Nematology, 2012, Vol. 14(4), 395-404

Bursaphelenchus firmae n. sp. (Nematoda: Aphelenchoididae), isolated from *Monochamus grandis* Waterhouse that emerged from dead firs, *Abies firma* Sieb. *et* Zucc.

Natsumi KANZAKI [1,*], Noritoshi MAEHARA [2], Takuya AIKAWA [2] and Kazuma MATSUMOTO [1]

[1] *Forestry and Forest Products Research Institute (FFPRI), 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan*
[2] *Tohoku Research Center, FFPRI, 92-25 Nabeyashiki, Shimo-Kuriyagawa, Morioka, Iwate 020-0123, Japan*

Received: 28 May 2011; revised: 7 September 2011
Accepted for publication: 7 September 2011; available online: 5 December 2011

Summary – *Bursaphelenchus firmae* n. sp. is described. This new species was isolated during a field survey of longhorn beetle-associated nematodes. The fourth-stage dispersal (dauer) juveniles of the new species were recovered from dissected bodies (tracheal system) of *Monochamus grandis*, which emerged from dead logs of Japanese fir, *Abies firma*, collected from Hachioji, Tokyo, Japan. The new species is mid-sized for the genus, with females 603-828 μm and males 530-698 μm long. Four lateral lines occur on the body surface and seven genital papillae are found in males (P1 ventral single papilla and P2-P4 pairs). A long and arcuate spicule forms a trapezium in lateral view and a rather large, sub-squared, bursal flap and a vulva with conspicuous vulval flap are present. The female tail is smoothly tapered and possesses a conspicuous and blunt mucro. Based upon its diagnostic morphological characters, the new species belongs to the *B. xylophilus* group of the genus, and is closely related to *B. fraudulentus*, *B. mucronatus*, *B. doui*, *B. macromucronatus* and *B. populi*. It is distinguished from these five species by the morphology of the male bursal flap and the female mucro and several morphometric values. Molecular phylogenetic analyses inferred from D2/D3 LSU suggest that the new species is close to *B. mucronatus* and *B. xylophilus*, *i.e.*, these three species form a well supported monophyletic clade within the genus. Although the new species has a weak pathogenicity to pine trees, it does not seem to be a severe risk to native pine forests.

Keywords – *Bursaphelenchus xylophilus* group, Cerambycidae, molecular, morphology, morphometrics, new species, phoresy, taxonomy.

The *Bursaphelenchus xylophilus* group *sensu* Braasch *et al.* (2009) in the genus *Bursaphelenchus* Fuchs, 1937 contains many cerambycid (longhorn beetle)-associated plant-parasitic/mycophagous nematodes. *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 and *B. mucronatus* Mamiya & Enda, 1979 are associated with *Monochamus* spp., and *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000 and *B. luxuriosae* Kanzaki & Futai, 2003 are associated with *Psacothea hilaris* (Pascoe) and *Acalolepta luxuriosa* (Bates), respectively (see Ryss *et al.*, 2005; Kanzaki *et al.*, 2009). During a field survey of cerambycid-associated *Bursaphelenchus* nematodes, an undescribed species from the *B. xylophilus* group was isolated from *Monochamus grandis* Waterhouse, which emerged from the Japanese fir *Abies firma* Sieb. *et* Zucc. The nematode was kept as a laboratory culture coded as "*Bursaphelenchus* sp. NK224". It was tested for its pathogenicity to pine trees in previous studies and is regarded to have weak pathogenicity (Kanzaki *et al.*, 2011a; Maehara *et al.*, 2011). The nematode formerly referred to as *Bursaphelenchus* sp. NK224 is taxonomically described and figured herein as *B. firmae* n. sp.

Materials and methods

NEMATODE ISOLATION

Several Japanese firs planted at the Tama Forest Science Garden of the Forestry and Forest Products Research Institute (FFPRI), Hachioji, Tokyo, Japan, were felled and cut into *ca* 1 m logs in the early summer of 2006. The logs were then left at the site until early spring 2007, and transferred to an insect cage facility at the experimental field FFPRI, Tsukuba, Ibaraki, Japan.

* Corresponding author, e-mail: nkanzaki@affrc.go.jp

DOI:10.1163/156854111X602974

Adults of *M. grandis* emerged from those logs and were collected in the cage from 4-25 June 2007, and dissected under a light microscope to determine their nematode-harbouring organ. The nematodes were isolated from dissected insects using the Baermann funnel technique. The number and associated stage (*i.e.*, dauer juvenile, parasitic juvenile or parasitic adult form) of the nematodes were determined for each individual beetle under a light microscope. The nematodes isolated from the insects were transferred to a Petri dish where a *Botrytis cinerea* lawn was growing on 2% malt extract agar. The successfully propagated nematodes were kept as a laboratory culture with the culture code *Bursaphelenchus* sp. NK224.

Morphological observation and micrography

Adult nematodes from 1- and 2-week-old cultures were used for morphological observation, micrography and drawings. Microscopic morphological observations were conducted using living material obtained from 1- and 2-week-old cultures with the aid of a light microscope facilitated with DIC/Nomarski devices (Eclipse 80i; Nikon, Tokyo, Japan), and micrographs were taken with a digital camera system (DS-Ri1; Nikon) connected to the microscope. The permanent slides (type materials), which were used for drawings and measurements of morphometrics were prepared as follows: the nematodes from a 2-week-old culture were heat-killed and fixed in TAF, processed with a glycerin-ethanol series using Seinhorst's methods and mounted in glycerin according to the methods of Maeseneer and d'Herde (described in Hooper, 1986).

Molecular profiles

A DNA sample from *B. firmae* n. sp. was collected and prepared as described by Kikuchi *et al.* (2009). The DNA base sequences of partial ribosomal DNA (*ca* a 1.6-kb near-full-length small subunit (SSU), *ca* 1.0 kb of an internal transcribed spacer region, which contains ITS1, the 5.8S small RNA subunit ITS2 (ITS) and a 0.7-kb D2/D3 expansion segment of the large subunit (D2/D3 LSU)) were determined for *B. firmae* n. sp. following Kanzaki and Futai (2002a) and Ye *et al.* (2007). The SSU, D2/D3 LSU and ITS were stored in the GenBank database with accession numbers AB650014, AB650015 and AB663192, respectively.

The sequences obtained were compared to those of other *B. xylophilus* group species stored in the GenBank database. The sequences employed for the analysis (operational taxonomic unit: OTU) were selected according to Kanzaki *et al.* (2011b).

The molecular phylogenetic status of the new species within the *B. xylophilus* group was determined based upon the D2/D3 LSU sequence using Bayesian analysis as the SSU does not yield sufficiently good resolution to determine the species status among closely related species (Kanzaki & Futai, 2002a; Ye *et al.*, 2007). The compared sequences were aligned using the MAFFT program (Katoh *et al.*, 2002; web version available at: http://align.bmr.kyushu-u.ac.jp/mafft/software/) and the base substitution model was evaluated using MODELTEST version 3.7 (Posada & Crandall, 1998). The Akaike-supported model, the log likelihood ($\ln L$), the Akaike information criterion (AIC), the proportion of invariable sites and the gamma distribution shape parameters and substitution rates were used in the phylogenetic analyses. Bayesian analysis was performed using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001) by running the chain for 1×10^6 generations and setting the 'burn in' at 1000. We used Markov Chain Monte Carlo methods within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using a 50% majority rule.

Results

Bursaphelenchus firmae* n. sp.

= *Bursaphelenchus* sp. NK224 *apud* Kanzaki *et al.*, 2011a *et* Maehara *et al.*, 2011

(Figs 1, 2)

Measurements

See Table 1.

Description

Adults

Mid-sized species, *i.e.*, 600-830 μm and 530-700 μm in length for females and males, respectively. Body cylindrical, ventrally arcuate when killed by heat treatment. Male tail strongly recurved ventrally when killed by heat. Cuticle thin, annulated, lateral field with four incisures. Head

* This species name is derived from the name of its host tree, *Abies firma*.

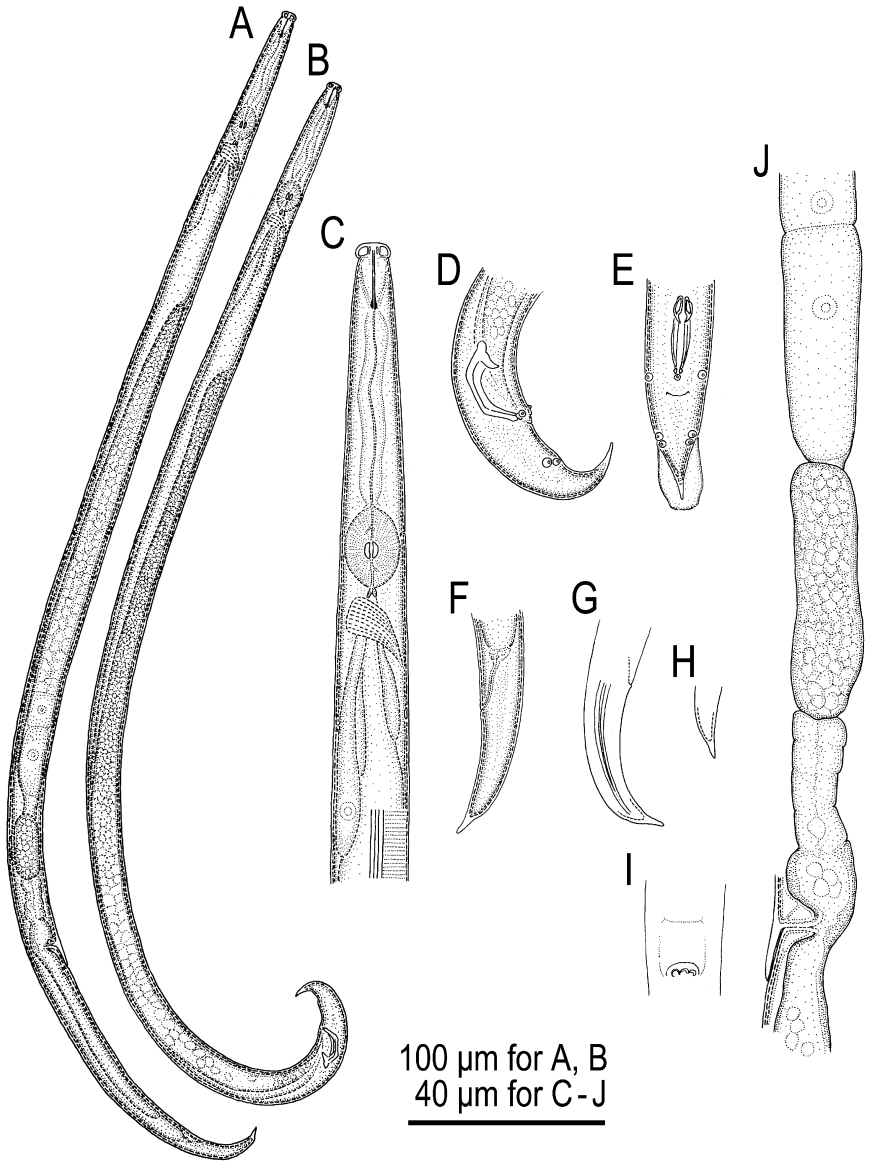

Fig. 1. Bursaphelenchus firmae *n. sp. A: Entire adult female; B: Entire adult male; C: Anterior region; D: Lateral view of male tail; E: Ventral view of male tail; F: Lateral view of female tail; G: Lateral view of female tail (surface); H: Variation in female tail tip; I: Ventral view of female vulval region; J: Lateral view of female reproductive tract (ovary, oviduct mostly occupied by developed oocytes, spermatheca filled with sperm, crustaformeria, uterus containing sperm, vagina and post-uterine sac containing sperm from anterior).*

distinctly offset from body, separated by a clear constriction, lip region in lateral view squarish-round, about twice as broad as high. Stylet with narrow lumen, in two parts with a short cone *ca* one-third of total stylet length and a shaft with a clear basal swelling. Procorpus cylindrical, *ca* three stylet lengths or three metacorpal lengths long, ending in a well developed metacorpus (median bulb). Dorsal pharyngeal gland orifice opening into lumen of metacorpus midway between anterior end of metacorpal valve and anterior end of metacorpus. Pharyngo-intestinal junction immediately posterior to metacorpus. Dorsal pharyngeal gland overlapping intestine dorsally and *ca* four metacorpal lengths long. Nerve ring surrounding pharyngeal glands and intestine at *ca* one-third of stylet length (equivalent to *ca* one-third of metacorpal length) posterior to pharyngo-intestinal junction. Hemizonid distinc-

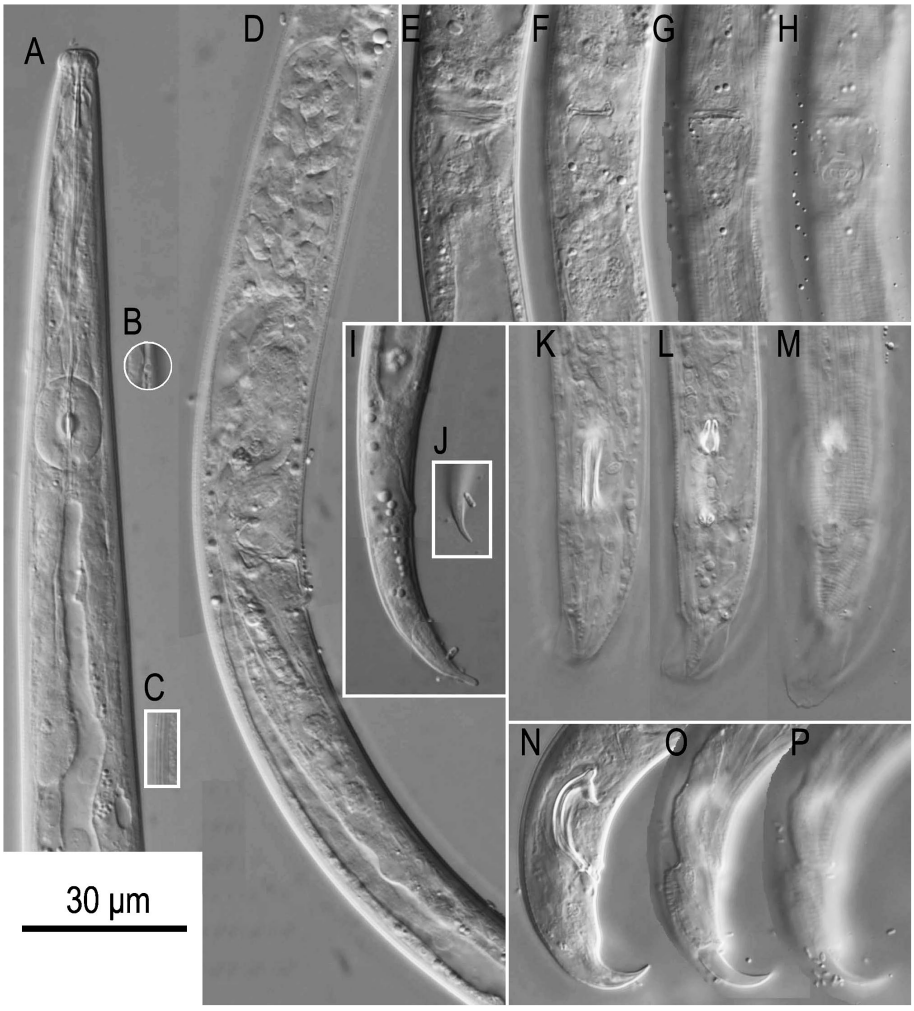

Fig. 2. Bursaphelenchus firmae *n. sp. A: Anterior region of adult female; B: Secretory-excretory pore (encircled); C: Lateral field; D: Lateral view of female vulval region (spermatheca filled with sperm, crustaformeria, uterus containing sperm, vagina and post-uterine sac containing sperm from anterior; E-H: Ventral view of female vulval region in different focal planes; I: Lateral view of female tail; J: Variation in female tail tip; K-M: Ventral view of male tail; N-P: Lateral view of male tail in different focal planes.*

tive in permanent mount materials, *ca* two stylet lengths or two metacorpal lengths posterior to nerve ring. Secretory-excretory pore located at, or a little less than, one stylet length or one metacorpal length posterior to metacorpus.

Female

Reproductive tract to right of intestine, comprising ovary, oviduct, spermatheca, crustaformeria, uterus, vagina + vulva and post-uterine sac. Ovary single, anteriorly outstretched. Oocytes present in multiple (2-4) rows in most parts of ovary and a single well developed oocyte sometimes located at posterior end of ovary or in oviduct. Oviduct tube-like, sometimes occupied by well developed oocytes. Spermatheca formed by distinctive, thick tissue, slightly irregular elongated oval in shape, sometimes filled with well developed sperm. Crustaformeria not conspicuous, formed by relatively large cells. Uterus with thick walls. Vagina perpendicular to body surface, with uterine wall nearest to vagina (uterus/post-uterine sac junction) thickened. Vulva with a conspicuous vulval flap, with a pair of conspicuous vulval papillae located immediately posterior to vulval flap. Post-uterine sac long, *ca* 5-7 vulval body diam., extending for *ca* 75% of vulval-anal distance, sometimes filled with sperm.

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

	Female		Male
	Holotype	Paratypes	Paratypes
n	–	15	16
L	674	671 ± 52 (603-828)	623 ± 43 (530-698)
a	33.1	31.4 ± 2.3 (27.1-34.0)	31.8 ± 2.3 (28.9-36.2)
b	10.1	10.1 ± 0.6 (9.3-11.9)	9.6 ± 0.6 (8.5-10.8)
c	18.5	20.6 ± 1.9 (18.0-24.1)	21.3 ± 1.4 (18.7-23.4)
c′	3.5	3.4 ± 0.2 (2.9-3.8)	2.2 ± 0.2 (1.9-2.7)
M	40.0	37.8 ± 3.0 (32.5-42.1)	36.6 ± 2.2 (33.3-40.0)
V or T	70.3	70.7 ± 1.2 (68.8-72.8)	61.1 ± 7.6 (45.0-70.5)
Max. body diam.	21.5	21.5 ± 2.2 (18.0-25.0)	19.5 ± 1.8 (17.0-22.5)
Lip diam.	7.5	7.0 ± 0.4 (6.5-7.5)	6.5 ± 0.4 (6.0-7.5)
Lip height	4.0	3.5 ± 0.3 (3.0-4.0)	3.5 ± 0.2 (3.0-4.0)
Stylet conus	5.0	5.0 ± 0.5 (4.5-6.5)	5.0 ± 0.4 (4.0-5.5)
Stylet length	14.0	14.0 ± 0.7 (12.5-15.5)	13.5 ± 0.7 (12.0-15.0)
Median bulb length	15.5	15.5 ± 0.7 (14.5-17.5)	14.5 ± 1.2 (11.0-16.0)
Median bulb diam.	12.0	11.5 ± 0.7 (10.5-13.5)	11.0 ± 0.7 (9.5-12.0)
Median bulb length/diam.	1.3	1.4 ± 0.1 (1.2-1.6)	1.3 ± 0.1 (1.0-1.4)
Excretory pore[1)]	57	61 ± 5.0 (55-72)	62 ± 5.3 (51-73)
Excretory pore[2)]	8.5a	4.0a ± 4.3 (10.5a-3.0p)	1.0a ± 5.8 (14.0a-8.0p)
Hemizonid[1)]	87	86 ± 4.4 (77-92)	86 ± 3.1 (81-91)
Hemizonid[2)]	21.5	20.5 ± 3.1 (14.5-26.5)	23.0 ± 3.3 (17.5-28.0)
Ovary or testis length[3)]	235	233 ± 34 (174-324)	381 ± 58 (296-479)
Anterior gonad length[4)]	337	325 ± 42 (254-449)	–
Anal or cloacal body diam.	10.5	9.5 ± 0.7 (8.5-10.5)	13.0 ± 0.7 (12.0-14.0)
Tail length	36	33 ± 2.9 (28-38)	29 ± 2.7 (23-34)
Vulva-anus distance	164	164 ± 13 (144-197)	–
Post-uterine sac length	115	122 ± 7.7 (110-137)	–
Post-uterine sac/vulva to anus (%)	70.4	75.0 ± 4.1 (68.9-82.9)	–
Mucro length	4.0	4.0 ± 0.7 (3.0-5.0)	–
Spicule length[5)]	–	–	22.5 ± 0.8 (21.0-24.0)
Spicule length[6)]	–	–	19.5 ± 0.5 (19.0-20.5)
Capitulum length	–	–	7.5 ± 0.4 (7.0-8.5)
P1, P2 papillae from cloaca	–	–	3.0 ± 0.5 (2.5-4.0)
P3 papillae from cloaca	–	–	12.0 ± 1.3 (9.0-14.0)
P4 papillae from cloaca	–	–	13.0 ± 1.3 (10.0-15.0)
P2-P3/P3-P4 distance ratio	–	–	21.8 ± 1.7 (19.0-24.0)

[1)] Distance from anterior end.
[2)] Distance from posterior end of median bulb, a: anterior to, p: posterior to.
[3)] Ovary length does not include the other organs in female genital tract.
[4)] Length between vulva to anterior end of ovary.
[5)] Curve along arc from bottom of capitulum depression to distal end.
[6)] Condylus tip to distal end measured in a straight line.

Anus a small, dome-shaped slit in ventral view. Tail *ca* 3.5 times longer than anal body diam., weakly recurved ventrally when killed by heat, cylindrical, smoothly tapering to a tail tip possessing a thick and bluntly pointed mucro.

Male

Gonad to right of intestine, outstretched in most individuals. Spermatozoa arranged in 3-4 rows for most of its length. *Vas deferens* containing developed sperm, merg-

ing with distal part of intestine to form a simple tube connected to cloacal opening. Sperm amoeboid. Tail region strongly ventrally arcuate. Spicules large, arcuate, forming trapezium in lateral view, paired, separate. Capitulum triangular, consisting of short, rounded condyles and a short, pointed rostrum, shallow depression present at mid-point. Lamina-calomus complex thin, strongly ventrally bent at one- and two-thirds of distance from anterior end. Cucullus present at distal end, small, triangular, conspicuous. Gubernaculum absent. Bursal flap present, covering distal half of tail, sub-square in shape, possessing an irregular distal end. Seven (a single ventral and three pairs) conspicuous genital papillae present with single ventral papilla (P1) immediately anterior to cloacal opening. First subventral pair (P2) located immediately anterior to cloacal slit, almost at same level with P1. Second subventral pair (P3) located at *ca* 60% of tail length from cloacal slit. Third ventral pair (P4) immediately posterior to or almost at same level as P3. P2-P3 distance clearly (>20 times) longer than P3-P4 distance.

Type material

Type materials were obtained from a 2-week-old culture. Holotype female, five paratype males and five paratype females deposited in the United States Department of Agriculture Nematode Collection (USDANC), Beltsville, MD, USA; five paratype males and five paratype females deposited at FERA, Sand Hutton, York, UK; five paratype males and five paratype females deposited in the Forest Pathology Laboratory collection, Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Japan; and two paratype males and two paratype females deposited in the Canadian National Collection (CNC), Ottawa, ON, Canada. In addition to the type materials, mass-fixed materials (fixed in formalin-glycerin or dehydrated glycerin) were deposited at the USDANC, the Fort Lauderdale Research and Education Center, University of Florida/IFAS, the CNC and the FFPRI.

Type host carrier insect and locality

The holotype female and paratypes of *B. firmae* n. sp. were isolated from a 2-week-old culture on a *Botrytis cinerea* lawn on 2% malt extract agar. The culture was established from dauer (dispersal fourth-stage) juveniles isolated from *M. grandis* that emerged from a log of *A. firma* collected from the Tama Forest Science Garden, FFPRI, Hachioji, Tokyo, Japan, on 13 June 2007.

Diagnosis and relationships

Bursaphelenchus firmae n. sp. is characterised by four lateral incisures in males and females, a large spicule forming a trapezium in lateral view, seven conspicuous genital papillae and a sub-square bursal flap with irregular posterior ends in the male, and a long and conspicuous vulval flap, long post-uterine branch, and a tapering tail possessing a rather thick and bluntly pointed mucro in the female. The new species is also distinguished from other species of *Bursaphelenchus* by its unique molecular sequences of ribosomal RNA genes.

Currently, the *B. xylophilus* group *sensu* Braasch *et al.* (2009) contains 12 species: *B. xylophilus*; *B. fraudulentus* Rühm, 1956; *B. mucronatus*; *B. kolymensis* Korentchenko, 1980; *B. conicaudatus*; *B. baujardi* Walia, Negi, Bajaj & Kalia, 2003; *B. luxuriosae*; *B. doui* Braasch, Gu, Burgermeister & Zhang, 2005; *B. singaporensis* Gu, Zhang, Braasch & Burgermeister, 2005; *B. macromucronatus* Gu, Zhang, Braasch & Burgermeister, 2008; *B. populi* Tomalak & Filipiak, 2010; and *B. trypophloei* Tomalak & Filipiak, 2011 (Braasch *et al.*, 2009; Tomalak & Filipiak, 2010, 2011). Of these 12 species, *B. kolymensis* is considered to be a junior synonym of *B. mucronatus* (Magnusson & Kulinich, 1996; Hunt, 2008).

Based upon the female tail, which is smoothly tapered with a rather thick and bluntly pointed mucro, the new species is typologically similar to the following five species, which have tapered female tails with conspicuous mucro: *B. fraudulentus*, *B. mucronatus*, *B. doui*, *B. macromucronatus* and *B. populi* (Rühm, 1956; Mamiya & Enda, 1979; Magnusson & Kulinich, 1996; Braasch *et al.*, 2005; Gu *et al.*, 2008; Kanzaki *et al.*, 2008; Tomalak & Filipiak, 2010).

Bursaphelenchus firmae n. sp. is distinguished from *B. fraudulentus* by the male bursal flap (sub-square with irregular distal end *vs* rounded), the female mucro (blunt *vs* sharply pointed) and the female V value (68.8-72.8 *vs* 73.6-74.7) (Rühm, 1956; Kanzaki & Futai, 2002b); and from *B. mucronatus* by the male bursal flap (sub-square with irregular distal end *vs* sub-square with smooth distal end and possessing two blunt projections on both sides) and the female mucro (thick and blunt *vs* sharply pointed). Morphometric values of *B. mucronatus* are highly divergent and are not applicable to distinguishing the new species (Mamiya & Enda, 1979; Korentchenko, 1980; Magnusson & Kulinich, 1996); from *B. doui* by the male bursal flap (sub-square with irregular distal end *vs* sub-square to round with smooth distal end), the female mucro (thick and blunt *vs* irregularly curved with

a sharply pointed tip), and male spicule length (curved along an arc; 21-24 *vs* >30 μm) (Braasch *et al.*, 2005; Kanzaki *et al.*, 2008); from *B. macromucronatus* by the male bursal flap (sub-square with irregular distal end *vs* sub-square with smooth distal end and possessing two blunt projections on both sides), the female mucro (thick and blunt *vs* conical and pointed), and the female V value (68.8-72.8 *vs* 73.5-77.3; Gu *et al.*, 2008); and from *B. populi* by the male bursal flap (sub-square with irregular distal end *vs* rounded) and the female tail (tapered with thick and blunt mucro *vs* variable (broad tail with rounded tip to tapered tail with more or less pointed mucro)), and male spicule length (curved along an arc; 21-24 *vs* 26-31 μm; Tomalak & Filipiak, 2010). The new species is also molecularly close to *B. xylophilus* (see below), but is distinguished from the species by the male bursal flap (sub-square with irregular distal end *vs* rounded with pointed distal end), the female tail (tapered with thick and blunt mucro *vs* variable (broad tail with rounded tip with or without more or less pointed mucro)) and male spicule length (curved along an arc; 21-24 *vs* 25-30 μm; Mamiya & Kiyohara, 1972). Although the morphometric values of *B. xylophilus* are rather divergent, the new species is more stout than *B. xylophilus* (*i.e.*, a = 33-36 and 36-47 for females and males of *B. xylophilus*, respectively, while the values are 27.1-34.0 and 28.9-36.2, respectively, for *B. firmae* n. sp.

Molecular profiles and phylogeny

Based upon near full-length SSU and D2/D3 LSU sequences, *B. firmae* n. sp. is closest to *B. mucronatus* and *B. xylophilus*, and these three species form a well supported monophyletic clade in the D2/D3 LSU tree (Fig. 3). Molecularly, the new species differs from closely related species and genotypes in the near full-length SSU sequences, with 3-4 substitutions compared to *B. xylophilus* and a single substitution compared to *B. mucronatus*, and in the D2/D3 LSU sequences with 13 substitutions compared to the European genotype of *B. mucronatus*, 20-21 substitutions compared to the Asian genotypes of *B. mucronatus* and 15-17 substitutions compared to *B. xylophilus*. Although the molecular base difference is not always a definitive character supporting species status, the D2/D3 sequence differences between *B. firmae* n. sp. and its sister species (genotype), European type *B. mucronatus*, are almost the same as those between *B. xylophilus* and *B. mucronatus* (13-23 substitutions in D2/D3 LSU).

Insect and plant associations

Ninety-one (47 males and 44 females) adults of *M. grandis* were collected from the logs. The nematode was associated with about 19% (nine individuals) of males and 23% (ten individuals) of females, and the number of nematodes recovered from male and female nematode harbouring insects was 98.8 $\pm$ 125.6 (9-377) and 38.1 $\pm$ 45.5 (4-135), respectively. The nematodes isolated from the insects were fourth-stage dispersal (dauer) juveniles and were recovered from the tracheal system of the insects. The 'parasitic adult' reported in *B. luxuriosae* (Kanzaki *et al.*, 2009) was not observed in the new species.

Monochamus grandis usually feeds on the bark tissue of conifer trees and prefers *Abies* spp. and *Picea* spp. but sometimes feeds on *Pinus* spp. (Makihara, 2007). Therefore, *B. firmae* n. sp. has some opportunities to enter pine trees susceptible to pine wilt disease. Because *B. firmae* n. sp. is a close relative of the pathogenic *B. xylophilus*, Kanzaki *et al.* (2011a) and Maehara *et al.* (2011) conducted an inoculation test of *B. firmae* n. sp. in Japanese native pine tree species. Kanzaki *et al.* (2011a) demonstrated that *B. firmae* n. sp. caused a certain level of tissue damage to the stems of 3-year-old seedlings (equivalent to twigs of adult trees) of *P. thunbergii* Parl., although external wilting symptoms were not observed. Maehara *et al.* (2011) inoculated the nematode in adult *P. thunbergii*, and examined the external wilting symptoms of inoculated trees and the long-term survival of nematodes in living pines, and reported that the nematode did not cause wilting nor survive in the living pine trees.

Under current conditions, *B. firmae* n. sp. does not seem to be a major risk to native pine forests because the number of nematodes harboured by the carrier beetle and the pathogenicity are almost the same as those of other less-pathogenic nematodes.

Remarks

The species boundary between *B. xylophilus* and *B. mucronatus*, two closely related species, is sometimes vague. Taga *et al.* (2011) reported that a *B. xylophilus* isolate "T4" and a European genotype *B. mucronatus* isolate "TCS-00" can interbreed to generate fully fertile offspring, and the population originating from the interbreeding is long lasting, although the Asian-type isolate "Un" yielded only infertile F_1 with any of the *B. xylophilus* iso-

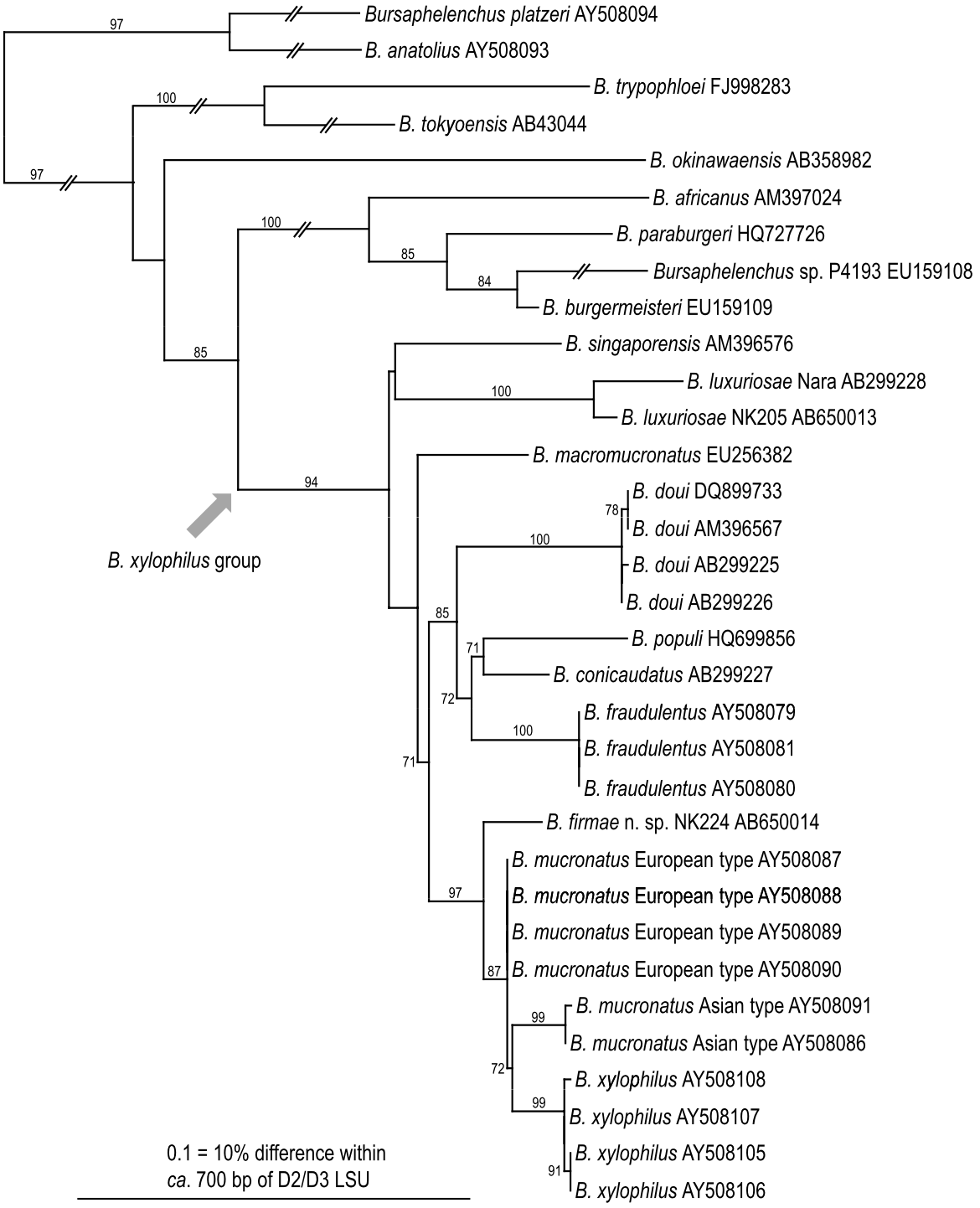

Fig. 3. *Molecular phylogenetic relationships among species in the* Bursaphelenchus xylophilus *group. The 10 001st Bayesian tree inferred from D2/D3 LSU with the TrN + G model (lnL = 4904.5273; freqA = 0.1945; freqC = 0.2043; freqG = 0.3375; freqT = 0.2636; R(a) = 0.4101; R(b) = 2.7192; R(c) = 0.7543; R(d) = 0.5555; R(e) = 6.0751; R(f) = 1; Pinva = 0; Shape = 0.03). Posterior probability values exceeding 50% are given for appropriate clades.*

lates. Based upon the molecular closeness among *B. firmae* n. sp., *B. mucronatus* and *B. xylophilus*, similar interbreeding could occur between the new species and *B. mucronatus* and/or *B. xylophilus*.

In the present study, a sufficient number of populations (isolates) of these three species was not available. More sampling to obtain a larger number of populations and detailed biological comparison will be necessary to clarify the relationship among these three closely related species.

Acknowledgements

We thank Prof. Dr Katsumi Togashi, the University of Tokyo, for providing a paper in press, and Ms Ami Akasaka, FFPRI, for technical assistance. This study was supported by a Grant-in-Aid for Young Scientists (B) (No. 21770094), Grants-in-Aid for Scientific Research (A) (No. 23248024) and (B) (No. 23380092) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Projective Fund of "Development of Mitigation and Adaptation Techniques to Global Warming in the Sectors of Agriculture, Forestry, and Fisheries" by the Ministry of Agriculture, Forestry and Fishery, Japan.

References

BRAASCH, H., GU, J., BURGERMEISTER, W. & ZHANG, J. (2005). *Bursaphelenchus doui* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Taiwan and South Korea – a new species of the *xylophilus* group. *Russian Journal of Nematology* 13, 19-27.

BRAASCH, H., BURGERMEISTER, W. & GU, J. (2009). Revised intra-generic grouping of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae). *Journal of Nematode Morphology and Systematics* 12, 65-81.

FUCHS, A.G. (1937). Neue arasitische und halbparasitische Nematoden bei Borkenkäfern und einige andere Nematoden. 1 Teil. *Zoologische Jahrbücher, Abteilung für Systematik, Öekologie und Geographie der Tiere* 70, 291-380.

GU, J., ZHANG, J., BRAASCH, H. & BURGERMEISTER, W. (2005). *Bursaphelenchus singaporensis* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Singapore – a new species of the *B. xylophilus* group. *Zootaxa* 988, 1-12.

GU, J., ZHANG, J., BRAASCH, H. & BURGERMEISTER, W. (2008). Description of *Bursaphelenchus macromucronatus* sp. n. (Nematoda: Parasitaphelenchidae) in packing wood from Taiwan and India – a new species of the '*xylophilus*' group. *Journal of Nematode Morphology and Systematics* 11, 31-40.

HOOPER, D.J. (1986). Handling, fixing, staining and mounting nematodes. In: Southey, J.F. (Ed.). *Laboratory methods for work with plant and soil nematodes*. London, UK, Her Majesty's Stationery Office, pp. 59-80.

HUELSENBECK, J.P. & RONQUIST, F. (2001). MR BAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 1754-1755.

HUNT, D.J. (2008). A checklist of the Aphelenchoidea (Nematoda: Tylenchina). *Journal of Nematode Morphology and Systematics* 10 (2007), 99-135.

KANZAKI, N. & FUTAI, K. (2002a). A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus* group. *Nematology* 4, 35-41.

KANZAKI, N. & FUTAI, K. (2002b). Observation on the arrangement of caudal papillae of *Bursaphelenchus conicaudatus* and *B. fraudulentus*. *Japanese Journal of Nematology* 32, 21-23.

KANZAKI, N. & FUTAI, K. (2003). Description and phylogeny of *Bursaphelenchus luxuriosae* n. sp. (Nematoda: Aphelenchoididae) isolated from *Acalolepta luxuriosa* (Coleoptera: Cerambycidae). *Nematology* 5, 565-572.

KANZAKI, N., TSUDA, K. & FUTAI, K. (2000). Description of *Bursaphelenchus conicaudatus* n. sp. (Nematoda: Aphelenchoididae), isolated from the yellow-spotted longicorn beetle, *Psacothea hilaris* (Coleoptera: Cerambycidae) and fig trees, *Ficus carica*. *Nematology* 2, 165-168.

KANZAKI, N., AIKAWA, T., MAEHARA, N. & MATSUMOTO, K. (2008). *Bursaphelenchus doui* Braasch, Gu, Burgermeister & Zhang, 2005 (Aphelenchida: Parasitaphelenchidae), an associate of *Monochamus subfasciatus* Bates (Coleoptera: Cerambycidae) and *Pinus densiflora* Sieb. & Zucc. *Nematology* 9, 69-78.

KANZAKI, N., MAEHARA, N., AIKAWA, T., GIBLIN-DAVIS, R.M. & CENTER, B.J. (2009). The first report of a putative "entomoparasitic adult form" of Bursaphelenchus. *Journal of Parasitology* 95, 113-119.

KANZAKI, N., AIKAWA, T., MAEHARA, N. & ICHIHARA, Y. (2011a). An inoculation experiment of Japanese *Bursaphelenchus* nematodes on Japanese black and red pine, *Pinus thunbergii* and *P. densiflora*. *Journal of Forest Research* 16, 325-330.

KANZAKI, N., MAEHARA, N., AIKAWA, T., MASUYA, H. & GIBLIN-DAVIS, R.M. (2011b). Description of *Bursaphelenchus kiyoharai* n. sp. (Tylenchina: Aphelenchoididae) with remarks on the taxonomic framework of the Parasitaphelenchinae Rühm, 1956 and Aphelenchoidinae Fuchs, 1937. *Nematology* 13, 787-804.

KATOH, K., MISAWA, K., KUMA, K. & MIYATA, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* 30, 3059-3066.

KIKUCHI, T., AIKAWA, T., OEDA, Y., KARIM, N. & KANZAKI, N. (2009). A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophi-*

lus by loop-mediated isothermal amplification (LAMP). *Phytopathology* 99, 1365-1369.

KORENTCHENKO, E.A. (1980). New species of nematodes from the family Aphelenchoididae, parasites of stem pests of the Dahurian larch. *Zoologichesky Zhurnal* 59, 1768-1780.

LARGET, B. & SIMON, D.L. (1999). Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* 16, 750-759.

MAEHARA, N., AIKAWA, T. & KANZAKI, N. (2011). Inoculation of several *Bursaphelenchus xylophilus* group nematodes into adult trees of *Pinus thunbergii* and their survival in the trees. *Forest Pathology* 41, in press, DOI:10.1111/j.1439-0029.2010.00707.x.

MAGNUSSON, C. & KULINICH, O.A. (1996). A taxonomic reappraisal of the original description, morphology and status of *Bursaphelenchus kolymensis* Korentchenko, 1980 (Aphelenchida: Aphelenchoididae). *Russian Journal of Nematology* 4, 155-161.

MAKIHARA, H. (2007). Tribe Lamiini Mulsant, 1839. In: Ohbayashi, N. & Niisato, T. (Eds). *Longicorn beetles of Japan*. Tokyo, Japan, Tokai University Press, pp. 576-605.

MAMIYA, Y. & ENDA, N. (1979). *Bursaphelenchus mucronatus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and its biology and pathogenicity to pine trees. *Nematologica* 25, 353-361.

MAMIYA, Y. & KIYOHARA, T. (1972). Description of *Bursaphelenchus lignicolus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and histopathology of nematode-infested trees. *Nematologica* 18, 120-124.

NICKLE, W.R. (1970). A taxonomic review of the genera of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). *Journal of Nematology* 2, 375-392.

POSADA, D. & CRANDALL, K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817-818.

RÜHM, W. (1956). *Die Nematoden der Ipiden. Parasitologische Schriftenreihe 6*, 435 pp.

RYSS, A., VIEIRA, P., MOTA, M.M. & KULINICH, O. (2005). A synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. *Nematology* 7, 393-458.

STEINER, G. & BUHRER, E.M. (1934). *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. *Journal of Agricultural Research* 48, 949-951.

TAGA, Y., GOTO, S., MATSUNAGA, K. & TOGASHI, K. (2011). Temporal changes in characteristics of populations originating from interbreeding between *Bursaphelenchus xylophilus* and *B. mucronatus*. *Nematology* 13, 701-712.

TOMALAK, M. & FILIPIAK, A. (2010). Description of *Bursaphelenchus populi* sp. n. (Nematoda: Parasitaphelenchidae), a new member of the *xylophilus* group from aspen, *Populus tremula* L., in Europe. *Nematology* 12, 399-416.

TOMALAK, M. & FILIPIAK, A. (2011). *Bursaphelenchus trypophloei* sp. n. (Nematoda: Parasitaphelenchinae) – an associate of the bark beetle, *Trypophloeus asperatus* (Gyll.) (Coleoptera: Curculionidae, Scolytinae), in aspen, *Populus tremula* L. *Nematology* 13, 619-636.

WALIA, K.K., NEGI, S., BAJAJ, H.K. & KALIA, D.C. (2003). Two new species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae) from pine wood and insect frass from India. *Indian Journal of Nematology* 33, 1-5.

YE, W., GIBLIN-DAVIS, 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.