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Bursaphelenchus masseyi sp. n. (Nematoda: Parasitaphelenchinae) – a nematode associate of the bark beetle, *Trypophloeus populi* Hopkins (Coleoptera: Curculionidae: Scolytinae), in aspen, *Populus tremuloides* Michx. affected by sudden aspen decline in Colorado

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Summary – *Bursaphelenchus masseyi* sp. n. is described from trunks of unhealthy trembling aspen, *Populus tremuloides*, affected by sudden aspen decline (SAD) in Colorado, USA. All propagative stages of the nematode were present in larval galleries of a bark beetle, *Trypophloeus populi.* The dauer juveniles occupy the haemocoel of older larvae, pupae and adult beetles. The characteristic morphology of the male spicules with small but distinct cucullus, extended anterior vulval lip in female, lateral fields with four incisures, and number and arrangement of male caudal papillae, indicate that *B. masseyi* sp. n. is closely related to the *xylophilus* group. This relation has been confirmed by DNA sequencing and phylogenetic analysis of the 28S rDNA region. The new species is characterised by the body length of 958 (765-1203) μm in female and 874 (691-1122) μm in male, moderately slender body (a = 38.2 (33.5-41.1) and 38.0 (33.1-44.1) in female and male, respectively), and spicules 29.2 (26.2-34.7) μm long with a small cucullus (1.3-1.5 μm in diam.) at their tips. *Bursaphelenchus masseyi* sp. n. can be separated from other species in the *xylophilus* group by the morphology of spicules which have a short capitulum and unique rostrum that is pointed somewhat anteriorly, relatively thick vulval flap, which is straight, parallel to the body long axis and bent towards the body wall at its distal end, and other morphological and morphometric characters. The new species most closely resembles *B. trypophloei*, but differs by the morphology of spicules (short but distinct condylus *vs* condylus in a continuous line with dorsal lamina, and smaller cucullus). The taxonomic separation of the new species is also confirmed by the unique molecular profile of the ITS region (ITS-RFLP). Diallelic cross-breeding *in vitro* revealed also that *B. masseyi* sp. n. and *B. trypophloei* are reproductively incompatible. The new species showed poor ability to develop and reproduce on *Botrytis cinerea* cultures, although it grew vigorously on laboratory cultures of Polish and Colorado isolates of *Cytospora chrysosperma*, the fungus naturally associated with galleries of the nematode vector, *T. populi.*

Keywords – *Bursaphelenchus trypophloei*, *Bursaphelenchus xylophilus* group, *Cytospora chrysosperma*, description, ITS-RFLP, molecular, morphology, morphometrics, new species, phylogeny, taxonomy, USA.

The bionomics of *Bursaphelenchus trypophloei* Tomalak & Filipiak, 2011, the wood nematode recently described from European aspen, *Populus tremula* L. in Poland (Tomalak & Filipiak, 2011), is unique among *Bursaphelenchus* species. This nematode is closely related to the *xylophilus* group and is associated with the bark beetle, *Trypophloeus asperatus* (Gyll.). Its infective (dauer) juveniles invade the haemocoel of the beetle larvae, pupae and adults, and remain there until invasion of new trees by the beetles in the following season. Interestingly, a similar behaviour has been recently observed in wood nematodes transmitted by a North American bark beetle, *Trypophloeus populi* Hopkins. Our 2010-2012 study on nematodes associated with sudden aspen decline (SAD)-affected trembling aspen, *P. tremuloides* Michx. in Colorado, USA, revealed the frequent presence of *Bursa-*

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phelenchus sp. nematodes, which morphologically closely resembled *B. trypophloei*. All developmental stages of this nematode were found in larval galleries of *T. populi* and their infective juveniles could be dissected from the haemocoel of larvae, pupae and adults of this bark beetle. As the two nematodes are isolated from geographically distant localities, they are associated with closely related species of host trees and insect-vectors, and their infective juveniles present a unique behaviour of invading the haemocoel of the vector's larvae, we undertook a detailed study to elucidate the relationship between the new North American population of *Bursaphelenchus* sp. from Colorado and that of *B. trypophloei* from Poland. The research reported here provides a detailed account of our morphological, molecular and cross-breeding compatibility study on taxonomic status of the *Bursaphelenchus* sp. CO-03 population, isolated from trembling aspen in Colorado, USA, which we propose herein as *B. masseyi* sp. n.

Materials and methods

Isolation and morphological examination of nematodes

The study was conducted on the CO-03 population of *B. masseyi* sp. n. isolated for the first time in June and July of 2010 and then re-isolated in August of 2011 and in November of 2012 from the bark of SAD-affected trembling aspen, *P. tremuloides*, grown in a forest stand of the Gunnison National Forest in south-western Colorado, USA. The samples were taken from trunks of trees infested with *T. populi* by cutting small pieces of bark (*i.e.*, 5-10 cm in diam.) with visible entrance holes recently made by the beetles. The bark was stored in plastic bags at 4°C until further processing. In the laboratory, all samples were individually chopped into 5-10 mm 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, individual live females or groups of five females and five males were transferred to 2- or 7-day-old cultures of *Botrytis cinerea* Pers. or *Cytospora chrysosperma* Pers. on PDA, and reared at 20 or 25°C, as described earlier (Tomalak & Filipiak, 2011). The remaining individuals were processed for detailed morphological analysis.

For the morphological analysis, adult nematodes extracted from the bark and dauer juveniles dissected from the haemocoel of *T. populi* larvae, pupae or adults 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 with an Olympus BX50 microscope with Differential Interference Contrast (DIC) optics. Micrographs were taken with an Olympus CX50 digital camera. Thirty 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. masseyi* 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.

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 regions of rDNA. The obtained PCR products were purified (Qiaex II Gel extraction kit, Qiagen) and suitable aliquots of the amplified DNA digested with 3 units of restriction endonucleases *Rsa*I, *Hae*III, *Msp*I, *Hin*fI and *Alu*I following the manufacturer's instructions. Restriction fragments were resolved by electrophoresis in 2% agarose gel using TBE buffer, stained with 1 μg ml^{-1} ethidium bromide and photographed under UV light.

The ITS-RFLP profiles of *B. masseyi* 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 (Hall, 1999).

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 sequences of ribosomal DNA of *B. masseyi* sp. n. (ITS regions and D2/D3 expansion re-

gion of 28S rDNA) were determined. For this purpose the PCR products were cloned into pGEM-T easy vector (Promega) and used to transform into *Escherichia coli* DH5α cells for further sequencing. Nucleotide sequences were determined (IBB) and contigs assembled using SeqMan program from Dnastar software package (DNASTAR, USA).

The molecular phylogenetic status of *B. masseyi* sp. n. within *Bursaphelenchus* was determined using the computer program ClustalX for multiple sequence alignments. Phylogenetic trees were generated using MEGA 5.05 by maximum likelihood (ML) and neighbour-joining (NJ) algorithms. The species names and GenBank accession numbers of the sequences compared to *B. masseyi* sp. n. are shown in the phylograms.

Rearing of nematodes on *B. cinerea* and *C. chrysosperma* on PDA plates

In vitro rearing of *B. masseyi* for additional morphological analysis was started on PDA plates with *B. cinerea* (non-sporulating laboratory strain) and *C. chrysosperma* (two isolates naturally associated with bark beetles *T. asperatus* on European aspen, *P. tremula* and *T. populi* on North American trembling aspen, *P. tremuloides*). As in the preliminary tests, the *B. cinerea* plates did not provide a suitable environment for vigorous development of the nematode population and in the subsequent rearing and cross-breeding experiments, only *C. chrysosperma* cultures were used. The diallelic cross-breeding was carried out between individuals of *B. masseyi* sp. n. and those of the Polish population of *B. trypophloei* (PL-08). The procedures for preparation of culture plates, handling of nematodes and rearing conditions were as described previously (Tomalak & Filipiak, 2010). Twenty nematode pairs in three replicates were examined for each of the breeding variants, *i.e.*, inter-specific (*B. trypophloei* × *B. masseyi* sp. n.) and intra-specific (*B. trypophloei*; *B. masseyi* sp. n.) breeding.

Identification of *B. masseyi* sp. n. natural vector

In order to confirm the relationship of adult individuals of *B. masseyi* sp. n. present in larval galleries of *T. populi* with nematode infective juveniles occupying the haemocoel of the insect, larvae, pupae and adult beetles extracted from the bark were surface-washed in distilled water and dissected in M9 buffer (Sulston & Hodgkin, 1988). All nematode juveniles found in the beetle haemocoel were counted, examined morphologically in a drop of buffer under a compound microscope with DIC optics, and transferred to 2-day-old cultures of *C. chrysosperma* on PDA for further development. The plates were then incubated at 20 or 25°C until at least some nematodes reached adult stage.

Results

Bursaphelenchus masseyi[*] **sp. n.**
(Figs 1, 2)

Measurements

See Table 1.

Description

Female

Body slender, cylindrical, usually strongly ventrally arcuate, occasionally conspicuously curved posterior to vulva when heat-killed. 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, 40-46% of total stylet length. Median bulb usually ovoid with greatest diam. at *ca* 70% from anterior edge, but individuals with more uniform bulb dimension over entire central region were also found. Posterior edge of bulb often flattened or even somewhat concave. Median bulb (13-17) × (10-14) μm in size, valve plate located at 53.9 ± 3.6% (50.0-61.7%) from posterior edge. Valve plate (3.3-4.5) × (2.5-3.7) μm in size. Positions of pharyngeal gland orifice and pharyngo-intestinal junction not clearly seen, but latter usually located 2-4 μm posterior to median bulb. Pharyngeal gland lobe 1.8-3.4 body diam. long, dorsally overlapping intestine. Nerve ring located at 15-65% body diam. posterior to median bulb. Excretory pore always posterior to nerve ring, 23-97% of body diam. posterior to its distal edge. Vulva postmedian. Anterior vulval lip elongated, directed posteriorly to form a 7-11 μm long flap (*i.e.*, 33-49% 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. Vagina always directed somewhat

[*] The specific epithet honours Dr Calvin L. Massey, an American nematologist who dedicated most of his work to nematodes associated with insects in forest trees.

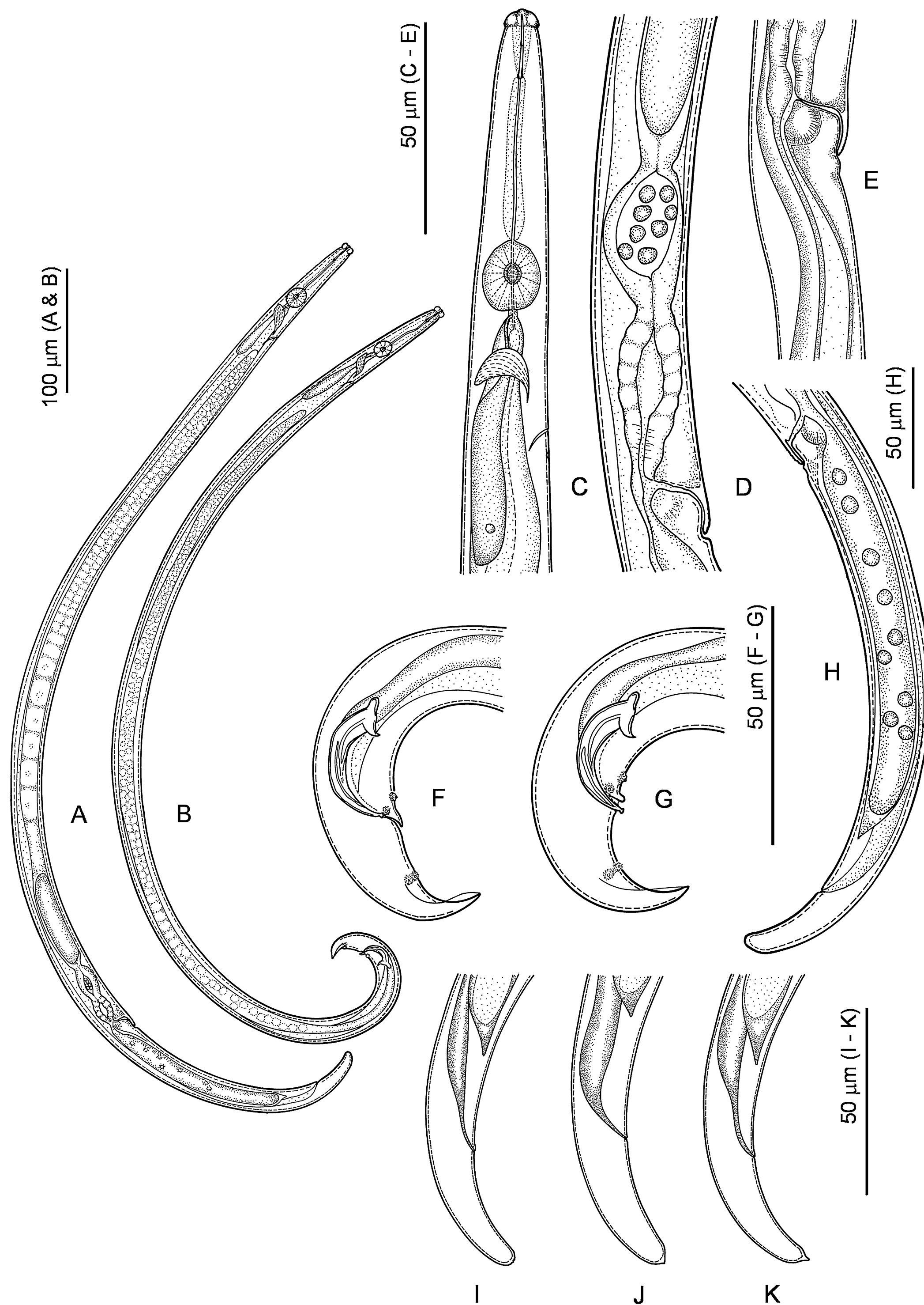

Fig. 1. *Bursaphelenchus masseyi* sp. n. A: Entire female; B: Entire male; C: Female anterior region; D: Female mid-body region; E: Vulva; F, G: Male tail showing spicules, bursa and positions of caudal papillae (lateral view – range of spicule forms); H: Female posterior region showing post-uterine sac; I-K: Female tail region.

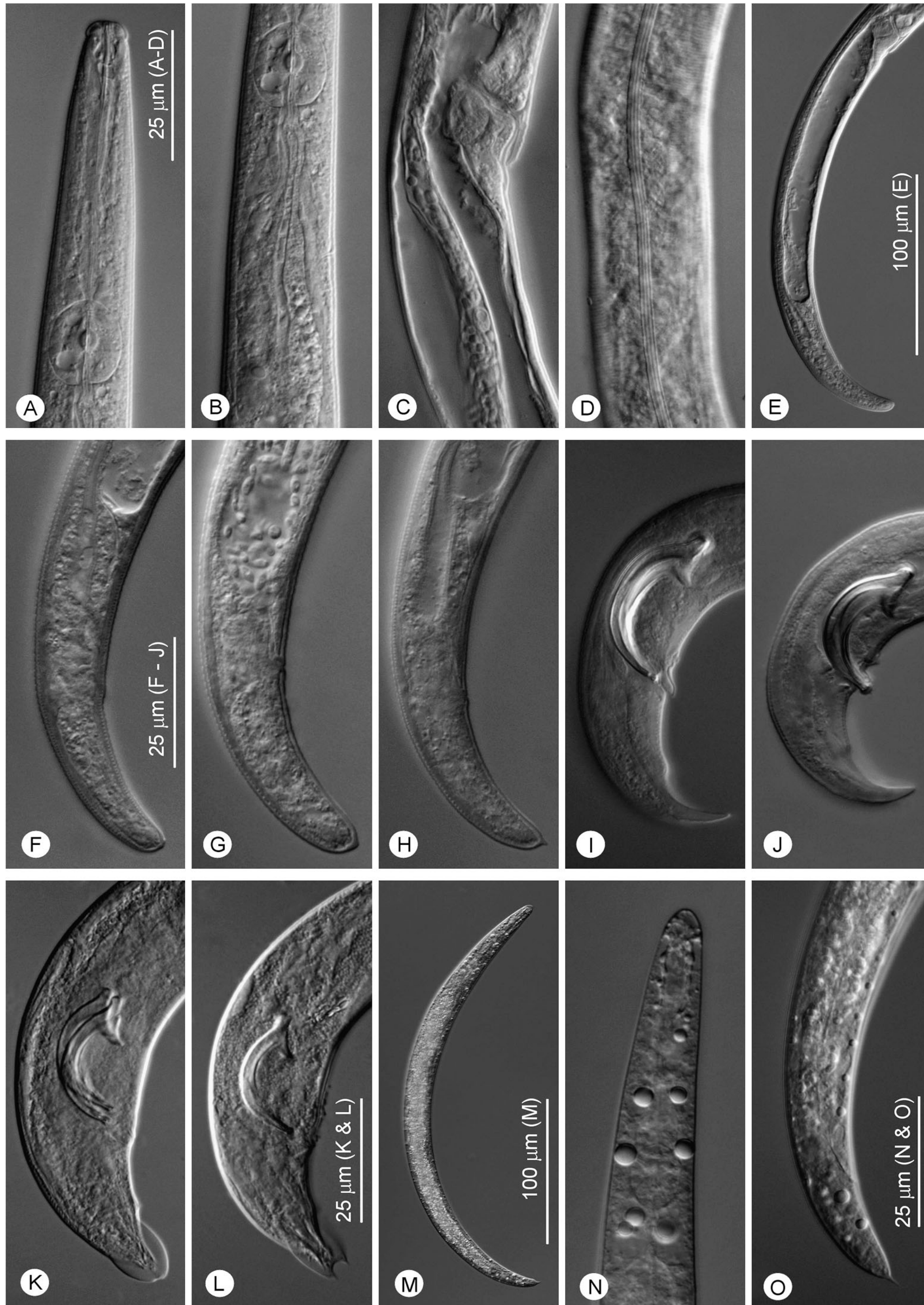

Fig. 2. Light micrographs of *Bursaphelenchus masseyi* sp. n. A-L: Adults. A: Head showing stylet and median bulb; B: Median bulb, nerve ring, excretory pore and pharyngeal gland; C: Vulva; D: Lateral field with four incisures; E: Female posterior region with post-uterine sac; F-H: Female tail region; I, J: Male tail with spicules and distinct cucullus (lateral view – range of forms); K, L: Male tail with bursa in partially ventral view (somewhat flattened – range of forms). M-O: Dauer juveniles. M: Juvenile (entire); N: Anterior region with faintly marked median bulb; O: Tail with mucro.

Table 1. Morphometrics of adults and dauer juveniles of *Bursaphelenchus masseyi* sp. n. extracted from bark of *Populus tremuloides* and haemocoel of larval *Trypophloeus populi*, respectively. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Female	Male		Dauer juvenile
	Paratypes	Paratypes	Holotype	
n	30	30	–	30
L	958 ± 130 (765-1203)	874 ± 130 (691-1122)	1073	327 ± 16 (316-350)
a	38.2 ± 3.9 (33.5-41.1)	38.0 ± 3.1 (33.1-44.1)	42.1	19.0 ± 0.7 (18.2-20.0)
b	14.0 ± 1.8 (11.3-17.2)	12.9 ± 1.5 (10.5-16.8)	15.6	5.7 ± 0.3 (5.4-6.0)
c	25.7 ± 2.7 (20.9-31.4)	25.7 ± 2.7 (20.9-31.4)	30.7	13.9 ± 2.5 (11.8-18.7)
c′	2.8 ± 0.3 (2.4-3.5)	2.2 ± 0.2 (1.9-2.5)	2.2	–
m	43.4 ± 1.7 (40.0-46.3)	42.9 ± 2.1 (38.5-46.8)	41.7	–
V	74.9 ± 1.3 (72.4-77.7)	–	–	–
Max. body diam.	25.0 ± 1.9 (19.6-28.3)	23.1 ± 2.4 (19.2-27.1)	25.5	17.2 ± 1.3 (15.4-18.8)
Pharynx length[1)]	68.4 ± 2.1 (65.2-72.5)	67.9 ± 6.1 (59.5-79.0)	68.8	54.6 ± 2.8 (52.3-60.8)
Excretory pore position[2)]	92 ± 5.9 (83-101)	87.0 ± 5.6 (77-97)	88	–
Nerve ring position[2)]	78 ± 3.9 (72-89)	77 ± 7.1 (64-90)	78.6	–
Post-uterine sac length	141 ± 19.4 (93-174)	–	–	–
Stylet length	13.2 ± 0.6 (12.0-14.3)	13.2 ± 0.5 (12.0-14.2)	13.9	–
Tail length	37.3 ± 4.2 (29.7-44.8)	33.9 ± 3.1 (30.2-39.7)	34.9	23.5 ± 3.1 (17.8-27.5)
Anal body diam.	13.1 ± 0.8 (11.8-15.0)	15.6 ± 1.2 (12.7-17.5)	16.2	–
Spicule length (arc)	–	29.2 ± 2.3 (26.2-34.7)	33.2	–

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

anteriorly. Female reproductive system located on left side of intestine. Ovary monoprodelphic, outstretched. Developing oocytes in two irregular to multiple rows in distal 25% of ovary length with two rows in central region and single row in proximal 40-45% of ovary length. Spermatheca elongated, oval. Crustaformeria partially obscured. Uterus thick-walled. Post-uterine sac extending for 58-87% of vulva-anus distance. Ratio of post-uterine sac length to body diam. = 5.6 ± 0.6 (4.7-6.8). 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. Spermatocytes arranged in a single row in proximal third to half of testis and irregularly to multiple

rows in remaining part. Sperm cells amoeboid. Spicules paired, large, measuring 26-35 μm along arc, arcuate, with capitulum almost parallel to shaft axis. Ratio of spicule length along arc to its width measured posterior to rostrum = 5.0-6.7. Angle subtended between lines along capitulum and extending spicule = 54-68°. Capitulum 8.8-11.7 μm long, flat to slightly concave on both sides of mid-region. Condylus short but distinct with bluntly rounded or occasionally squared tip. Rostrum relatively short, usually pointed somewhat anteriorly. Distal third to fourth of spicule dorsal contour usually straight. Localised widening or small cucullus present at spicule terminus, 1.3-1.5 μm diam. Tail conoid, terminus pointed. Small terminal bursa present, broadly rounded or occasionally with concavity at tip 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, and two postcloacal pairs located ventrally and ventrosublaterally near base of bursa and almost at the same level.

Dauer juvenile

Lip region high, conical, not offset. Stylet thin, poorly visible, *ca* 6-7 μm long. Median bulb circular to oval. Internal organs partially obscured by granular contents. Tail conical to slightly sub-cylindrical, pointed, mucro-like terminus, 2-3 μm long.

Type host and locality

Bark of trunks of standing live or dying trembling aspen, *P. tremuloides*, found in a forest stand in the Terror Creek watershed of the Gunnison National Forest, north of Paonia, CO, USA (GPS: 38°59′26″N 107°34′48″W).

Type material

Holotype male (slide no. MIZ 3/2013/1), ten female paratypes (slide no. MIZ 3/2013/2-3/2013/11) and ten male paratypes (slide no. MIZ 3/2013/12-3/2013/21) 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 ten female paratypes and one with ten male paratypes 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 3/13, and in the USDA Nematode Collection, Agricultural Research Service, Nematology Laboratory, Beltsville, MD, USA, under accession numbers T-5984p and T-5985p.

Diagnosis and relationships

Bursaphelenchus masseyi sp. n. is characterised by the body length of 958 (765-1203) μm in female and 874 (691-1122) μm in male, its moderately slender body (a = 38.2 (35.5-41.1) and 38.0 (33.1-44.1) in female and male, respectively), lateral fields with four incisures, spicules 29.2 (26.2-34.7) μm long with a small cucullus (1.3-1.5 μm diam.) at their tips, and the arrangement of the seven caudal papillae in the male (single papilla anterior to cloacal aperture, one pair adcloacal and two pairs at the base of the bursa). Males of *B. masseyi* sp. n. are unique amongst *Bursaphelenchus* species by their combination of spicule length and morphology. They have a short but distinct condylus, with bluntly rounded or occasionally squared tip and a relatively short rostrum usually pointed somewhat anteriorly. The distal third to fourth of the spicule dorsal contour is usually straight. Females of *B. masseyi* sp. n. have a distinct, elongated, anterior vulval lip that is directed posteriorly to form a 7-11 μm long flap, and a long post-uterine sac (140.6 (97.9-173.8) μm) extending for over 58-87% of the vulva to anus distance.

In these characteristics, *B. masseyi* sp. n. is closely related to species of the *xylophilus* group (*sensu* Braasch, 2008) and 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 masseyi* 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. masseyi* sp. n. differ by having a short condylus and a relatively short rostrum which is somewhat pointed anteriorly. The angular contour of the dorsal lamina is distinct only in some individuals.

In its morphology, *B. masseyi* sp. n. most closely resembles *B. trypophloei* Tomalak & Filipiak, 2011. The two species can be distinguished by the shape of the male spicules. Although the overall contour of the spicules is similar, they differ in the shape of the condylus, which is short but clearly separated from the dorsal lamina and bluntly rounded or squared at the tip in the former *vs* indistinct and in an almost continuous line with the dorsal lamina in the latter. The spicule cucullus in *B. masseyi* sp. n. is also smaller and less distinct than that of *B. trypophloei*. The species differ in mean values of many examined morphometric characters. However, the ranges of almost all of them widely overlap. Most distinctive are pharynx length and position of the female excretory

pore although, in the males, the ranges of these characters partially overlap.

As *B. masseyi* sp. n. and *B. trypophloei* are almost identical in their remaining morphological characters, *B. masseyi* sp. n. can be separated from all other species in the *xylophilus* group using, with only minor changes, the diagnosis presented for the relationships of *B. trypophloei* (see Tomalak & Filipiak, 2011).

The subcylindrical tail with a broadly rounded tail terminus and the absence of a mucro, or with only a short (1-2 μm long) conical projection at the tail tip in *B. masseyi* sp. n., is similar to the non-mucronate form of *B. xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Mamiya & Kiyohara, 1972) and *B. populi* Tomalak & Filipiak, 2010, reared *in vivo*, in the host tree (Tomalak & Filipiak, 2010). *Bursaphelenchus masseyi* sp. n. can be easily separated from *B. xylophilus* by the arrangement of the vagina which is always directed somewhat anteriorly *vs* at a right angle to the body axis in the latter.

Bursaphelenchus masseyi sp. n. can be separated from *B. populi* by the excretory pore which is always posterior to, or sometimes at, nerve ring level *vs* variable from level with the anterior end of the median bulb to just anterior to the nerve ring in the latter species (Tomalak & Filipiak, 2010).

In having a subcylindrical female tail, *B. masseyi* sp. n. can also resemble *B. fraudulentus* Rühm, 1956, *B. doui* Braasch, Gu, Burgermeister & Zhang, 2004, *B. kolymensis* Korentchenko, 1980, and *B. mucronatus* Mamiya & Enda, 1979. It can be separated from *B. fraudulentus* by the female tail mucro either being absent or sometimes represented by a very small conical projection at the tail tip *vs* a distinct mucro *ca* 2 μm long (Rühm, 1956) and the excretory pore is always posterior to the nerve ring, a feature which can also help to distinguish *B. masseyi* sp. n. from *B. fraudulentus* where it is located anterior to the nerve ring, usually at the median bulb or just posterior to this organ.

By the absence of, but sometimes with, a very small conical projection at the female tail tip, *B. masseyi* sp. n. can be easily separated from *B. doui* which always has a distinct, 2-4 μm long and ventrally located terminal mucro. The latter species has also larger spicules (34-43 *vs* 26-35 μm long as measured along arc) (Braasch *et al.*, 2004).

In having the female tail mucro either absent or sometimes represented by a very small conical projection at the tip, *B. masseyi* sp. n. can be distinguished from *B. kolymensis* which has a distinct, 3.5-4.4 μm long mucro (Magnusson & Kulinich, 1996; Korentchenko, 1980) and also from *B. mucronatus*, which usually has a 3-7 μm long mucro (Mamiya & Enda, 1979).

The subcylindrical female tail with a broadly rounded or shortly conical tail terminus separates *B. masseyi* 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, Zhang, Braasch & Burgermeister, 2005 or with a mucro in *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000, *B. macromucronatus* Gu, Zheng, Braasch & Burgermeister, 2008, *B. firmae* Kanzaki, Maehara, Aikawa & Matsumoto, 2012, and *B. baujardi* Walia, Negi, Bajaj & Kalia, 2003. *Bursaphelenchus masseyi* sp. n. also differs from the latter species by the presence *vs* absence of swellings at the stylet base (Walia *et al.*, 2003).

Molecular characterisation

The amplification of the ITS regions of *B. masseyi* sp. n. resulted in a PCR product of 985 bp. Restriction fragments produced by digestion of the PCR product with *Rsa*I, *Hae*III, *Msp*I, *Hin*fI and *Alu*I are listed in Table 2. Electrophoretic separation of restriction fragments showed a distinctive profile for the ITS-RFLP pattern (Fig. 3), which was different from profiles of all other *Bursaphelenchus* species obtained with the same PCR primers and the same set of restriction enzymes (see Gu *et al.*, 2008; Burgermeister *et al.*, 2009).

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. masseyi* sp. n. were deposited in GenBank with the accession numbers JQ287494 for 18S, ITS1, 5.8S, ITS2 and 28S and JQ287495 for the D2/D3 region of 28S rDNA.

Two molecular phylogenetic trees generated from partial 28S rDNA region with maximum likelihood (Fig. 4) and neighbour-joining algorithms (Fig. 5), show that *B. masseyi* sp. n. is a new species in the genus *Bursaphelenchus*. On the presented trees, *B. masseyi* sp. n. is positioned most closely to *B. trypophloei* and *B. tokyoensis* – two species located at the edge of the *xylophilus* group. and next to the *africanus* group (Kanzaki *et al.*, 2009; Tomalak & Filipiak, 2011).

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

PCR product (bp)	Restriction fragments (bp)				
	*Rsa*I	*Hae*III	*Msp*I	*Hinf*I	*Alu*I
985	483	400	340	445	447
	480	257	272	402	181
	22	216	219	114	172
		89			92
		19			79
		4	154	24	14

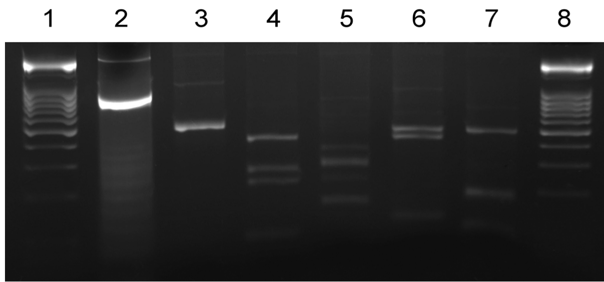

Fig. 3. ITS-RFLP profile of *Bursaphelenchus masseyi* sp. n. Lanes 1, 8 = DNA marker (100 bp ladder, MassRuler™, Fermentas); Lane 2 = rDNA amplification product; Lanes 3-7 = Restriction fragments: Lane 3 = *Rsa*I; Lane 4 = *Hae*III; Lane 5 = *Msp*I; Lane 6 = *Hinf*I; Lane 7 = *Alu*I.

Isolation and morphological examination of nematodes

Bursaphelenchus masseyi sp. n. was isolated from the bark on trunks of live or dying trembling aspen, *P. tremuloides*, previously infested with the bark beetle, *T. populi*. Infested trees were generally unhealthy, with some degree of crown loss, but beetle galleries with nematodes were surrounded by living bark. The nematode was found in stands affected by sudden aspen decline, but also in individual, unhealthy trees in otherwise healthy stands. The nematodes were very numerous in the bark and all developmental stages could be concurrently found in active larval galleries of the beetle. The nematode infective juveniles occupied the insect's haemocoel. In the examined material 2-42 (16.2 ± 11.53) infective juveniles could be extracted per insect. When subsequently reared *in vitro*, only a small proportion (3-5%) of infective juveniles originally dissected from the haemocoel recovered on *C. chrysosperma* cultures and then developed into adult *B. masseyi* sp. n. No further development of the infective juveniles was observed on *B. cinerea* cultures. The nematodes were found in bark samples from six out of ten aspen trees examined in June and July of 2010, from four out of six trees examined in August 2011, and from two out of four trees examined in November 2012. All sampled trees were located in forest stands of the Gunnison National Forest in south-western Colorado, USA.

The studied population of *B. masseyi* sp. n. developed and reproduced poorly on *B. cinerea* cultures on PDA. Only a small proportion of the introduced nematodes (less than 8%) could be found after 14-day incubation at 20-25°C, with a very few juveniles of the new generation present in the culture. The cultures on *B. cinerea* declined quickly and did not recover after a transfer to fresh rearing plates. In contrast, they developed and reproduced vigorously on cultures of *C. chrysosperma* with equally good development on the fungus strains originally isolated from larval galleries of *T. populi* in trembling aspen, *P. tremuloides*, and from galleries of *T. asperatus* in European aspen, *P. tremula*. The whole mature cultures, or nematodes extracted to water, could be stored for several weeks at 1-2°C. In such storage conditions, juveniles survived much better than adults.

Diallelic cross-breeding of *B. masseyi* sp. n. and *B. trypophloei in vitro*

Diallelic cross-breeding of *B. masseyi* sp. n. with *B. trypophloei* (PL-08) conducted *in vitro* on *C. chrysosperma*/PDA plates did not produce any offspring. In control plates with pairs of nematode individuals from *B. masseyi* sp. n. or *B. trypophloei*, intra-specific cross-breeding produced new generation offspring in 50 and 42% of dishes, respectively, after 14 days of incubation at 25°C.

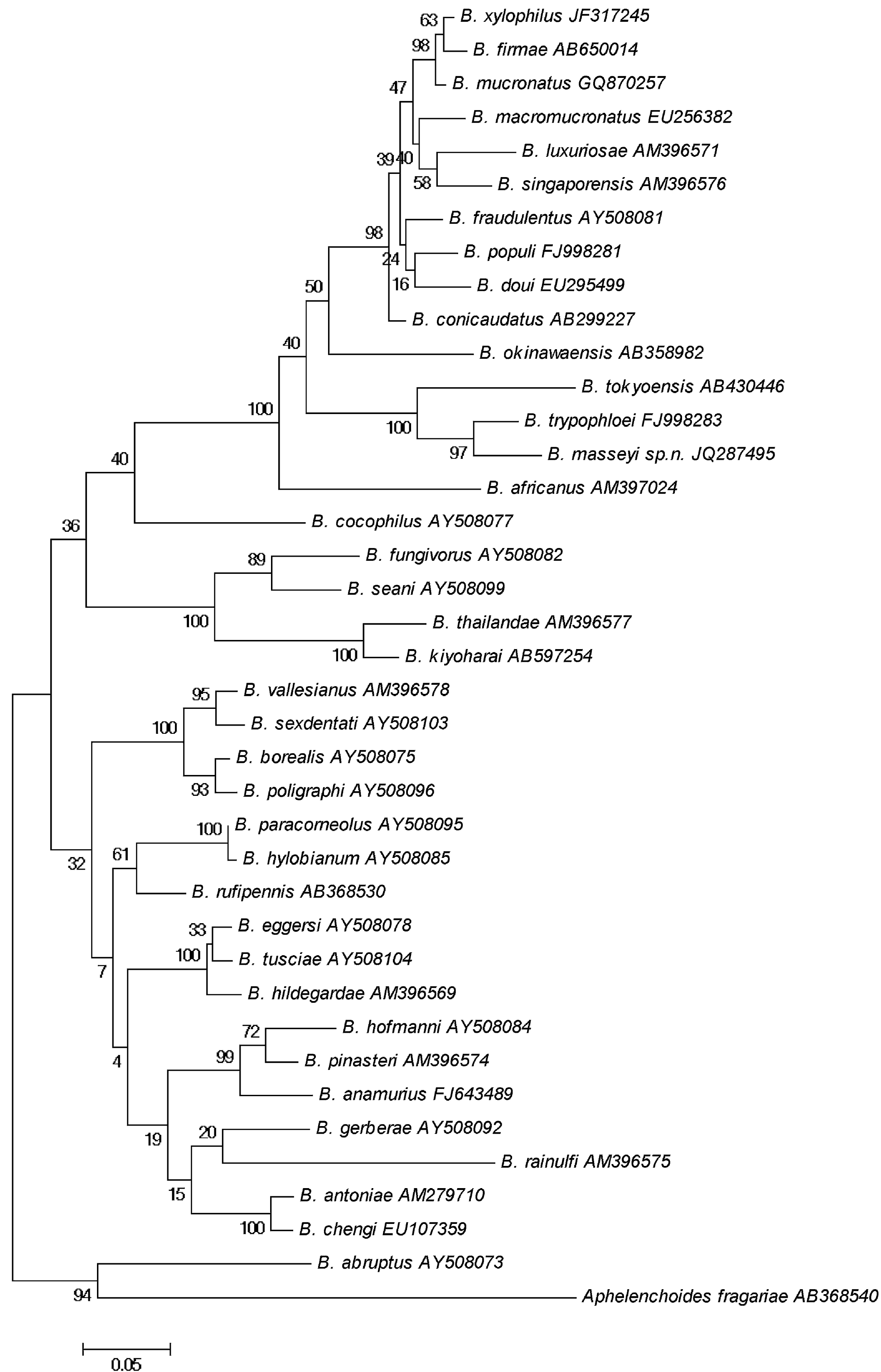

Fig. 4. Maximum likelihood (ML) analysis of *Bursaphelenchus masseyi* sp. n. and 37 other *Bursaphelenchus* species based on partial 28S rDNA. *Aphelenchoides fragariae* served as outgroup species. Phylogenetic trees were generated with 1000 bootstrap replications.

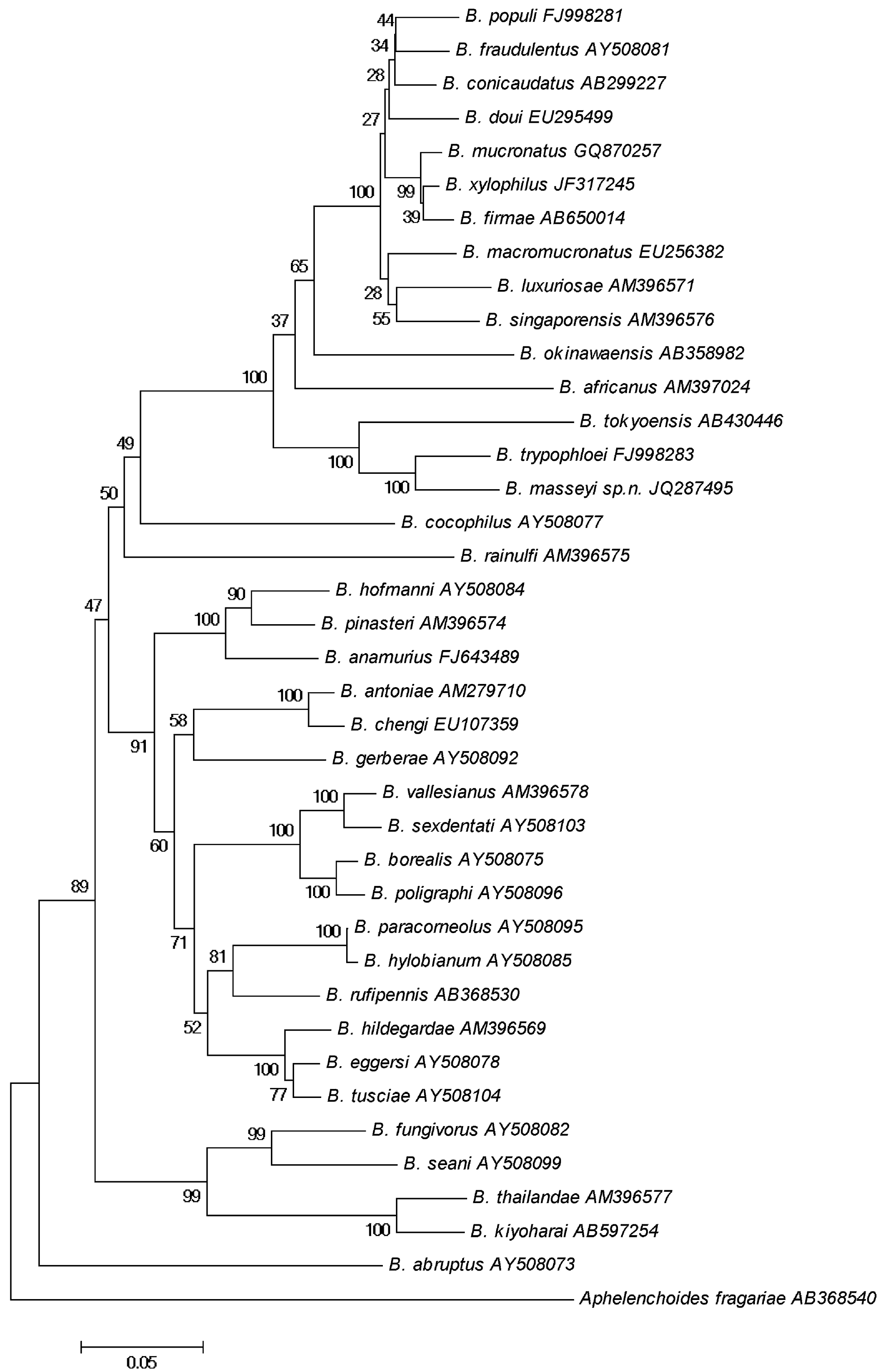

Fig. 5. Neighbour-joining (NJ) analysis of *Bursaphelenchus masseyi* sp. n. and 37 other *Bursaphelenchus* species based on partial 28S rDNA. *Aphelenchoides fragariae* served as outgroup species. Phylogenetic trees were generated with 1000 bootstrap replications.

Identification of natural vector(s) of *B. masseyi* sp. n.

Dissection of all larval developmental stages of *T. populi* revealed that infective juveniles of *B. masseyi* started to penetrate into the haemocoel of second instar larvae and continued into the third instar. The nematodes remained in pupae and adult beetles until the latter invaded new host trees in the following season. The infective juveniles emerged from the beetles during egg laying by the female and the excavation of larval galleries. No apparent negative effects of the nematode infective juveniles on overall vigour and internal organs of *T. populi* were observed during dissection of adult beetles and larvae or by examination of early larval galleries in the bark. The dauer juveniles of *B. masseyi* sp. n. extracted from the insect haemocoel only rarely (3-5%) initiated further development into adult nematodes *in vitro* on *C. chrysosperma* cultures.

Discussion

Bursaphelenchus masseyi sp. n. has striking similarities in its bionomics and morphology with *B. trypophloei* – a species recently described from Poland (Tomalak & Filipiak, 2011). Although the two species were isolated from geographically distant locations (*i.e.*, Colorado, USA and Poland, respectively), they are associated with closely related species of host-tree (*i.e.*, trembling aspen, *P. tremuloides* and European aspen, *P. tremula*, respectively), are transmitted by closely related vector-insects (*i.e.*, bark beetles, *T. populi* and *T. asperatus*, respectively), and their infective juveniles present a unique behaviour among *Bursaphelenchus* species in invading the haemocoel of the vector's larvae and remaining there until the beetles mature and start gallery construction in new host trees. However, the two species can be readily distinguished from one another by specific morphological characters, selected morphometric characters (pharynx length, position of nerve ring in females), and molecular characteristics of their DNA (ITS-RFLP, sequencing). The cross-breeding study revealed that the species are also reproductively isolated.

Although in our earlier study the phylogenetic analysis revealed that *B. trypophloei* was located at the edge of the *xylophilus* group and in the vicinity of *B. tokyoensis* and the *africanus* group, the presence of such distinctive morphological characters as the large size and shape of the spicules with a distinct cucullus, long vulval flap, lateral fields with four incisures, and the number and position of the male caudal papillae, clearly indicated that the nematode was more closely related to the *xylophilus* group than the *africanus* group (Tomalak & Filipiak, 2011). In the present study the same is true for *B. masseyi* sp. n. Molecular analysis of the DNA shows a close similarity with *B. trypophloei* and, together with this species, *B. masseyi* sp. n. is located at the edge of the *xylophilus* group, near *B. tokyoensis* and *B. africanus*. In fact, the obtained dendrograms suggest the formation of a distinct sub-clade consisting of *B. tokyoensis*, *B. trypophloei* and *B. masseyi* sp. n., the phylogenetically most closely related species. However, the morphological characters indicate that *B. masseyi* sp. n. is more similar to the *xylophilus* group than to *B. tokyoensis*. Further field research and identification of further new species may clarify whether *B. masseyi* sp. n. and *B. trypophloei* should remain in the *xylophilus* group, or whether they should be treated as a separate group, as the association with bark beetles as vectors and the bionomics with the juveniles penetrating the haemocoel of the vector's second instar larvae make these nematodes unique among species of the *xylophilus* group.

In an earlier study on nematode parasites and associates of bark beetles in Manitoba, Canada, a similar association between the bark beetle *T. populi* and a *Bursaphelenchus* sp. was found (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. Since at that time molecular methods for nematode identification were not available, the isolate was, based exclusively on morphological characters, determined as *B. fraudulentus* Rühm, 1956. However, in the light of our present experience on the range of morphological and host plant variation of *B. fraudulentus*, the correctness of the species identification in the Canadian samples of aspen is questionable (Tomalak & Filipiak, 2011). The bionomics of the Canadian *Bursaphelenchus* sp., which is unique among species of this group and identical with the presently described *B. masseyi* sp. n., suggests that the Canadian population of *Bursaphelenchus* sp. associated with *T. populi* in *P. tremuloides* was probably *B. masseyi* sp. n. Since the reference material from the Canadian study was not successfully stored, and as the authors were unable to obtain aspen bark or nematode samples from the original isolation sites in Manitoba, the true taxonomic identity of the Canadian population in question remains uncertain, despite their evident similarity to the present species.

Bursaphelenchus masseyi sp. n. and *B. trypophloei* can feed and reproduce well on mycelium of *C. chrysosperma*, a fungus frequently present in larval galleries of both species of the vector-beetles and apparently transmitted by these insects between trees (Tomalak & Filipiak, 2011). In the present research, each species reproduced equally well on isolates of *C. chrysosperma* originating from bark samples of *P. tremuloides* from Colorado, and *P. tremula* from Poland. Interestingly, their development and reproduction on *B. cinerea*, a standard laboratory fungus successfully used in rearing many *Bursaphelenchus* species, proved not to be effective.

Many species of *Cytospora* Ehrenb, 1881, are important causal agents of tree cankers worldwide (Davison & Tay, 1983; Adams *et al.*, 2006; Worrall *et al.*, 2010). *Cytospora chrysosperma* is frequently found pathogenic to poplars and willows (Biggs *et al.*, 1983; Kepley & Jacobi, 2000). In Colorado, this species also contributes to sudden aspen decline (SAD) which results in massive loses of trembling aspen (Marchetti *et al.*, 2011). Our earlier study on the association of *B. trypophloei* with *C. chrysosperma* revealed that the nematode could transmit the fungus spores *in vitro* (Tomalak & Filipiak, 2011). A similar *in vitro* transmission on PDA plates has been found in our research on *B. masseyi* sp. n. Although none of the presently studied species is apparently pathogenic to trees by itself, this phenomenon might be significant in the epidemiology of Cytospora canker. It seems probable that the nematode's ability to transmit *C. chrysosperma* may contribute to the disease by facilitating within-tree dispersal. This hypothesis, however, needs to be substantiated with laboratory and field experiments conducted *in vivo* in aspen trees. Since, under natural conditions, the nematode is always associated with the bark beetle as a vector and the fungus as a food source, it is difficult precisely to explain the roles played by each of these organisms in relation to the host tree. A series of experiments on artificial inoculation of aspen seedlings with the fungus alone, with surface-sterilised individuals of *B. masseyi* sp. n., and with non-sterilised nematodes extracted from *C. chrysosperma* cultures should distinguish the potential direct damage to the plant tissue caused by fungus or nematode, as well as help better to understand the role played by the nematode in dispersion of *C. chrysosperma* within the tree.

The morphological, molecular and cross-breeding studies reported here have provided data on two intriguing, very closely related, nematode species, species that occupy distant geographic regions yet share most of their biological characteristics. These species could be interesting models for more detailed zoogeographical and evolutionary analyses. Their relatedness is expressed not only by their close morphological and molecular similarities, but also by the similarity of their natural association with the plant pathogenic fungus *C. chrysosperma*, the host plant species, the insect vector species, and the niche occupied in the vector's body during transmission. This similarity seem to be an effect of divergent evolution from a common ancestor, but whether the subsequent species separation should be attributed to the separation of the host plant, insect vector or the nematode itself, remains to be elucidated.

With the addition of *B. masseyi* sp. n., the number of nominal species assigned to the *xylophilus* group (*sensu* Braasch, 2008) is now 14, most of which originate from Asia and Europe. Until now, only *B. xylophilus* was originally described from North America where it is relatively common. Two other species in the *xylophilus* group, *i.e. B. mucronatus* and *B. fraudulentus*, have occasionally been reported from Canada (Bolla & Boschert, 1993; Harmey & Harmey, 1993) and the USA (after Ryss *et al.*, 2005), respectively, but it is not clear if they are native to these regions. *Bursaphelenchus masseyi* sp. n. expands the *xylophilus* group reported from North America to four species. Repeated isolation of this nematode over three consecutive years suggests that its population is well established in the Rocky Mountain region. As the host tree (*P. tremuloides*) is present across Canada, as well as in central and western USA (Little, 1971), and the bark beetle (*T. populi*) is distributed transcontinentally in Canada, and in Arizona, Colorado, Nevada and Utah in the USA (Bright, 1976), it is probable that the nematode is also widely distributed in North America. This suggestion seems to be supported by our earlier study conducted in Manitoba, Canada (Tomalak, 1984). Further research on *T. populi*-infested aspen across the continent should provide more precise information on distribution and potential association of *B. masseyi* sp. n. with sudden aspen decline.

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