

***Bursaphelenchus doui* sp.n. (Nematoda: Parasitaphelenchidae) in packaging wood from Taiwan and South Korea – a new species of the xylophilus group**

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Summary. *Bursaphelenchus doui* sp. n. isolated in China from coniferous packaging wood imported from Taiwan and Korea, is described and illustrated. The new species belongs to the xylophilus group. It is characterized by a body length averaging 876 and 811 μ m for females and males, respectively, a=33 and 29, a 15 μ m long stylet, four lateral lines, a long postuterine branch and conoid female tail (c=23) with slightly ventrally bent terminus and a distinct mucro in ventral position, male with large spicules (34-43 μ m) with distinct rostrum and cucullus, dorso-ventrally visible terminal bursa and three pairs and a single caudal papillae. The morphological differentiation from other group species is mainly based on the shape of the female tails and the size of the spicules. The new species can be differentiated from *B. abruptus*, *B. xylophilus*, *B. mucronatus*, *B. fraudulentus*, *B. conicaudatus* and *B. luxuriosae* by means of ITS-RFLP patterns.

Key words: *Bursaphelenchus doui* sp. n., *Bursaphelenchus* spp., China, distribution, ITS-RFLP analysis, Korea, morphology, morphometrics, packaging wood, Parasitaphelenchidae, Singapore, taxonomy.

A high percentage of loaded shipping containers is equipped with packaging wood from both hardwood or coniferous wood. Solid wood packaging material is usually milled from unprocessed raw wood from very low quality trees without commercial value, or from dead or dying trees. These are more likely than healthy trees to harbour insects and other organisms including dangerous pests, such as the pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 and/or its vectors of the genus *Monochamus*. The likelihood of entry of *Monochamus* beetles carrying *B. xylophilus* is very high when imports come from countries where the pine wood nematode is present and widespread. Most of the pine wood nematodes and *Monochamus* spp. intercepted on imported materials have been found in packaging wood. Therefore, packaging wood is considered the most

likely pathway for the introduction of the pine wood nematode. Unprocessed wood packaging material, which has not been treated by chemicals or heat, may also contain other *Bursaphelenchus* species (Braasch *et al.*, 2001; Tomiczek *et al.*, 2003) and can serve as a pathway for their introduction.

Following the FAO Guidelines for Regulating Wood Packaging Material in International Trade, plant quarantine border or post-border inspections of packaging wood are carried out in many countries in order to ensure that the import requirements for packaging wood have been met by the exporting country. In China, almost all imported wooden packages were inspected and sampled in recent years. Several *Bursaphelenchus* spp. were repeatedly found among the nematode species detected in wooden packaging material originating in East Asian countries, at the Ningbo

Entry-exit Inspection and Quarantine Bureau. An undescribed *Bursaphelenchus* species of the xylophilus group (Braasch, 2001) was found in packaging wood from Taiwan and South Korea (Republic of Korea). Morphological features and ITS-RFLP patterns of this new species are presented in this paper.

MATERIAL AND METHODS

Samples taken from solid wooden packaging materials imported with other goods were sawn into about 10 cm long pieces which were subsequently cut into smaller pieces no more than 1 cm wide. Nematodes were extracted by the modified Baermann funnel technique for 48 h at 25°C and morphologically studied using both an Olympus BX50 microscope fitted with a Furi CCD camera and a Zeiss Axioscope microscope. The new species multiplied on *Botrytis cinerea* growing on malt agar. A multiple specimen isolate culture was established. Measurements were made on heat-killed and TAF-fixed specimens from a culture, mounted in glycerine.

Nematode DNA was extracted from samples containing one to about 20 nematode specimens and purified by alcohol precipitation as described by Braasch & Burgermeister (2002). Alternatively, DNA was extracted using the QIAamp DNA Micro Kit (Qiagen) according to the instructions provided by the manufacturer. In the latter procedure, DNA is purified by reversible adsorption to a silica matrix. DNA concentration of samples obtained from more than one specimen was determined fluorimetrically using a DyNa Quant 200 fluorimeter (Amersham Biosciences) and the fluorescent dye, Hoe 33258.

For ITS-RFLP analysis, a segment of nematode ribosomal DNA was amplified by PCR as described by Braasch & Burgermeister (2002). Suitable aliquots of the amplified DNA were digested with 3 units of the restriction endonucleases *Alu* I, *Hae* III, *Hinf* I, *Msp* I and *Rsa* I following the manufacturer's instructions. Restriction fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

The *Bursaphelenchus* isolates used in ITS-RFLP analysis for comparison with *B. doui* sp. n. were *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000, *B. fraudulentus* Rühm, 1956, *B. mucronatus* Mamiya & Enda, 1979 (European and East Asian types) and *B. xylophilus* (Steiner & Buhner, 1934) Nickle, 1970. They were cultured on *Botrytis cinerea* on malt agar.

DESCRIPTION

Bursaphelenchus doui sp.n. (Figs. 1-4)

Measurements: Table 1.

Female. Body cylindrical and slim tapering at both ends. Heat relaxed form ventrally arcuate. Cuticle marked by fine annules. Lateral field 2-3 µm wide with four lines. Lip region convex, 4 µm high, 8-9 µm wide, and offset by a distinct constriction. Stylet slender with only very small basal swellings, shaft constituting about 65 % of total stylet length. Procorpus cylindrical. Median bulb well developed, round or slightly oval. Valve plates in the middle of the median bulb. Oesophageal gland lobe overlapping intestine dorsally for the length of two, to three body widths. Excretory pore position at level of median bulb. Reproductive system prodelphic, gonad outstretched, occupying about half of the body length. Oocytes first arranged as multiple rows, further down the body occur in single file. Spermatheca irregular ovoid. Anterior vulval lip posteriorly prolonged forming a 10-16 µm long vulval flap. Body slightly narrowing behind the vulva. Postuterine branch extending up to 80% of vulva to anus distance, acting as a seminal receptacle. Tail moderately narrowing to a slightly ventrally bent terminus with a distinct ventral mucro of 2 to 4 µm length that tapers, sometimes appearing hairlike.

Male. Anterior body region and cuticle similar to that of female. Body bent ventrally when killed by heat. Testis outstretched, occupying about half to three quarters of the body length. Spermatocytes arranged in multiple rows. Spicules paired, large and arcuate with pointed rostrum. The middle part of the spicules nearly straight. Distal ends of spicules with small cucullus. Tail ventrally arcuate with a pointed, talon-like terminus bearing a distinct, oval-shaped terminal bursa, which can be seen in dorso-ventral position. There are three pairs of ventro-lateral caudal papillae (one adanal and two adjacent subventral postanal papillae at the origin of the bursal flap) and a single mid-ventral papilla before the anus. Their position is shown in Figures 1 and 3.

Diagnosis and relationships. *Bursaphelenchus doui* sp. n. clearly belongs to the xylophilus group having males with the typically shaped spicules with a cucullus at their distal extremity, the typical number and position of caudal papillae (particularly two adjacent pairs postanal just before the bursa) and the anterior vulval lip of the

females developed as a distinct flap. The new species possesses a body length of 876 (634 - 1143) μm and 811 (629-948) μm of females and males, respectively, robust body ($a=33$ and 38 , resp.) tapering anteriorly as well as posteriorly, $15\ \mu\text{m}$ long stylet, lateral field with four lines, long postuterine branch extending up to 80% of vulva to anus distance and a conoid female tail ($c=23$) with only slightly ventrally-bent terminus showing a distinct mucro in ventral position, male with large spicules ($34\text{--}43\ \mu\text{m}$ long), distinct rostrum and small cucullus, and a dorso-ventrally visible oval terminal bursa.

Having the diagnostic characters of the xylophilus group, *B. doui* sp. n. is easily distinguishable from all other *Bursaphelenchus* species except species of this group. It is morphologically most similar to *B. mucronatus* Mamiya and Enda, 1979, *B. kolymensis* Korenchenko, 1980, *B. fraudulentus* Rühm, 1956 and *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000, but also to *B. baujardi* Walia, Negi, Bajaj & Kalia, 2003, *B. luxuriosae* Kanzaki & Futai, 2003, *B. xylophilus* (Steiner & Buhner, 1934) Nickle, 1970 and *B. abruptus* Giblin-Davis, Mundo-Ocampo, Baldwin, Norden & Batra, 1993. The differentiation is mainly based on the shape of the female tails, whereas the spicules of xylophilus group males widely resemble each other. However, *B. doui* sp. n. differs from the other species of the xylophilus group by the larger spicules.

B. doui sp. n. differs also from *B. abruptus*, which is molecularly different from the xylophilus group, by the larger spicules ($34\text{--}43$ vs $23\text{--}30\ \mu\text{m}$) and the longer postuterine sac of females averaging only 28% of the vulva-anus distance in *B. abruptus* (Giblin-Davis *et al.*, 1993).

B. doui sp. n. differs from *B. luxuriosae* by size of the spicules ($34\text{--}43$ vs $27\text{--}30\ \mu\text{m}$) and the presence of a mucro.

B. doui sp. n. differs from *B. xylophilus* mainly by the conoid female tail in comparison to the cylindrical tail with rounded tail terminus without mucro in *B. xylophilus*. The mucronate form of *B. xylophilus* and the mucronate species *B. mucronatus*, *B. kolymensis*, *B. fraudulentus*, *B. conicaudatus* and *B. baujardi* are very difficult to distinguish morphologically from *B. doui* sp. n. However, they all have smaller spicules and deviations in the form of spicules lacking the straight area in the spicules' central part. *B. fraudulentus* has usually an indistinct mucro and is reported as having spicules of $21\text{--}22\ \mu\text{m}$ length (Rühm, 1956). *B. conicaudatus* and *B. baujardi*, which both possess a short mucro have shorter

spicules of $23\text{--}28\ \mu\text{m}$ and $25\text{--}28\ \mu\text{m}$ length, respectively, than *B. doui* sp. n. (Kanzaki *et al.*, 2000; Walia *et al.*, 2003). The bursa of *B. doui* sp. n. is not trapezoid as described for *B. conicaudatus*. In addition, *B. doui* sp. n. differs from *B. conicaudatus* by the overall shape of the female tail, which tapers strongly after the anus in *B. conicaudatus*, whereas the tail of *B. doui* sp. n. is broader as in *B. mucronatus*. The most similar species is *B. mucronatus* and particularly its European type. The bursa of *B. mucronatus* and the location of the excretory pore are variable, but *B. doui* sp. n. distinctly differs from this species by the larger spicules and the straight area in the central part of the lamina. *B. kolymensis* is possibly synonymous with *B. mucronatus*.

Molecular differentiation of *B. doui* sp. n. from similar species. An ITS-PCR product of approximately 1000 bp was obtained with *B. doui* sp. n. (Fig. 4). Digestion of the PCR product with five restriction enzymes resulted in a typical ITS-RFLP pattern with fragments of 450 bp, 310 bp, 240 bp (*Rsa* I); 650 bp, 210 bp, 80 bp (*Hae* III); 330 bp, 280 bp, 170 bp, 120 bp (*Msp* I); 300 bp, 240 bp, 170 bp (*Hinf* I); 620 bp, 360 bp (*Alu* I). The ITS-RFLP pattern of *B. doui* sp. n. is different from the patterns of the four morphologically most similar species of the xylophilus group (Fig. 4). It is also distinct from the ITS-RFLP patterns of the following *Bursaphelenchus* species obtained in earlier investigations: *B. leoni* Baujard, 1980, *B. sexdentati* Rühm, 1956 (see Hoyer *et al.*, 1998); *B. eggersi* Rühm, 1956, *B. poligraphi* Fuchs, 1937, *B. borealis* Korenchenko, 1980, *B. fungivorus* Franklin & Hooper, 1962, *B. hofmanni* Braasch, 1998 (see Braasch *et al.*, 1999); *B. paracorneolus* Braasch, 2000 (= *Bursaphelenchus* spec. DE-14(w) in Braasch *et al.*, 1999); *B. hylobianum* Korenchenko, 1980, *B. abietinus* Braasch & Schmutzenhofer, 2000, *B. rainulfi* Braasch & Burgermeister, 2002, *B. hellenicus* Skarmoutsos, Braasch & Michalopoulou, 1998 (see Braasch & Burgermeister, 2002); *B. vallesianus* Braasch, Schönfeld, Polomski & Burgermeister, 2004, *B. pinophilus* Brzeski & Baujard, 1997 (see Braasch *et al.*, 2004); *B. thailandae* Braasch & Braasch-Bidasak, 2002, *B. lini* Braasch 2004, *B. tusciae* Ambrogioni & Marinari Palmisano, 1998, *B. abruptus* Giblin-Davis, Mundo-Ocampo, Baldwin, Norden & Batra, 1993 and *B. luxuriosae* Kanzaki & Futai, 2003 (Burgermeister & Braasch, unpublished).

Type locality and habitat. *B. doui* sp. n. was isolated in June 2004 by Gu Jianfeng from coniferous packaging wood exported from Taiwan and inspected at the Ningbo Entry-exit Inspection and

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

	Females		Males	
	Holotype	Paratype	Allotype	Paratype
n	1	15	1	15
L	667.0	875.9 \pm 112.9 (634-1143)	754.0	811.3 \pm 84.1 (629.0-948.0)
a	26.6	32.6 \pm 3.5 (26.6-37.3)	26.0	28.8 \pm 4.5 (25.0-33.0)
b	7.8	9.5 \pm 1.8 (6.5-12.9)	8.9	8.4 \pm 0.8 (6.3-9.7)
c	19.7	23.4 \pm 2.2 (19.7-28.6)	20.7	21.9 \pm 3.4 (17.5-30.7)
c'	3.5	3.6 \pm 0.3 (2.8-4.2)	1.9	1.9 \pm 0.2 (1.5-2.3)
V	74.8	74.9 \pm 1.7 72.1-78.3	—	—
Postuterine sac	100.3	111.2 \pm 10.7 (100.0-120.4)	—	—
Stylet	15.2	15.1 \pm 0.8 (13.0-16.2)	14.5	15.2 \pm 0.7 (14.1-15.9)
Spicules as bow	—	—	38.0	39.6 \pm 2.5 (33.8-43.3)

Quarantine Bureau, China. Gu Jianfeng detected the same species from coniferous packaging wood exported from Korea to China in August 2004.

Type specimens. Collected from a culture on *Botrytis cinerea* on malt agar, developed from a sample taken in China from packaging wood arriving from Taiwan with other commodities. Slides are deposited in the nematode collection of Ningbo Entry-exit Inspection and Quarantine

Bureau, China, in the nematode collection of Helen Braasch, Germany, and in the nematode collection of the Federal Biological Research Centre for Agriculture and Forestry, Germany. A culture is available in the Federal Biological Research Centre for Agriculture and Forestry in Braunschweig, Germany.

Etymology. *B. doui* sp. n. was named after Doudou, the son of Gu Jianfeng.

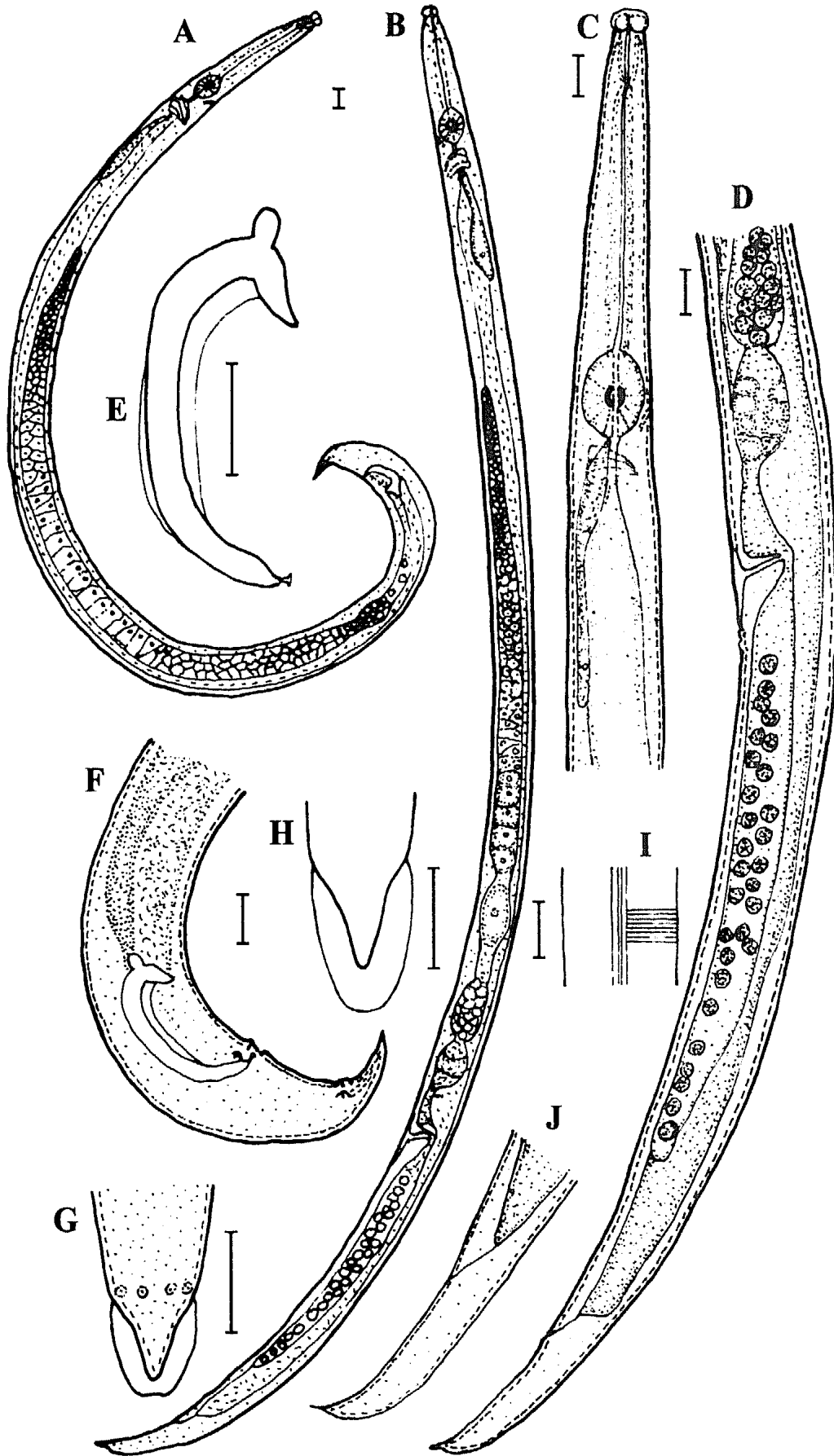


Fig. 1. *Bursaphelenchus doui* sp. n. A: Male; B: Female; C: Head region; D: Female vulval region and tail; E: Spicules; F: Male tail; G & H: Bursa; I: Lateral field; J: Female tail. (Scale bars=10 μ m)

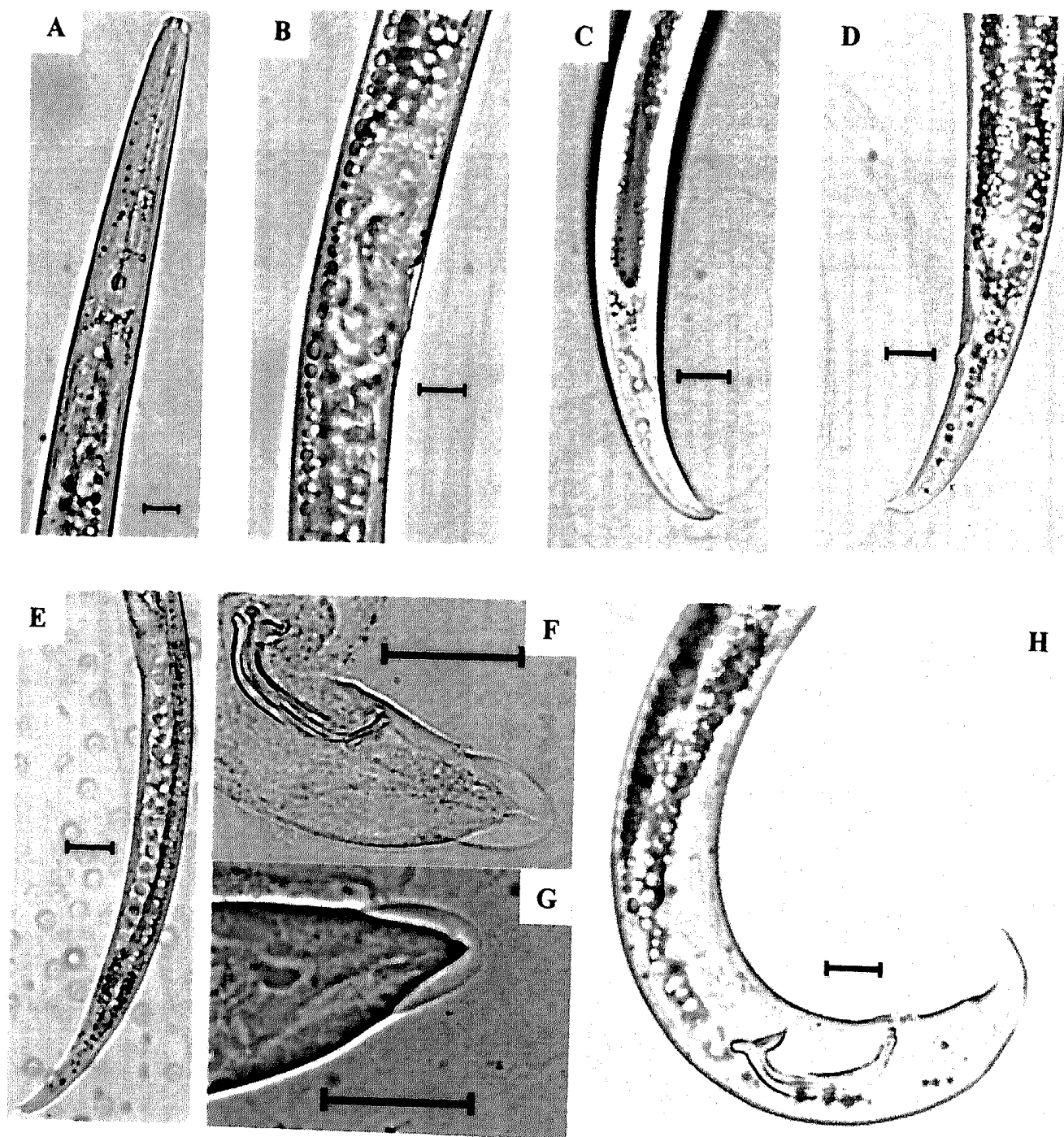


Fig. 2. Light photomicrographs of *Bursaphelenchus doui* sp. n. A: Head region; B: Vulval region. C, D & E: Female tail; F & G: Bursa; H: Male tail. (Scale bars: A-E, H – 10 μ m, E – 20 μ m F,G – 25 μ m).

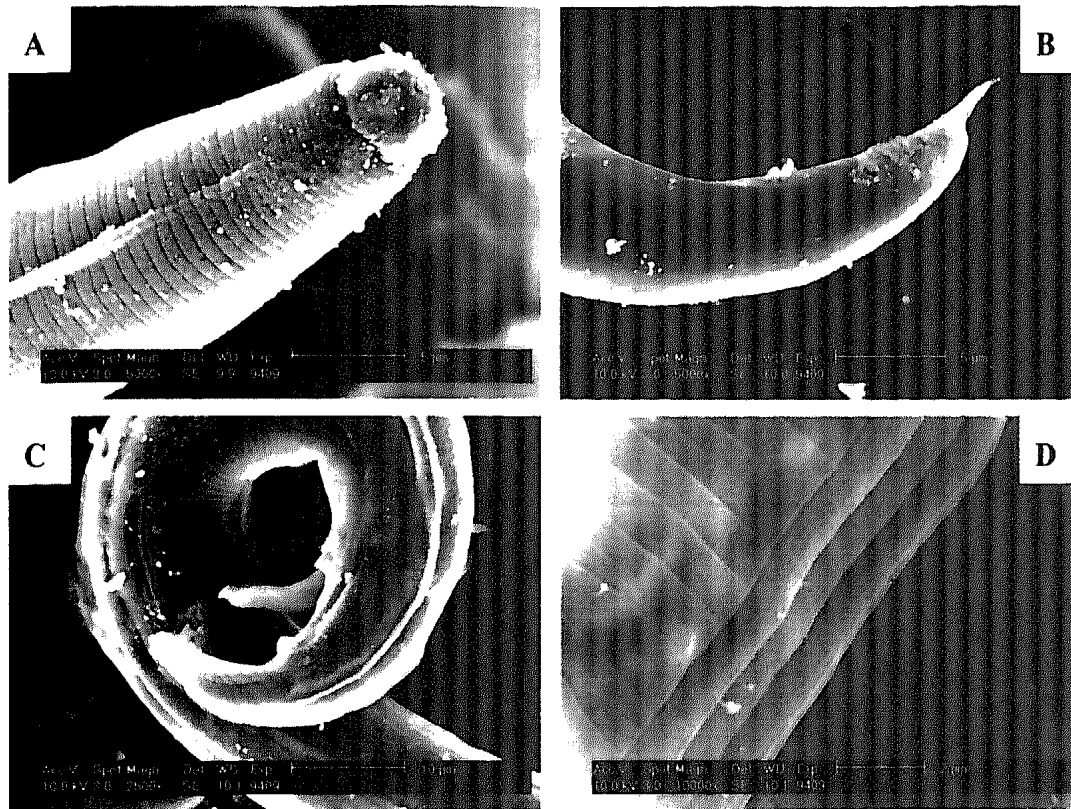


Fig. 3. Scanning electron microscope (SEM) observations of *Bursaphelenchus doui* sp.n. A: Head region; B: Female tail; C: Details of male tail showing spicule tip (cucullus) and papillae; D: Female cuticle showing four lateral lines.

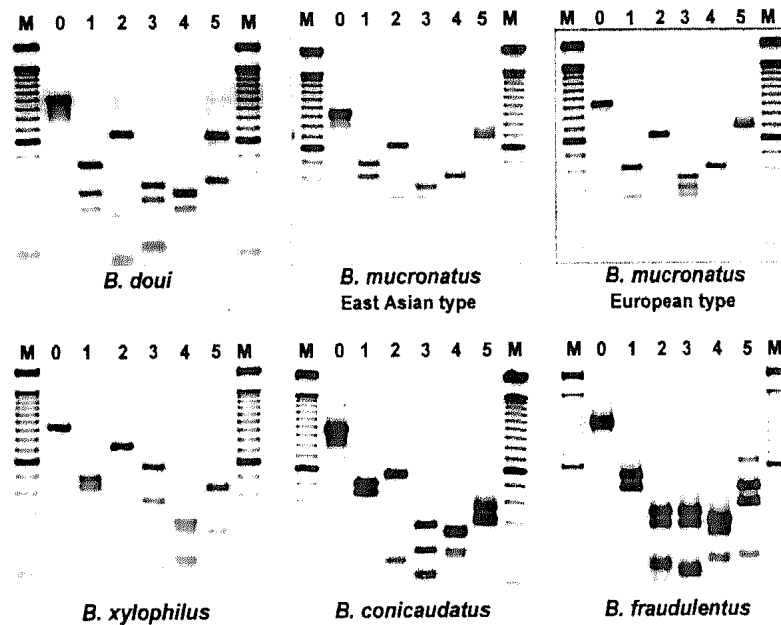


Figure 4. ITS-RFLP patterns of *Bursaphelenchus doui* sp. n. and four morphologically similar species of the xylophilus group. Each picture shows (from left to right) DNA marker (100 bp ladder, Invitrogen), rDNA amplification product, restriction fragments obtained with *RsaI*, *HaeIII*, *MspI*, *HinfI*, *AluI*, and again DNA marker.

DISCUSSION

The damage caused by the pine wood nematode on three continents and the recognition of packaging wood as an important pathway for its international distribution has led to increased wood sampling. Other species of the *xylophilus* group of the genus *Bursaphelenchus* were found in the course of these surveys. Whereas the widely distributed species *B. xylophilus*, *B. fraudulentus*, *B. mucronatus* and also *B. kolymensis* (possibly synonymous with *B. mucronatus*) have been known for a long time, three other species of the *xylophilus* group were described in recent years. They all were detected in Asia (Kanzaki et al. 2000; Kanzaki & Futai 2003; Walia et al., 2003) and two of them on non-coniferous trees (*B. conicaudatus*, *B. luxuriosae*). *Bursaphelenchus xylophilus*, *B. mucronatus*, *B. kolymensis* and the recently described species *B. baujardi* live in coniferous trees. *Bursaphelenchus fraudulentus* has been found in both coniferous and deciduous trees. The description of a further species of the *xylophilus* group, *B. doui* sp. n., again from East Asia seems to indicate that this region has a remarkable range of species in this group of the genus *Bursaphelenchus*. *B. doui* sp. n. was also found in packaging wood arriving from South Korea to China, and the species status of the two provenances was confirmed molecularly. Further investigation is necessary to explore its actual distribution, life cycle and biology. The important differentiation of the quarantine pest *B. xylophilus* from the other species of the *xylophilus* group is reliably supported by use of the ITS-RFLP technique. The description of more mucronate species of the *xylophilus* group beside *B. mucronatus* and *B. fraudulentus* and the occasional presence of a mucro on females of *B. xylophilus* (special strains or variation in round-tailed natural populations) make the ITS-RFLP method an indispensable tool of species identification.

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Braasch H., Gu J., Burgermeister W., Zhang J. *Bursaphelenchus doui* sp.n. (Nematoda: Parasitaphelenchidae) из упаковочной древесины с Тайваня и Южной Кореи – новый вид группы *xylophilus*.

Резюме. Дано первоописание и иллюстративный материал для *Bursaphelenchus doui* sp. n., изолированного в Китае из упаковочной древесины хвойных пород импортированной с Тайваня и из Южной Кореи. Новый вид принадлежит к группе *xylophilus* и характеризуется средней длиной тела самок - 876 μm и, самцов - 811 μm , значением индекса «а» 33 и 29, соответственно, стилетом длиной 15 μm , четырьмя линиями латерального поля, длинным рудиментом задней матки и коническим хвостом самок («с» = 23) со слабо загнутым на вентральную сторону терминусом, а также хорошо различимым мукро смещенным на вентральную сторону, крупными спикулами самцов (34 - 43 μm) с хорошо различимым рострумом и кукулюсом, различимой в дорсо-вентральном аспекте терминальной бурсой и тремя парами хвостовых папилл. Морфологическая дифференциация нового вида от других видов группы основывается в основном на форме хвостового конца самки и размере спикул. Новый вид также может быть дифференцирован от *B. abruptus*, *B. xylophilus*, *B. mucronatus*, *B. fraudulentus*, *B. conicaudatus* и *B. luxuriosae* по профилям рестрикции ITS-участка рибосомальной ДНК.
