**

The nematode specimens originally found in Romania were collected from sawdust samples (Picea spp.) in a wood factory located in Covasna county. Wood samples (150 g) were placed into polythene bags and incubated at approximately 25°C for 14 days, according to the sampling guidelines for B. xylophilus (Schröder et al., 2009). Nematodes were then extracted using a modified Baermann funnel method, and collected after 48 h in distilled water. Measurements were made on specimens collected directly from sawdust samples, using a Leica DMLB light microscope fitted with a Leica DC300 camera, and the Leica DFC 295 image processing software (Leica Microsystems).

In Turkey, trap logs were prepared in the Mediterranean and Aegean regions during the survey investigating the insect vectors of Bursaphelenchus species. Each trap tree was cut down and left in place. Study locations were selected according to the results of previous nematode surveys. In each location, trap logs from both Pinus spp. and Abies spp. were established to capture possible insect vectors. After their flight period, trap logs were cut into small logs (40-50 cm length and 30-50 cm diam.) and transported to the laboratory in Düzce University. There, samples of 40-80 g were collected from opposing sides of the logs using a Pressler borer. Nematodes were extracted from wood chips using a modified Baermann funnel technique and processed within 48 h. The collected nematodes were inoculated on Botryotinia fuckeliana (de Bary) growing on malt agar and incubated for 2 weeks at 25°C. Specimens from this culture were morphologically studied using a Zeiss Axioskop (Carl Zeiss Microscopy) and a Leitz Diaplan microscope. Specimens were killed by heat and fixed in TAF and morphological measurements taken. A culture of the new species isolated in Turkey is kept in the Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for National and International Plant Health, Braunschweig, Germany, under the collection number NE 9/12. The description of the new species is mainly based on the Turkish populations.

**Molecular analyses**

DNA samples of specimens collected in Turkey were prepared according to Li et al. (2008), whereas DNA extraction in Romania was carried out using a single specimen following the methodology described in Calin et al. (2013). DNA for ITS-RFLP was extracted using the QIAmp DNA Micro Kit (Qiagen) as described in detail by Burgermeister et al. (2005).

Three sets of primers were used in the PCR analyses to amplify the partial SSU region (Penas et al., 2006), the ITS1/2 region (Ferris et al., 1993; Vrain, 1993) and the D2/D3 LSU region of rDNA (De Ley et al., 1999), respectively. PCR conditions applied followed the same conditions reported by Li et al. (2008) and Ye et al. (2007). PCR products were separated on 1% agarose gels and visualised by staining with ethidium bromide.

ITS-RFLP analysis followed the protocol of Burgermeister et al. (2009). For ITS-RFLP profiles, suitable aliquots of the amplified ITS rDNA were digested with three units of the restriction endonucleases (RsaI, HaeIII, MspI, HinfI and Alul) (Fermentas) following the manufacturer’s instructions. Fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

The partial 18S and D2/D3 sequences were analysed and aligned using the program ClustalW implemented in MEGA version 5.1 (Tamura et al., 2007). The tree topology was obtained by maximum likelihood (ML) and neighbour joining (NJ) analyses with 1000 bootstrap replications. The species names and respective NCBI accession number are shown in each tree.
**Results**

*Bursaphelenchus andrassyi* sp. n.  
= *Bursaphelenchus* sp. NR512 of Calin et al., 2013  
(Figs 1, 2)

**MEASUREMENTS**

See Table 1 (differences in the measurements of the Turkish and Romanian material may be explained by the lower number of measured specimens from Romania, and the fact that these came directly from wood samples, whereas the Turkish specimens were collected from fungus cultures).

**DESCRIPTION**

**Male**

Body C-shaped with curved tail when killed by heat. Cuticle thin, marked by fine annules. Lateral field *ca* 2 μm wide with four lateral lines in mid-body region. Lip region convex, *ca* 4 μm high and 7-8 μm broad, offset by a distinct constriction. Stylet with small basal swellings. Stylet shaft comprising *ca* two-thirds of total stytel length. Procorpus cylindrical, median bulb oval or pear-shaped, with central valves. Excretory pore located at median bulb level, or closely posterior to it at nerve ring level. Pharyngeal glands forming a dorsally overlapping lobe 2-3 body diam. long over intestine. Pharyngo-intestinal junction located immediately posterior to median bulb. Testis occupying *ca* one-third to half of body length, germinal zone outstretched but often reflexed. Developing spermatocytes arranged in a single row. Spicules paired, relatively stout and strongly ventrally arcuate, with a pointed prominent rostrum *ca* 3-4 μm long in proximal half of spicules and a line parallel to dorsal limb. Condylus compact, dorsally slightly kinked posteriad, more seldom almost straight in dorsal line, square at its end, extending *ca* 1-2 μm above the dorsal kink. Capitulum without distinct protuberances, distance from tip of condylus to tip of rostrum in direct line 6-7 μm. Distal ends of spicules with very weak cucullus-like thickening or a small blunt extension. Ryss *et al.* (2005) mentioned the angle between imaginary lines drawn along capitulum (condylus to tip of rostrum) and extending spicule distal end as diagnostic character. These lines are practically parallel in the new species. Additionally, some ratios are of interest (*n* = 10): ratio of spicule length (along arc) to its width measured posterior to rostrum = 3.6 (2.7-4.6); ratio of distance of a line between rostrum and condylus from bottom of capitulum depression to rostrum-condylus line length = 0.12 (0.07-0.16); ratio of spicule length to rostrum-condylus distance = 2.1 (2.0-2.5). Tail ventrally arcuate with pointed, talon-like terminus bearing an oval or sometimes bilobed terminal bursa *ca* 10 μm long. Single precloacal papilla (P1) in ventral mid-line, an adcloacal ventro-lateral pair, sometimes slightly posterior to cloacal aperture (P2), and two postcloacal subventral pairs of caudal papillae present, one such pair (P4) at beginning of bursa, other (P3) *ca* 5-6 μm anterior to P4 and more latero-ventral. P3 and P4 of different structure, with P4 resembling ‘gland papillae’.

**Female**

Body slim, slightly ventrally curved when killed by heat. Anterior body region and cuticle similar to male. Lips *ca* 4 μm high and 6-7 μm broad. Reproductive system prodelphic, occupying *ca* one-third to half of body length, consisting of ovary, oviduct, axial spermatheca, columella, uterus, vagina and post-uterine sac. Developing oocytes arranged in a single file. Spermatheca usually elongated oval, sometimes rounded, up to 40 μm from vulva, containing amoeboid sperm 4-5 μm diam. Vulva at three-quarters of body length. Vagina positioned almost at right angles to body axis and at junction of uterus and post-uterine sac, anterior vulval lip sometimes slightly extending over vulva (= ‘vulval flap’). Posterior vulval lip sometimes slightly swollen, sometimes not, no distinct post-vulval constriction. Post-uterine branch occupying *ca* half of vulva to anus distance, often containing sperm, sometimes an egg. Tail subcylindrical, sometimes slightly conoid, with rounded, sometimes wedge-shaped terminus (like a weakly sharpened pencil) which may occasionally show a very small protuberance. Tail on average 2.6 times longer than anal body diam. In two cases a hatched juvenile observed in body of mature female.

**TYPE HABITAT AND LOCALITY**

Wood of *Abies cilicica* growing in a forest site in Bucak-Burdur (37°20′04″N, 30°37′40″E), Turkey.

**OTHER HABITATS AND HOSTS**

Sawdust (from *Picea* spp.) collected in Covasna County, Romania, and *Pinus brutia* wood growing in a...
Fig. 1. *Bursaphelenchus andrassyi* sp. n. A: Female; B: Male; C: Anterior body; D: Female tail region; E: Male tail region with bursa in dorso-ventral view; F: Spicules; G: Male tail, lateral view (P1-P4 = papillae); H: Vulval region; I: Lateral lines. (Scale bars = 10 μm.)
Bursaphelenchus andrassyi sp. n. from Romania and Turkey

Fig. 2. Light microscope observations of *Bursaphelenchus andrassyi* sp. n. A: Anterior body; B: Detailed image of labial region and stylet; C: Median bulb region showing excretory pore (arrow); D: Female vulval region in lateral view (Vf = ‘vulval flap’); E: Lateral lines in mid-body, F: Male tail with bursa in dorso-ventral view and position of P3 and P4 papillae; G, H: Male spicules; I: Reflexed testis; J: Details of female reproductive tract (Vf = ‘vulval flap’, PUS = post-uterine sac with sperm); K-N: Male tail in lateral view (P1 = precloacal single papilla, P3 and P4 = postcloacal pairs of papillae); O-S: Variation of female tail. (Scale bars = 10 μm.)
Table 1. Morphometrics of *Bursaphelenchus andrassyi* sp. n. from Turkey (type locality) and Romania. All measurements are in μm and in the form: mean ± s.d. (range).

<table>
<thead>
<tr>
<th>Character</th>
<th>Turkey Male</th>
<th>Turkey Female</th>
<th>Romania Male</th>
<th>Romania Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holotype</td>
<td>Paratypes</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>n</td>
<td>–</td>
<td>18</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>L</td>
<td>827</td>
<td>759 ± 49.4 (640-842)</td>
<td>847 ± 66.5 (699-1018)</td>
<td>648 ± 32.4 (605-697)</td>
</tr>
<tr>
<td>a</td>
<td>43.1</td>
<td>43.9 ± 3.7 (36.9-49.8)</td>
<td>43.1 ± 4.5 (36.5-52.6)</td>
<td>35.2 ± 4.6 (32.2-44.6)</td>
</tr>
<tr>
<td>b</td>
<td>5.7</td>
<td>5.5 ± 0.6 (4.8-7.3)</td>
<td>6.0 ± 0.4 (5.7-7.0)</td>
<td>–</td>
</tr>
<tr>
<td>c</td>
<td>30.4</td>
<td>31.9 ± 3.4 (26.5-37.8)</td>
<td>33.5 ± 2.5 (30.7-39.3)</td>
<td>21.2 ± 3.5 (14.8-25.7)</td>
</tr>
<tr>
<td>c′</td>
<td>2.3</td>
<td>2.0 ± 0.2 (1.7-2.4)</td>
<td>2.7 ± 0.3 (2.2-3.2)</td>
<td>2.7 ± 0.6 (2.0-4.0)</td>
</tr>
<tr>
<td>V</td>
<td>–</td>
<td>–</td>
<td>76.3 ± 5.3 (72-90)</td>
<td>–</td>
</tr>
<tr>
<td>T</td>
<td>49</td>
<td>47.4 ± 8.9 (33.5-62.9)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pharynx length</td>
<td>144</td>
<td>140 ± 13.9 (104-158)</td>
<td>125 ± 26.9 (87-154)</td>
<td>–</td>
</tr>
<tr>
<td>Tail length</td>
<td>27</td>
<td>24 ± 2.9 (19-29)</td>
<td>26 ± 2.2 (21-29)</td>
<td>32 ± 7.05 (24.3-45)</td>
</tr>
<tr>
<td>Anterior end to median bulb</td>
<td>50</td>
<td>47 ± 3.2 (40-54)</td>
<td>46 ± 3.3 (42-54)</td>
<td>–</td>
</tr>
<tr>
<td>Vulva to anus distance (VA)</td>
<td>–</td>
<td>–</td>
<td>192 ± 14.8 (173-224)</td>
<td>–</td>
</tr>
<tr>
<td>Post-uterine sac length (PUS)</td>
<td>–</td>
<td>–</td>
<td>100 ± 20.1 (67-131)</td>
<td>–</td>
</tr>
<tr>
<td>PUS/VA (%)</td>
<td>–</td>
<td>–</td>
<td>54 ± 10.6 (39-68)</td>
<td>–</td>
</tr>
<tr>
<td>PUS/vulval body diam.</td>
<td>–</td>
<td>–</td>
<td>5.3 ± 1.2 (3.6-7.6)</td>
<td>–</td>
</tr>
<tr>
<td>Stylet</td>
<td>12.8</td>
<td>13.5 ± 0.77 (12-14.4)</td>
<td>14.2 ± 0.52 (12.8-15.2)</td>
<td>12.1 ± 0.35 (11.5-12.5)</td>
</tr>
<tr>
<td>Spicules (median line)</td>
<td>14.4</td>
<td>14 ± 0.88 (11.2-14.4)</td>
<td>–</td>
<td>11.7 ± 1.28 (10.6-13.5)</td>
</tr>
</tbody>
</table>

forest site in Bucak-Burdur (37°20′07″N, 30°37′44″E), Turkey.

**Type Material**

Collected from a culture on Botryotinia fuckeliana growing on malt agar and originating from a sample collected in 2012 from Cilician fir, *A. cilicica*, in Bucak-Burdur, Turkey. Holotype male, together with 14 male and 16 female paratypes deposited in the Forest Entomology and Protection Laboratory of Düzce University Faculty of Forestry, Turkey. Two male and two female paratypes deposited in the German nematode collection at the Julius Kühn-Institut, Institute for Epidemiology and Pathogen Diagnostics, Münster, Germany, and two male and two female paratypes deposited in the USDA Nematode Collection, Beltsville, MD, USA.

**Diagnosis and Relationships**

*Bursaphelenchus andrassyi* sp. n. is characterised by a relatively small stylet with basal swellings, a lateral field with four lines, and the excretory pore located at, or posterior to, the median bulb. The female has a very small extension of the anterior vulval lip over the vulva (= a ‘vulval flap’), a mostly subcylindrical tail with a rounded or wedge-shaped terminus, more seldom with a small protuberance. The male spicules are strongly ventrally arcuate, have a prominent pointed rostrum and squat condylus, lack a distinct cucullus, and there is a dorso-ventrally elongated, oval terminal bursa.

Because of the presence of four lateral lines, spicule shape, the presence of a very small ‘vulval flap’ and the position of the caudal papillae, *B. andrassyi* sp. n. is affiliated to the *sexdentati* group (sensu Braasch et al., 2009) within *Bursaphelenchus*, a group which also includes *B. vallesianus* Braasch, Schönfeld, Polomski & Burgermeister, 2004; *B. sexdentati* Rühm, 1960; *B. pinophilus* Brzeski & Baujard, 1997; *B. poligraphi* Fuchs, 1937; *B. fuchsi* Kruglik & Eroschenko, 2004; and probably also *B. incurvus* Rühm, 1956; *B. piniperdae* Fuchs, 1937 (Rühm, 1956) and *B. pityogeni* Massey, 1974. A German isolate belonging to the *sexdentati* group was originally identified...
Bursaphelenchus andrassyi sp. n. from Romania and Turkey

Fig. 3. ITS-RFLP patterns of Bursaphelenchus andrassyi sp. n. and five other Bursaphelenchus species of the sexdentati group. M = molecular size marker (100 bp ladder); Lane P = rDNA amplification product; Lanes 1-5 = digestion products obtained with RsaI, HaeIII, MspI, Hinfi and AluI, respectively.

as B. borealis Korentchenko, 1980, but was subsequently considered to be B. fuchsi (Braasch et al., 2009). Morphological differentiation of species within the sexdentati group is difficult.

Due to the spicule shape, B. andrassyi sp. n. is most similar to B. vallesianus and B. sexdentati. It can be distinguished from B. vallesianus by the female tail shape (usually subcylindrical vs conical), position of excretory pore (at median bulb level or posterior to it vs at, or anterior to, median bulb), slightly different spicule shape (sharply pointed vs ‘more or less pointed’ rostrum, condylus shorter and square and not hook-like dorsally bent vs dorsally bent), and shorter spicule length of 14 (11-14) vs 16 (14-19) μm (Braasch et al., 2004). The new species differs from B. sexdentati in lacking a distinct post-vulval constriction (Rühm, 1960), shorter stylet of 14 (12-14) and 14 (13-15) μm for males and females, respectively, vs 18 (16-19) μm, shorter spicules of 14 (11-14) vs 20 (19-22) μm.

Bursaphelenchus andrassyi sp. n. differs from B. pinophilus by shorter spicules of 14 (11-14) vs 19 (15-21) μm, differently shaped spicules (B. pinophilus has a high, variously shaped condylus), shorter distance from the tip of condylus to the tip of rostrum (ca 6 vs 8-10 μm), and by the female tail shape (B. pinophilus has a pointed conoid and sometimes mucronate tail) (Brzeski & Baujard, 1997); from B. poligraphi by spicule shape (prominent rostrum vs small and narrow rostrum) and length of 14 (11-14) vs 15-18 μm (Fuchs, 1937; Rühm, 1956); from B. fuchsi by the shorter body length of 759 (640-842) and 849 (699-1018) vs 936 (748-1024) and 1055 (922-1227) μm for males and females, respectively, shorter stylet of 14 (12-14) and 14 (13-15) μm for males and females, respectively, vs 18 (16-19) μm, shorter spicules of 14 (11-14) vs 20 (18-22) μm, and a slightly lower female ratio c’ range of 3 (2-3) vs 3 (3-4) (Kruglik & Eroshenko, 2004); from B. incurvus, which was described rather poorly by Rühm (1956), by spicule rostrum in the proximal half vs almost in the middle of spicules, condylus short vs longer in B. incurvus (as interpreted from the drawings provided by Rühm, 1956), bursa oval vs square-shaped, and a higher ratio a value of 44 (37-50) and 43 (37-53) for males and females, respectively vs 21-25; from B. piniperdae by the shape of the female tail (subcylindrical and rounded or wedge-shaped vs cylindrical and bluntly rounded), lack of a distinct post-
vulval constriction, shorter stylet of 14 (12-14) and 14 (13-15) µm for males and females, respectively, vs 18-19 and 16-18 µm for males and females, respectively, and spicule length of 14 (11-14) vs 17-19 µm and spicule shape (condylus small and square-shaped vs thicker and proximally button-like) (Rühm, 1956).

*Bursaphelenchus pityogeni* Massey, 1974 was originally described as having two lateral lines and was therefore not listed in the *sexdentati* group (sensu Braasch et al., 2009). R.M. Giblin-Davis (USA), who studied type material of this species and found four lateral lines, suggested (pers. comm.) that this species also belonged within the *sexdentati* group. *Bursaphelenchus andrassyi* sp. n. differs from *B. pityogeni* in female tail shape (subcylindrical vs much narrower posteriorly), testis often reflexed vs outstretched and arrangement of the developing oocytes (single row vs three rows) (Massey, 1974).

**Molecular differentiation of *B. andrassyi* sp. n. from similar morphological species**

The ITS-RFLP pattern of *B. andrassyi* sp. n. is different from the patterns of the morphologically similar species *B. vallesianus, B. sexdentati, B. pinophilus, B. poligraphi* and *B. fuchsi* (Fig. 3; Table 2). It is also distinct from the ITS-RFLP patterns of numerous other conifer-inhabiting *Bursaphelenchus* species obtained in earlier investigations (Burgermeister et al., 2009). On the other hand, the DNA fragments shown in Figure 3 and Table 2 exhibit some common features among the six species examined. In all cases, a PCR product of the same size (ca 980 bp) was obtained, this containing no recognition sites for *Msp* I and *Alu* I. *Bursaphelenchus andrassyi* sp. n. differs from *B. vallesianus* only in restriction fragments obtained with *Hae* III and *Hin* II, from *B. sexdentati* only in restriction fragments obtained with *Hae* III and *Hin* II, and from *B. fuchsi, B. poligraphi* and *B. pinophilus* in fragments obtained with *Rsa* I, *Hae* III and *Hin* II.

**Molecular profiles and phylogenetic status**

Figures 4 and 5 show phylogenetic trees based on D2/D3 (28S rDNA gene) and partial 18S rDNA sequences. In all trees, *B. andrassyi* sp. n. clustered very close to *B. vallesianus* and *B. sexdentati*. Sequencing results also show that the ITS1/2 region of *B. andrassyi* sp. n. collected from Turkey and Romania differ only in two nucleotides, the 28S D2/D3 sequences differ in one nucleotide, whereas the partial 18S sequences are identical.

The range of pair-wise sequence divergence values obtained at the three different loci is illustrated in Table 3. Among the ribosomal sequences, the ITS1/2 region exhibits much higher divergence than the 28S D2/D3 region and partial 18S region. The range of ITS1/2 sequence divergence between *B. andrassyi* sp. n. and *B. vallesianus* is 2.6%, which is slightly smaller than the difference between *B. andrassyi* sp. n. and *B. sexdentati* (3.9-4.1%). The sequence divergence of five species of the *sexdentati* group is 3.1-9.2% (with the new species 2.6-9.2%), whereas the divergence between the Romanian and Turkish populations of *B. andrassyi* sp. n. is only 0.2%, and the difference between *B. andrassyi* sp. n. and *B. xylophilus* (*xylophilus* group) reached much a higher value of 46.5%.

![Downloaded from Brill.com](https://via-free-access.com/06/16/2019/06/42:44PM)

Table 2. Size of DNA fragments obtained by ITS-RFLP analysis of the ITS1/2 region sequences of *Bursaphelenchus andrassyi* sp. n. and closely related species.

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR product (bp)</th>
<th><em>Rsa</em> I</th>
<th><em>Hae</em> III</th>
<th><em>Msp</em> I</th>
<th><em>Hin</em> I</th>
<th><em>Alu</em> I</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. andrassyi</em> sp. n.</td>
<td>980</td>
<td>543</td>
<td>477</td>
<td>980</td>
<td>464</td>
<td>967</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>118</td>
<td>159</td>
<td>22</td>
<td>70</td>
<td>13</td>
</tr>
<tr>
<td><em>B. vallesianus</em></td>
<td>981</td>
<td>543</td>
<td>862</td>
<td>981</td>
<td>465</td>
<td>967</td>
</tr>
<tr>
<td></td>
<td>416</td>
<td>119</td>
<td>280</td>
<td>416</td>
<td>280</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>212</td>
<td>24</td>
<td>22</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td><em>B. sexdentati</em> (type A)*</td>
<td>981</td>
<td>543</td>
<td>585</td>
<td>981</td>
<td>465</td>
<td>967</td>
</tr>
<tr>
<td></td>
<td>416</td>
<td>277</td>
<td>280</td>
<td>416</td>
<td>280</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>119</td>
<td>212</td>
<td>22</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td><em>B. sexdentati</em> (mixture of types A + B)+</td>
<td>981</td>
<td>543</td>
<td>862</td>
<td>981</td>
<td>465</td>
<td>967</td>
</tr>
<tr>
<td></td>
<td>416</td>
<td>584</td>
<td>280</td>
<td>416</td>
<td>280</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>279</td>
<td>212</td>
<td>22</td>
<td>212</td>
<td></td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>122</td>
<td>24</td>
<td>122</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

* ITS-RFLP profiles of different *B. sexdentati* isolates may show different *Hae* III restriction fragment patterns (Burgermeister et al., 2009). Lange et al. (2006) was able to show that amplified and cloned ITS2 from a single nematode of *B. sexdentati* revealed two ITS2 sequence variants (A and B) by the presence or absence of a *Hae* III site within the DNA tandem repeats (sequence microheterogeneity).
Fig. 4. Maximum likelihood (ML) and neighbour joining (NJ) analyses of Bursaphelenchus andrassyi sp. n. with other Bursaphelenchus species based on partial D2D3 region (28S rDNA gene). Aphelenchoides besseyi was used as outgroup species. The phylogenetic trees were generated with 1000 bootstrap repetitions. Scale bar = substitutions/site.

The range of 28S D2/D3 sequence divergence between B. andrassyi sp. n. and B. vallesianus is 1.3%, which is somewhat smaller than between B. andrassyi sp. n. and B. sexdentati (2.9–3.2%). The sequence divergence of five species of the sexdentati group is 2.0–5.5%, whereas the divergence between the Romanian and Turkish populations of B. andrassyi sp. n. is only 0.3%, between B. andrassyi sp. n. and B. xylophilus 26.5%, and between B. andrassyi sp. n. and B. pinasteri (hofmanni group) 17.3%. The relatively low divergences of the partial 18S region can be seen in Table 3.

Discussion

Species of the sexdentati group of Bursaphelenchus are widely distributed in Central and Southern Europe. Braasch et al. (2009) listed nine species, of which six have been found lately. Due to their very similar characters, the morphological differentiation of species belonging to the sexdentati group is difficult. ITS-RFLP analysis proved to be a well established technique for identification of Bursaphelenchus species, including those of the sexdentati group (Burgermeister et al., 2009). The ITS-RFLP pattern...
Fig. 5. Maximum likelihood (ML) and neighbour joining (NJ) analyses of *Bursaphelenchus andrassyi* sp. n. with other *Bursaphelenchus* species based on partial 18S region (18S rDNA gene). *Aphelenchoides besseyi* was used as outgroup species. The phylogenetic trees were generated with 1000 bootstrap repetitions. Scale bar = substitutions/site.

The morphological similarities with related species, the ITS-RFLP patterns and the phylogenetic trees support the affiliation of *B. andrassyi* sp. n. with the *sexdentati* group. Equally, both phylogenetic and pair-wise sequence differences support the distinct status of *B. andrassyi* sp. n. The isolates from Romania and Turkey show residual nucleotide differences in the three rDNA loci investigated (zero, one or two nucleotides in 18S, 28S D2/D3 or ITS1/2, respectively) and clearly belong to the same species. The interspecific genetic distances within the *sexdentati* group, known from five species so far, are...
similar as the distances between the new species and the other species of the *sexdentati* group, with *B. andrassyi* sp. n. being closer to *B. vallesianus* (Table 3).

The records of *B. andrassyi* sp. n. in sawdust of a wood processing factory in Romania and in two samples from Turkey indicate a wide distribution of the new species in the southern regions of Europe, associated with different host species, such as *Pinus brutia*, *Abies cilicica* and *Picea* sp. It is quite possible that the new species feeds on fungi in wood, as is the case with most *Bursaphelenchus* species. However, it must be remembered that species of the *sexdentati* group (namely *B. vallesianus*) caused high mortality in 3-year-old pines in inoculation experiments in Greece (Skarmoutsos & Michalopoulou-Skarmoutsos, 2000), Switzerland (Polomski et al., 2009; Polomski & Rigling, 2010) and Turkey (Dayı & Akbulut, 2012). It should be also noted that some *B. sexdentati* isolates (GR-7w, GR-8w) used in inoculation experiments in Greece were later determined to be *B. vallesianus* (Lange et al., 2006). Since the isolates of *B. andrassyi* sp. n., which is closely related to *B. vallesianus*, originate from a region with damaged conifer trees in Turkey, careful observations and inoculation experiments in adult trees may clarify the possible role of this species in tree wilting.

### Acknowledgements

The scientists from Turkey were supported by Düzce University Research Fund Project Number: BAP-2012.02.02.076. M. Dayı and S. Akbulut kindly thank İ. Halil Elekcioğlu and Ece B. Kasapoğlu (Çukurova University-Adana, Turkey) for their help on morphometric studies. T. Schröder and P. Vieira were partially supported by the EC 7th Framework project REPHRAME (KBBE.2010.1.4-09, ‘Analysis of the potential of the pinewood nematode, *Bursaphelenchus xylophilus*, to spread, survive and cause pine wilt in European coniferous forests in support of EU plant health policy’). J. Gu was partially supported by the National Science and Technology Support Program of China (2012BAK11B03). The authors wish to thank Silvia Urban, JKI Braunschweig, Germany for careful technical assistance. The authors would like to thank Dr Marc J. Linit, University of Missouri, USA, for reading the manuscript.

### Table 3. Sequence divergences at different rDNA loci of *Bursaphelenchus* species and isolates belonging to different morphological groups.

<table>
<thead>
<tr>
<th>Bursaphelenchus isolates compared</th>
<th>Range of pairwise sequence divergence (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITS1/2 of rDNA</td>
</tr>
<tr>
<td>Five species of the <em>sexdentati</em> group (without <em>B. andrassyi</em> sp. n.)</td>
<td>3.1 (<em>Bp/Bv</em>) to 9.2 (<em>Bs/Bf</em>)</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. from Romania vs Turkey</td>
<td>0.2</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. and <em>B. vallesianus</em></td>
<td>2.6</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. and <em>B. sexdentati</em></td>
<td>3.9/4.1</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. and <em>B. pinophilus</em></td>
<td>3.8</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. and <em>B. fuchsi</em></td>
<td>9.5</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. and <em>B. poligraphi</em></td>
<td>8.1</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. and <em>B. xylophilus</em> (<em>xylophilus</em> group)</td>
<td>46.5</td>
</tr>
<tr>
<td><em>B. andrassyi</em> sp. n. and <em>B. pinasteri</em> (<em>hofmanni</em> group)</td>
<td>–</td>
</tr>
</tbody>
</table>

*Bp* = *B. poligraphi*, *Bv* = *B. vallesianus*, *Bs* = *B. sexdentati*, *Bf* = *B. fuchsi.*

### References


tológische Schriftenreihen 6, 1-435.


