

***Bursaphelenchus parathailandae* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Taiwan**

Jianfeng Gu, Jiangling Wang and Xianfeng Chen

Technical Centre, Ningbo Entry-exit Inspection and Quarantine Bureau, 9 Mayuan Road,
Ningbo 315012, Zhejiang, China,
e-mail: chenxf@nbciq.gov.cn

Accepted for publication 12 January 2012

Summary. *Bursaphelenchus parathailandae* sp. n., isolated in Ningbo, China from packaging wood of *Pinus* sp. from Taiwan is described. It is characterised by a slim body ($a = 30-42$), lateral field with four lines, excretory pore 1-1.5 body diam. behind median bulb, vulva at 71-75% of total body length, vulval lips distinctly protruding but not forming a vulval flap, post-uterine branch one-third to two-thirds of the vulva-anus distance long, female tail five to six anal body diam. long, filiform, almost straight with bluntly pointed terminus, spicules relatively small and delicate (15-18 μm) with blunt pointed rostrum and rounded distal end without cucullus, condylus high, not dorsally bent, dorsal edge of spicules along condylus with characteristic darker section, bursa small and indistinct. The new species belongs to the *fungivorus* group of the genus *Bursaphelenchus* and is most similar to *B. thailandae*, from which it can be distinguished as well as from other *Bursaphelenchus* species by morphology, ITS-RFLP patterns and sequencing results.

Key words: molecular taxonomy, morphology, morphometrics, phylogeny.

During nematological inspections of imported packaging wood in Ningbo, China, an undescribed species of *Bursaphelenchus* Fuchs, 1937 was detected in coniferous packaging material from Taiwan, China. It is described herein as *B. parathailandae* sp. n. using morphological characters, ITS-RFLP patterns and sequencing results.

MATERIALS AND METHODS

Nematode culturing and morphological observations. Sawn samples taken from packaging wood were cut into small pieces no more than 1 cm wide. Nematodes were extracted by a modified Baermann funnel technique for 24 h and multiplied successfully on *Botryotinia fuckeliana* growing on PDA. Measurements were made on specimens cultured on PDA for 1 month, then fixed in TAF and processed to glycerin following the method of Seinhorst (1959). Light micrographs were made using a Zeiss Imager Z1 microscope equipped with a Zeiss AxioCam MRm CCD camera.

Molecular analyses. DNA samples were prepared according to Li *et al.* (2008). Four sets of primers (synthesized by Invitrogen, Shanghai,

China) were used in the PCR analyses to amplify the partial SSU region, the ITS1/2 region and the D2D3 LSU region of rDNA and partial mtCOI gene, respectively (Wang & Gu, 2012). PCR conditions were as described by Li *et al.* (2008) and Ye *et al.* (2007). 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 (*Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I) (Takara, Japan) following the manufacturer's instructions. Fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

The 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) method using the Tajima-Nei distance option. Bootstrapping analysis was performed with 1000 replicates.

DESCRIPTION

***Bursaphelenchus parathailandae* sp. n.**
(Figs 1-2)

Measurements. See Table 1.

Female. Body slightly ventrally arcuate or strait when heat-relaxed. Cuticle marked by fine transverse striations, ca 1 μm wide. Lateral field ca. 5 μm wide, with four equally spaced lines at mid-body. Lip region convex, offset. Stylet with small basal swellings, conus forming ca. 40% of total length. Procorpus cylindrical. Median bulb oval or pear-shaped, conspicuous valve plates situated almost centrally. Pharyngo-intestinal junction ca. one metacorporeal valve length posterior to metacarpus. Pharyngeal gland lobe ca. three to four body diam. long, overlapping intestine dorsally. Nerve ring located ca. 3 μm posterior to median bulb. Excretory pore 1-1.5 body diam. behind median bulb, 68-90 μm from anterior end. Hemizonid just anterior to excretory pore. Reproductive system prodelphic, gonad outstretched, occupying about 40% of body length. Developing oocytes arranged in multiple rows, developed oocytes arranged as single row in posterior third to quarter of ovary. Oviduct present but obscure. Spermatheca oblong or irregular, sometimes full of sperm. Quadricolumella rectangular. Uterus roundish, thick walled. Vulva at 71-75% of body length, vagina slightly inclined anteriorly, vulval lip usually distinctly protruding, not forming a vulval flap. Body slightly narrowing behind vulva. Post-uterine sac well developed, 38-69% of vulva to anus distance long, sometimes containing sperm. Rectum and anus visible. Tail five to six anal body diam. long, almost straight, conoid at first and then narrowing rapidly to a finely rounded terminus.

Male. Anterior body region and cuticle similar to those of female. Tail hooked ventrally, terminus claw-like, but sometimes only slightly curved. Testis occupying over half of body length, outstretched. Spermatocytes arranged in multiple rows. Cloacal lips slightly protruding. Spicules delicate, condylus 5-6 μm high, rostrum bluntly pointed. Distal ends of spicules without cucullus. Spicules with a characteristic sector opposite rostrum, extending dorsally from condylus for almost half the calomus and appearing darker than rest of spicule. Tail ventrally arcuate with a pointed, talon-like terminus bearing a very small (2-4 μm long) bursa. Three pairs of ventro-lateral caudal papillae: one pre-anal, one ca. middle of tail and another pair in the second half of tail. The position of the papillae is shown in Fig. 1G, Fig. 2G-J.

Diagnosis and relationships. *Bursaphelenchus parathailandae* sp. n. is characterised by a slim body ($a = 30-42$), lateral field with four lines, excretory pore 1-1.5 body diam. behind median bulb, vulva at 71-75% of total body length, vulval lips distinctly protruding but not forming a vulval flap, post-uterine branch ca. one-third to two-thirds of the vulva-anus distance long, female tail five to six anal body diam. long, almost straight with bluntly pointed terminus, spicules relatively small and delicate (15-18 μm) with blunt pointed rostrum and rounded distal end without cucullus, condylus high, not dorsally bent, dorsal edge of spicules along condylus with characteristic darker section, bursa small and indistinct.

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, *Bursaphelenchus parathailandae* sp. n. is affiliated to the *fungivorus* group of the genus (Braasch *et al.*, 2009).

According to Braasch *et al.* (2009) and Gu & Wang (2010), the *fungivorus* group contains 10 species: *B. hunti* (Steiner, 1935) Giblin & Kaya, 1983, *B. sychnus* 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, *B. willibaldi* Schönfeld, Braasch & Burgermeister, 2006 and *B. braaschae* Gu & Wang, 2010.

Bursaphelenchus parathailandae n. sp. is most similar to *B. thailandae*. It differs from *B. thailandae* by the size and shape of spicules (spicules average 16.9 μm vs 14 μm ; rostrum bluntly conical vs rostrum blunt and short), by c' value (mean $c' = 5.2$ vs $c' = 4.1$) and female tail shape (filiform vs conical).

Bursaphelenchus parathailandae sp. n. is distinguished from: *B. braaschae* by different body shape (mean $a = 36$ and 35 for males and females vs $a = 24$), spicules shape (rostrum bluntly pointed vs rostrum small and low, bluntly conical), caudal papillae position (P3 and P4 about 6 μm vs 1-2 μm), and the length of bursa (2-4 μm vs 6-8 μm); *B. willibaldi* by different body shape (mean $a = 36$ and 35 for males and females vs $a = 32$ and 29), spicules shape (condylus not bent vs condylus slightly dorsally bent), caudal papillae position (P3 and P4 about 6 μm vs close to each other), and the length of bursa (2-4 μm vs 8-12 μm); *B. sychnus* by size of spicules (15-18 μm vs 19-23 μm) and female tail shape (slim conical vs conical); *B. steineri* by the shape

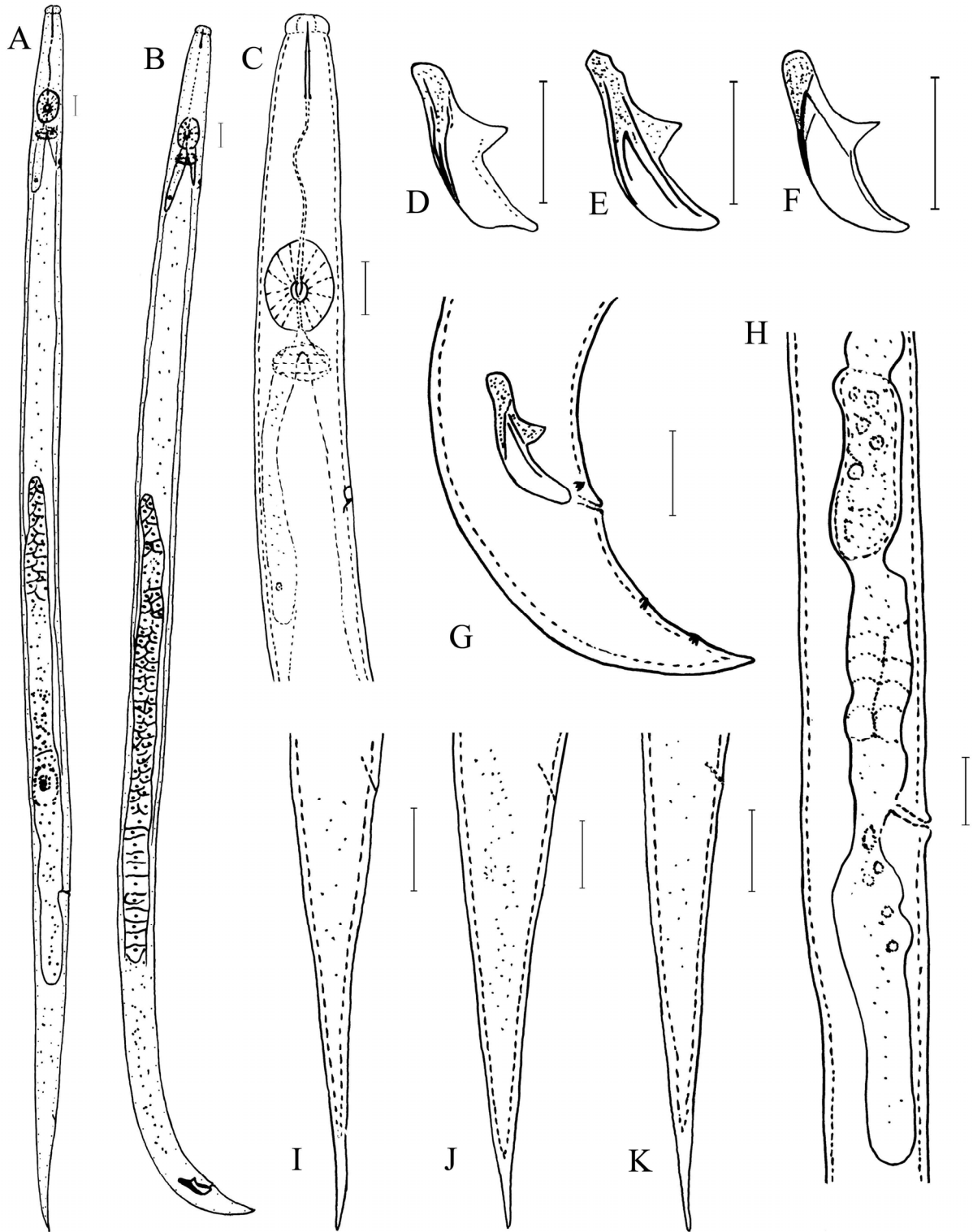


Fig. 1. *Bursaphelenchus parathailandae* sp. n. A: Female; B: Male; C: Head region; D-F: Spicules; G: Male tail in lateral view; H: Vulval region; I-K: Female tail. (Scale bars = 10 μ m.)

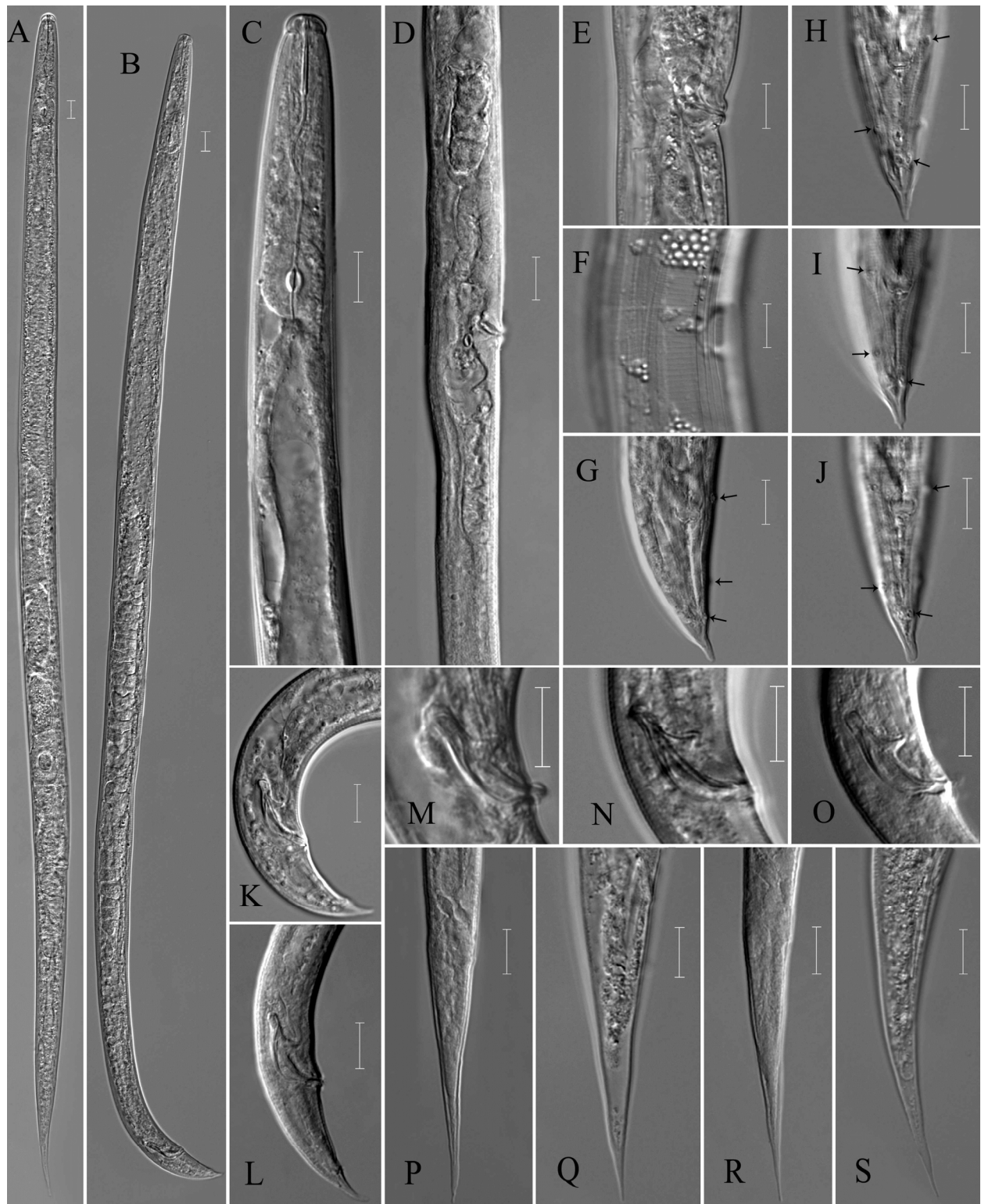


Fig. 2. Light microscope observations of *Bursaphelenchus parathailandae* sp. n. A: Female; B: Male; C: Head region; D, E: Vulval region; F: Lateral field; G-J: Male tail in ventral view (show caudal papillae position and bursa); K, L: Male tail in lateral view; M-O: Spicules; P-S: Female tail. (Scale bars = 10 μ m.)

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

Characters	Female		Male
	Holotype	Paratypes	Paratypes
n		20	20
L	643	615 \pm 34.3 (552-696)	619 \pm 84.0 (475-714)
a	36.1	34.5 \pm 2.5 (29.8-39.1)	36.1 \pm 3.0 (29.4-41.6)
b	10.5	10.7 \pm 0.6 (9.8-11.8)	10.5 \pm 0.9 (9.0-11.9)
b'	5.5	5.6 \pm 0.3 (5.1-6.4)	5.3 \pm 0.5 (4.7-6.1)
c	12.3	11.6 \pm 0.7 (10.5-13.0)	22.6 \pm 2.4 (18.7-26.1)
c'	5.0	5.2 \pm 0.3 (4.8-5.8)	2.2 \pm 0.1 (2.0-2.3)
V or T	71.8	72.0 \pm 1.1 (70.7-74.7)	57.1 \pm 6.2 (40.6-64.5)
Max body diam.	17.8	17.9 \pm 1.4 (15.3-20.7)	17.3 \pm 2.0 (13.9-19.9)
Stylet length	13.9	13.7 \pm 0.6 (12.9-15.0)	13.5 \pm 0.7 (12.5-15.0)
Excretory pore position	78.0	78.1 \pm 7.0 (68.0-90.0)	75.5 \pm 7.2 (65.0-90.0)
Spicule (chord)	–	–	16.9 \pm 1.0 (15.1-18.0)
Ovary (from anterior end to vulva) or testis length	290	256 \pm 22.0 (225-290)	354 \pm 66.1 (255-455)
Post-uterine sac length	65.5	60.1 \pm 9.7 (46.0-78.0)	–
Post-uterine sac length/ Vulva to anus (%)	50.6	50.3 \pm 7.1 (38.3-69.0)	–
Tail length	52.0	52.8 \pm 2.4 (48.8-59.0)	27.4 \pm 1.2 (25.0-29.0)
Anal or cloacal body diam.	10.4	10.1 \pm 0.5 (9.3-11.0)	12.7 \pm 0.6 (11.4-13.5)

Table 2. Sizes of DNA restriction fragments obtained for *Bursaphelenchus parathailandae* and related species in ITS-RFLP analysis and calculated on sequencing results of the ITS regions.

<i>Bursaphelenchus</i> species	PCR product (bp)	Restriction fragments (bp)				
		<i>Rsa</i> I	<i>Hae</i> III	<i>Msp</i> I	<i>Hinf</i> I	<i>Alu</i> I
<i>B. parathailandae</i> sp. n.	879	480 399	879	879	379 219 162 46 40 25 8	639 121 106 13
<i>B. thailandae</i>	880	482 333 65	880	880	382 226 202 46 24	555 273 52
<i>B. braaschae</i>	1060	552 508	852 208	1060	358 325 212 94 47 24	622 332 92 14
<i>B. willibaldi</i>	1132	543 301 288	1132	731 401	488 359 215 46 24	534 379 136 93

of spicules (rostrum bluntly conical *vs* sharp pointed), and by different shape of female tail terminus (bluntly pointed *vs* pointed with a cuticular mucron).

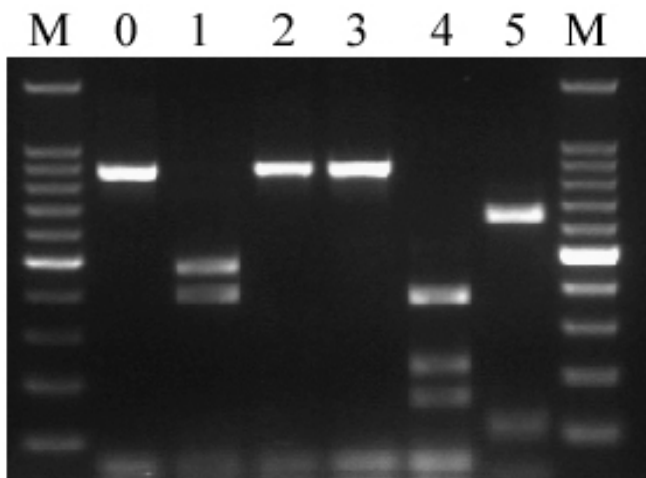


Fig.3. RFLP profiles of *Bursaphelenchus parathailandae* sp. n. Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa* I (1), *Hae* III (2), *Msp* I (3), *Hinf* I (4) and *Alu* I (5). M: DNA marker (100 bp ladder, Invitrogen Life Technologies).

All the other species of the fungivorus group have seven male papillae and high and sharply pointed rostrum, which can be easily distinguished from the above mentioned species.

Type locality and habitat. Packaging wood (*Pinus* sp.) from Taiwan and inspected in Ningbo Entry-exit Inspection and Quarantine Bureau, China (April, 2011).

Type material. Holotype female, 35 male and 41 female paratypes (slide numbers 00349-1 to 00349-12) deposited in the nematode collection of Ningbo Entry-exit Inspection and Quarantine Bureau, China. 5 paratype females and 4 paratype males (slide numbers 10002 and 10003) deposited in the Canadian National Collection of Nematodes, Ottawa, Canada.

Molecular profiles and phylogenetic status. The partial 18S, ITS1/2, 28S D2/D3 region and the mitochondrial cytochrome oxidase subunit I (mtCOI) sequences are deposited in the GenBank database with the accession numbers JN377724 (1707 bp), JN377723 (879 bp), JN377722 (765 bp) and JN377725 (709 bp), respectively. The molecular phylogenetic status of the new species is shown in Figures 4-6. *Bursaphelenchus parathailandae* sp. n. clearly belongs to the *fungivorus* group, and it is closely related to *B. thailandae*.

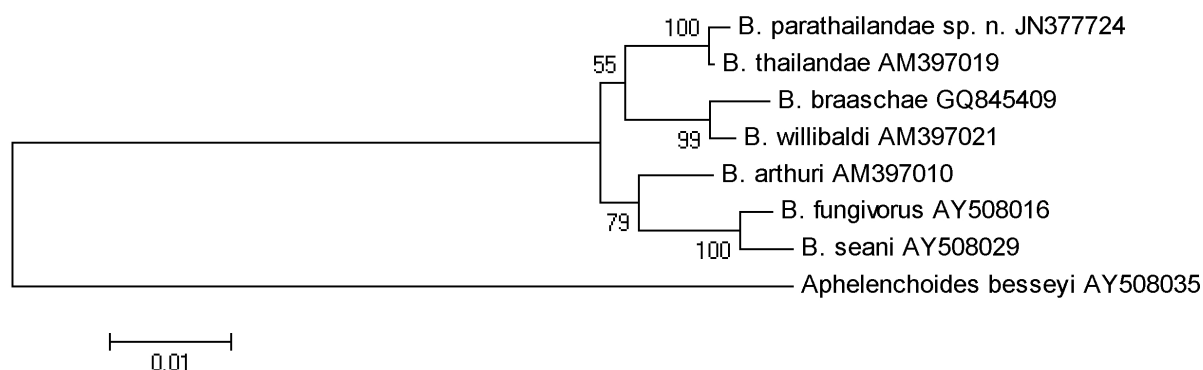


Fig. 4. Phylogenetic relationships of *Bursaphelenchus parathailandae* sp. n. using NJ tree construction method of aligned sequences of partial 18S gene. EMBL accession numbers are listed with the species names. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

Amplification of the ITS1/2 region of *B. parathailandae* sp. n. resulted in a PCR product of 879 bp. The patterns of restriction fragments produced by digestion of the PCR product with *Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I (Fig. 3, Table 2) were different from ITS-RFLP patterns of 44 *Bursaphelenchus* species obtained using the same primers for amplification and the same set of restriction enzymes (Burgermeister *et al.*, 2009).

Ethymology. Specific epithet formed from the species name *thailandae*, and reflecting its close similarity.

DISCUSSION

For *Bursaphelenchus parathailandae* sp. n. and *B. thailandae*, the bursa is very small; sometimes it is even not visible or difficult to see under light microscope.

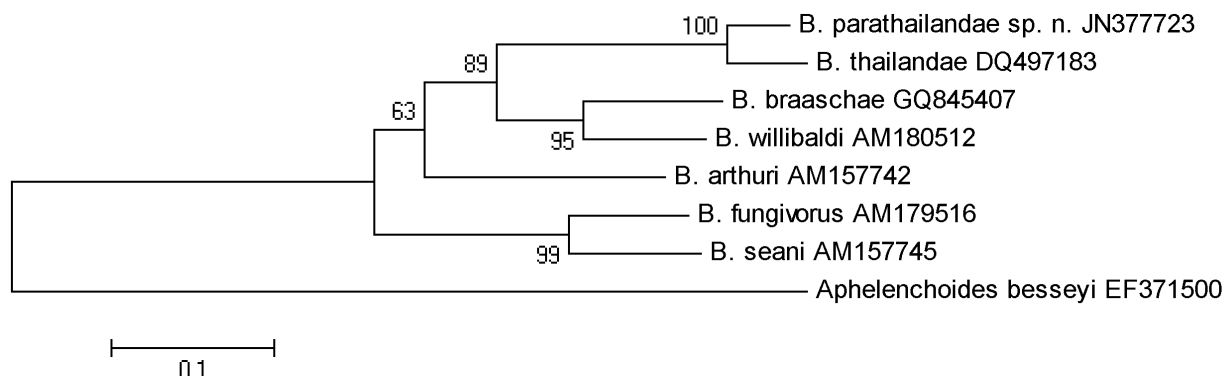


Fig. 5. Phylogenetic relationships of *Bursaphelenchus parathailandae* sp. n. using NJ tree construction method of aligned sequences of ITS1/2. EMBL accession numbers are listed with the species names. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

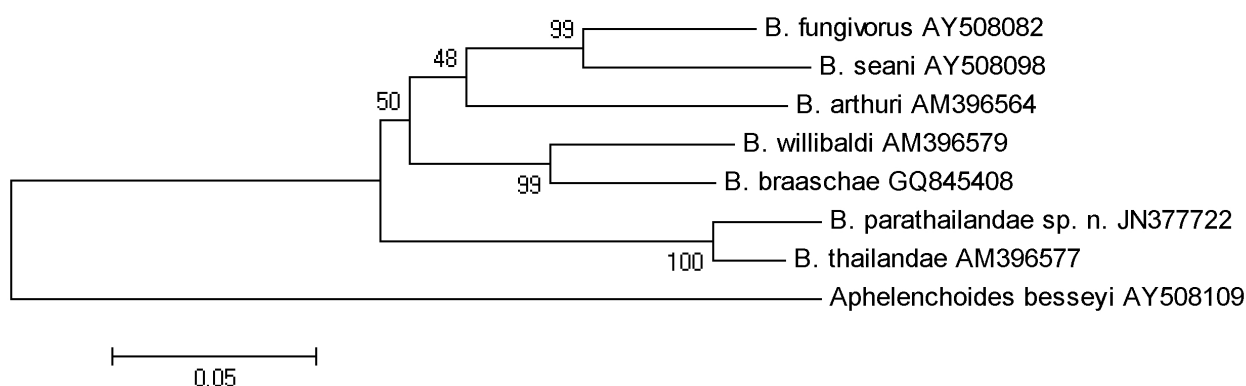


Fig. 6. Phylogenetic relationships of *Bursaphelenchus parathailandae* sp. n. using NJ tree construction method of aligned sequences of the D2/D3 region of LSU. EMBL accession numbers are listed with the species names. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

In the *fungivorus* group, *B. braaschae*, *B. willibaldi*, *B. sychnus*, *B. steineri*, *B. thailandae* and *B. parathailandae* sp. n. have six male papillae and relatively low and bluntly rostrum, while other species of this group have seven male papillae and high and sharply pointed rostrum. Possibly, these species represent two subgroups of the *fungivorus* group as supported by the phylogenetic trees (Figs 4-6).

Because packaging wood is a circulating product and there is no phytosanitary treatment mark, the geographic origin of the new species remains unclear. The vector of the new species is also unknown.

ACKNOWLEDGEMENT

The research presented here was supported by National Science and Technology Support Program

(2012BAK11B03), General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (2010IK269) and Ningbo Natural Science Foundation (2010A610018).

REFERENCES

- BRAASCH, H. & BRAASCH-BIDASAK, R. 2002. First record of the genus *Bursaphelenchus* Fuchs, 1937 in Thailand and description of *B. thailandae* sp. n. (Nematoda: Parasitaphelenchidae). *Nematology* 4: 853-863.
- BRAASCH, H., BURGERMEISTER, W. & GU, J. 2009. Revised intra-generic grouping of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae). *Journal of Nematode Morphology and Systematics* 12: 65-88.
- BURGERMEISTER, W., GU, J. & BRAASCH, H. 2005. *Bursaphelenchus arthuri* sp. n. (Nematoda:

- Parasitaphelenchidae) in packaging wood from Taiwan and South Korea - a new species belonging to the *fungivorus* group. *Journal of Nematode Morphology and Systematics* 8: 7-17.
- BURGERMEISTER, W., BRAASCH, H., METGE, K., GU, J., SCHRÖDER, T. & WOLDT, E. 2009. ITS-RFLP analysis, an efficient tool for differentiation of *Bursaphelenchus* species. *Nematology* 11: 649-668.
- FRANKLIN, M.T. & HOOPER, D.J. 1962. *Bursaphelenchus fungivorus* n. sp. (Nematoda, Aphelenchoidea) from rotting *Gardenia* buds infected with *Botrytis cinerea* Purs. Ex Fr. *Nematologica* 8: 136-142.
- GIBLIN, R.M. & KAYA, H.K. 1983. *Bursaphelenchus seani* n. sp. (Nematoda, Aphelenchoididae), a phoretic associate of *Anthophora bomboidea stanfordiana* Cockerell, 1904 (Hymenoptera, Anthophoridae). *Revue de Nématologie* 6: 39-50.
- GU, J. & WANG, J. 2010. Description of *Bursaphelenchus braaschae* sp. n. (Nematoda: Aphelenchoididae) found in dunnage from Thailand. *Russian Journal of Nematology* 18: 59-68.
- LI, H., TRINH, P.Q., WAEYENBERGE, L. & MOENS, M. 2008. *Bursaphelenchus chengi* sp. n. (Nematoda: Parasitaphelenchidae) isolated at Nanjing, China, in packaging wood from Taiwan. *Nematology* 10: 335-346.
- LOOF, P.A.A. 1964. Free-living and plant parasitic nematodes from Venezuela. *Nematologica* 10: 201-300.
- RÜHM, W. 1956. Die Nematoden der Ipiden. *Parasitologische Schriftenreihe* 6: 1-435.
- SCHÖNFELD, U., BRAASCH, H. & BURGERMEISTER, W. 2006. *Bursaphelenchus* spp. (Nematoda: Parasitaphelenchidae) in wood chips from sawmills in Brandenburg and description of *Bursaphelenchus willibaldi* sp. n. *Russian Journal of Nematology* 14: 119-126.
- SEINHORST, J.W. 1959. A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4: 67-69.
- STEINER, G. 1935. Opuscula miscellanea nematologica, II. 2. *Aphelenchoides hunti* n. sp., a new nematode parasitic in tiger lily bulbs (*Lilium tigrinum*) and fruits of the tomatillo (*Physalis ixocarpa*). *Proceedings of the Helminthological Society of Washington* 2: 104-110.
- TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.
- YE, W., GIBLIN, R.M., BRAASCH, H., MORRIS, K. & THOMAS, W.K. 2007. Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution* 43: 1185-1197.
- WANG, J. & GU, J. 2012. *Bursaphelenchus paraburgeri* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Malaysia. *Nematology* 14: 39-50.

Jianfeng Gu, Jialing Wang, Xianfeng Chen. *Bursaphelenchus parathailandae* sp. n. (Nematoda: Parasitaphelenchidae) из упаковочной тайваньской древесины.

Резюме. Описан *Bursaphelenchus parathailandae* sp. n., изолированный в Нингбо (Китай) из упаковочной древесины сосны, импортированной из Тайваня. Новый вид характеризуется сравнительно тонким телом ($a = 30-42$), латеральными полями с 4 линиями, экскреторной порой на расстоянии 1-1,5 диаметра тела за медианным бульбусом, вульварным процентом 71-75%, четко выступающими, но не формирующими вульварную складку вульварными губами, отростком задней матки, составляющим от 1/3 до 2/3 длины участка вульва-анус, хвостовым концом самки, составляющим от 5 до 6 анальных диаметров, длинным нитевидным хвостовым отростком с тупо округленной оконечностью, сравнительно мелкими и тонкими спикулами (15-18 мкм) с тупо оканчивающимся рострумом и дистальным концом без кукулюса, высоким, но не загнутым на спинную стороны кондилусом, характерной темной частью края спикулы, обращенной к кондилусу, небольшой и плохо различимой бурсой. Новый вид принадлежит к группе видов *fungivorus* в пределах рода *Bursaphelenchus* и наиболее близок к *B. thailandae*, от которого, как и от других видов *Bursaphelenchus*, отличается своей морфологией и последовательностью ITS- участка рибосомальной ДНК.
