**Bursaphelenchus trypophloei** sp. n. (Nematoda: Parasitaphelenchinae) – an associate of the bark beetle, *Trypophloeus asperatus* (Gyll.) (Coleoptera: Curculionidae, Scolytinae), in aspen, *Populus tremula* L.

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**Summary** – *Bursaphelenchus trypophloei* sp. n. is described from weakened and dead twigs of living or recently fallen aspen trees, *Populus tremula*, in Poland. All propagative stages of the nematode were present in larval galleries of a bark beetle, *Trypophloeus asperatus*, and its dauer juveniles could be found in the haemocoel of older larvae, pupae and adult beetles. Characteristic morphology of the male spicules with distinct cucullus, extended anterior vulval lip, lateral fields with four incisures, and number and arrangement of male caudal papillae indicate that *B. trypophloei* sp. n. is closely related to the *xylophilus* group. This relation has been further confirmed by DNA sequencing and phylogenetic analysis of the 28S and ITS-1 rDNA region. *Bursaphelenchus trypophloei* sp. n. can be separated from other species in the *xylophilus* group by the morphology of spicules which have a shorter capitulum and unique rostrum which is pointed somewhat anteriorly, relatively thick vulval flap, which is straight, parallel to the body long axis or bent towards the body wall at its distal end, and other morphological and morphometric characters, *i.e.*, shape of female tail, position of excretory pore at or posterior to nerve ring, spicule length of 25.7 (23.1-28.0) μm (as measured along arc) and shape, moderate length, *i.e.*, L = 702 (603-946) μm in females and L = 679 (543-828) μm in males, and slender body (a = 39.2 (33.0-45.2) and 40.9 (32.0-46.4) in female and male, respectively). The taxonomic separation of the new species is also confirmed by the unique molecular profile of the ITS region (ITS-RFLP). Full reproductive incompatibility of *B. trypophloei* sp. n. with *B. xylophilus*, *B. mucronatus*, *B. fraudulentus*, *B. populi* and *B. doui* has been demonstrated in *in vitro* diallelic cross-breeding. The newly described nematode revealed limited ability to develop and reproduce on *Botrytis cinerea* cultures although it produced large populations on laboratory cultures of *Cytospora chrysosperma* – a fungus naturally associated with galleries of the nematode vector, *T. asperatus*.

**Keywords** – *Bursaphelenchus xylophilus* group, *Cytospora chrysosperma*, molecular, morphology, morphometrics, phylogeny, taxonomy.

Recent expansion of the pine wilt nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Nematoda: Parasitaphelenchinae), from North America and East Asia (Japan, China, South Korea and Taiwan) to new localities in Europe (Mota et al., 1999) has brought a real threat to coniferous forests in this region and alerted phytosanitary services throughout the world (Webster & Mota, 2008). The close morphological similarity of *B. xylophilus* to a number of other tree-inhabiting *Bursaphelenchus* species from the *xylophilus* group makes the problem particularly important, as any misidentification of nematodes extracted from the material subjected to phytosanitary inspection may lead to disturbances in the international wood trade and serious economic consequences. As this situation calls for an urgent response, the precision and reliability of taxonomic identification of *Bursaphelenchus* species has been recently improved through a wide deployment of advanced molecular methods (Matsumaga & Togashi, 2004; Leal et al., 2007; Burgermeister et al., 2009; Kikuchi et al., 2009; Filipiak et al., 2010). As a consequence, several new *Bursaphelenchus* species have been identified in the last decade, greatest progress being made in the *xylophilus* group where seven out of 11 known species have been described since the begin-

Our field observations on *B. populi*, the most recently described species from the *xylophilus* group, which is associated with a longhorn beetle, *Saperda perforata* Pall., in trunks of aspen (Tomalak & Filipiak, 2010), have drawn our attention to a population of another *Bursaphelenchus* species, tentatively designated as PL-08, which, in contrast to the previous species, lives exclusively in thin twigs of these trees and is apparently associated with larval galleries of a bark beetle, *Trypophloeus asperatus* (Gyll.). The close morphological, molecular and bionomic examination of these nematodes has revealed that they represent another, as yet undescribed, species. The research reported here provides an account of our taxonomic and bionomic studies on the *Bursaphelenchus* sp. PL-08 isolate, which we propose herein as *B. trypophloeus* sp. n.

**Materials and methods**

**ISOLATION AND MORPHOLOGICAL EXAMINATION OF NEMATODES**

The study was conducted on the PL-08 population of *B. trypophloeus* sp. n. isolated for the first time in 1999 and occasionally re-isolated from fallen or standing aspen, *Populus tremula* L., grown in a forest stand within urban forest in Poznan, Poland. The locality of this nematode was identical with that of *B. populi*, a nematode recently described from trunks of aspen trees (Tomalak & Filipiak, 2010). In our earlier screening, the presence of the new nematode was limited to weakened or dying aspen twigs infested with a bark beetle, *T. asperatus*. Therefore, for the present laboratory examination, 10 cm long samples of tree branches with diameters ranging between 0.5 and 10.0 cm were cut in the field. Since *T. asperatus* mainly invades thin twigs, selected material included 50% of samples taken from twigs with a diam. of up to 2 cm which contained active larval galleries of this bark beetle.

In the laboratory, all samples were individually chopped into 5-10 mm pieces and subjected to nematode extraction in water on 18 cm diam. sieves with a mesh size of 0.2 mm. The obtained suspension of nematodes was concentrated by sedimentation and washed three times in distilled water. After preliminary identification under a compound light microscope, subsamples of live males and females were transferred to laboratory cultures of *Botrytis cinerea* Pers. and, since 2009, also to cultures of *Cylindrocladium botryosum* Pers. on PDA or malt agar media, and reared at 20 or 25°C. The remaining individuals were processed for detailed morphological analysis.

For the morphological analysis, adult nematodes and dauer juveniles were picked with a fine pipette or mounted eyelash, placed on a glass slide, killed with gentle heat over a flame and examined morphologically in water mounts or processed further by fixation in TAF and gradual dehydration to pure glycerin (Seinhorst, 1959) for subsequent examination in permanent mounts. Morphological observations and all measurements were done with an Olympus BX50 microscope with Differential Interference Contrast (DIC) optics. Micrographs were taken with an Olympus C7070 digital camera. Forty randomly picked individuals were examined for each morphological category. Male spicule length was measured along the arc (Ryss et al., 2005).

**MOLECULAR CHARACTERISATION**

DNA samples of *B. trypophloeus* sp. n. were prepared following the protocol of Iwahori et al. (1998), with minor modifications, namely, the composition of lysis buffer was: 100 mM Tris, pH 8.5, 100 mM NaCl, 50 mM EDTA, 1% SDS, 1% β-mercaptoethanol and 100 μg ml⁻¹ proteinase K per 100 μl buffer. Before incubation the mixture was frozen at −80°C for 40 min.

The ITS-RFLP analysis was done as described by Burgermeister et al. (2005). Forward primer F194 5′-CGT AAC AAG GTA GCT GTA G-3′ (Ferris et al., 1993) and reverse primer 5368r 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (Vrain, 1993) were used to amplify the ITS region of rDNA. The obtained PCR products were purified (Qiaex II Gel extraction kit, Qiagen, Hilden, Germany) and suitable aliquots of the amplified DNA digested with 3 units of restriction endonucleases *RsaI*, *HaeIII*, *MspI*, *HinII* and *AluI* following the manufacturer’s instructions. Restriction fragments were resolved by electrophoresis in 2% agarose gel using TBA buffer, stained with 1 μg ml⁻¹ ethidium bromide and photographed under UV light.
The ITS-RFLP profiles of *B. tryphloehi* sp. n. were calculated with the computer program BioEdit Sequence Alignment Editor v. 7.0.5.3 based on the DNA base sequence of the rDNA.

For amplification of the D2/D3 expansion region of 28S rDNA, the forward primer D2A 5′-ACA AGT ACC GTG AGG GAA AGT TG-3′ and reverse primer D3B 5′-TCG GAA GGA ACC AGC TAC TA-3′ (De Ley et al., 1999) were used. The amplification was done according to Li-Qin et al. (2007).

The DNA base sequence of ribosomal DNA of *B. tryphloehi* sp. n. (ITS region and D2/D3 expansion region of 28S rDNA) were determined. For this purpose, the PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and used to transform into DH5α *Escherichia coli* cells for further sequencing. Nucleotide sequences were determined (IBB, Warsaw, Poland) and contigs assembled using SeqMan program from Dnastar software package (DNASTAR, Madison, WI, USA).

The molecular phylogenetic status of *B. tryphloehi* sp. n., both within the genus and within the *xylophilus* group, was determined using the computer program ClustalX for multiple sequence alignments. Automatic alignment was then manually improved in order to eliminate improper phylogenetic signals. Maximum likelihood tree was calculated with the Phylm program and the Bayesian tree with MrBayes (Guindon & Gascuel, 2003; Ronquist & Huelsenbeck, 2003). The substitution model for ML analysis was chosen by the Findmodel program. The species names and GenBank accession numbers of the sequences compared with those of the *B. tryphloehi* sp. n. are shown in the phylograms.

The sequence identity matrix of ITS regions in *B. tryphloehi* sp. n. and other species of the *xylophilus* group was determined at the nucleotide level. It was done by generating consensus alignments with ClustalW and incorporating results into the computer program BioEdit Sequence Alignment Editor v. 7.0.5.3. Multiple nucleotide sequence alignments were generated using one sequence of each of the following species: *B. mucronatus* (GenBank accession numbers AM179514), *B. singaporen-sis* (AM180514), *B. conicaudatus* (AM179513), *B. tryphloehi* sp. n. (FJ998282), *B. populi* (FJ888483), *B. doui* (AM157743), *B. macromucronatus* (EU256381), *B. xylophilus* (AB294736), *B. fraudulentus* (AB067758) and *B. luxuriosae* (AB097864).

REARING OF NEMATODES ON *B. CINEREA* AND *C. CHRYSOSPERMA* ON PDA OR MALT AGAR PLATES

For establishment of laboratory cultures and examination of the morphological trait stability of *B. tryphloehi* sp. n., a series of cultures was started from individual mature females extracted from aspen twigs. The rearing was done in 7 cm diam. Petri dishes half-filled with a sterile 2% agar and a 5 × 5 mm piece of mature *B. cinerea* culture on PDA added to the centre of the agar surface, as described earlier for rearing of *B. populi* (Tomalak & Filipiak, 2010). The cultures were periodically examined under a dissecting microscope and, when justified, individual nematodes withdrawn for detailed inspection under a compound light microscope. After 2-3 weeks of incubation at 20 or 25°C the nematode offspring were washed off the plates with distilled, sterilised water, washed three times and the suspension concentrated by sedimentation. Then, the nematodes were transferred in 25-50 µl water to 2-week-old cultures of *B. cinerea* on PDA or malt agar for further rearing in vitro. As only a small proportion of individuals, wild females initiated their reproduction on *B. cinerea* plates, four independent series of cultures, each with 30 plates, were used for starting nematode rearing on this fungus.

Identical rearing plates with *B. cinerea* were used for intra- and interspecific cross-breeding of the nematodes. In this part of the research, pairs of juveniles (J3/J4), i.e., one each from each parental population, were transferred with a mounted eyelash to the agar surface in individual plates. Since in the earlier rearing experiments only a small proportion of wild females of the new nematode could initiate reproduction and establish viable populations on *B. cinerea* plates, only individuals originating from these populations were used for the subsequent cross-breeding. In these experiments, individuals of *B. tryphloehi* sp. n. (PL-08) were crossed with two isolates of *B. fraudulentus* from Poland (PL-01) and Germany (H26), two isolates of *B. mucronatus* from Germany (i.e., DE-5(w) and DE-7(w) of East Asian and European genotypes, respectively), and single isolates of *B. populi* (PL-03) from Poland, *B. xylophilus* (China) originating from Nanjing, China, and *B. doui* (Ne9/06) from Taiwan. Control crosses were done between individuals of the same nematode species and strains. The isolates of *B. mucronatus* (DE-5(w) and DE-7(w), *B. xylophilus* (China), *B. fraudulentus* (H26), and *B. doui* (Ne9/06) were kindly provided by Dr T. Schröder from the Julius Kühn Institute, Federal Research Centre for Cultivated Plants (formerly Federal Biological Research Centre for Agriculture and
Forestry – BBA), Braunschweig, Germany. The *B. xylophilus* isolate had been reared in our laboratory for over 4 years. At least 30 individual crosses were performed for each combination of the nematode parental lines. The presence of new generation offspring was examined after a 14-day incubation period at 25°C.

Due to the erratic performance of *B. trypophloei* sp. n. (PL-08) in initiation and establishment of its populations *in vitro* on PDA or malt agar with *B. cinerea* cultures, another type of rearing medium with *C. chrysosperma* grown on PDA or malt agar was also used for the nematode *en masse* rearing in 2009. This fungus is a conidial form of *Valsa sordida* Nitschke which is naturally present and predominant in larval galleries of *T. asperatus*, and apparently transmitted by these bark beetles to new host trees. Shortly after invasion of the tree by *T. asperatus* the fungus can be found in galleries constructed by adult beetles. It caused a local characteristic brown coloration of the tree phloem. *Cytospora chrysosperma* was selected after examination of the nematode reproductive response to a series of fungi isolated from dying and dead aspen. It can be easily isolated from beetle-infested thin twigs of aspen, and cultured on both of the above fungigrowing media at 20 and 25°C. In our research, the isolation was done with non-sterilised males of *B. trypophloei* sp. n. (PL-08) which were individually transferred from a fresh (lasting max. 5 min) suspension of the beetle gallery frass in sterilised water to Petri dishes with sterilised PDA or malt agar. Usually, one or two additional transfers of the inoculum were needed before obtaining bacteria-free, monoxenic culture of *C. chrysosperma*. For the *en masse* rearing, *ca* 30 wild adult individuals of the new nematode were picked from the *T. asperatus* gallery frass extract and, after additional microscopic examination, transferred to growing plates with a 7-day-old culture of *C. chrysosperma* on PDA or malt agar. The cultures were then incubated at 20-25°C for 14-21 days. Subsequent cultures were started with *ca* 100 adult nematodes extracted from older plates and transferred to fresh, 1-week-old cultures of the fungus.

**In vivo** rearing of nematodes in tree twigs

As the life cycle of *B. trypophloei* sp. n. seemed to be very closely related to the life cycle of its insect vector it was decided to ascertain if the nematode could develop and reproduce in healthy aspen branches with no previous infestation with the bark beetle. The inoculation experiment was set up in the laboratory and ran for 2.5 months (27/10/2009-12/01/2010) at 20 ± 1°C. Ten 35-cm-long fresh twigs, with a maximum diameter of 2 cm, were cut from aspen trees and placed in a glass bucket with their lower ends immersed 5 cm deep in water. The nematodes were obtained from *en masse* rearing *in vitro* on *C. chrysosperma* malt agar plates. For the nematode inoculation, an upside-down U-shaped wound was made with a scalpel in the bark of each twig, *ca* 10 cm above the water level. *Circa* 1000 nematodes at various developmental stages, in 30-50 μl of distilled water per twig, were pipetted into a small cotton plug inserted into the wound. After injection the wound was sealed with a parafilm to protect water in the cotton plug from evaporation. The experimental twigs were left undisturbed in the laboratory with only a weekly change of water in the bucket. After 1 and 2.5 months, five twigs each were withdrawn from the bucket and subjected to examination for the presence of nematodes. Each twig was cut into four 5-cm-long sections: one below, one at the inoculation site, and two above the inoculation site. The sections were then separately chopped into 1 cm pieces and placed in 9 cm Petri dishes with distilled water for nematode extraction. The presence of living nematodes and their position in the stem were recorded after 24 h extraction. The juveniles and adults of the new nematode generation extracted from the experimental twigs were examined morphologically under a compound microscope equipped with DIC optics.

**Identification of *B. trypophloei* sp. n. natural vectors**

Since dispersion of nematodes from the genus *Bursaphelenchus* to new tree hosts depends on the availability of insect vectors, in order to understand in full the biomics of the new nematode species, it was necessary to elucidate the relationship(s) between its dispersal developmental stages and insects occupying the same niche in the tree.

To date, the new nematode has been isolated exclusively from larval galleries of the bark beetle, *T. asperatus*, which breeds in thin twigs of aspen, *P. tremula*. Therefore, searching for potential insect vectors of this species was limited to this niche.

Larvae, pupae and adult beetles found throughout the year in galleries under the bark of aspen were examined in detail with the aid of a dissecting microscope. After decapitation, the insects were individually dissected in
M9 buffer (Sulston & Hodgkin, 1988). All external and internal body parts that could potentially serve as hiding sites for the nematodes were inspected for the presence of dauer juveniles with characteristic structures, such as aphelenchoid bulbus and head shape.

In order to examine the association between *T. asperatus* and juvenile nematodes found in its haemocoel, a series of dauer recovery experiments was done in 2008 and 2009. Batches of 20-30 dauer juveniles of nematodes dissected from the haemocoel of older larvae, pupae, and adult beetles were transferred to fresh 2% agar/B. cinerea rearing plates, prepared as described earlier. The plates were then incubated for 4 weeks at 20 or 25°C to initiate dauer recovery and development of adults to assist identification. In 2009, in half of the rearing plates *B. cinerea* was replaced by the new fungus *C. chrysosperma*, which had proved to be more effective in laboratory rearing of the new nematode. In total, over 80 culture plates were examined in these experiments.

Due to the inability of dauer juveniles extracted from the haemocoel of *T. asperatus* to recover and develop on artificial media, further examination had to be done in a more natural setting. For this purpose, adult beetles of *T. asperatus* were gently withdrawn from the bark of aspen twigs during their natural invasion of the tree host in the field on 7 July 2009. On the same day, bunches of four, freshly cut, 30-cm-long and up to 2 cm in diameter aspen twigs, were placed into five glass cylinders with screened tops, and left in insectary conditions at 20 ± 2°C. Five control twig samples were chopped into small pieces and examined for potential presence of nematodes after extraction on nematological sieves. Twenty of the field-collected beetles of *T. asperatus* were then subjected to a detailed dissection analysis as described earlier. The remaining beetles, randomly divided into groups of 20 individuals, were then released into each cylinder with aspen twigs and allowed to deposit eggs in the bark for 1 month. Once a week, one aspen twig with nuptial chambers, and later with the beetle larval galleries, was taken from each cylinder. Samples (5 cm long), with visible gallery entrance holes made by the beetle, were cut off from the experimental twigs and, after dividing into small pieces, were subjected to nematode extraction in water on nematological sieves. All extracted nematodes were then examined under a compound microscope with DIC optics.

### Results

#### Isolation and Morphological Examination of Nematodes

In our previous study, the newly described nematodes had been isolated only from aspen, *P. tremula*. In the examined forest stand they were widely distributed and present in freshly broken, dying, or dead branches and twigs with active galleries of the bark beetle, *T. asperatus*. The adult and juvenile nematodes were numerous in the insect galleries, usually from the summer (late June) until April-May of the following year. The dauer juveniles were present in older larvae, pupae and adult beetles. The newly described nematodes occasionally shared the same aspen tree with another, recently described nematode species, *B. populi* (Tomalak & Filipiak, 2010). However, each of these nematode species occupied separate niches on the tree, i.e., thin twigs and trunks, respectively. *Bursaphelenchus tryphloei* sp. n. was isolated mainly from the frass of beetle galleries and only very rarely from the undamaged bark and phloem in areas between individual gallery systems.

The present population had a limited ability to reproduce on *B. cinerea/PDA* or malt agar plate cultures at 20-25°C. Only 7-11% of wild females initiated reproduction on these cultures, but once established the populations remained viable for at least six generations. All wild nematodes could, however, vigorously reproduce on laboratory cultures of another fungus species, *C. chrysosperma*, originally isolated from galleries of *T. asperatus* in aspen bark and phloem. The whole mature cultures or nematodes extracted to water could be stored from several weeks up to 6 months at 1-4°C.

![Bursaphelenchus tryphloei sp. n.](Figs 1, 2)

#### Measurements

See Table 1.

#### Description

**Female**

Body slender, cylindrical, ventrally arcuate, frequently conspicuously curved posterior to vulva when heat-killed.

*The specific epithet is derived from the genus of the insect vector.*
Fig. 1. Bursaphelenchus tryphloei sp. n. A: Female; B: Male; C: Anterior region (female); D: Mid-body region (female); E: Head with stylet (female); F: Vulva; G: Male tail with spicules, bursa and positions of caudal papillae (lateral view); H-K: Female tail (range of forms).
Fig. 2. Light (Differential Interference Contrast) micrographs of Bursaphelenchus tryphloeoi sp. n. Adults. A, B: Head region; C: Position of excretory pore; D: Vulva and tail region (female); E: Vulva; F: Lateral field with four incisures; G-J: Female tail (range of forms); K, L: Male tail with spicules and distinct cucullus (lateral view); M: Male tail with bursa in ventral view (somewhat flattened). Dauer juveniles. N: Anterior region; O: Tail.
### Table 1. Morphometrics of adults and dauer juveniles of *Bursaphelenchus trypophloei* sp. n. extracted from bark of *Populus tremula* and haemocoel of adult *Trypophloeus aspratus*, respectively. All measurements are in μm and in the form: mean ± s.d. (range).

<table>
<thead>
<tr>
<th>Character</th>
<th>Male</th>
<th>Female</th>
<th>Dauer juveniles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holotype</td>
<td>Paratypes</td>
<td>Paratypes</td>
</tr>
<tr>
<td>n</td>
<td>–</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>L</td>
<td>632 (543-828)</td>
<td>679 ± 79</td>
<td>702 ± 88</td>
</tr>
<tr>
<td>a</td>
<td>42.1 (32.0-46.4)</td>
<td>40.9 ± 3.3</td>
<td>39.2 ± 3.1</td>
</tr>
<tr>
<td>b</td>
<td>11.1 (8.4-13.0)</td>
<td>11.4 ± 1.1</td>
<td>11.9 ± 1.4</td>
</tr>
<tr>
<td>c</td>
<td>22.7 (19.2-29.2)</td>
<td>24.8 ± 2.3</td>
<td>25.4 ± 2.3</td>
</tr>
<tr>
<td>c'</td>
<td>2.4 (1.6-2.4)</td>
<td>1.9 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>m</td>
<td>40.5 (39.6-45.8)</td>
<td>43.3 ± 2.1</td>
<td>42.0 ± 2.2</td>
</tr>
<tr>
<td>V</td>
<td>–</td>
<td>–</td>
<td>73.7 ± 1.3</td>
</tr>
<tr>
<td>Max. body diam.</td>
<td>14.5 (14.0-20.0)</td>
<td>16.6 ± 1.4</td>
<td>17.9 ± 1.4</td>
</tr>
<tr>
<td>Pharynx length&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.2 (55.0-67.5)</td>
<td>59.7 ± 3.1</td>
<td>59.1 ± 2.4</td>
</tr>
<tr>
<td>Excretory pore position&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.4 (67.5-87.5)</td>
<td>76.1 ± 5.5</td>
<td>80.0 ± 4.8</td>
</tr>
<tr>
<td>Nerve ring position&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.8 (57.5-68.8)</td>
<td>62.9 ± 3.1</td>
<td>65.3 ± 3.3</td>
</tr>
<tr>
<td>Post-uterine sac length</td>
<td>–</td>
<td>–</td>
<td>147 ± 19.4</td>
</tr>
<tr>
<td>Stylet length</td>
<td>11.3 (11.0-13.5)</td>
<td>12.9 ± 0.5</td>
<td>12.6 ± 0.6</td>
</tr>
<tr>
<td>Tail length</td>
<td>27.8 (22.5-32.5)</td>
<td>27.4 ± 2.5</td>
<td>27.7 ± 2.4</td>
</tr>
<tr>
<td>Anal body diam.</td>
<td>11.6 (11.3-13.8)</td>
<td>12.3 ± 0.8</td>
<td>11.3 ± 0.8</td>
</tr>
<tr>
<td>Spicule length (arc)</td>
<td>25.9 (23.1-28.0)</td>
<td>25.7 ± 1.5</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> Distance from anterior end to base of median bulb.  
<sup>b</sup> Distance from anterior end.

Cuticle finely annulated. Lateral fields with four incisures. Cephalic region high, offset by constriction. Stylet well developed, 12-14 μm long, slightly swollen at base, conus short, 38-46% of total stylet length. Median bulb oval (14-16) × (9-11) μm with almost centrally located valve plates (3.3-4.5) × (2.5-3.7) μm in size. Positions of pharyngeal gland orifice and pharyngo-intestinal junction not clearly seen. Latter usually located 4-6 μm posterior to median bulb. Pharyngeal gland lobe 2.6-4.2 body diam. long, dorsally overlapping intestine. Nerve ring located at 20-43% of body diam. posterior to median bulb. Excretory pore at or usually posterior to nerve ring, up to more than one body diam. posterior to its distal edge. Vulva post-median. Anterior vulval lip elongated, directed posteriorly to form a 7-10 μm long flap (i.e., 40-58% of body diam. as measured just anterior to vulva). Flap relatively thick, straight, parallel to body long axis or directed towards body wall at its distal end. Vagina always directed somewhat anteriorly. Female reproductive system located on left side of intestine.
Ovary monoprodelphic, outstretched. Developing oocytes in two irregular to multiple rows at distal 20-25% of ovary length, two rows in central region and single row at proximal 40% of ovary length. Spermatocheca elongated, ovoid. Crustaformerria partially obscured. Uterus thick-walled. Post-uterine sac extending for 72-89% of vulva-anus distance. Ratio of post-uterine sac length to body diam. = 8.7 ± 1.4 (7.1-11.7). Tail sub-cylindrical to slightly conoid, with a broadly rounded terminus or, in some individuals, with a short, 1-2 μm, narrowly conical projection.

**Male**

Body ventrally arcuate with tail region sharply curved ventrally when heat-killed. Anterior region similar to that of female. Testis expanded anteriorly, located on left side of intestine. Spermatoocytes arranged in two rows in distal third to half part of testis and in one row in remaining part. Sperm cells amoeboid. Spicules paired, large, 23-28 μm along arc, arcuate, with capitulum almost parallel to shaft axis. Ratio of spicule length along arc to its width measured posterior to rostrum = 4.8-6.3. Angle subtended between lines along capitulum and extending spicule = 42-58°. Capitulum 6.3-8.0 μm long, slightly concave on both sides of mid-region. Condylus rounded, in almost continuous line with dorsal lamina. Rostrum relatively short, usually somewhat pointed anteriorly. Distal third to fourth of spicule dorsal contour usually straight. Distinct disc-like cucullus present at spicule terminus, 1.3-1.8 μm diam. Width of spicule terminus abruptly narrowed shortly before cucullus. Tail conoid, terminus pointed. Small terminal bursa present, broadly rounded in ventro-dorsal view. Seven caudal papillae arranged as follows: one pair of adcloacal ventrosublateral papillae, a single precloacal ventromedian papilla just anterior to cloacal opening, two postcloacal pairs located ventrally and ventrosublaterally near base of bursa and almost at the same level.

**TYPE HOST AND LOCALITY**

Isolated from thin (up to 2 cm diam.) weakened or dead twigs of just fallen or live standing aspen, *P. tremula*, found in an urban forest at the Eastern edge of Poznan, Poland (GPS 52°24′49″N, 17°0′50″E). The locality is identical with that of the recently described *B. populi* (Tomalak & Filipiak, 2010).

**TYPE MATERIAL**

Holotype male (slide no. MIZ 216142), 16 female paratypes (slide nos MIZ 216143 and MIZ 216144) and 15 male paratypes (slide no. MIZ 216141) deposited in the nematode collection of the Museum and Institute of Zoology, Polish Academy of Science, Warsaw, Poland. Sets of two slides, each including one slide with 15 female paratypes and one with 15 male paratypes of *B. tryphlolei* sp. n. also deposited in the nematode collection of the Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Braunschweig, Germany, under the accession number Ne 9/09; in the USDA Nematode Collection, Agricultural Research Service, Nematology Laboratory, Beltsville, MD, USA, under accession numbers T-6014p and T-6015p, and at the Herbarium and Insect Museum of the National Institute of Agro-Environmental Science, Tsukuba, Japan. Plate cultures of the nematode are available upon request from the authors at the Institute of Plant Protection – National Research Institute, Poznan, Poland.

**DIAGNOSIS AND RELATIONSHIPS**

*Bursaphelenchus tryphlolei* sp. n. is characterised by the body length of 702 (603-946) μm in female and 679 (543-828) μm in male, its moderately slender body (*a* = 39.2 (33.0-45.2) and 40.9 (32.0-46.4) in female and male, respectively), lateral fields with four incisures, spicules 25.7 (23.1-28.0) μm long with a relatively large, disc-like cucullus (1.3-1.8 μm in diam.) at their tips, and the arrangement of the seven caudal papillae in the male (single papilla anterior to cloacal aperture, one pair anal and two pairs at the base of the bursa). Males of *B. tryphlolei* sp. n. are unique amongst *Bursaphelenchus* species by the combination of spicule length and their morphology. They have a rounded condylus situated in an almost continuous line with the dorsal lamina and a relatively short rostrum somewhat pointed anteriorly. Distal third to fourth of spicule dorsal contour usually straight. Females of *B. tryphlolei* sp. n. have a distinct, elongated, anterior vulval lip which is directed posteriorly to form a 7-10 μm long flap and long post-uterine sac (147 (130-190) μm) extending for over 72-89% of the vulva to anus distance.

In these characteristics, *B. tryphlolei* sp. n. is closely related to species of the *xylophilus* group (*sensu* Braasch, 2008). It can be easily separated from all other *Bursaphelenchus* species, including those from groups that partially share morphological characteristics of the *xylophilus* group (Braasch, 2001; Ryss et al., 2005).
**Bursaphelenchus trypophloei** sp. n. can be separated from all other species of the *xylophilus* group by the unique spicule morphology. Although they fit into the general plan for the *xylophilus* group, the spicules of *B. trypophloei* sp. n. differ in having a very short condylos only slightly offset from the line of the dorsal lamina and by the relatively short rostrum somewhat pointed anteriorly. The angular contour of the dorsal lamina is only distinct in some individuals. In the continuous line of the condylus and dorsal lamina, the spicules of *B. trypophloei* sp. n. may resemble those of *B. xylophilus* of the Condylus and dorsal lamina, the spicules of *B. trypophloei* sp. n. may resemble those of *B. xylophilus* of the East Asian genotype (Braasch, 2008). However, both species can be distinguished by the rostrum shape, which is short and somewhat pointed anteriorly in *B. xylophilus* taste terminus and absence of mucro or with only a 2-4 μm long mucro (Mamiya & Enda, 1979; Braasch, 2008). In having the female tail mucro either being absent or sometimes represented by a very small conical projection at the tail tip, *B. trypophloei* sp. n. can be easily separated from *B. kolymensis* which has a distinct, 3.5-4.4 μm long mucro (Korentchenko, 1980; Magnusson & Kulinch, 1996) and also from *B. mucronatus*, which usually has a 3-7 μm long mucro (Mamiya & Enda, 1979; Braasch, 2008).

The subcylindrical female tail with a broadly rounded or shortly conical tail terminus separates *B. trypophloei* sp. n. from all the remaining species in the *xylophilus* group which have gradually tapering tails with a narrowly rounded terminus in *B. luxuriosae* (Kanzaki & Futai, 2003) and *B. singaporensis* (Gu et al., 2005) or with a mucro in *B. conicaudatus* (Kanzaki et al., 2000), *B. macromucronatus* (Gu et al., 2008) and *B. baujardi* (Walia et al., 2003). *Bursaphelenchus trypophloei* sp. n. differs also from the latter species by more slender body (*a* = 32-46 vs. 25-36, respectively) and presence vs absence of swellings at the stylet base (Walia et al., 2003).

**Molecular Characterisation**

The amplification of the ITS regions of *B. trypophloei* sp. n. yielded one fragment with a length of 930 bp. ITS-RFLP profiles obtained by digestion of the PCR product with *RsaI, HaeIII, MspI, HinI* and *AluI* are shown in Figure 3 and Table 2. The patterns are clearly different from the ITS-RFLP patterns of all other *Bursaphelenchus*.
species summarised by Burgermeister et al. (2005), Gu et al. (2008) and Burgermeister et al. (2009).

In silico digestion of the amplified rDNA fragment (930 bp) using BioEdit software (Hall, 1999) was done. Analysis using MspI restriction enzyme revealed DNA fragments of 219, 218, 217, 122, 84 and 70 bp. However, electrophoretic separation of the obtained PCR products resulted in only two bands representing 218 and 122 bp, respectively. We were not able to distinguish fragments ranging between 217 and 219 bp on agarose gel.

The DNA base sequences of B. trypophloei sp. n. were deposited in GenBank with the accession numbers FJ998282 (ITS region) and FJ998283 (D2/D3 region of 28S rDNA).

The molecular phylogenetic analysis conducted with Bayesian and maximum likelihood algorithms was successful when based on partial 28S, and ITS-1 regions of rDNA only (Figs 4, 5). Unfortunately, of the examined species, the variability of the ITS-2 region was so great that we could not logically interpret the obtained relationships. Results of the phylogenetic analysis place B. trypophloei sp. n. close to the xylophilus group. Based on examination of both 28S and ITS-1 regions B. trypophloei sp. n. shares its position with B. tokyoensis, a species which is located between the xylophilus and africanus group (Kanzaki et al., 2009a). Bayesian analysis of 28S and maximum likelihood analysis of ITS1 regions confirm this placing. However, analyses of 28S and ITS1 regions with maximum likelihood and Bayesian algorithms, respectively, show a closer relationship of both species to the xylophilus, rather than to the africanus group.

Among all the examined species from the xylophilus group, consensus alignment of ITS-1 regions showed 69.61% identity, while B. trypophloei sp. n. revealed only 46.63% identity with other species of this group. The alignment of ITS-2 regions was more variable. The level of identity among species of the xylophilus group was 47.94%, while B. trypophloei sp. n. was only 34.40% identical with other species of this group.

### Rearing of B. trypophloei sp. n. on B. cinerea or C. chrysosperma/PDA-malt agar plates

**Reproduction in vitro**

Botrytis cinerea cultures on PDA or malt agar proved to be unreliable for culturing B. trypophloei sp. n. Only a small proportion of adult nematodes transferred from larval galleries of T. asperatus in aspen twigs to in vitro cultures with B. cinerea continued their development and reproduction. In vitro experiments on reproduction of individual, field-collected females revealed that only 7 and 11% of plates yielded a new generation offspring after 2 weeks rearing at 20 or 25°C, respectively. The reproduction rate was 78 ± 43 (32-219) nematodes produced per female at 20°C and 124 ± 71 (46-259) per female at 25°C. In our 2007-2008 cultures, these nematodes, when transferred to new plates, vigorously reproduced for a few (up to 6) subsequent generations before their populations gradually declined. Interestingly, in spite of apparent good viability, nematodes kept in cool storage (1-4°C) in the original rearing plates for more than 2 months, were unable to continue development and reproduction at 20 or 25°C, when transferred to new plates.

By contrast, PDA or malt agar plates inoculated by C. chrysosperma originally isolated from T. asperatus galleries in aspen proved to be a much more reliable and effective growing medium with both culture establishment and quantity of obtained nematodes being excellent. Within 2-3 weeks at 25°C, large populations of B. trypophloei sp. n. could easily be established on 1-week-old cultures of this fungus, a small initial inoculum of 30-100 individuals producing 4730-63 260 of new generation offspring in every 2-3 weeks rearing at 20 or 25°C. In vitro experiments on reproduction of individual, field-collected females revealed that only 7 and 11% of plates yielded a new generation offspring after 2 weeks rearing at 20 or 25°C, respectively. The reproduction rate was 78 ± 43 (32-219) nematodes produced per female at 20°C and 124 ± 71 (46-259) per female at 25°C. In our 2007-2008 cultures, these nematodes, when transferred to new plates, vigorously reproduced for a few (up to 6) subsequent generations before their populations gradually declined. Interestingly, in spite of apparent good viability, nematodes kept in cool storage (1-4°C) in the original rearing plates for more than 2 months, were unable to continue development and reproduction at 20 or 25°C, when transferred to new plates.

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In such cultures, B. trypophloei could be reared indefinitely, providing the nematodes were periodically (every 2-3 weeks) transferred to a fresh medium.

Interestingly, the second Bursaphelenchus species isolated from aspen, i.e., B. populi, either could not develop and reproduce on C. chrysosperma or only weak populations of a few individuals each were produced within a 2-3 week period. By contrast, cultures of B. populi showed excellent growth on B. cinerea.

In vitro recovery of dauer juveniles dissected from the haemocoel of T. asperatus

The aphelenchoid dauer juveniles that were dissected from the haemocoel of immature T. asperatus did not re-

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**Table 2. Sizes of DNA restriction fragments obtained in ITS-RFLP analysis of Bursaphelenchus trypophloei sp. n. and calculated from sequencing of the ITS region.**

<table>
<thead>
<tr>
<th>PCR product (bp)</th>
<th>Rsal</th>
<th>HaeIII</th>
<th>MspI</th>
<th>HinfI</th>
<th>Alu</th>
</tr>
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<tbody>
<tr>
<td>930</td>
<td>478</td>
<td>402</td>
<td>219</td>
<td>506</td>
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<td>70</td>
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</tbody>
</table>
Fig. 4. Molecular phylogenetic relationship of Bursaphelenchus tryrophloei sp. n and 25 other Bursaphelenchus species based on partial 28S rDNA. Aphelenchoides fragariae served as outgroup species. Phylogenetic trees were generated with 1000 bootstrap replications. A: Bayesian analysis with substitution model GTR; B: Maximum likelihood (ML) analysis with substitution model GTR.
Fig. 5. Molecular phylogenetic relationship of *Bursaphelenchus trypophloei* sp. n and 25 other *Bursaphelenchus* species based on ITS-1 region. *Aphelenchoides fragariae* served as outgroup species. Phylogenetic trees were generated with 1000 bootstrap replications. A: Bayesian analysis with substitution model GTR; B: Maximum likelihood (ML) analysis with substitution model GTR.
cover or develop further on any of the examined rearing media. However, 7-day-old cultures of *C. chrysosperma* supported further development of the juvenile nematodes shortly after their active emergence from adult *T. asperatus* into nuptial chambers and larval galleries in the following season. The juveniles developed into adult *B. trytophloei* sp. n. and produced new generation offspring within 7-10 days *in vitro*.

**Diallelic cross-breeding**

Diallelic cross-breeding of *B. trytophloei* sp. n. with selected isolates of *B. fraudulentus* (PL-01, and H26), *B. mucronatus* (DE-5(w) and DE-7(w)), *B. doui* (Ne 9/06), *B. populi* (PL-03), and *B. xylophilus* (China) done *in vitro* on *B. cinerea/PDA-2%* agar plates did not produce any offspring. In intraspecific cross-breeding, control plates with pairs of nematode individuals from the same isolate and from independent isolates of the same species were observed to have new generation offspring in 17-54% of dishes after 14 days of incubation at 25°C.

**IN VIVO REARING OF NEMATODES IN ASPEN TWIGS**

Examination of the experimental aspen twigs 1 and 2.5 months after artificial inoculation with *T. asperatus* revealed that nematodes established only in 3 (60%) and 2 (40%) twigs, respectively. The nematodes were recovered only from the vicinity of the inoculation wound. In this region, the bark tissue died off and turned brown. From all the nematode-infested sites the fungus *C. chrysosperma* was also isolated. In samples with no nematode development, the bark remained yellow and green. In nematode positive samples 1 month after inoculation, all developmental stages of *B. trytophloei* sp. n could be extracted from the bark. After 2.5 months adult nematodes were found only occasionally, while the remaining immature individuals represented only one early juvenile stage (J2 with broadly rounded tail apex) with no evidence of any further development.

**IDENTIFICATION OF NATURAL VECTOR(S) OF B. TRYTOPHLOEI SP. N.**

In the examined forest stand *B. trytophloei* sp. n. was always associated with thin (5-20 mm in diam.) twigs of aspen which were concurrently infested with the bark beetle, *T. asperatus*. Therefore, this insect was considered as the most probable vector. Detailed examination of all developmental stages of the beetle did not show any nematodes on their body surface. However, dissection of older larvae, pupae and adult beetles just after their final moult revealed numerous (37.5 ± 31.4 (11-126) juvenile nematodes in the insect haemocoel. The prevalence of infection was 82.5%. The nematodes occupied the haemocoel, swimming freely amongst the lobes of the fat body. They had morphological structures characteristic of aphelenchoid dauer juvenile, *i.e.*, slightly flattened bulbous and cup-like head region with indistinct lips. Similar dauer juveniles were found in the haemocoel of adult *T. asperatus* collected during their construction of nuptial chambers and egg deposition on new tree hosts in the following season. However, in these insects the number of extracted nematodes was usually lower (11.6 ± 7.3 (2-26)) than in beetles collected from their original breeding sites. At the same time, similar juveniles and a few adult *B. trytophloei* sp. n. were recovered from the frass of the beetle nuptial chambers and early larval galleries under the bark.

Dauer juveniles extracted from the haemocoel of larvae, pupae or adult beetles did not recover or develop further when transferred to 2% agar/PDA-*B. cinerea* or *C. chrysosperma* plates, the nematodes dying within a few days.

In the insectary experiment performed on isolated, fresh aspen twigs subjected to infestation with adults of *T. asperatus*, weekly examination of frass taken from nuptial chambers and then from larval galleries of the new generation offspring revealed that the only nematode released by the beetle was *B. trytophloei* sp. n. Shortly after the beetle oviposition mature juveniles (J4) of *B. trytophloei* sp. n. could be recovered from the nuptial chamber, followed by adults and new generation juveniles together with development of the beetle larvae in the bark and phloem. No nematodes were observed in the wood. Mature nematode juveniles (J4) isolated from the beetle nuptial chambers continued their development *in vivo* on *C. chrysosperma* malt agar plates and within 2 weeks produced large populations of various developmental stages of *B. trytophloei* sp. n.

Interestingly, *B. trytophloei* sp. n. was the only nematode found in larval galleries of *T. asperatus*. Consecutive dissection of all developmental stages of the beetle revealed that the nematode juveniles started to invade late second instar larvae and continued penetration into the third instar. Then, they remained in the beetle haemocoel with no apparent signs of further development until its invasion of a new tree host and egg deposition in the next season. The dauer juveniles of *B. trytophloei* sp. n. extracted from the insect haemocoel are characterised as follows: lip region high, conical, not offset.
Styllet invisible. Bulbus ovoid, slightly flattened. Internal organs mostly partially obscured by granular contents. Tail conical to slightly sub-cylindrical, macro-like terminus pointed. Body measurements are presented in Table 1.

Discussion

Taxonomic separation of *B. tryphlolei* sp. n. has been based on a series of unique morphological, molecular, ecological and reproductive characteristics. In its morphology the nematode presents all the distinctive characters of the *xylophilus* group (sensu Braasch, 2008). A detailed phylogenetic analysis confirmed such a close relationship of *B. tryphlolei* sp. n. with other species of the *xylophilus* group. Interestingly, this analysis revealed also its close molecular similarity to *B. tokyoensis*, a recently described species, which phylogenetically is located between the *xylophilus* and *africanaus* groups (Kanzaki et al., 2009a). However, the major morphological characters of *B. tryphlolei* sp. n., such as the large size and shape of the spicules which have a distinct cucullus, long vulval flap and position of the male caudal papillae, indicate that the newly described species is more closely related to the *xylophilus* group than to *B. tokyoensis*.

The data available on vectors of *Bursaphelenchus* species from the *xylophilus* group indicate that these nematodes are usually transmitted by long-horned beetles (Coleoptera: Cerambycidae) belonging to the subfamily Lamiinae (*Enda* & *Mamiya*, 1972; Mamiya & *Enda*, 1979; Kanzaki et al., 2000, 2008; Kanzaki & *Futai*, 2003; Tomalak & *Filipiak*, 2010). Such associations have been suggested as being due to a close co-evolution between the nematodes and their vectors (Kanzaki & *Futai*, 2001). In its association with insects *B. tryphlolei* sp. n. differs from the remaining species of the *xylophilus* group for which the vectors are known. This nematode is transmitted to new tree hosts by a bark beetle, *T. asperatus*. Although bark beetles are common vectors of *Bursaphelenchus* species belonging to other groups (Rühm, 1956; Massey, 1974), this is the first report of such an association with a *Bursaphelenchus* species from the *xylophilus* group.

Interestingly, in the case of the presently described species, not only the vector insect but also the niche occupied in the vector’s body is unique in this group of nematodes. The juveniles of *B. tryphlolei* sp. n. penetrate into the haemocoel of the vector insect larvae (L2/L3) and, without any apparent development or damage to the host’s tissue, remain there for the rest of the beetle’s life cycle. The nematodes leave the insect only next season, after its invasion of a new tree and deposition of eggs. The ecological status of the nematode-insect relationship described here has not yet been clearly explained. Such a long (up to several months) presence inside the insect’s haemocoel would suggest a form of parasitism. This type of nematode-vector (host) relationship is characteristic in other asphelenchoids invading bark beetles, mainly *Parasitaphelenchus* spp. (Rühm, 1956; Massey, 1974; Tomalak et al., 1988). However, our morphological and developmental investigations excluded any association of *Parasitaphelenchus* species with *T. asperatus*, which could lead to potential misidentification of the nematode juveniles. Based on differences between sizes of dauer juveniles found inside the insect and in its external environment, or on further development inside the insect, some authors (Korentchenko, 1980; Giblin et al., 1984; Kanzaki et al., 2009b) have also suggested parasitism of *Bursaphelenchus* species in the insect vectors/hosts. However, to verify such conclusion, further study on actual uptake of food from the host tissue would be needed.

A similar relationship between the vector (host) and a *Bursaphelenchus* nematode was previously described in a bark beetle, *Trypophloeus populi* Hopkins, living in twigs of American aspen, *Populus tremuloides* Michx., in Canada (Tomalak, 1984). All propagative stages of the nematode developed in galleries of the insect whilst its dauer juveniles were present in the haemocoel of older larvae, pupae and adult beetles. At that time, based on similarity of morphological characters, host plants and insect vectors to those originally reported by Rühm (1956), the nematode was identified as *B. fraudulentus*. It should be noticed that in the original description of *B. fraudulentus* Rühm (1956) claimed isolation of this species from galleries of a long-horned beetle, *Cerambyx scopolii* Fuesslins, in bird cherry, *Prunus avium* L., and from a bark beetle, *Trypophloes granulatus* Ratz., under the bark of poplars, *Populus nigra* L. and *P. tremula*. However, in the light of our earlier (Tomalak & *Filipiak*, 2010) and the presently reported research, the correctness of the species identification of both referred studies could be challenged. As the fine morphology of spicules was not studied in the Canadian population, such subtle characters as direction of rostrum and shape of dorsal lamina could have been overlooked by the author. Unfortunately, at the time of the study (Tomalak, 1984) no molecular methods were available for verification of the morphology-based taxonomic identification of *Bursaphelenchus* species. It is also probable that Rühm’s (1956) population of *B.
fraudulentus isolated from aspen could be misidentified as morphologically similar to populations isolated from other tree species, since, in the more recent studies, B. fraudulentus has been isolated mainly from oak and alder (Schauer-Blume & Sturhan, 1989; Braasch et al., 1995; Schönfeld et al., 2001; Filipiak et al., 2010), while morphologically similar populations isolated from aspen turned out to be B. populi (Tomalak & Filipiak, 2010) or the newly described B. trypophloei sp. n. (for extended discussion see Tomalak & Filipiak, 2010). Therefore, it is probable that the Canadian population of Bursaphelenchus sp. associated with T. populi in P. tremulooides is more closely related (if not identical?) to the presently described B. trypophloei sp. n. than to B. fraudulentus, since both populations use closely related bark beetles as vectors and occupy the same niche in the vector’s body. Since the reference material from the Canadian study has not been successfully stored, the problem should be further elucidated by comparative study on nematodes freshly isolated from the original locality in the field.

The niche occupied by B. trypophloei sp. n. is directly related to the vector-beetle’s preferences for a breeding site on the tree. The nematodes are released into the living bark of P. tremula and limit their dispersion to the beetle’s gallery system in thin twigs. In our research, shortly after invasion of the new tree by T. asperatus the beetle galleries became infested with the fungus C. chrysosperma. The nematodes remained within the fungus-infested area of the gallery system throughout the entire developmental cycle of the beetle. This observation suggests that, under natural conditions, B. trypophloei has a close trophic association with C. chrysosperma, a fungus that is naturally transmitted by its vector insect. These findings were clearly supported by our laboratory experiments, where B. trypophloei sp. n. much more effectively developed and reproduced on monoxenic cultures of C. chrysosperma than on B. cinerea – the fungus which is commonly used in rearing of many other Bursaphelenchus species. It seems probable that still other, as yet unidentified, insect- or host-tree-derived signals are needed to initiate further development of dauer juveniles since no dauer recovery was observed on any of the examined fungi. Similar preferences for particular species of fungi or specific cues might be a more common phenomenon among tree-inhabiting nematodes, since we frequently experienced failures in rearing of other Bursaphelenchus species on B. cinerea-based cultures.

In contrast to the vector of B. trypophloei sp. n., the vector of B. populi, a long-horned beetle, Saperda perforata, mainly invades trunks of dying or dead aspen trees (Tomalak & Filipiak, 2010). Although both nematode species can occasionally live on the same tree they have never been found together. Since galleries of T. asperatus are predominantly infested with C. chrysosperma, the lack or poor ability of B. populi to develop and reproduce on this fungus, as repeatedly observed in our laboratory study, could be one of potential barriers that keep both species spatially separated within the tree.

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References


Bursaphelenchus tryphophilei sp. n. from Populus tremula


MAMIYA, Y. & ENDA, N. (1979). Bursaphelenchus mucronatus n. sp. (Nematoda: Aphelenchoididae) from pine wood and...


