

Description of *Bursaphelenchus braaschae* sp. n. (Nematoda: Aphelenchoididae) found in dunnage from Thailand

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Summary. *Bursaphelenchus braaschae* sp. n. is described and figured. The new species was isolated from deciduous dunnage from Thailand. The new species belongs to the *fungivorus* group of the genus. It is characterised by relatively stout body ($a = 23.5$ and 24.0 for males and females, respectively); four lines in the lateral field; spicules relatively small and delicate (14.2 - 16.3 μm) with blunt rostrum and rounded distal end without cucullus, condylus thin and high, not dorsally bent, dorsal edge of spicules along condylus with characteristic darker section; and by females with strongly protruding vulva lips and a slim, attenuated tail with rounded terminus. The new species is morphologically closest to *B. willibaldi* and can be distinguished by the shape and size of spicules, post-uterine sac length and vulva position. The new species status is supported by ITS-RFLP patterns and molecular phylogenetic analysis based on partial SSU, ITS1/2 and partial LSU sequences, which revealed that *B. braaschae* sp. n. is closest to *B. willibaldi*.

Key words: *Bursaphelenchus braaschae* sp. n., DNA sequencing, morphology, molecular taxonomy, PCR-RFLP, SEM.

To prevent the spread of the pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 and other pests through packaging wood, almost all packaging wood imported through Ningbo Harbour, Zhejiang, China has been inspected and sampled since 1997 (Gu *et al.*, 2006).

In June 2009, big dunnage pieces made from deciduous wood without a phytosanitary mark were sampled and inspected during inspection of medium density fibreboard from Thailand. In addition to *Cryptaphelenchus* sp., *Ruehmaphelenchus* sp. and *B. fraudulentus*, a new species of *Bursaphelenchus* was detected. It is described and figured herein as *Bursaphelenchus braaschae* sp. n.

MATERIAL AND METHODS

Sawn samples taken from packaging wood were cut into small pieces no more than 1 cm wide. Nematodes were extracted by the modified Baermann funnel technique for 24 h and reared successfully on *Botryotinia fuckeliana*. Measurements were made on permanent slides fixed in TAF and processed to glycerol following the method of Seinhorst (1959). The light micrographs

were made using a Zeiss Imager Z1 microscope equipped with a Zeiss AxioCam MRm CCD camera. The SEM micrographs were taken with a scanning electron microscope Hitachi TM1000.

DNA samples of *Bursaphelenchus braaschae* sp. n. were prepared according to Li *et al.* (2008). Three sets of primers (synthesised by Invitrogen, Shanghai, China) were used in the PCR analyses to amplify the partial SSU region, the ITS1/2 region and the D2/D3 LSU region of rDNA, respectively. Primers for amplification of SSU were forward primer K4f (5'-ATG CAT GTC TAA GTG GAG TAT TA -3') and reverse primer K1r (5'- TTC ACC TAC GGC TAC CTT GTT ACG ACT -3') (Penas *et al.*, 2006). Primers for amplification of ITS1/2 were 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). Primers for amplification of D2/D3 LSU were forward primer D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and reverse primer D3Br (5'-TCG GAA GGA ACC AGC TAC TA-3') (De Ley *et al.*, 1999). PCR conditions were as described by Li *et al.* (2008). PCR products were separated on 1% agarose gels and visualised by staining with ethidium bromide.

PCR products of sufficiently high quality were purified for cloning and sequencing by Invitrogen, Shanghai, China.

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 (*RsaI*, *HaeIII*, *MspI*, *HinfI* and *AluI*) (Takara, Japan) following the manufacturer's instructions. Fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

Partial SSU, ITS1/2 and partial LSU sequences of many other *Bursaphelenchus* species were available as GenBank accessions which had been contributed by various research teams (Ye *et al.*, 2007). Sequences were analysed and aligned using the program Clustal W implemented in MEGA version 4.0 (Tamura *et al.*, 2007). Phylogenetic trees were generated with the Neighbor Joining (NJ) method using the Tajima-Nei distance option. Bootstrapping analysis was performed with 1000 replicates.

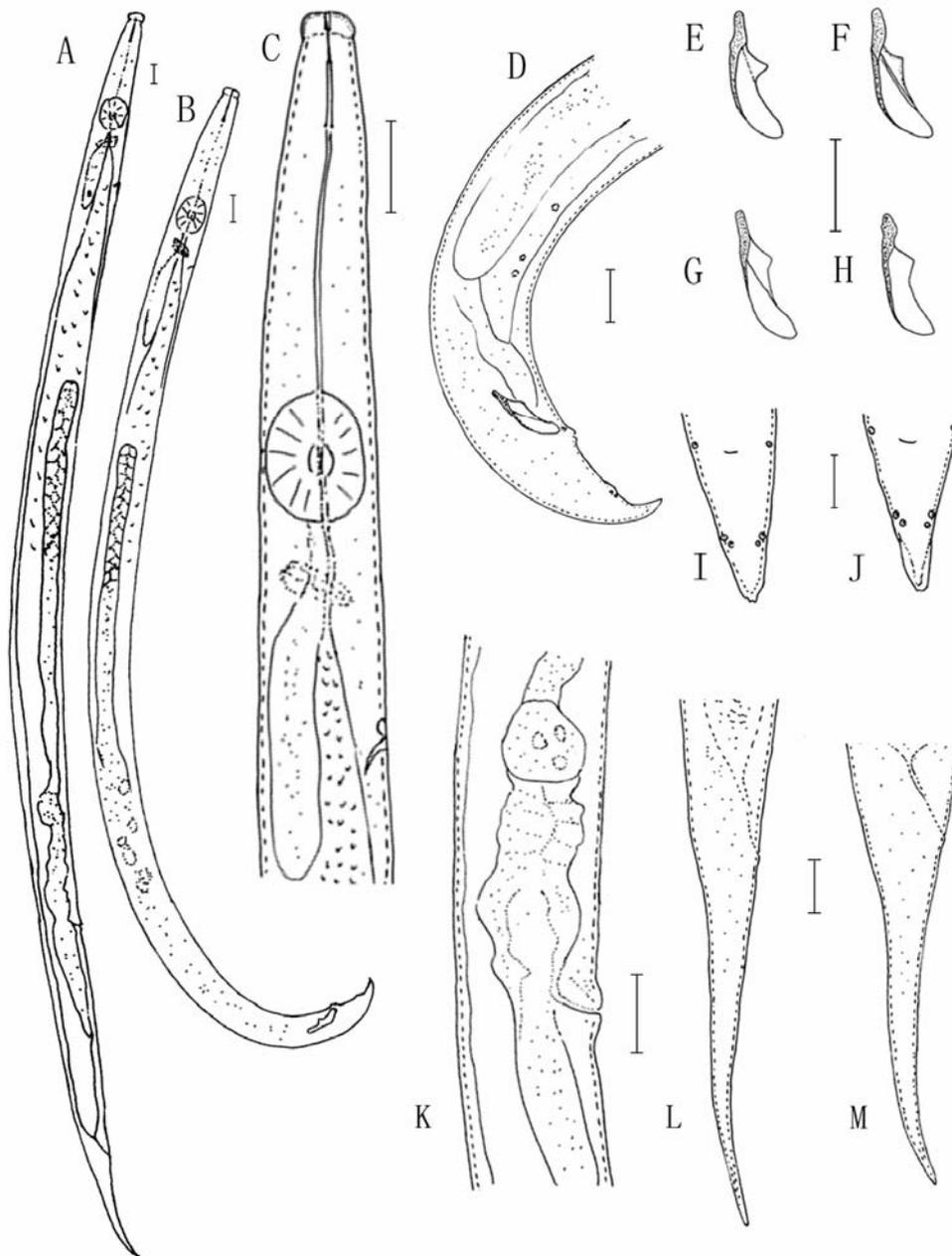


Fig. 1. *Bursaphelenchus braaschae* sp. n. A: Female; B: Male; C: Anterior body; D: Lateral view of male tail; E-H: Variation in shape of spicules; I, J: Ventral view of male tail; K: Vulva region; L, M: Variation of female tail. (Scale bars=10 μm).

DESCRIPTIONS

Bursaphelenchus braaschae sp. n. (Figs. 1, 2, 3)

Measurements. Table 1.

Male. Body stout, cylindrical, posterior region sharply curved ventrally when heat-killed. Cuticle weakly annulated, lateral field with four incisures (*i.e.*, three ridges). Lip region rounded, offset, about 3.5 μm high and 6.8 μm wide. Stylet with small basal swellings, conus *ca.* 39% of total length. Procorpus cylindrical. Median bulb strongly developed, almost oval, with valve plates placed slightly posteriorly. Pharyngeal gland lobe slender and well developed, about two to three body diameters long, overlapping intestine dorsally. Nerve ring located at *ca.* 6 μm posterior to median bulb. Excretory pore located at *ca.* 15 μm posterior to nerve ring. Hemizonid just anterior to excretory pore. Testis single, spermatocytes arranged in multiple rows. Cloaca opening lips slightly protruding. Spicules small (14.2-16.3 μm long) and separate, with blunt rostrum and rounded distal end without cucullus. Condylus thin and high, not dorsally bent. Rostrum small and low, bluntly conical. Junction of rostrum and calomus smoothly curved. Dorsal edge of spicules along condylus with characteristic darker section in light microscopy. Tail strongly recurved, terminus sharply pointed. Three pairs of caudal papillae present: the first lateral pair located slightly anterior to cloacal slit, the second and the third pairs about 1-2 μm apart from each other at the base of bursa, the third pair more ventral than the second. A single P1 papilla in front of cloaca has not been detected. Bursa 6-8 μm long, often truncated with two to three points.

Female. Body slightly arcuate ventrally when heat-relaxed. Cuticle and lip region similar to male. Ovary outstretched, developing oocytes in multiple rows. Vulva slightly inclined anteriorly, vulva lips often strongly protruding, but sometimes only slightly thickened. Spermatheca oval, sometimes containing round sperms. Post-uterine sac well developed, about half vulva to anus distance, sometimes containing round sperms. Tail slim, attenuated with finely rounded terminus.

Diagnosis and relationships. *Bursaphelenchus braaschae* sp. n. is characterised by relatively stout body ($a = 23.5$ and 24.0 for males and females, respectively); four lines in the lateral field; spicules relatively small and delicate (14.2-16.3 μm) with blunt rostrum and rounded distal end without cucullus, condylus thin and high, not dorsally bent, dorsal edge of spicules along condylus with

characteristic darker section; and by females with strongly protruding vulva lips and a slim, attenuated tail with rounded terminus. Based on number of lateral lines, spicule shape (broad spicules with highly rounded apex, conspicuous ventral and dorsal limb, rounded distal end without cucullus), number and arrangement of caudal papillae of males and the other characters mentioned above (Figs. 1-3), *B. braaschae* sp. n. is affiliated to the *fungivorus* group of the genus (Braasch *et al.*, 2009).

According to Braasch *et al.*, (2009), the *fungivorus* group contains nine species: *B. huntii* (Steiner, 1935) Giblin & Kaya, 1983, *B. sychmus* Rühm, 1956 (J.B. Goodey, 1960), *B. steineri* Rühm, 1956 (J.B. Goodey, 1960), *B. fungivorus* Franklin & Hooper, 1962, *B. gonzalezi* Loof, 1964, *B. seani* Giblin & Kaya, 1983, *B. thailandae* Braasch & Braasch-Bidasak, 2002, *B. arthuri* Burgermeister, Gu & Braasch, 2005 and *B. willibaldi* Schönfeld, Braasch & Burgermeister, 2006.

Bursaphelenchus braaschae sp. n. is most similar to *B. willibaldi*. It differs from *B. willibaldi* by the size and shape of spicules (spicules average 15.3 μm vs 17 μm ; condylus not dorsally bent vs condylus slightly dorsally bent; rostrum in obtuse angle and indistinct vs rostrum in sharp angle and conspicuous), by the post-uterine sac length (average 50.4 μm , which occupies 55.3% of the vulva-to-anus distance vs average 95 μm , which occupies 68% of the vulva-to-anus distance), by slightly stouter body ($a = 23.5$ and 24.0 for males and females, respectively vs $a = 32$ and 29), and also the vulva lips protrude more in the new species.

Bursaphelenchus braaschae sp. n. is distinguished from: *B. huntii* by the shape of spicules (blunt rostrum vs sharp rostrum); *B. sychmus* by the shape and size of spicules (broadly rounded distal end vs pointed distal end; 14.2-16.3 μm vs 19-23 μm); *B. steineri* by the shape and size of spicules (blunt rostrum vs sharp rostrum; 14.2-16.3 μm vs 17 μm), and by different shape of female tail terminus (rounded vs pointed with a cuticular mucron); *B. fungivorus* by body size (average 535 μm and 554 μm for males and females vs 850 μm and 980 μm , respectively) and shape of spicules (blunt rostrum vs sharp and long rostrum), and by the shape of female tail (slightly ventrally curved vs ventrally hooked); *B. gonzalezi* by the shape of spicules (condylus high vs condylus low); and by the shape of female tail terminus (rounded vs pointed); *B. seani* by the shape and size of spicules (blunt rostrum vs sharp rostrum; 14.2-16.3 μm vs 18-27 μm) and the ratio c' in females (5.7 vs 3.4 on average); *B. thailandae* by different body shape ($a=23.5$ and 24.0 for males and females vs $a=39$ and 38 , respectively), ratio c' (5.7

Table 1. Measurements of *Bursaphelenchus braaschae* sp. n. Measurements in μm and in format: mean \pm s.d. (range).

	Female		Male
	Holotype	Paratypes	Paratypes
n	-	15	15
L	629.0	554.1 \pm 46.9 (466.5-623.6)	534.5 \pm 42.8 (467.0-615.0)
a	25.6	24.0 \pm 2.3 (18.6-28.0)	23.5 \pm 2.4 (19.3-26.9)
b	8.2	7.4 \pm 1.0 (5.6-8.9)	7.0 \pm 0.7 (5.5-8.6)
b'	6.0	5.4 \pm 0.5 (4.4-6.1)	4.7 \pm 0.6 (3.8-6.2)
c	11.1	9.3 \pm 0.7 (8.2-11.0)	22.2 \pm 1.8 (19.5-25.8)
c'	4.9	5.7 \pm 0.4 (5.1-6.4)	1.8 \pm 0.1 (1.5-2.0)
V or T	70.7	70.5 \pm 0.8 (69.3-71.9)	67.5 \pm 7.2 (50.4-79.5)
Max. body diam.	24.6	23.8 \pm 3.7 (17.8-30.7)	23.2 \pm 2.8 (19.1-27.9)
Lip diam.	6.9	6.9 \pm 0.5 (6.1-7.5)	6.8 \pm 0.5 (5.9-7.5)
Lip height	3.1	3.5 \pm 0.2 (3.1-3.8)	3.5 \pm 0.3 (3.1-3.9)
Stylet length	14.8	14.6 \pm 0.7 (13.4-15.7)	13.7 \pm 0.8 (12.1-15.0)
Median bulb length	16.7	16.8 \pm 1.0 (14.8-18.1)	16.8 \pm 0.9 (14.6-18.0)
Median bulb diam.	13.7	13.7 \pm 1.1 (12.1-15.8)	14.5 \pm 1.4 (12.7-16.4)
Median bulb length/diam.	1.2	1.2 \pm 0.1 (1.1-1.4)	1.2 \pm 0.1 (1.0-1.4)
Excretory pore position	92.5	86.9 \pm 5.0 (80.7-99.0)	88.0 \pm 5.1 (82.0-99.0)
Spicule (chord)	-	--	15.3 \pm 0.6 (14.2-16.3)
Spicule (dorsal limb)	-	--	17.4 \pm 0.8 (16.1-18.6)
Ovary or testis length	191.3	248.7 \pm 46.8 (156.0-318.0)	345.3 \pm 66.6 (205.0-455.0)
Post-uterine sac length	76.8	50.4 \pm 16.4 (29.0-78.3)	-
Post-uterine sac length/ Vulva to anus (%)	-	55.3 \pm 14.8 (34.4-78.3)	-
Tail length	56.6	60.1 \pm 4.9 (50.1-69.9)	24.2 \pm 1.8 (21.5-27.1)
Anal body diam.	11.5	10.6 \pm 1.3 (8.9-13.6)	13.3 \pm 1.1 (11.7-16.2)

vs 4.2 on average), shape of female tail terminus vs (rounded pointed), and the length of bursa (only 2-4 μm in *B. thailandae*); *B. arthuri* by different shape and size of spicules (blunt rostrum vs sharp rostrum; 14.2-16.3 μm vs 16.0-20.9 μm), body length (average 535 μm and 554 μm for males and females vs 923 μm and 961 μm , respectively), and ratio c' of females (5.7 vs 4.8 on average).

Key to the *fungivorus* group

1. Six male papillae, rostrum low.....2

Seven male papillae, rostrum high and sharply pointed.....7

2. Rostrum sharply conical, spicules tip pointed or narrow.....3

Rostrum bluntly conical, spicule tip broad and blunt.....4

3. Spicules broad, female tail terminus sharply pointed.....*B. sychnus*

Spicules slim, female tail terminus mucronate.....*B. sternerii*

4. Female tale terminus pointed.....5

Female tale terminus finely rounded.....6

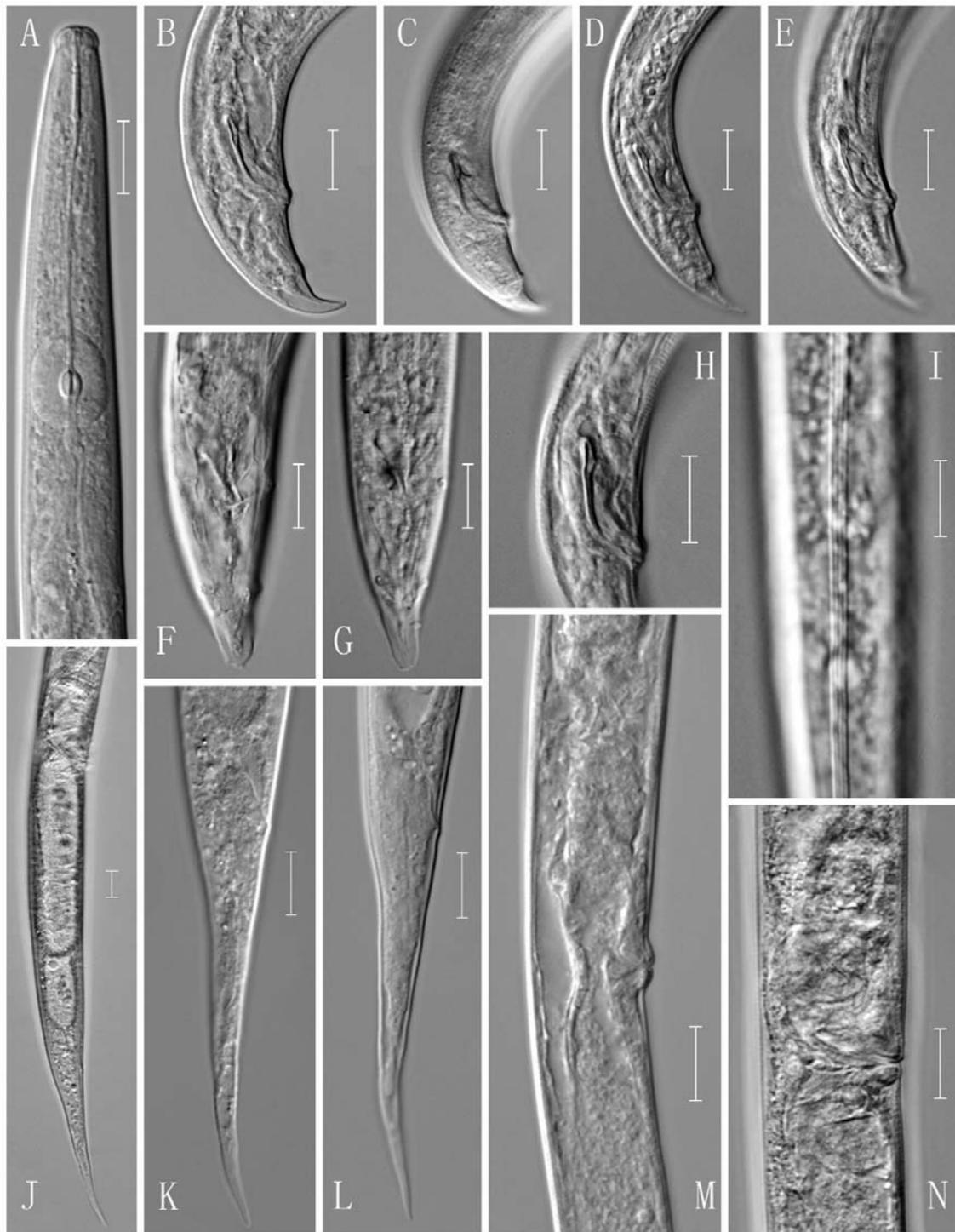


Fig. 2. Light photomicrographs of *Bursaphelenchus braaschae* sp. n. A: Female anterior region; B-E & H: Lateral view of male tail; F, G: Ventral view of male tail with variations of terminal bursa; I: Lateral lines; J-L: Female tail; M, N: Vulva region. (Scale bars=10 μ m).

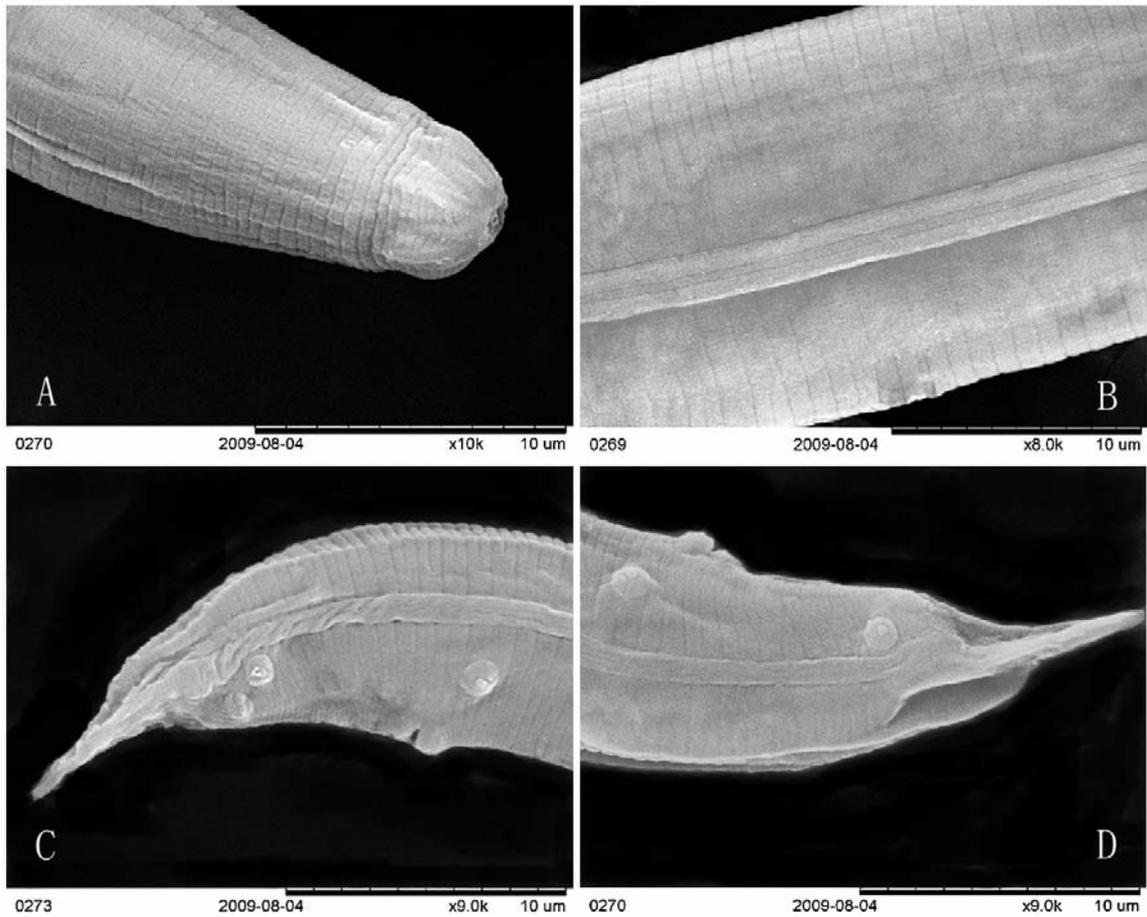


Fig. 3. Scanning electron microscope (SEM) observations of *Bursaphelenchus braaschae* sp. n. A: Head region; B: Lateral view; C, D: Male tail.

Table 2. Sizes of PCR products and DNA restriction fragments obtained in ITS-RFLP analysis and calculated on sequencing results of the ITS1/2 regions.

<i>Bursaphelenchus</i> species	PCR product (bp)	Restriction fragments (bp) ¹				
		<i>Rsa</i> I	<i>Hae</i> III	<i>Msp</i> I	<i>Hinf</i> I	<i>Alu</i> I
<i>B. braaschae</i> sp. n.	1060	552	852	1060	358	622
		508	208		325	332
					212	92
					94	14
					47	
<i>B. willibaldi</i>	1132	543	1132	731	488	534
		301		401	359	379
		288			215	136
					46	93
					24	
<i>B. thailandae</i>	880	482	880	880	382	555
		333			226	273
		65			202	52
					46	
					24	

¹Fragment sizes (bp) were calculated with the computer program DNASTAR MapDraw 5.01.

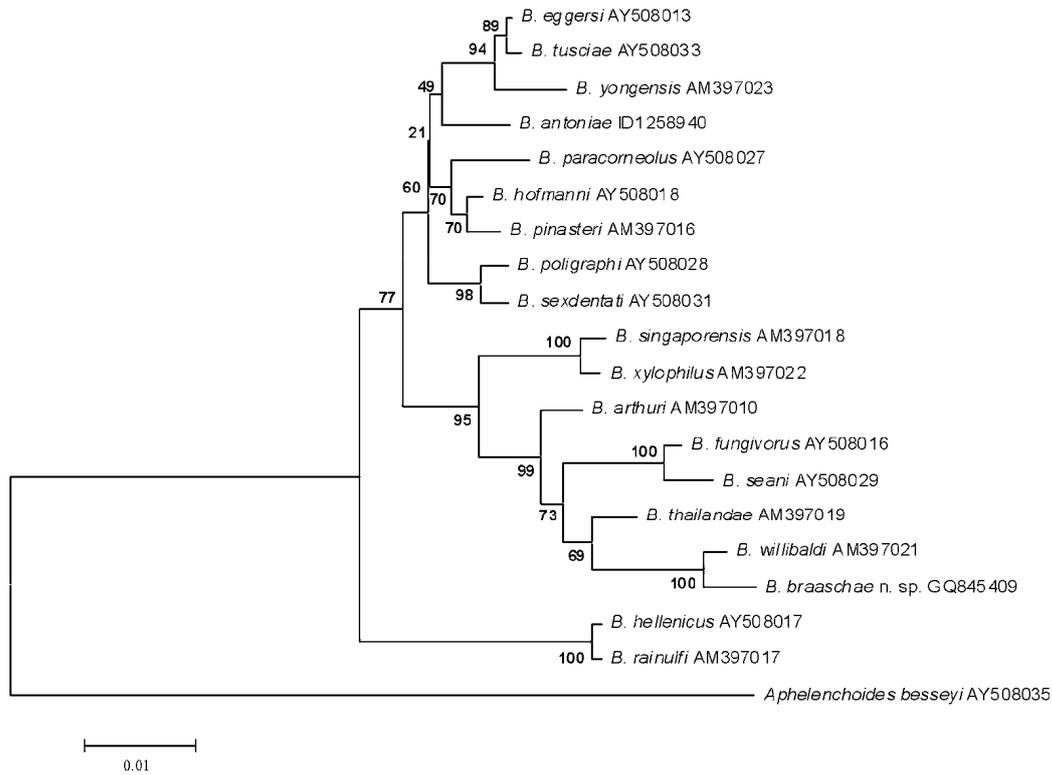


Fig. 4. Molecular phylogenetic status of *Bursaphelenchus braaschae* sp. n. based on partial SSU sequences. *Aphelenchus besseyi* served as outgroup species. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

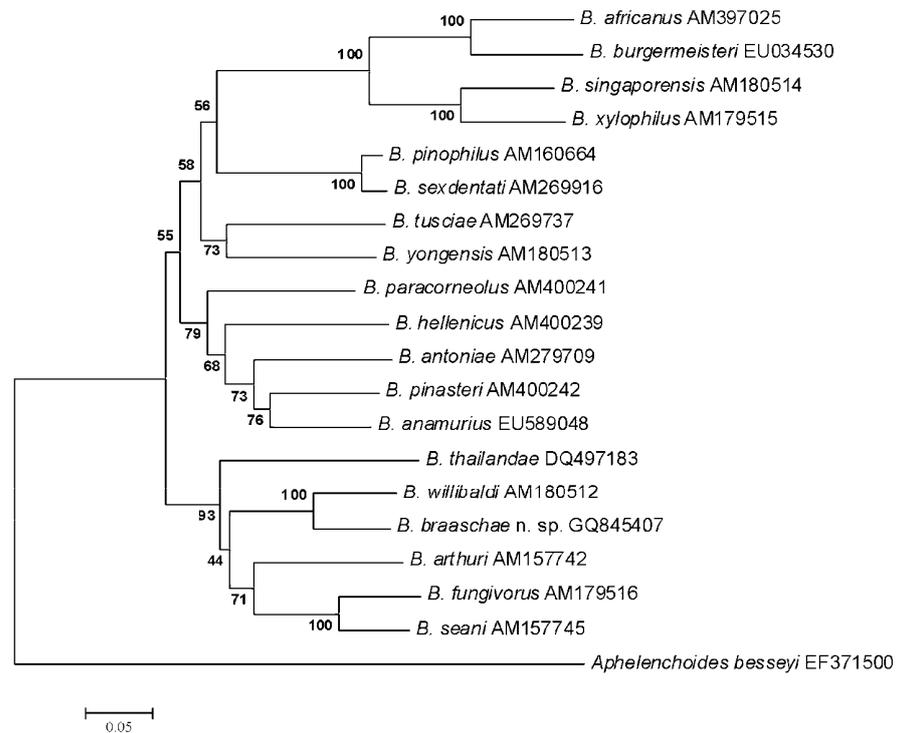


Fig. 5. Molecular phylogenetic status of *Bursaphelenchus braaschae* sp. n. based on ITS1/2 sequences. *Aphelenchoides besseyi* served as outgroup species. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

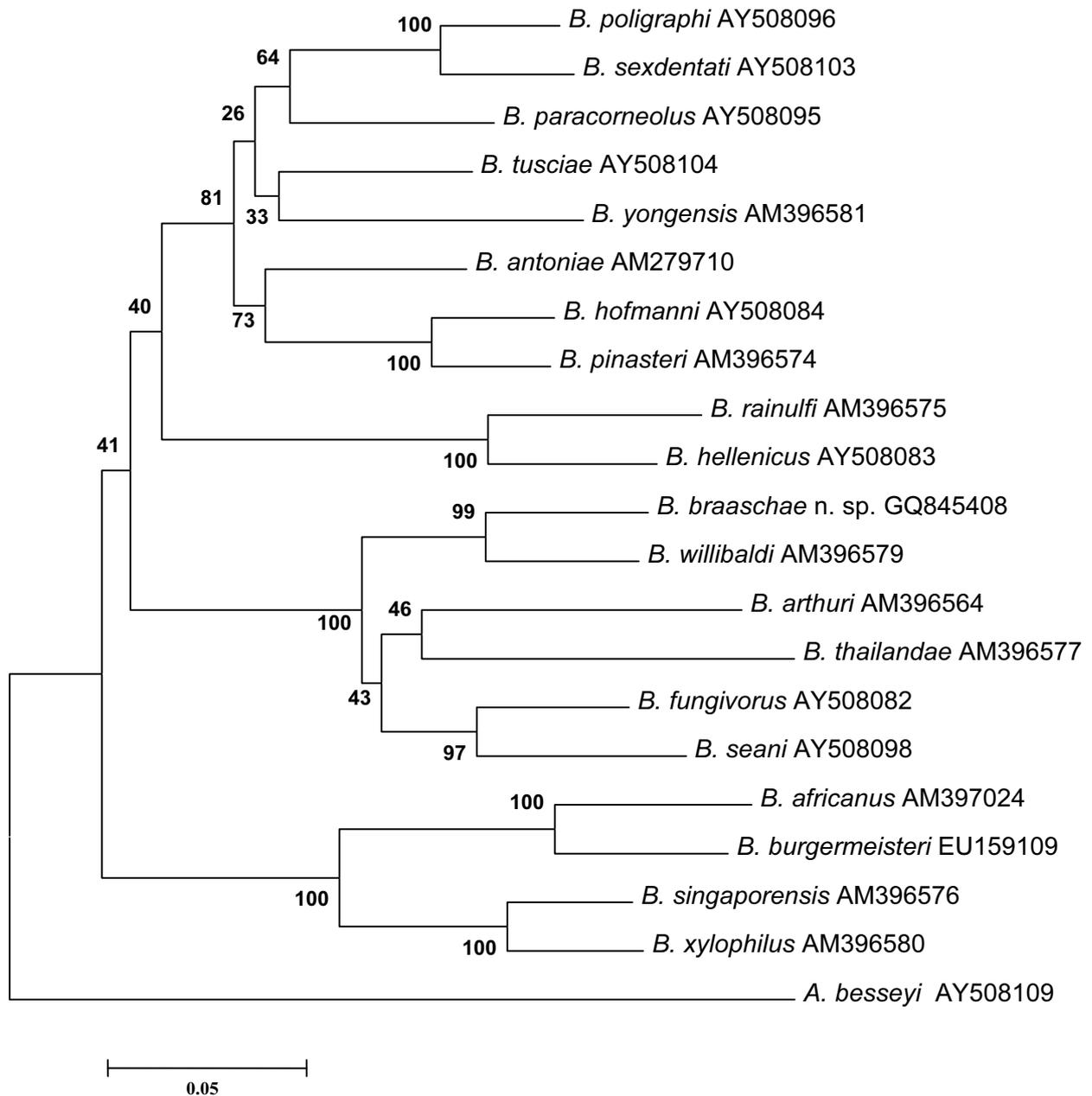


Fig. 6. Molecular phylogenetic status of *Bursaphelenchus braaschae* sp. n. based on partial LSU sequences. *Aphelenchus besseyi* served as outgroup species. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

- | | |
|--|--|
| 5. Condylus low, female tail ventrally curved..... <i>B. gonzalezi</i> | 8. Condylus low..... <i>B. hunti</i> |
| Condylus high, female tail straight..... <i>B. thailandae</i> | Condylus high.....9 |
| 6. Mean spicules length 15 μm, vulva lips protruding..... <i>B. braaschae</i> sp. n. | 9. Female tail relatively short (c'=4-5) and slightly ventrally bent, post-uterine branch length occupies 75% of the vulva-to-anus distance..... <i>B. arthuri</i> |
| Mean spicules length 17μm, vulva lips not protruding..... <i>B. willibaldi</i> | |
| 7. Female tale terminus pointed..... <i>B. seani</i> | Female tail relatively long (c'=6-7) and ventrally hooked, post-uterine branch length occupies 50% of the vulva-to-anus distance..... <i>B. fungivorus</i> |
| Female tale terminus finely rounded.....8 | |

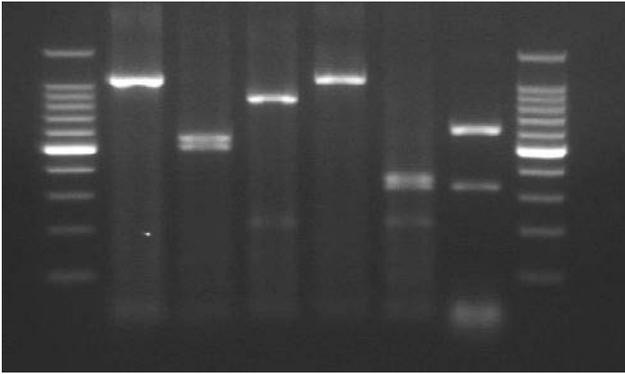


Fig. 7. ITS-RFLP pattern of *Bursaphelenchus braaschae* sp. n. M = Molecular size marker (100 bp ladder); Lane 1: rDNA amplification product; Lanes 2-6: Digestion products obtained with *RsaI*, *HaeIII*, *MspI*, *HinfI* and *AluI*. Sizes of PCR product and its restriction fragments are shown in Table 2.

Molecular profiles and phylogenetic status.

The rDNA base sequences of partial SSU, ITS1/2 and D2D3 LSU are deposited in the GenBank database with the accession numbers GQ845409, GQ845407 and GQ845408, respectively. The molecular phylogenetic status of the new species is shown in Figures 4, 5 and 6, and the ITS-RFLP profiles of rDNA are shown in Figure 7 and Table 2. The ITS-RFLP pattern of *B. braaschae* sp. n. is different from the patterns of known *fungivorus* group species, such as *B. fungivorus*, *B. seani*, *B. thailandae*, *B. arthuri* and *B. willibaldi* as shown in Burgermeister *et al.* (2009).

In all the molecular trees, the new species is close to *B. willibaldi* and the species of the *fungivorus* group distinctly grouped in one clade and separated from other species of the genus. The paired sequence similarities of *B. braaschae* sp. n. compared to *B. willibaldi* are 0.988, 0.751 and 0.924 for partial SSU, ITS1/2 and D2D3 LSU sequences.

Type locality and habitat. Deciduous dunnage from Thailand, inspected in Ningbo Entry-exit Inspection and Quarantine Bureau, China, in 2009.

Type specimens. Holotype male, eighty-three female and ninety male paratypes (slide numbers 14190-1 to 14190-30) deposited in the nematode collection of Ningbo Entry-Exit Inspection and Quarantine Bureau, China. Eight paratype males and eleven paratype females (slide numbers 14190-31 and 14190-32) deposited in the Canadian National Collection of Nematodes, Ottawa, Ontario, Canada. Nine paratype males and ten paratype females (slide numbers 14190-33 and 14190-34) deposited in the Institute of Biotechnology, College

of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China.

Etymology. *Bursaphelenchus braaschae* sp. n. was named after Dr Helen Braasch from the former Federal Biological Research Center for Agriculture and Forestry of Germany for her outstanding contributions to the understanding of the genus *Bursaphelenchus* and her great help to the first author in many nematological investigations.

DISCUSSION

According to Braasch *et al.* (2009), position and number of male caudal papillae are important in grouping of *Bursaphelenchus*, apart from other characters. Males of the *fungivorus* group appear to have seven preanal and postanal papillae as observed in *B. seani*, *B. fungivorus*, *B. hunti* and *B. arthuri*, with the single P1 papilla occurring relatively far above the cloacal slit, and the two postanal pairs close to each other (1-2 μ m distance). However, the single P1 papilla has not been observed in *B. sychmus*, *B. steineri*, *B. gonzalezi*, *B. thailandae*, *B. willibaldi* and *B. braaschae* sp. n. Possibly, these species represent two subgroups of the *fungivorus* group as supported by the phylogenetic trees based on rDNA sequences (Figs. 4 and 5), which show that *B. fungivorus*, *B. seani* and *B. arthuri* group together (seven papillae, rostrum high and sharply pointed), whereas *B. thailandae*, *B. willibaldi* and *B. braaschae* sp. n. cluster together in another branch (six papillae, rostrum low and bluntly conical). However, it is also possible that the P1 papilla is relatively indistinct or does not open through the cuticle in the species in question.

Without further molecular evidence, the grouping status of *B. sychmus*, *B. steineri*, *B. gonzalezi* and *B. hunti* is still questionable.

The vector of the new species is unknown.

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Jianfeng Gu, Jiangling Wang. Описание *Bursaphelenchus braaschae* sp. n. (Nematoda: Aphelenchoididae) из материала упаковочных подстилок, изготовленных в Таиланде.

Резюме. Дано описание и иллюстрации для *Bursaphelenchus braaschae* sp. n. Новый вид был выделен из подстилок под морские грузы, изготовленных из древесины лиственных пород в Таиланде. Новый вид относится к группе видов *fungivorus* и характеризуется сравнительно мощным телом ($a = 23.5$ и 24.0 для самцов и самок, соответственно); четырьмя линиями в латеральном поле, сравнительно короткими и тонкими спикулами ($14.2-16.3 \mu\text{m}$) с притупленным роостромом, округлой дистальной частью и без кукулюса, тонким и высоким кондилюсом без загиба на спинную сторону, дорсальным краем спикул вдоль кондилюса с характерной затемненной частью, самками с сильно выступающими губам вульвы и тонким вытянутым хвостом с закругленным терминусом. Новый вид морфологически близок к *B. willibaldi*, но отличается от него формой и размером спикул, длиной заднего маточного мешка и положением вульвы. Статус отдельного вида для него подтвержден спектром ITS-RFLP, а также результатами молекулярно-филогенетического анализа, основанного на частичной последовательности SSU, полной ITS и частичной последовательности LSU рибосомальной ДНК. Этот же анализ подтвердил близость *B. braaschae* sp. n. к *B. willibaldi*.