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Bursaphelenchus gillanii sp. n. (Nematoda: Aphelenchoididae) – a new species of the *xylophilus* group in packaging wood imported from China

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Summary – A new *Bursaphelenchus* species of the *xylophilus* group was detected in coniferous packaging wood imported with goods from China in 2011. The new species is described herein and compared with other species of the *xylophilus* group. *Bursaphelenchus gillanii* sp. n. has a slim body (a = 31 (28-34) and 33 (29-36) in females and males, respectively), c′ = 3.8 (3.2-4.5) and 2.1 (1.7-2.5) in females and males, respectively, a large vulval flap, a 5-7 μm long digitate mucro as a continuation of the female tail, excretory pore at or closely posterior to the median bulb, strongly arcuate spicules, 34 (31-37) μm long as measured along the median line, with prominent pointed rostrum and small cucullus. The ITS-RFLP pattern of the new species obtained by digestion of the PCR product with *Rsa*I, *Hae*III, *Msp*I, *Hin*fI and *Alu*I is different from other known *Bursaphelenchus* species. Results of sequencing the ITS1/2 region demonstrate the close relationship of the new species to *B. mucronatus* and *B. xylophilus*.

Keywords – distribution, ITS-RFLP, molecular, morphology, morphometrics, new species, pine, taxonomy, *xylophilus* group.

Wooden packaging imported with diverse consignments is being investigated in international trade for the presence of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, in the EU Member States, in order to prevent the introduction and spread of this dangerous quarantine pest. During nematological inspection of imported packaging wood by the plant protection service in the State Office for Rural Development, Agriculture and Land Reallocation, Germany, an undescribed *Bursaphelenchus* species was detected in a pallet made of pine wood and imported from China *via* the Chinese port of Dachan Bay. The wood also contained bore holes of longhorn beetles. The morphological characters (aphelenchoid bulb, vulval flap, presence of a small terminal bursa in males, typical shape of spicules), clearly show that the nematode found in the wood belonged to the *xylophilus* group *sensu* Braasch *et al.* (2009). It was also most similar to *B. mucronatus mucronatus* in having a distinct mucro on the female tail end and in female tail shape. However, the ITS-RFLP pattern of this species shows differences compared with *B. mucronatus* as well as with other species of the *xylophilus* group, including *B. xylophilus*. The new species is described herein as *B. gillanii* sp. n. on the basis of morphological characters, ITS-RFLP patterns and sequencing results and compared with related species of the *xylophilus* group.

Materials and methods

NEMATODE CULTURING AND MORPHOLOGICAL OBSERVATIONS

Sawn samples taken from packaging wood were cut with a laboratory wood mill (RETSCH) into small pieces *ca* 1 cm long. The nematodes were extracted using a modified Baermann funnel technique for 24 h. Besides a few juveniles, one male and one female were obtained from 600 g of wood. Measurements were made after culturing the nematodes on *Botryotinia fuckeliana* on malt agar, fixation in TAF and processing to glycerin following the methods of Seinhorst (1959). Measurements

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of individuals directly extracted from wood are not available. Morphological observations were performed using a Leica DMLB light microscope. Light micrographs were taken using a Leica DFC 320 camera with camera software LAS 3.2.

Molecular analyses

For extraction of DNA, 5-10 cultured nematodes frozen in 10 μl Bidest were homogenised in a beadbeater (RETSCH). DNA was extracted using the QIAamp DNA Micro Kit following the manufacturer's instructions. For ITS-RFLP profiles, suitable aliquots of the amplified ITS rDNA were digested for at least 3 h at 37°C using 10 U of each of the five restriction endonucleases (*Rsa*I, *Hae*III, *Msp*I, *Hin*fI, *Alu*I) (Hoyer *et al.*, 1998; Burgermeister *et al.*, 2005). Fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide. For ITS sequencing, the PCR product of the primers F194 (Ferris *et al.*, 1993) and 5368R (Vrain, 1993) was concentrated to 40 ng μl^{-1} using MSB Spin PCRapace (Stratec Molecular) and sent to LGC Genomics for sequencing (Flexi Run).

The ITS1/2 sequences were analysed and aligned using the program ClustalW implemented in MEGA version 4.0 (Tamura *et al.*, 2007). Phylogenetic trees were generated with the Neighbour Joining (NJ) and UPGMA method using the Tajima-Nei distance option by MEGA software. Bootstrapping analysis was performed with 1000 replicates.

Results

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

Measurements

See Table 1.

Description

Female

Body slim (a = 31), slightly ventrally arcuate when heat-relaxed, cuticle marked by fine transverse striations, lateral field with four incisures, *ca* 3 μm wide. Lip region convex, offset by a constriction, *ca* 4 μm high and 8 μm broad. Stylet well developed, 15 μm long on average, with very small basal swellings, conus forming *ca* one-third of total stylet length. Procorpus cylindrical. Median bulb rounded with well-developed central valve plates. Pharyngeal glands overlapping intestine dorsally for 2-3 body diam. Nerve ring located just posterior to median bulb. Excretory pore situated at level of median bulb or up to 0.5 body diam. posterior to bulb. Reproductive system prodelphic. Gonad straight, occupying *ca* half of body length. Developing oocytes arranged in two rows. Spermatheca elongated ovoid. Vagina usually perpendicular to body axis. Anterior vulval lip prolonged into straight and prominent vulval flap *ca* 12-15 μm long. Post-uterine branch extending two-thirds to four-fifths of vulva-anus distance. Tail conoid, 3-4 anal body diam. long, more strongly curved dorsally than ventrally. Tail with pointed digitate mucro, mucro wide at base, appearing as a continuation of tail, 5-7 μm long, resembling beak of a bird.

Male

Anterior body region and cuticle similar to female. Tail strongly curved ventrally when heat-killed. Testis expanded anteriorly, occupying almost half or less of body length. Spermatocytes arranged in two rows. Spicules paired, strongly arcuate, 25-30 μm long measured as straight line from condylus tip to distal end (31-37 μm along arc or median line), contour of dorsal lamina angular in posterior fourth of its length, capitulum *ca* 9 μm long, almost parallel to spicule shaft axis, slightly concave with small, rounded and remote condylus, elongated more or less pointed rostrum, and distinct disc-like cucullus almost 2 μm wide. A straight line can be drawn between cucullus, tip of rostrum and condylus. Three pairs of papillae and one single papilla present. Single mid-ventral papilla located anterior to cloacal aperture, P2 ventro-lateral pair located slightly anterior to cloacal aperture, postcloacal pairs P3 and P4 adjacent and at commencement of terminal oval bursal flap.

Type habitat and locality

Packaging wood (pine) imported from China *via* the Chinese port of Dachan Bay and the German entry point port of Hamburg and inspected in Hoppegarten on 22 June, 2011.

[*] The new species is named after the great Persian religious teacher and Sufi saint of the 12th century, Abdul Qadir Gillani, and dedicated to the revered physician of Eastern Medicine, Prof. Dr Said Ahmed Gill.

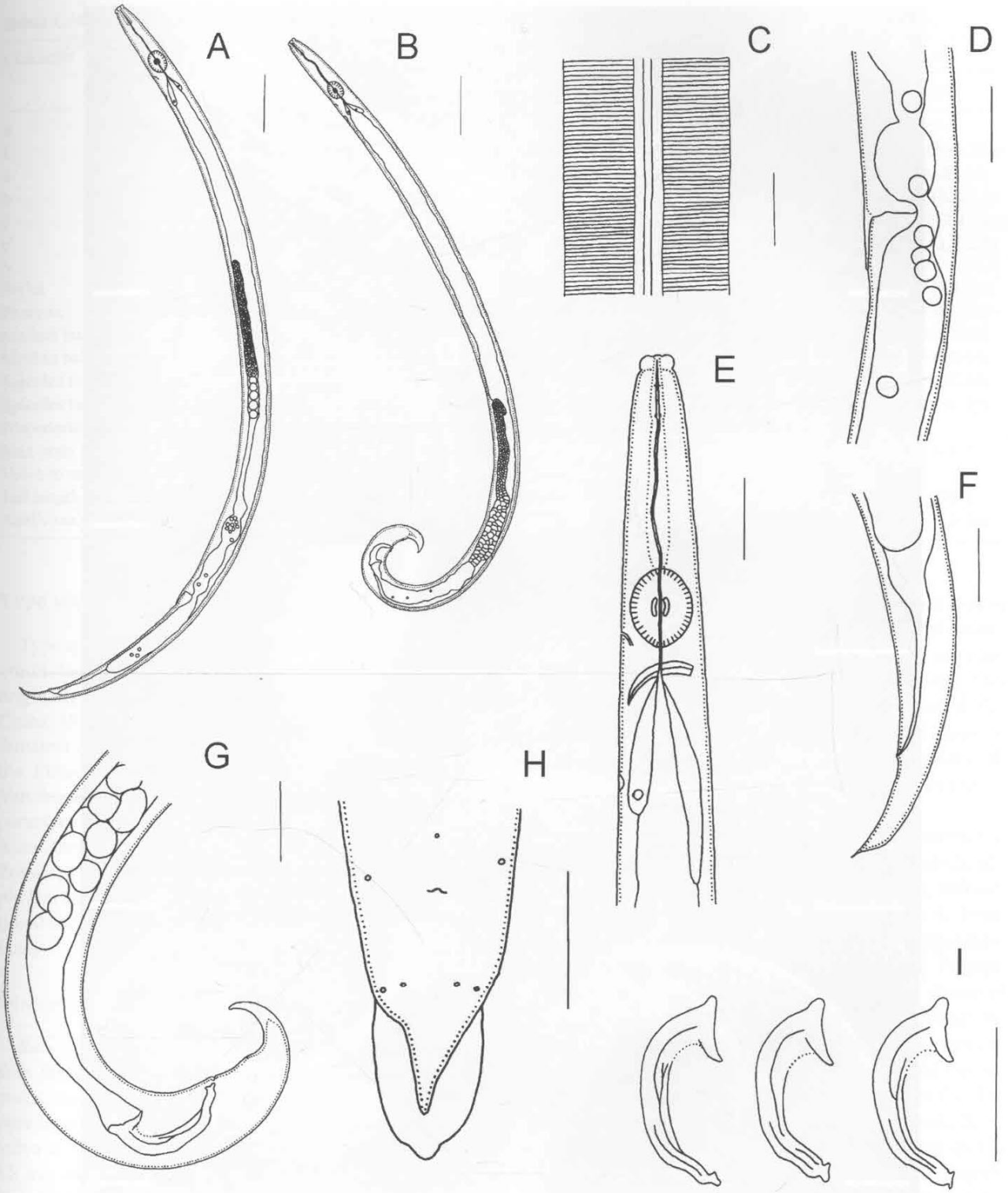

Fig. 1. *Bursaphelenchus gillanii* sp. n. A: Female; B: Male; C: Lateral field; D: Vulval region; E: Female anterior end; F: Female tail; G: Male tail, ventral view; H: Male tail, dorsal view; I: Spicules. (Scale bars: A, B = 50 μm; C-I = 20 μm.)

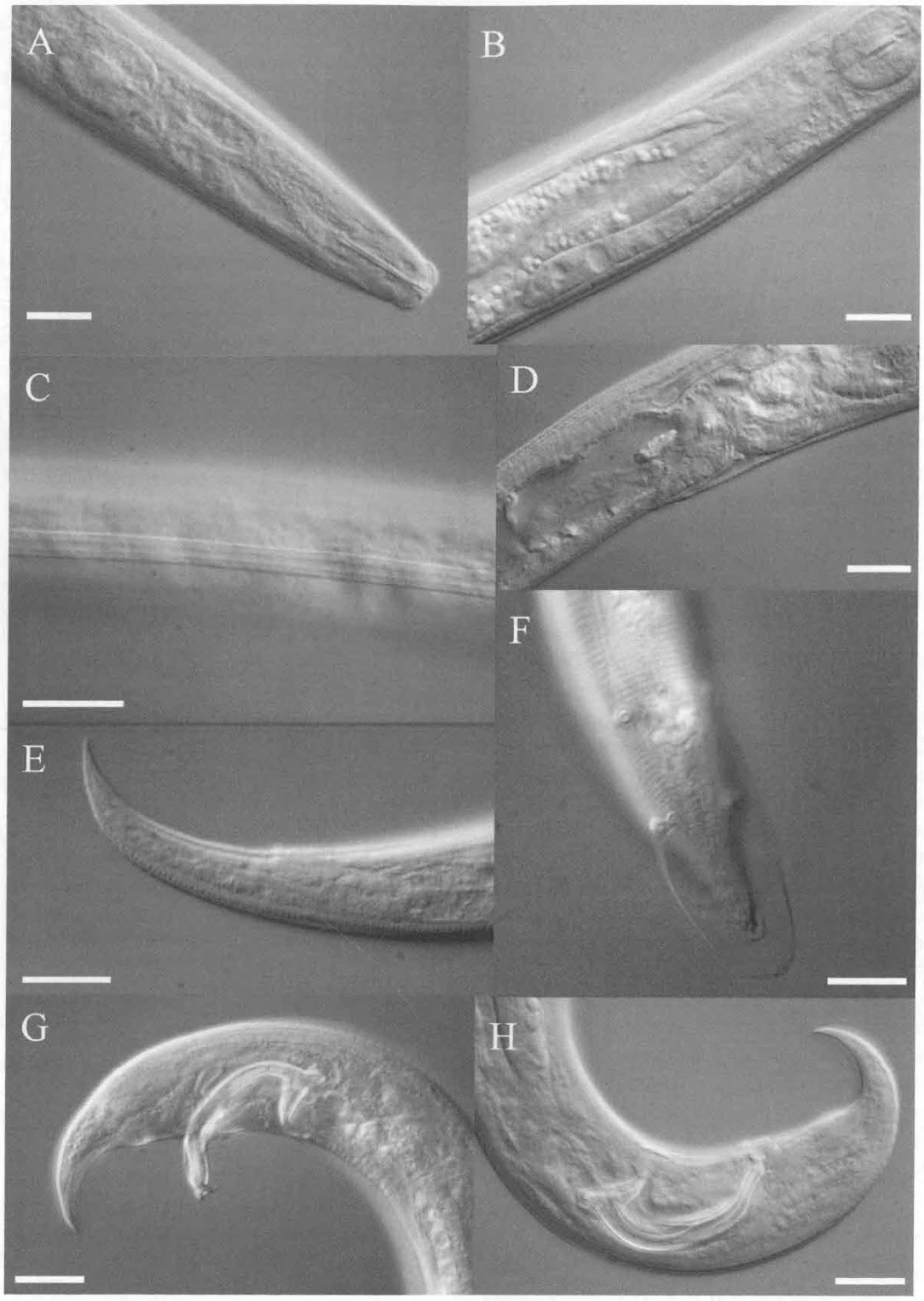

Fig. 2. Light micrographs of *Bursaphelenchus gillanii* sp. n. Female, A-E. A: Anterior end; B: Pharynx; C: Lateral field; D: Vulva; E: Tail. Male, F-H. F: Tail papillae; G, H: Spicules. (Scale bars = 10 μm.)

Table 1. Morphometrics of *Bursaphelenchus gillanii* sp. n. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Female		Male
	Holotype	Paratypes	Paratypes
n	–	15	15
L	808	797 ± 78 (607-890)	698 ± 53 (596-794)
a	28	30.6 ± 1.8 (28-34)	33 ± 1.9 (29-36)
b	9.6	10.0 ± 1.0 (8.1-12.3)	8.7 ± 0.5 (7.8-9.7)
c	25.3	23.3 ± 2.2 (19.6-27.8)	20.0 ± 1.8 (17.4-24.0)
c′	3.6	3.8 ± 0.4 (3.2-4.5)	2.1 ± 0.2 (1.7-2.5)
V	75	75 ± 0.9 (73-77)	–
Stylet	14	15 ± 0.7 (14-16)	15 ± 0.7 (13-16)
Pharynx	84	80 ± 4.3 (71-87)	80 ± 4.4 (71-87)
Median bulb length	18	17 ± 1.1 (15-19)	16 ± 1.0 (15-18)
Median bulb diam.	15	14 ± 1.0 (12-15)	14 ± 0.8 (12-15)
Spicules (chord)	–	–	27 ± 1.4 (25-30)
Spicules (arc)	–	–	34 ± 1.8 (31-37)
Post-uterine sac	100	108 ± 13 (82-133)	–
Max body diam.	29	26 ± 3.2 (22-31)	21 ± 1.6 (19-24)
Vulva to anus distance	166	164 ± 15 (131-185)	–
Tail length	32	34 ± 2.2 (30-38)	35 ± 2.9 (32-41)
Anal/cloacal body diam.	9	9.0 ± 0.9 (8-11)	17 ± 1.2 (15-19)

Type material

Type specimens were collected from a culture on *Botryotinia fuckeliana* growing on malt agar, the nematodes originally being isolated from pine wood imported from China. Holotype female and paratypes (15 males and 15 females) deposited in the German nematode collection at the Julius Kühn Institute, Institute for Nematology and Vertebrate Research, Münster, Germany. An additional 20 paratypes of each sex are deposited in the State Office for Rural Development, Agriculture and Land Reallocation, Zossen, Germany. A culture is maintained at the *Bursaphelenchus* culture collection of the JKI-Institute for National and International Plant Health, Braunschweig, Germany.

Diagnosis and relationships

Bursaphelenchus gillanii sp. n. is characterised by a slim body (a = 31 and 33 on average for females and males, respectively), lateral field with four lines, excretory pore located at level of median bulb or slightly posterior, vulva at 75% of total body length, distinct vulval flap 12-15 μm long visible in lateral view, post-uterine branch two-thirds to four-fifths of vulva-anus distance, female tail 3-4 anal body diam. long, conoid and with a distinct, broad-based mucro, spicules large (25-30 μm long in a line between tip of condylus and distal end) and arcuate with distinct cucullus and rostrum, three pairs of ventrosublateral papillae (one pair slightly precloacal, two pairs postcloacal) and a single precloacal papilla present. Due to the presence of four lateral lines, a large vulval flap in the female, size of the post-uterine branch, shape of spicules and number and position of the male papillae, *B. gillanii* sp. n. clearly belongs to the *xylophilus* group *sensu* Braasch *et al.* (2009).

Among the 13 species of the *xylophilus* group known so far, *B. gillanii* sp. n. can be distinguished morphologically from *B. xylophilus*, *B. singaporensis* Gu, Zhang, Braasch & Burgermeister, 2005, *B. luxuriosae* Kanzaki & Futai, 2003, *B. paraluxuriosae* Gu, Wang, Braasch, Burgermeister & Schröder, 2012 and *B. populi* Tomalak & Filipiak, 2010 by the presence *vs* absence of a distinct mucro of 5-7 μm length on the female tail terminus, although the round-tailed species *B. xylophilus* and *B. populi* may occasionally have a small projection on the female tail terminus (Tomalak & Filipiak, 2010; Gu *et al.*, 2011). The same applies to *B. trypophloei* Tomalak & Filipiak, 2011, a species closely related to the *xylophilus* group and in which the tail shape varies from subcylindrical to slightly conoid with a rounded terminus to a more conical form with a distinct short, 1-2 μm projection in some individuals (Tomalak & Filipiak, 2011). The new species can

also be distinguished from *B. xylophilus* by the more robust body (a = 31 (28-34) or 33 (29-36) in females and males, respectively *vs* >40 (35-64) in both sexes), tail shape (conoid *vs* cylindrical with broadly rounded terminus) and excretory pore position (at, or just posterior, to median bulb *vs* much posterior); from *B. paraluxuriosae* by the conoid mucronate female tail *vs* conical tail with irregular or roughened terminus (Gu *et al.*, 2012); from *B. singaporensis* by the shorter spicule length of 27 (25-30) *vs* 45 (41-48) μm (Gu *et al.*, 2005) and from *B. populi* mainly by the vulval flap shape (almost straight *vs* bent) (Tomalak & Filipiak, 2010).

The new species is also morphologically similar to *B. fraudulentus* Rühm, 1956, *B. mucronatus* Mamiya & Enda, 1979, *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000, *B. baujardi* Walia, Negi, Bajaj & Kalia, 2003, *B. doui* Braasch, Gu, Burgermeister & Zhang, 2004, *B. macromucronatus* Gu, Zheng, Braasch & Burgermeister, 2008 and *B. firmae* Kanzaki, Maehara, Aikawa & Matsumoto, 2012 in having a mucro on the female tail terminus. *Bursaphelenchus gillanii* sp. n. is most similar to *B. mucronatus mucronatus* in terms of female tail shape and length of the pointed mucro and morphological differentiation from this subspecies is difficult. The new species is best distinguished from *B. m. mucronatus* by the less slim body (a = 31 (28-34) or 33 (29-36) *vs* 41 (36-46) or 44 (39-51), in females and males, respectively), and spicule shape (condylus offset *vs* almost continuous with the dorsal spicule line in most specimens) (Braasch *et al.*, 2011). The excretory pore position may also be helpful (usually distinctly posterior to bulb in *B. m. mucronatus*). The new species can be distinguished from *B. m. kolymensis* by female tail shape (conoid *vs* subcylindrical) and shape and length of the mucro (5-7 *vs* usually <5 μm) (Braasch *et al.*, 2011).

The new species can be distinguished from *B. fraudulentus* by female tail shape (conoid *vs* subcylindrical), the longer mucro (5-7 *vs* 2 μm), higher female c′ ratio (3.8 (3.2-4.5) *vs* 2.8 – see Kanzaki *et al.*, 2000) and position of the spicule capitulum, which is not parallel to the shaft axis in *B. fraudulentus*. The new species can be distinguished from *B. conicaudatus* by the subcylindrical *vs* conical female tail and a longer mucro (see drawing in Kanzaki *et al.*, 2000); from *B. baujardi* by presence *vs* absence of swellings at the stylet base and absence *vs* presence of prominent vulva sclerotisation (Walia *et al.*, 2003); from *B. doui* mainly by the mucro length (5-7 *vs* 2-4 μm); from *B. macromucronatus* by the digitate *vs* more hair-like mucro which appears as a continuation of the tail (Gu *et al.*, 2008); and from *B. firmae* by a somewhat longer mucro (5-7 *vs* 3-5 μm) and longer spicules (34 (31-37) *vs* 22 (21-24) μm, as measured along the median line) (Kanzaki *et al.*, 2012).

Molecular profiles and phylogenetic status

Amplification of ITS1/2 region of the new species resulted in a PCR product of 950 bp (almost the same size as for related species of the *xylophilus* group). The differentiation of the new species from other species of the *xylophilus* group is possible on the basis of restriction fragments (Fig. 3; Table 2) obtained by digestion of the PCR product with *Rsa*I, *Hae*III, *Msp*I, *Hin*fI and *Alu*I (Burgermeister *et al.*, 2009). Table 2 shows the approximate sizes of restriction fragments for the new species compared with *B. xylophilus*, *B. mucronatus* and *B. firmae*. The complete sequence covering ITS1 and ITS2 is deposited in GenBank (Accession No. KC347020). The phylogenetic trees of the ITS1/2 region generated by NJ and UPGMA methods give similar results regarding the position of the new species within the *xylophilus* group.

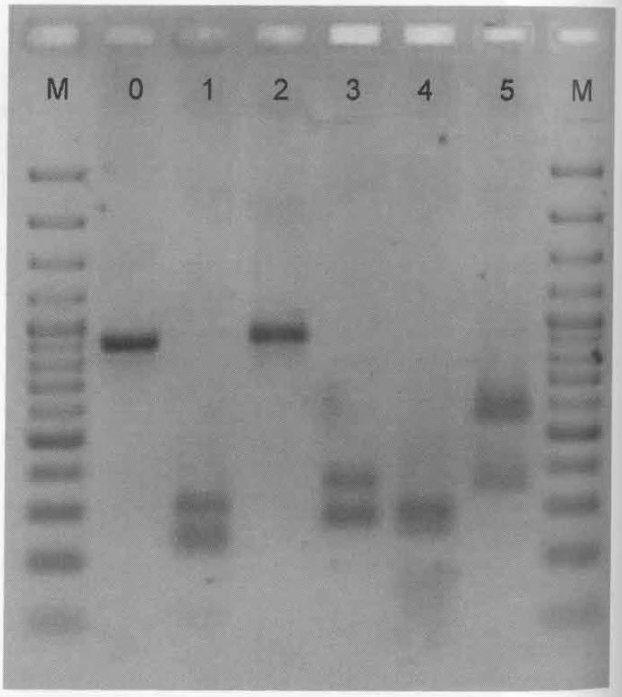

Fig. 3. ITS-RFLP patterns of *Bursaphelenchus gillanii* sp. n. and similar species of the *xylophilus* group. Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa*I (1), *Hae*III (2), *Msp*I (3), *Hin*fI (4) and *Alu*I (5). M = Gene Ruler 100 bp Plus DNA Ladder.

Table 2. Approximate size of DNA fragments observed in ITS-RFLP analysis of *Bursaphelenchus gillanii* sp. n. and related species.

Bursaphelenchus species	PCR product (bp)	Restriction fragments (bp)				
		*RSA*I	*HAE*III	*Msp*I	*Hin*fI	*Alu*I
B. gillanii sp. n.	950	300	950	330	280	540
		240		280	150	370
		100		?	110	
					?	
B. xylophilus	950	500	730	570	270	460
		420	200	380	260	250
					140	140
						100
B. macromucronatus[1, 2)]	967	525	468	361	301	967
		420	245	253	279	
		22	95	243	217	
			89	110	146	
			70		24	
B. mucronatus mucronatus	950	500	620	370	410	700
		410	310	310	250	250
				280	130	
					90	
B. mucronatus kolymensis	950	410	620	370	410	700
		290	220	310	250	250
		230	110	280	130	
					90	
B. firmae[1)]	915	470	347	549	412	791
		423	291	366	232	124
		22	277		216	
					50	

[1)] Virtual ITS-RFLP pattern after sequencing.
[2)] Restriction fragment sizes of *Msp*I and *Hin*fI: corrected version of the data of Gu *et al.* (2008).

They also show that the new species is closely related to *B. mucronatus*, *B. firmae* and *B. xylophilus*.

Discussion

Since the discussion of the *xylophilus* group by Braasch (2008), when there were nine nominal species, several additional species have been described. In 2008, *B. macromucronatus* was described as a member of the *xylophilus* group (Braasch *et al.*, 2009). The list of the known species has been supplemented by *B. populi*, *B. firmae*, *B. paraluxuriosae* and, very recently, by *B. koreanus* Gu, Wang & Cheng, 2013. Since 2000, most of the newly described species of the *xylophilus* group have their origin in East Asia, the exception being *B. populi* from Poland. The number of known species was reduced by the proposal of a new rank for *B. kolymensis* Korentchenko, 1980, when it was made a subspecies of *B. mucronatus* by Braasch *et al.* (2011).

Among the 13 species of the *xylophilus* group known to date, seven (both subspecies of *B. mucronatus*, *B. fraudulentus*, *B. conicaudatus*, *B. baujardi*, *B. doui*, *B. macromucronatus* and *B. firmae*) always have a mucro on the female tail as in the new species. Even the round-tailed females of *B. xylophilus* may sometimes have a small mucro when directly isolated from trees or in case of mucronate populations (Gu *et al.*, 2011). The separate species status of *B. gillanii* sp. n. was confirmed by morphological comparison, ITS-RFLP technique and sequencing results. The new species clustered closely to *B. xylophilus* and *B. mucronatus* in the phylogenetic trees (Figs 4, 5). Due to the economic significance of the pine wood nematode being a serious threat to coniferous forests in Europe, a clear distinction of all species of the *xylophilus* group is invaluable. Because

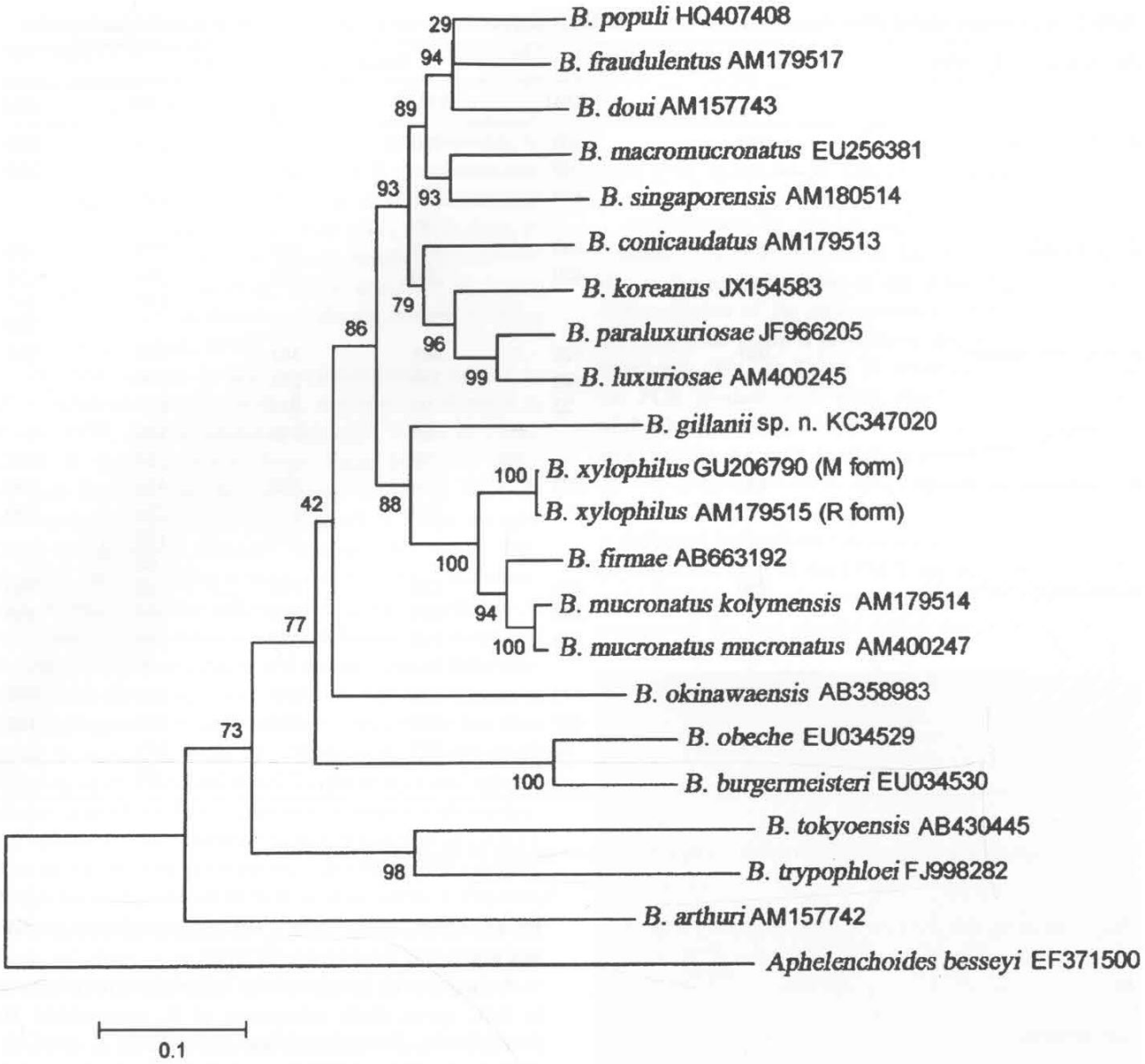

Fig. 4. Phylogenetic relationships of *Bursaphelenchus gillanii* sp. n. using NJ tree construction method of aligned sequences of ITS1/2. EMBL accession numbers are listed with the species names. Numbers at branching points are bootstrap values obtained using 1000 repetitions. (Scale bar = substitutions/site.)

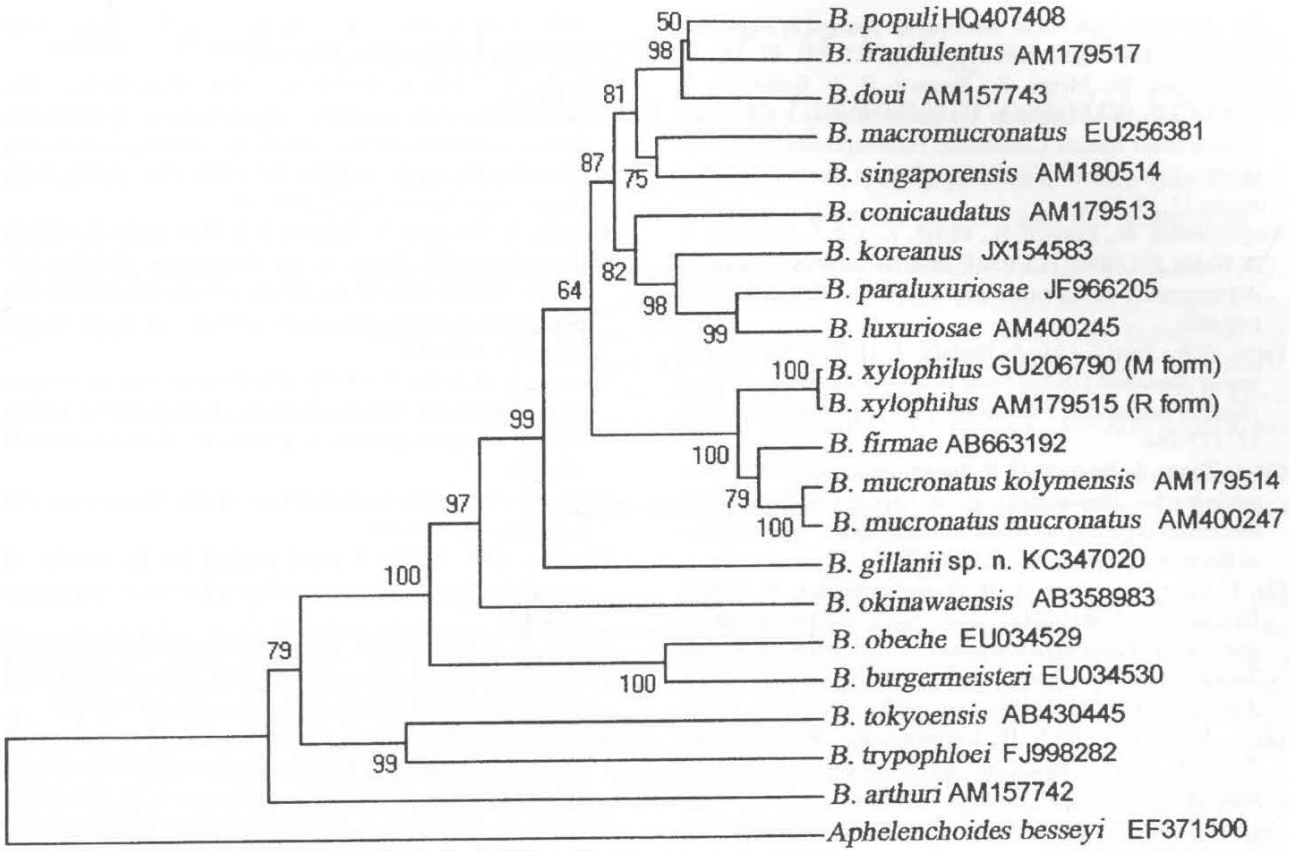

Fig. 5. Phylogenetic relationships of *Bursaphelenchus gillanii* sp. n. using UPGMA tree construction method of aligned sequences of ITS1/2. EMBL accession numbers are listed with the species names. Numbers at branching points are bootstrap values obtained using 1000 repetitions.

of their characteristic and unique morphology, they can be relatively easily distinguished from *Bursaphelenchus* species of other groups, but distinction of species within the *xylophilus* group is based on minute morphological details. Fortunately, molecular methods are now available to improve diagnostic processes and have drawn attention to several new species, including the one described herein. Ecology, including hosts and vectors of the new species, are unknown and further investigations on its distribution, life cycle and possible pathogenicity are advisable.

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