Bursaphelenchus fagi sp. n. (Nematoda: Parasitaphelenchidae), an insect-pathogenic nematode in the Malpighian tubules of the bark beetle, Taphrorychus bicolor (Herbst.) (Coleoptera: Curculionidae, Scolytinae), in European beech, Fagus silvatica L.

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Summary – Bursaphelenchus fagi sp. n. is described from the bark of European beech, Fagus silvatica. All propagative stages of the nematode are numerous in larval galleries of the beech bark beetle, Taphrorychus bicolor, while dauer juveniles aggregate in Malpighian tubules of adult beetles. The new species is characterised by the body length of 871 (763-1110) μm in female and 852 (718-992) μm in male, very slender body (a = 56.8 (50.4-67.1) and 64.5 (56.5-73.8) in female and male, respectively), and spicules 15.2 (14.0-17.0) μm long. The extended anterior vulval lip in female, lateral fields with four incisures, and number (7) and arrangement of male caudal papillae may indicate that B. fagi sp. n. is closely related to the xylophilus group. It differs from this group by the relatively small, claw-like spicules with narrow capitulum, indistinct condylus in almost continuous line with dorsal lamina, and lack of distinct cucullus at the spicule tip. The close relation of B. fagi sp. n. with the xylophilus group has been confirmed by DNA sequencing and phylogenetic analysis of the 28S rDNA region.

Keywords – beech bark beetle, Botryotinia fuckeliana, Bursaphelenchus idius, Bursaphelenchus tokyoensis, Bursaphelenchus xylophilus group, ITS-RFLP, molecular, morphology, morphometrics, phylogeny, taxonomy.

Nematodes living in wood and bark are transmitted to new breeding sites on the surface, or in the internal organs, of insects infesting trees. This association between the nematode and its insect vector can be exclusively phoretic or/and parasitic (Massey, 1974; Tomalak et al., 1989a, b, 1990). The wood-inhabiting nematodes of Bursaphelenchus are generally mycophagous with a few exceptions, such as the pine wilt nematode, Bursaphelenchus xylophilus (Steiner & Buhrer, 1934) Nickle, 1970 and B. cocophilus (Cobb, 1919) Baujard, 1989, which feed directly on the host plant tissue. In relation to their insect vectors, such as cerambycid, scolytid and curculionid beetles, they are mostly phoretic. However, potential parasitic relationships of some Bursaphelenchus species with their insect vectors are also suggested for juveniles and adults found in the haemocoel of beetles and bees (Korentchenko, 1980; Giblin et al., 1984; Gerber et al., 1989; Kanzaki et al., 2009a). During our recent survey of nematodes associated with wood-boring insects in Poland, aggregations of dauer juveniles of unidentified aphelenchoid species were frequently observed in Malpighian tubules of young adults of beech bark beetle, Taphrorychus bicolor (Herbst.) (Coleoptera: Curculionidae, Scolytinae), colonising European beech, Fagus silvatica L. Examination of the beetle galleries during subsequent development of its larvae revealed the presence of numerous adults and juveniles of nematodes belonging to Bursaphelenchus and these could not be assigned to any of the presently known species. As all Malpighian tubules invaded by the nematode juveniles revealed clear morphological and histolog-
ical changes, we decided to identify the responsible nematode and elucidate its relationship with T. bicolor. The research reported here provides a detailed account of our morphological, molecular and bionomic study on taxonomic and ecological status of the new Bursaphelenchus species which we propose herein as B. fagi sp. n.

Materials and methods

ISOLATION AND MORPHOLOGICAL EXAMINATION OF NEMATODES

The study was conducted on a Buc-01 population of B. fagi sp. n. isolated for the first time in July 2011 and then re-isolated on many occasions in 2011, 2012 and 2013. The nematode dauer juveniles could be found in Malpighian tubules of adult bark beetles, T. bicolor, while the remaining juvenile and adult stages were numerous in larval galleries of the beetle in the European beech, F. sylvatica, growing in the State Forest, Łopuchówko District, Poland. Logs 50 cm long with bark were cut off from larger branches (> 10 cm in diam.) of beech infested with larvae of the beetle then stored in the open under shade trees until further processing. In the laboratory, the bark was stripped from the logs, chopped into 3-6 cm² pieces and subjected to nematode extraction in distilled water on 18 cm diam. nematological sieves with a mesh size of 0.2 mm. The obtained suspension of nematodes was concentrated by sedimentation and washed in three changes of distilled water. After preliminary identification under a compound light microscope, the nematodes were processed for detailed morphological and molecular analysis.

For the morphological analysis, adult nematodes extracted from the bark, dauer juveniles still remaining inside the Malpighian tubules of young adults of T. bicolor, and those dissected from the tubules were killed with gentle heat over a flame and examined 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 on an Olympus BX50 microscope with Differential Interference Contrast (DIC) optics. The nematode morphometric characters were measured with the aid of Olympus CellSens Standard software. Micrographs were taken with an Olympus CX50 digital camera. Thirty randomly taken individuals were examined for each morphological category. Male spicule length was measured along the arc (Ryss et al., 2005).

Since the visibility of caudal papillae in males of B. fagi sp. n. examined under the light microscope was partially obscured, the nematodes were also examined under a scanning electron microscope (SEM). For the SEM examination the nematodes were fixed in a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde. Washed three times in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide, and serial dehydrated in ethanol (10, 30, 50 and 70%) and acetone (70, 80, 90, 96 and 100%). After subsequent critical point drying, the specimens were coated with gold and palladium. The observations were conducted with a Hitachi S-3000N SEM.

MOLECULAR CHARACTERISATION

DNA samples of B. fagi 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 (Filipiak et al., 2010). Before incubation the mixture was frozen at −80°C for 40 min.

Different sets of primers were used for PCR reactions of each examined region of rDNA. For amplification of the ITS1 and ITS2 rDNA regions the forward primer F194 5′-CGT AAC GTC GTA GCT G-3′ (Ferris et al., 1993) and reverse primer 5368r 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (Vrain, 1993) were used. Amplification of the D2/D3 region of 28S rDNA was done with 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). For ITS-RFLP analysis, a ribosomal DNA section containing the ITS1 and ITS2 regions was amplified by PCR as described by Burgermeister et al. (2005). The obtained PCR products were purified according to the protocol provided by the manufacturer (Qiaex II Gel extraction kit, Qiagen). Suitable aliquots of the amplified DNA were digested with three units of the restriction endonucleases RsaI, HaeIII, MspI, HinfI and Alul. 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. fagi sp. n. were calculated based on the DNA base sequence of the ITS region by using the restriction site/fragment lengths analysis of the computer program BioEdit Sequence Alignment Editor v. 7.0.5.3.

For rDNA partial sequences of B. fagi sp. n., PCR products were cloned into pGEM-T easy vector (Promega)
and used to transform into DH5α *Escherichia coli* cells for further sequencing. Nucleotide sequences were determined (IBB) and contigs assembled using the SeqMan program from the Dnastar software package (DNASTAR).

The molecular phylogenetic status of *B. fagi* sp. n. within the genus *Bursaphelenchus* was determined using the computer program ClustalX for multiple sequence alignments. Phylogenetic trees were generated using MEGA 5.05 (Tamura et al., 2011) by maximum likelihood (ML), neighbour-joining (NJ), and maximum parsimony (MP) algorithms. The base substitution model was determined for D2/D3 region of 28S rDNA using MEGA 5.05 under the Bayesian information criterion and the model GTR + G + I was selected to the analysis. The General Time Reversible model, log likelihood (ln L), Akaike information criterion values, proportion of invariable sites, gamma distribution shape parameters, and substitution rates were examined in the phylogenetic analysis. Phylogenetic trees were generated with 1000 bootstrap replications. The species names and GenBank accession numbers of the sequences compared to *B. fagi* sp. n. are shown in the phylograms.

The sequence identity matrix of 28S and ITS regions in *B. fagi* sp. n. and *B. tokyoensis* 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.

**Rearing of the Nematodes on *Botryotinia fuckeliana***

*In vitro* rearing of *B. fagi* sp. n. for additional morphological and molecular analyses was conducted on PDA plates with non-sporulating laboratory strain of *B. fuckeliana*. The nematode cultures were started with individual females extracted from *T. bicolor* larval galleries, with mixed groups of 30 females and males, or with 50-100 dauer juveniles dissected from the beetle Malpighian tubules. The procedure for preparation of culture plates, handling of nematodes and rearing conditions were as described previously (Tomalak & Filipiak, 2010). At least 20 replicates (plates) were examined for cultures started with adult nematodes, and 53 for cultures with dauer juveniles.

**Identification of the Natural Vector of *B. fagi* sp. n.**

In order to confirm the relationship of the adult individuals of *B. fagi* sp. n. found in larval galleries of *T. bicolor* with nematode dauer juveniles present in Malpighian tubules of young adults of the beetle, the insects were individually dissected in M9 buffer (Sulston & Hodgkin, 1988). Then their Malpighian tubules containing nematodes were transferred to a drop of fresh M9 buffer and dissected in order to release the nematodes. The nematode dauer juveniles were subsequently transferred to 1-week-old cultures of *B. fuckeliana* on PDA for dauer recovery and further development. The plates were incubated at 25°C for 3-4 weeks.

As the *in vitro* rearing of dauer juveniles of *B. fagi* sp. n. was successful for only a small proportion of examined nematodes in individual plates, the juveniles extracted from Malpighian tubules were also subjected do detailed molecular analysis (ITS-RFLP) as described above.

**Results**

*Bursaphelenchus fagi*<sup>∗</sup> sp. n.

(Figs 1, 2)

**Measurements**

See Table 1.

**Description**

**Female**

Very slender nematode with exceptionally high ratio a. Body cylindrical, usually strongly ventrally arcuate when heat-killed. Cuticle finely annulated. Lateral fields with four incisures. Cephalic region offset by constriction. Stylet 11-12 μm long, slightly swollen at base, conus 41-48% of total stylet length. Procorpus cylindrical, 3.6-4.0 stylet lengths long. Median bulb ovoid (14-15) × (8-11) μm, with centre of valve plate located at 51.6 ± 2.8% (46.7-56.0%) from posterior margin. Valve plate (3.0-3.8) × (2.3-3.0) μm in size. Positions of pharyngeal gland orifice and pharyngo-intestinal junction not clearly seen. Latter usually located 2-4 μm posterior to median

<sup>∗</sup> Specific epithet derived from the European beech, *Fagus silvatica*, the host tree of the nematode and its insect vector, the bark beetle *Taphrophyes bicolor*. 
Fig. 1. Bursaphelenchus fagi sp. n. A: Female; B: Male; C: Anterior region (female); D: Vulval region (female); E: Male tail with spicules, bursa and positions of papillae (lateral view); F, G: Male tail showing shape of bursa and positions of two postcloacal pairs of papillae (partially ventral view, somewhat flattened); H-J: Female tail (range of forms); K: Dauer juvenile (dissected from a Malpighian tubule of the vector bark beetle, Taphrorychus bicolor).
Bursaphelenchus fagi sp. n. from Fagus silvatica

Fig. 2. Light (DIC) and SEM micrographs of Bursaphelenchus fagi sp. n. A-L: Adults. A, B: Anterior region: A: Head with stylet, median bulb and nerve ring; B: Median bulb and position of excretory pore; C: Vulva with extended anterior lip (flap); D: Vulva and postuterine sac with large sperm cells; E: Large sperm cells in male gonad; F: Lateral field with four incisures; G-I: Male tail; G: Spicules, lateral view; H: Shape of bursa (partially ventral view, somewhat flattened); I: Positions of caudal papillae; spicule terminus with somewhat widened and rounded tip (SEM); J-L: Female tail (range of forms); M-O: Dauer juvenile. M: Juvenile (entire view); N: Anterior region with median bulb; O: Tail.
Table 1. Morphometrics of adults and dauer juveniles of *Bursaphelenchus fagi* sp. n. extracted from bark of *Fagus silvatica* and Malpighian tubules of adult *Taphrorychus bicolor*, respectively. All measurements are in μm and in the form: mean ± s.d. (range).

<table>
<thead>
<tr>
<th>Character</th>
<th>Female Paratypes</th>
<th>Male Paratypes</th>
<th>Holotype</th>
<th>Dauer juvenile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
</tr>
<tr>
<td>L</td>
<td>871±106 (763-1110)</td>
<td>852±72 (718-992)</td>
<td>827</td>
<td>434±24 (420-462)</td>
</tr>
<tr>
<td>a</td>
<td>56.8±5.1 (50.4-67.1)</td>
<td>64.5±4.7 (56.5-73.8)</td>
<td>64.4</td>
<td>30.1±3.6 (27.8-34.7)</td>
</tr>
<tr>
<td>b</td>
<td>12.9±1.3 (11.2-16.4)</td>
<td>12.4±0.8 (11.1-13.9)</td>
<td>12.5</td>
<td>6.6±1.9 (5.5-8.9)</td>
</tr>
<tr>
<td>c</td>
<td>33.7±4.3 (29.0-45.5)</td>
<td>25.6±3.1 (29.8-42.1)</td>
<td>38.2</td>
<td>14.9±1.3 (14.1-16.4)</td>
</tr>
<tr>
<td>c'</td>
<td>2.9±0.3 (2.3-3.5)</td>
<td>2.4±0.2 (2.1-2.8)</td>
<td>2.2</td>
<td>–</td>
</tr>
<tr>
<td>m</td>
<td>44.0±2.3 (40.9-47.8)</td>
<td>43.8±3.6 (39.4-51.5)</td>
<td>41.5</td>
<td>–</td>
</tr>
<tr>
<td>V</td>
<td>75.7±1.7 (73.1-78.5)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Max. body diam.</td>
<td>15.4±1.5 (13.5-18.7)</td>
<td>13.3±1.5 (10.5-15.7)</td>
<td>12.8</td>
<td>14.2±0.9 (13.3-15.1)</td>
</tr>
<tr>
<td>Pharynx length1)</td>
<td>67.5±2.8 (63.7-74.8)</td>
<td>68.6±2.7 (63.7-73.7)</td>
<td>66.2</td>
<td>54.4±2.1 (52.2-56.3)</td>
</tr>
<tr>
<td>Excretory pore position2)</td>
<td>54.9±3.8 (49.7-61.7)</td>
<td>57.6±4.2 (48.0-65.7)</td>
<td>55.0</td>
<td>–</td>
</tr>
<tr>
<td>Nerve ring position2)</td>
<td>76.7±4.9 (68.3-86.5)</td>
<td>78.2±4.7 (69.3-90.3)</td>
<td>76.3</td>
<td>–</td>
</tr>
<tr>
<td>Post-uterine sac length</td>
<td>134±20.5 (107-181)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stylet length</td>
<td>11.4±0.3 (10.8-12.0)</td>
<td>11.0±0.6 (10.2-12.3)</td>
<td>10.8</td>
<td>6.9±0.3 (6.7-7.2)</td>
</tr>
<tr>
<td>Tail length</td>
<td>26.0±2.8 (19.7-31.7)</td>
<td>24.0±1.7 (20.7-26.7)</td>
<td>21.7</td>
<td>25.3±2.5 (23.3-28.2)</td>
</tr>
<tr>
<td>Anal body diam.</td>
<td>9.2±1.1 (7.2-11.7)</td>
<td>10.1±0.8 (9.0-12.0)</td>
<td>9.8</td>
<td>9.9±1.2 (8.6-10.9)</td>
</tr>
<tr>
<td>Spicule length (arc)</td>
<td>–</td>
<td>15.2±1.0 (14.0-17.0)</td>
<td>14.2</td>
<td>–</td>
</tr>
</tbody>
</table>

1) Distance from anterior end to base of median bulb.
2) Distance from anterior end.

bulb. Pharyngeal gland lobe 3.6-5.8 body diam. long, dorsally overlapping intestine. Nerve ring located at 24-114% body diam. posterior to median bulb. Excretory pore either at level of median bulb or up to 20% of body diam. anterior to proximal edge of bulb. Vulva postmedian. Anterior vulval lip elongated, directed posteriorly to form a 4.0 ± 0.8 μm (2.2-5.0 μm) long flap (i.e., 16-39% of body diam. as measured just anterior to vulva). Flap relatively thick, straight, parallel to body long axis or slightly bent and directed towards body wall at its distal end. Reproductive system composed of ovary, oviduct, spermatheca, crustaformeria, uterus, vagina and post-uterine sac. Ovary monoprodelpic, outstretched. Developing oocytes in multiple to two irregular rows at distal 30% of ovary length, two irregular to one row in central region and single row at proximal 50-60% of ovary-oviduct length. Spermatheca elongated, oval, usually empty or with a few large sperm cells. Crustaformeria partially obscured,
apparently formed from large cells arranged in more or less distinct four-cell rows. Uterus short, thick-walled. Vagina directed somewhat anteriorly. Junction between uterus and post-uterine sac with very small lumen and thickened wall. Post-uterine sac extending for 59.89% of vulva-anus distance, partially filled with large (6.3-7.5 μm) sperm cells. Ratio of post-uterine sac length to body diam. = 8.7 ± 1.3 (7.1-12.4). Tail sub-cylindrical to slightly conoid, with a broadly rounded terminus or, in some 20% individuals, with a short, 1-2 μm, narrowly conical projection.

**Male**

Body more slender than in females with very high ratio a, ventrally arculate with tail region sharply curved ventrally when heat-killed. Anterior region similar to that of female. Testis outstretched anteriorly, located ventrally on left side of intestine. Spermatocytes arranged in multiple to two irregular rows at distal third part of testis and irregular single row in central part. In remaining 30-40% of testis, sperm cells arranged irregularly. Sperm cells large (6.3-7.5 μm). Spicules paired, claw-like, relatively small, 14-17 μm along arc. Ratio of spicule length measured along arc to its width measured posterior to rostrum = 3.0-4.0. Capitulum 4.7-5.8 μm wide, flat to slightly concave on both sides of mid-region. Condylus indistinct, in almost continuous line with dorsal lamina. Rostrum very short, usually pointed somewhat anteriorly. Spicules with no distinct cucullus but in some individuals with local, shallow constriction just before terminus, followed by somewhat widened and rounded tip. Tail conoid, terminus pointed. Small bursa present, relatively narrow, rounded on both sides, with concave rounded abscission and two pointed tips at terminus in ventro-dorsal view. Seven caudal papillae arranged as follows: a single precloacal ventromedian papilla (P1) just anterior to cloacal opening, one pair of adcloacal ventrosublateral papillae (P2), and two postcloacal pairs (P3 and P4) located closely to each other ventrosublaterally, near base of bursa, at almost same level or second pair shifted less than one papilla diam. posterior to first pair.

**Dauer juvenile**

Found in Malpighian tubules of *T. bicolor* and characterised as follows: lip region high, conical, rounded, not offset. Stylet thin, poorly visible, ca 7 μm long. Median bulb circular to oval. Internal organs partially obscured by granular contents. Tail conical with pointed terminus.

**TYPE HABITAT AND LOCALITY**

Isolated from the bark of trunks and larger branches (>10 cm in diam.) of recently fallen or broken European beech, *Fagus silvatica*, infested with the bark beetle, *Taphrorychus bicolor* (Herbst.). All infested trees were found in a beech forest stand located in the Łopuchówko Forest District, State Forests, Poland (GPS: 52°39′41″N, 17°0′41″E).

**TYPE MATERIAL**

Holotype male (slide no. MIZ 9/2013/1), six female paratypes (slide no. MIZ 9/2013/2-9/2013/7) and six male paratypes (slide no. MIZ 9/2013/8-3/2013/13) deposited in the nematode collection of the Museum and Institute of Zoology, Polish Academy of Sciences, 00-679 Warsaw, Wilcza 64, Poland. A set of two slides, including one slide with six female paratypes and one with five male paratypes deposited in the nematode collection of the Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Messeweg 11/12, D-38104 Braunschweig, Germany, under the accession number Ne 30/13. Another set of a slide with seven female paratypes and another with seven male paratypes deposited in the USDA Nematode Collection, Agricultural Research Service, Nematology Laboratory, Beltsville, MD, USA, under accession numbers T-5986p and T-5987p, respectively.

**DIAGNOSIS AND RELATIONSHIPS**

*Bursaphelenchus fagi* sp. n. is characterised by the body length of 871 (763-1110) μm in female and 852 (718-992) μm in male, its distinctive, very slender body (a = 56.8 (50.4-67.1) and 64.5 (56.5-73.8) in female and male, respectively), lateral fields with four incisures, elongated anterior vulval lip directed posteriorly to form a 4.0 ± 0.8 (2.2-5.0) μm long flap, small claw-like spicules 15.2 (14.0-17.0) μm long, and the arrangement of the seven male caudal papillae (single papilla anterior to cloacal aperture, one pair adcloacal and two pairs at the base of bursa). Males of *B. fagi* sp. n. are unique amongst *Bursaphelenchus* species by the combination of the arrangement of their papillae and spicule length and morphology. The spicules do not have a cucullus at their tip. The condylus is indistinct and in an almost continuous line with the dorsal lamina. The rostrum is very short and somewhat pointed anteriorly. Females of *B. fagi* sp. n. have a long post-uterine sac (134 μm (107-181 μm)) extending for over 59-89% of the vulva to anus distance.
The characteristic shape of the male spicules easily separates B. fagi sp. n. from all other known species of Bursaphelenchus, with the exception of B. idius Rühm, 1956 (Rühm, 1956) and B. tokyoensis Kanzaki, Aikawa & Giblin-Davis, 2009 (Kanzaki et al., 2009b), which have spicules of similar form with an indistinct condylus in almost continuous line with the dorsal lamina, and with a very short rostrum. Bursaphelenchus fagi sp. n. can be distinguished from both of these species by the unique ratio a, which for female and male, respectively, is 56.8 (50.4-67.1) and 64.5 (56.5-73.8) in B. fagi sp. n. vs 32.1-34.0 and 33.8-34.4 in B. idius, and 36.9 (28.9-41.3) and 35.1 (31.8-38.3) in B. tokyoensis. A combination of several other morphological characters may also help to separate these species. With four ridges in the lateral fields, B. fagi sp. n. is similar to B. tokyoensis (Kanzaki et al., 2009a), but differs from B. idius, which has six ridges (Rühm, 1956). All three species show distinctive arrangements of seven caudal papillae present on the male posterior region. In B. fagi sp. n., the position of the first pair of ventrosublateral papillae just anterior to cloacal opening is similar to that in B. tokyoensis, but differs from B. idius, in which the first ventrosublateral pair is located one spicule length anterior the cloacal opening. Bursaphelenchus fagi sp. n. also differs from B. idius by the position of the two remaining pairs of caudal papillae which in B. fagi sp. n. are located ventrosublaterally, close to each other near the base of bursa and either at almost the same level or the third pair is shifted less than one papilla diam. posterior to the second, whereas in B. idius the second pair of papillae is placed slightly posterior to the cloacal opening, only 25% of the distance between the opening and tail tip, while the third pair is at the base of the bursa. In the close position of the second and third pair of papillae at almost the same level or with the third pair only slightly posterior to the second pair, B. fagi sp. n. also differs from B. tokyoensis, in which the second and third pair papillae are positioned in tandem along the body line, as depicted in the original drawings of Kanzaki et al. (2009a). In the small bursa with concave, rounded abscission and two pointed tips at the terminus, B. fagi sp. n. can be separated from B. idius, which has an almost square bursa, and from B. tokyoensis which has a rounded bursa with bluntly pointed terminus. The excretory pore located always at the level of the metacorpus or up to 20% of the body diam. anterior to proximal edge of the bulb can help to distinguish B. fagi sp. n. from B. tokyoensis, where it varies from being level with the metacorpus to ca one metacorpus length posterior (Kanzaki et al., 2009a). No position of the excretory pore was specified for B. idius in its original description (Rühm, 1956).

In the combination of four incisures in the lateral fields, distinct vulval flap in female, and the number (i.e., 7) and arrangement of male papillae, B. fagi sp. n. is also similar to the xylophilus group (sensu Braasch, 2008). It can be easily separated from species of this group by the small claw-like spicules 15.2 μm (14.0-17.0 μm) long which lack a cucculus at their tip vs arcuate, large spicules ranging from 21-22 μm in B. fraudulentus (Rühm, 1956) to 41-48 μm in B. singaporensis (Gu et al., 2005) with distinct, pointed rostrum and cucculus present.

**MOLECULAR CHARACTERISATION**

Amplification of the ITS regions of B. fagi sp. n. yielded a single PCR product with a length of 820 bp. Sizes of restriction fragments obtained in ITS-RFLP analysis by digestion of the PCR product with RsaI, HaeIII, MspI, HinII and AluI enzymes are shown in Table 2. Electrophoretic separation of restriction fragments revealed a distinctive profile for the ITS-RFLP pattern (Fig. 3) which was clearly different from profiles of all other known species of Bursaphelenchus obtained with the same PCR primers and the same set of restriction enzymes (Burgermeister et al., 2005, 2009). Interestingly, digestion of the PCR product with HaeIII restriction enzyme revealed DNA fragments of 462, 217 and 141 bp. However, electrophoretic separation of fragments obtained in repeated digestion of the PCR product resulted in visualisation of one additional band representing ca 350 bp. A segment of nematode rDNA containing the internal transcribed spacer regions ITS1 and ITS2 was used for the ITS-RFLP analysis. Due to the high variability of these regions, the appearance of an additional band might be caused by polymorphism of single nucleotides.

**Table 2.** Sizes of DNA restriction fragments (bp) obtained in ITS-RFLP analysis of Bursaphelenchus fagi sp. n. and calculated on sequencing results of the ITS regions.

<table>
<thead>
<tr>
<th>PCR product (bp)</th>
<th>Rsal</th>
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The new species can be distinguished from other sequenced *Bursaphelenchus* species by its characteristic sequence containing complete regions of ITS1, 5.8S and ITS2, and partial 18S and 28S regions of rDNA. The DNA base sequences of *B. fagi* sp. n. were deposited in GenBank with the accession numbers JX683685 (ITS region) and JX683686 (D2/D3 region of 28S rDNA).

The molecular phylogenetic trees, generated from partial 28S rDNA with maximum likelihood, neighbour-joining, and maximum parsimony algorithms (Figs 4-6), showed that *B. fagi* sp. n. is a new species belonging to the genus *Bursaphelenchus*. The molecular phylogenetic analysis revealed that this nematode is most closely related to *B. tokyoensis*. Consensus alignment of 28S and ITS regions showed, respectively, an 84.8 and 64.9% identity with *B. tokyoensis* – the species positioned on the phylogenetic tree between the *xylophilus* and *africanus* group (Kanzaki et al., 2009a). *Bursaphelenchus fagi* sp. n. also showed a close phylogenetic relationship with two other, recently described species, *viz.*, *B. tryphophloei* and *B. masseyi*, which have all the morphological characters distinctive of the *xylophilus* group sensu Braasch (2008) (Tomalak & Filipiak, 2011; Tomalak et al., 2013).

**Rearing of *B. fagi* sp. n. on *B. fuckeliana***

Both individual females of *B. fagi* sp. n. and small, mixed groups of 30 females and males transferred from the beech bark extract to 7-day-old cultures of *B. fuckeliana* on PDA continued their development and reproduction in vitro. The population development was, however, slower than that of other *Bursaphelenchus* species (*i.e.*, *B. xylophilus, B. mucronatus, B. fraudulentus and B. populi*) routinely reared in our laboratory on *B. fuckeliana*. The cultures of *B. fagi* sp. n. incubated at 20-25°C matured after 21-28 days with thousands of F2/F3 generation offspring in all developmental stages vs the usually 10-14 days observed in the remaining species. After this period the cultures rapidly declined unless the nematodes were transferred to fresh rearing plates. *B. fagi* sp. n. extracted to water could be stored for several weeks at 1-2°C. In such storage conditions, juveniles survived better than adults.

Serial rearing of the nematode in vitro apparently did not affect vigour of the offspring. Sub-populations started from a single female or small group of females and males successfully continued their development in vitro for at least ten rearing cycles.

The juveniles dissected from Malpighian tubules were generally reluctant to start further development in vitro on *B. fuckeliana*. New populations of *B. fagi* sp. n. were established in only two out of 53 rearing plates (*i.e.*, 3.8%).

**Identification of Natural Vector(s) of *B. fagi* sp. n. and Nematode Bionomics and Pathogenicity***

Repeated finding of numerous *B. fagi* sp. n. in active larval galleries of the beech bark beetle, *T. bicolor*, was indicative for the nematode association with this beetle or with any other predatory or parasitic insects regularly visiting galleries of this species. All developmental stages of the nematode could be found in the same tree shortly after the beginning of construction of galleries by the beetle larvae (July-August) until the following spring or early summer (May-June). In the galleries they were numerous throughout the beetle development. Dissection of *T. bicolor* larvae and pupae did not reveal any nematodes inside the insect body. The nematode infective juveniles colonised Malpighian tubules of young adult beetles shortly before their emergence from the breeding site and remained there until the insects invaded a new host tree. From 9-171 (52.3 ± 45.2) nematodes could be found per individual beetle. In the examined sample of 100 beetles collected at the beginning of tree invasion (*i.e.*, after boring of the entrance hole), 43% beetles were infested with the nematode dauer juveniles, which aggregated in 1-3 tubules. As pointed out above, dauer juveniles dissected from Malpighian tubules only occasionally responded to further rearing in vitro on *B. fuckeliana*.

Further evidence that dauer juveniles dissected from Malpighian tubules represented the *B. fagi* sp. n. was provided by DNA analysis (ITS-RFLP), which produced identical results with those obtained for adult nematodes present in larval galleries of *T. bicolor*. 

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**Fig. 3.** ITS-RFLP profile of *Bursaphelenchus fagi* sp. n. Lanes 1, 8 = DNA marker (100 bp ladder, MassRuler™, Fermentas); Lane 2 = rDNA amplification product; Lanes 3-7 = restriction fragments: Lane 3 = RsaI; Lane 4 = HaeIII; Lane 5 = MspI; Lane 6 = HinfI; Lane 7 = Alul.
Fig. 4. Maximum likelihood (ML) analysis of *Bursaphelenchus fagi* sp. n. and 38 other *Bursaphelenchus* species based on partial 28S rDNA. *Aphelenchoides subtenuis* served as outgroup species.
Fig. 5. Neighbour-joining (NJ) analysis of *Bursaphelenchus fagi* sp. n. and 38 other *Bursaphelenchus* species based on partial 28S rDNA. *Aphelenchoides subtenuis* served as outgroup species.
Fig. 6. Maximum parsimony (MP) analysis of *Bursaphelenchus fagi* sp. n. and 38 other *Bursaphelenchus* species based on partial 28S rDNA. *Aphelenchoides subtenuis* served as outgroup species.
The presence of dauer juveniles of *B. fagi* sp. n. in the beetle Malpighian tubules clearly affected the morphology and histology of the tubules. The severity of observed changes was directly related to the intensity of infestation. Uninfested tubules are composed of a circular layer of simple cuboidal epithelium externally covered with a basement membrane. Their diameter is similar throughout their length. The diameter of infested tubules increased 2-3 times in sections with aggregations of nematodes, *i.e.*, up to 143 μm vs 47-60 μm in uninfested tubules. Closer microscopic examination with DIC optics revealed partial or complete degradation of the tubule epithelial cells in regions invaded by the nematodes (Fig. 7). In tubules with a few dauer juveniles, tissue degradation was only partial and individual cells or their nuclei were still present. Degradation of the epithelial tissue was local and in the vicinity of the dauer juvenile aggregations, while more distant sections of the tubules remained unaffected. In heavily infested tubules, the presence of numerous dauer juveniles resulted in complete degradation of the cellular epithelium. In such cases, the nematode aggregation remained enclosed in a sac of a simple basement membrane of apparently nonfunctional Malpighian tubule.

**Discussion**

The recent spread of the pine wilt disease caused by *B. xylophilus*, and the necessity for precise identification of this quarantine pest, have renewed interest in research on the taxonomy of *Bursaphelenchus* spp. and, particularly, to those belonging to the *xylophilus* group. By a relatively uniform set of morphological characters, members of this group are easily separated from other species in the genus (Braasch, 2008). The same is true for molecular phylogenetic relationships, although with the continuous addition of newly described species, the gaps between morphologically defined groups gradually become less distinct. The recent description of *B. tokyoensis* (Kanzaki et al., 2009b) introduced an intermediate species between the *xylophilus* and *africanus* groups, which in some key morphological characters differs from species of both these groups. The subsequent description of *B. tryrophloei* (Tomalak & Filipiak, 2011) and *B. masseyi* (Tomalak et al., 2013) added two new species having their main morphological characters identical with the *xylophilus* group, but phylogenetically located at the edge of this group, in the vicinity of *B. tokyoensis*. The morphological and molecular data reported here provides evidence for the discovery of yet another *Bursaphelenchus* species which is phylogenetically intermediate between the *xylophilus* and *africanus* group and, except for the spicule shape, shares key morphological characters with the *xylophilus* group. *Bursaphelenchus fagi* sp. n. can be distinguished from all other *Bursaphelenchus* species by a unique set of morphological and molecular characters. In its morphology *B. fagi* sp. n. is most similar to *B. tokyoensis* and in its phylogeny it falls into a distinct cluster with three other species, *i.e.*, *B. tokyoensis*, *B. tryrophloei* and *B. masseyi*, which are located most closely to the *xylophilus* group yet clearly outside the main cluster. The lack of molecular data on *B. idius* (Rühm, 1956), which morphologically resembles *B. tokyoensis* and *B. fagi*, presently prevents us from placing this nematode together with the above listed species. Future re-isolation and molecular phylogenetic study should elucidate the position of *B. idius* within the genus.

Interestingly, all species in this cluster (and also *B. idius*) share some ecological similarities, which distinguish them from the *xylophilus* group. They all are associated with larval galleries of bark beetles (*Coleoptera: Curculionidae, Scolytinae*) and have adult bark beetles as vectors, although transmission of *B. tokyoensis* from galleries of *Cryphalus fulvus* Niijima was not directly observed (Kanzaki et al., 2009b). By contrast, in all the remaining *Bursaphelenchus* species of the *xylophilus* group which have a known insect association, the vectors are long-horned beetles (*Coleoptera: Cerambycidae*), mainly from the subfamily Lamiinae (Enda & Mamiya, 1972; Mamiya & Enda, 1979; Kanzaki et al., 2000, 2008, 2012; Kanzaki & Futai, 2003; Tomalak & Filipiak, 2010). It remains to be elucidated whether this distinction is related to different phylogenetic pathways in the nematodes and their insect vectors.

Pathogenicity of nematodes to their insect vectors has been reported in relation to a number of taxonomic groups, including many aphelenchoids. In wood-boring beetles, this phenomenon mostly concerns species of *Cryptaphelenchus* (Poinar, 1972) and *Aphelenchoides* (Tomalak et al., 1989b). Dauer juveniles of both genera can be found in Malpighian tubules of their insect vectors and cause pathological changes to these organs. The potential parasitic relationships of some *Bursaphelenchus* species with their vectors/hosts have also been reported for juveniles and adults found in the insect haemocoel and tracheal system (Korentchenko, 1980; Giblin et al., 1984; Gerber et al., 1989; Kanzaki et al., 2009a). However, no clear symptoms of pathogenicity were identified in these cases. Our research has revealed that dauer juve-
niles of *B. fagi* sp. n. are directly responsible for degradation of epithelial tissue of Malpighian tubules in the bark beetle vector. Mass presence of dauer juveniles inside the tubules led to elimination of epithelial cells and the magnitude of observed changes was related to the number of nematodes. Degradation of the epithelium was always local and in the vicinity of the nematodes. As no further development of dauer juveniles was observed inside the vector, it is possible that the histological damage may be caused by direct physical contact with ne-
matodes during their continuous, vigorous movement in the tubule and/or by potential nematode metabolites released to the tubule lumen. A similar degradation of epithelium in Malpighian tubules and expansion of their lumen was observed in another bark beetle, *Pityokeites sparsus*, mass infested with dauer juveniles of *Aphelenchoides pityokeini* Massey, 1974 (Tomalak et al., 1989b). In contrast to the presently described species, those nematodes clearly increased in size, suggesting some development inside the vector. Since Malpighian tubules are the main organ of the insect excretory system, which depends on the active transport of potassium and sodium ions (Stobbart & Shaw, 1974), degradation of the cellular epithelium may disturb this process and affect insect viability. With such a high natural infestation of *T. bicolor* with *B. fagi* sp. n., as recorded in the present study (i.e., 43%), the nematode may play an important, regulating role in the beetle’s population, although further research is needed to substantiate this hypothesis.

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References


