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Bursaphelenchus doui Braasch, Gu, Burgermeister & Zhang, 2005 (Aphelenchida: Parasitaphelenchidae), an associate of *Monochamus subfasciatus* Bates (Coleoptera: Cerambycidae) and *Pinus densiflora* Sieb. & Zucc.

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Summary – *Bursaphelenchus doui* was isolated from a dead Japanese red pine, *Pinus densiflora*, in Shizuoka, and from the tracheal system of a species of longhorn beetle, *Monochamus subfasciatus*, collected at Tama Forest Science Garden of Forestry and Forest Products Research Institute, Tokyo, Japan. The Japanese populations of *B. doui* were compared with the original description of material obtained from coniferous packaging materials imported from Taiwan and Korea to continental China. Additional characters from the Japanese population include a constricted female mucron with a step-like appearance and several morphometric values. The molecular profiles of the Japanese *B. doui* populations were determined by DNA sequencing and ITS-RFLP profiles and were compared with those of the Taiwanese and Chinese populations of *B. doui* and other species in the genus. The phylogenetic analysis of the small subunit and large subunit ribosomal DNA indicated that *B. doui* is clearly included in the *xylophilus*-group of the genus *Bursaphelenchus* and may be close to *B. conicaudatus* and *B. luxuriosae*. The potential risk of *B. doui* for pine species is considered to be relatively low because *B. doui* did not display any pathogenicity to Japanese black pine in an inoculation test.

Keywords – bionomics, Japan, molecular, morphology, morphometrics, pathology, phylogeny, vector, *xylophilus*-group.

Bursaphelenchus doui Braasch, Gu, Burgermeister & Zhang, 2005 was originally described from coniferous packaging material imported from Taiwan and Korea to continental China (Braasch *et al.*, 2005). However, its host tree species and vector insect have yet to be identified in nature.

We studied two Japanese isolates of *B. doui*. One population was obtained from the tracheal system of *Monochamus subfasciatus* Bates in Tokyo, and the other was isolated from a dead Japanese red pine, *Pinus densiflora* Sieb. & Zucc., in Shizuoka prefecture, Japan. Both isolates were observed by light microscopy and compared with the original description of Braasch *et al.* (2005). The DNA sequences of near full length small subunit (SSU), internal transcribed spacer (ITS) region (ITS1, ITS2 and 5.8S rDNA) and D2D3 expansion segment of large subunit (LSU) were determined for both isolates, and the SSU and LSU sequences were compared with those of some other *Bursaphelenchus* spp. to determine its phylogenetic position within the genus. The pathogenicity of *B. doui* to the Japanese black pine, *Pinus thunbergii* Parl., which is highly susceptible to pine wilt disease, was also tested by field inoculation.

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Materials and methods

Isolation of nematodes and morphological observations

Three adults of *M. subfasciatus* were collected from Tama Forest Science Garden of Forestry and Forest Products Research Institute (FFPRI), 1833-81 Todori, Hachiohji, 193-0843 Tokyo, Japan, on 28 June 2006. The beetles were dissected and examined for the presence of nematodes. Dauer juveniles were recovered from a female beetle and were transferred to a mat of *Botrytis cinerea* Pers. growing on 2% (w/v) malt extract agar (MEA). The successful culture was maintained in the laboratory (culture code NK204). Adult nematodes from the culture were observed with a light microscope and identified as *B. doui*. Another culture (code NK217), initiated from a population obtained in 1995 from a dead Japanese red pine, *P. densiflora*, in Izu region, Shizuoka prefecture, Japan, and kept at the FFPRI nematode culture collection, was also identified as *B. doui* based on its morphology and molecular sequence.

Adult nematodes of NK204 and NK217 were isolated from 2-week-old cultures on *B. cinerea*-MEA using the Baermann funnel technique. Most of the specimens isolated were heat-killed at 65°C, fixed in TAF, processed through a glycerol-ethanol series using Seinhorst's method and mounted in glycerin according to the method of Maeseneer and D'Herde (Hooper, 1986). Voucher specimens observed and measured in the present study were deposited in the FFPRI Nematology Collection with slide numbers: *Bursaphelenchus doui* #204 F-01-20 (20 females) and M-01-20 (20 males) and *Bursaphelenchus doui* #217 F-01-20 (20 females) and M-01-20 (20 males). Additional material from the same source as the vouchers is available upon request to the authors.

Molecular profiles

Samples of *B. doui* DNA were prepared as described by Ye *et al.* (2007). The DNA base sequences of partial ribosomal DNAs (= near full length SSU, 5.8S, ITS 1, and ITS 2: *ca* 2.5 kb, and the D2D3 expansion segment of LSU: *ca* 0.7 kb) were determined for both isolates of *B. doui* following the methods described by Iwahori *et al.* (1998) and Ye *et al.* (2007). Amongst the loci sequenced we chose SSU and D2D3 LSU rDNAs for phylogenetic comparison as these are suitable for comparison within the genus and group (Kanzaki & Futai, 2002a; Kanzaki, 2006; Ye *et al.*, 2007). The molecular phylogenetic status of *B. doui* both within the genus and within the *xylophilus*-group was determined by maximum likelihood analysis with 1000 bootstrap replications using the computer program TreeFinder (version: updated on May 2006; latest version available online at: http://www.treefinder.de). The species names and GenBank accession numbers of the sequences compared with those of the Japanese *B. doui* are shown in the phylograms.

The ITS and D2D3 of Japanese populations were compared with those of the other isolates of *B. doui* deposited in the GenBank database in order to examine intraspecific sequence variation. An ITS sequence of a Taiwanese population (accession number: AM157743; strain: NE26/04) and a D2D3 sequence from an unknown geographical source, probably China (accession number DQ899733) were used for comparison.

The ITS-RFLP profiles of *B. doui* were obtained following Burgermeister *et al.* (2005), and the DNA fragment sizes were calculated based on the DNA base sequence of the ITS region by using the restriction site/fragment lengths analysis of the computer program BioEdit v. 7.0.5 (available online at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The profiles were compared with those obtained in the original description (Braasch *et al.*, 2005) and those of NE26/04 calculated from the sequence.

Hybridisation tests

The occurrence of a F1 generation was examined using a hybridisation test. The test was conducted using a method of single-pair reciprocal crosses between NK204 and NK217. The pairs consisted of a fourth-stage female juvenile of NK204 and a male adult NK217 or a fourth-stage female juvenile of NK217 and a male adult NK204. Nematodes were placed individually on *B. cinerea* growing on PDA agar in a 3 cm diam. Petri dish and incubated at 23°C (n = 20 plates for each combination). The Petri dishes with nematodes were observed daily for 2 weeks to examine the occurrence of F1 and its subsequent propagation. When the F1 and successive generation propagated to more than 100 nematodes in each Petri dish, the cross was considered successful. The 20 pairs of NK204 J4 ♀×NK204 ♂ and NK217 J4 ♀×NK217 ♂ were also examined as control crosses. The ratio of successful crosses was compared between two interbreeding treatments and two control crosses using the χ^2 test.

Inoculation test

Two species of nematodes, *B. doui* NK217 and *B. xylophilus* (laboratory culture 'Ka-4', originally obtained from Kasama, Ibaraki, Japan), were used for this inoculation test. *Bursaphelenchus doui*, reared on a fungal mat of *B. cinerea*, was isolated using the Baermann funnel technique and a suspension (100 000 nematodes/1 ml distilled water) was prepared on 25 July 2002. The inoculum was composed of mixed-age fungal feeding (propagative) phase and the dispersal fourth-stage (dauer) juvenile was not included. Twelve 5-year-old Japanese black pine, *P. thunbergii*, trees (*ca* 2.5 m height) planted at the nursery of Chiyoda Experimental Station of FFPRI, Kasumigaura, Ibaraki, Japan, were inoculated with 10 000 individuals of *B. doui* (0.1 ml of the suspension). As positive and negative controls, 12 trees were inoculated with 10 000 individuals of *B. xylophilus*, and another 12 with 0.1 ml of distilled water, respectively. The trees were examined for external disease symptoms every month after inoculation for 5 months.

Results

Morphological observations

Morphometric values of the original *B. doui* description and the Japanese populations are compared in Table 1. There were no clear differences in morphometric values between populations. The general morphological features were as described by Braasch *et al.* (2005), although an additional morphological feature was found in the present study. In females, a distinctive ventral mucron was originally described as "... 2-4 μm length that tapers, sometimes appearing hairlike". In our observations, the mucron was relatively difficult to distinguish from the tapered tail, some individuals having a tapering mucron, narrowing in a step-like manner (Fig. 1).

Molecular profiles and phylogeny

The DNA base sequences in the present study were deposited in the GenBank database with the accession numbers, AB299223 (NK204) and AB299224 (NK217) for SSU-ITS1-5.8S-ITS2 rDNAs and AB299225 (NK204) and AB299226 (NK217) for D2D3 LSU rDNA. There was an ITS sequence variation within individual nematodes of NK204 and the variation affected the ITS-RFLP profile (Fig. 2; Table 2).

Both SSU and LSU rDNA phylogenetic trees (Figs 3, 4) show that *B. doui* is clearly included in the *xylophilus*-group and is probably close to *B. luxuriosae* and *B. conicaudatus* (Fig. 4), although the bootstrap support was too low to make a definite statement.

The ITS-RFLP profiles obtained in the present study are shown in Figure 2 and Table 2. The profiles derived from a sequence type of NK204 (ITS-RFLP Type I: see Fig. 2 and Table 2) were identical to those reported by Braasch *et al.* (2005) in the original description of the species (Type I), but were slightly different from those of the other sequence type of NK204 (Type II), NK217 (Type II) and Taiwanese NE26/04 (Type III) (Fig. 2; Table 2).

Tables 3 and 4 show the sequence differences in ITS and in D2D3 LSU between the two Japanese populations and NE26/04 (ITS) and DQ899733 (D2D3). In total, 14 substitution sites were found within 981 bp of ITS and three substitution sites were found within 742 bp of D2D3 LSU. The SSU sequences of both Japanese populations were identical.

Hybridisation tests

The ratio of successful crossing of NK204 ♀×NK217 ♂ (15 dishes: 75%) and NK217 ♀ × NK204 ♂ (18 dishes: 90%) did not differ significantly from those of NK204 ♀ × NK204 ♂ (19 dishes: 95%) and NK217 ♀ × NK217 ♂ (16 dishes: 80%) ($P = 0.27$; χ^2 test).

Inoculation test

Although an individual host was killed during the inoculation experiment by a natural infection of *B. xylophilus*, the other trees inoculated with *B. doui* did not show any wilting symptoms. *Bursaphelenchus doui* was not isolated from the tree killed by *B. xylophilus*, although many *B. xylophilus* individuals were. All hosts inoculated with *B. xylophilus* were killed within 3 months after inoculation (= until 23 October 2002), and trees inoculated with distilled water did not show any wilting symptoms.

Discussion

In the present study, two Japanese isolates of what appeared to be *B. doui* were obtained. These two populations were confirmed as conspecific based on their successful interbreeding and were attributed to *B. doui* on molecular and morphological traits. Furthermore, a morphological

Table 1. *Morphometrics of three populations of* Bursaphelenchus doui. *All measurements are in μm and in the form: mean ± s.d. (range).*

	Original description		Japan (Tokyo: NK204)		Japan (Shizuoka: NK217)	
	Female	Male	Female	Male	Female	Male
n	15	15	20	20	20	20
L	876 ± 112.9 (634-1143)	811 ± 84.1 (629-948)	820 ± 63 (722-1019)	718 ± 78 (556-886)	863 ± 152 (660-1226)	731 ± 87 (608-920)
a	32.6 ± 3.5 (26.6-37.3)	28.8 ± 4.5 (25.0-33.0)	33.3 ± 3.7 (24.9-36.7)	31.6 ± 3.4 (21.8-37.5)	32.4 ± 2.7 (26.1-37.3)	33.4 ± 2.0 (29.1-36.5)
b	9.5 ± 1.8 (6.5-12.9)	8.4 ± 0.8 (6.3-9.7)	11.3 ± 0.7 (10.0-12.9)	10.2 ± 0.7 (8.5-11.4)	10.8 ± 1.6 (8.5-14.5)	9.4 ± 0.8 (8.3-11.1)
c	23.4 ± 2.2 (19.7-28.6)	21.9 ± 3.4 (17.5-30.7)	20.2 ± 1.5 (18.0-22.5)	21.1 ± 1.5 (18.4-24.6)	23.2 ± 3.6 (18.4-30.5)	22.2 ± 2.2 (19.4-27.4)
c′	3.6 ± 0.3 (2.8-4.2)	1.9 ± 0.2 (1.5-2.3)	4.3 ± 0.4 (3.6-5.0)	2.0 ± 0.1 (1.7-2.2)	3.8 ± 0.3 (3.4-4.4)	2.0 ± 0.1 (1.8-2.1)
M	35		37.0 ± 2.2 (33.3-40.6)	36.8 ± 2.8 (31.4-42.4)	37.8 ± 3.0 (27.9-42.9)	37.6 ± 2.0 (34.4-42.4)
V or T	74.9 ± 1.7 (72.1-78.3)	50-75	73.1 ± 1.1 (70.3-74.8)	43.0 ± 8.6 (31.2-64.2)	73.9 ± 1.4 (70.8-77.1)	59.0 ± 6.2 (41.4-66.6)
Max. body diam.	not given	not given	25 ± 3.1 (21-33)	23 ± 2.6 (18-28)	27 ± 5.2 (21-38)	22 ± 3.4 (17-30)
Lip diam.	8-9		8.5 ± 0.4 (7.5-9.5)	8.5 ± 0.6 (7.5-9.5)	8.5 ± 0.7 (7.5-9.5)	8.5 ± 0.8 (7.5-9.5)
Lip height	4		4.0 ± 0.3 (3.5-4.0)	4.0 ± 0.3 (3.5-4.5)	4.0 ± 0.4 (3.5-4.5)	4.0 ± 0.3 (3.5-4.5)
Stylet length	15.1 ± 0.8 (13.0-16.2)	15.2 ± 0.7 (14.1-15.9)	16.5 ± 0.7 (15.0-18.5)	15.0 ± 1.1 (13.0-17.0)	16.0 ± 1.1 (15.0-20.0)	15.5 ± 0.5 (14.5-16.5)
Excretory pore position[1)]	not given	not given	57 ± 4.7 (47-68)	58 ± 8.3 (48-82)	67 ± 4.0 (59-74)	66 ± 5.2 (55-76)
Nerve ring position[1)]	not given	not given	80 ± 3.9 (73-90)	76 ± 4.6 (66-86)	86 ± 3.8 (80-92)	83 ± 3.2 (76-89)
Hemizonid position[1)]	not given	not given	93 ± 4.4 (87-102)	89 ± 5.3 (76-98)	98 ± 5.8 (89-108)	93 ± 5.3 (86-105)
Ovary or testis length	not given	not given	245 ± 65 (167-443)	305 ± 60 (219-429)	286 ± 44 (210-374)	429 ± 47 (352-514)
PUS[2)] length	111.2 ± 10.7 (100-120.4)	–	119 ± 11 (93-136)	–	120 ± 17 (92-147)	–
PUS[2)]/vulva to anus (%)	80	–	66.2 ± 6.3 (51.3-76.2)	–	64.6 ± 5.3 (53.7-74.4)	–
Vulval flap length	10-16	–	16 ± 0.2 (12-19)	–	15 ± 2.1 (12-19)	–
Spicule length[3)]	–	39.6 ± 2.5 (33.8-43.3)	–	37 ± 2.9 (30-42)	–	35 ± 1.4 (32-38)
Capitulum length[4)]	–	not given	–	10.5 ± 1.1 (8.0-12.0)	–	10.0 ± 0.9 (8.5-11.5)
Tail length	not given	not given	41 ± 2.5 (37-45)	34 ± 3.6 (26-40)	37 ± 2.4 (34-44)	33 ± 2.3 (29-38)
Anal body diam.	not given	not given	9.5 ± 0.8 (8.0-12.0)	17.0 ± 1.3 (14.0-19.5)	10.0 ± 0.5 (9.5-11.5)	17.0 ± 1.5 (15.0-20.0)

[1)] Distance from anterior end.
[2)] PUS = post-uterine sac.
[3)] Measured as curved median line.
[4)] Distance from dorsal tip of condylus to ventral tip of rostrum.

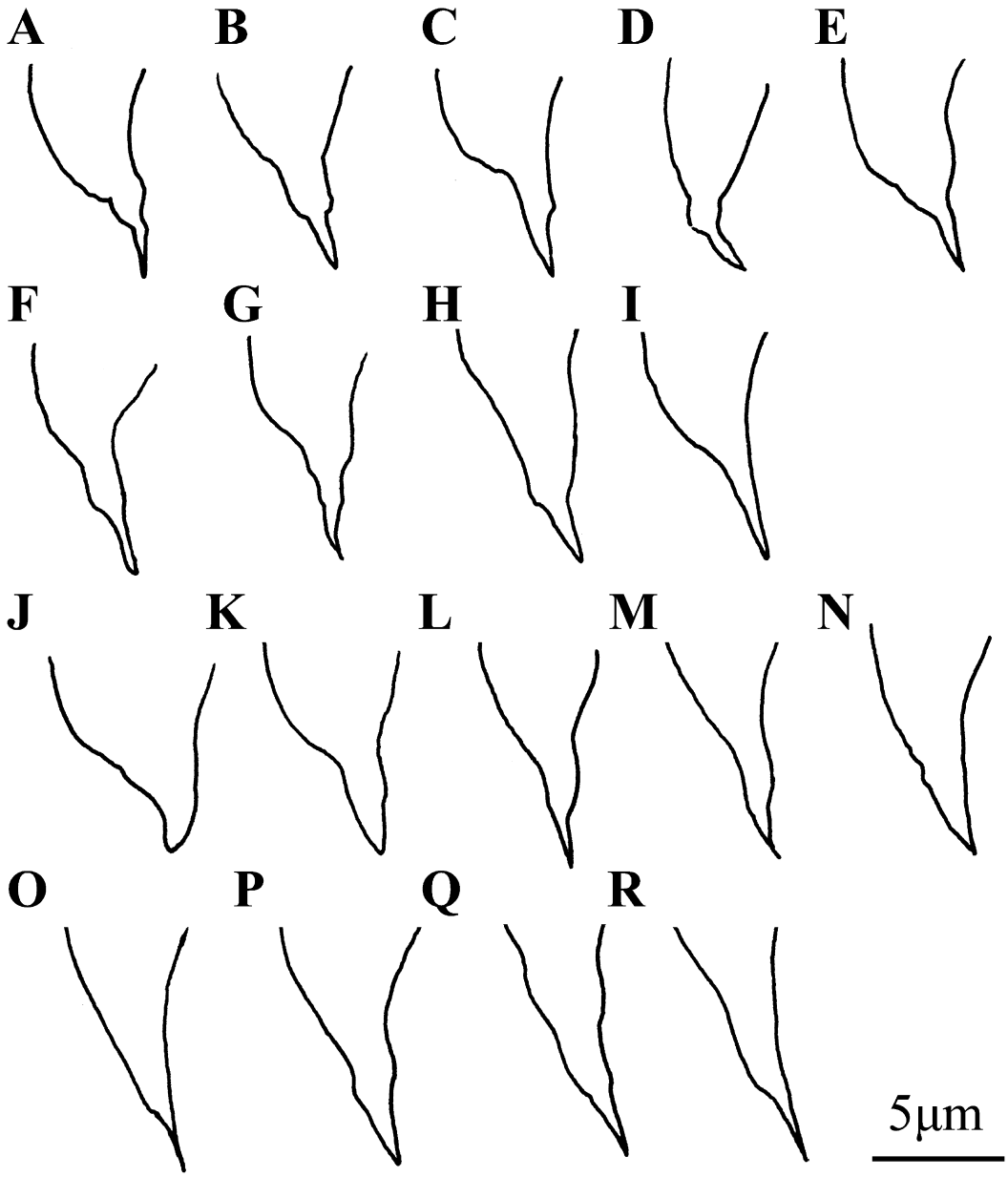

Fig. 1. *Morphological variation of female tail tip of* Bursaphelenchus doui*, observed in right lateral view. A-I: NK204; J-R: NK217.*

variation in the form of the female mucron, several morphometric values and several molecular sequence variations were ascribed to the species.

Variation of the female mucron shape within and amongst populations is known in *B. xylophilus*. For example, Bolla and Boschert (1993) examined the morphological variation, chromosome number and hybridisation affinity amongst Japanese, USA and Canadian isolates of *B. xylophilus* and confirmed that the difference between the 'R form' (possessing a rounded tail), and the 'M form' (possessing a mucronate tail) is an intraspecific variation. Mamiya and Kiyohara (1972) also described the occurrence of short mucron in a Japanese isolate of *B. xylophilus* which is basically an 'R form' population. Brzeski and Baujard (1997) and Kanzaki and Futai (2007) reported several intraspecific morphometrical variations

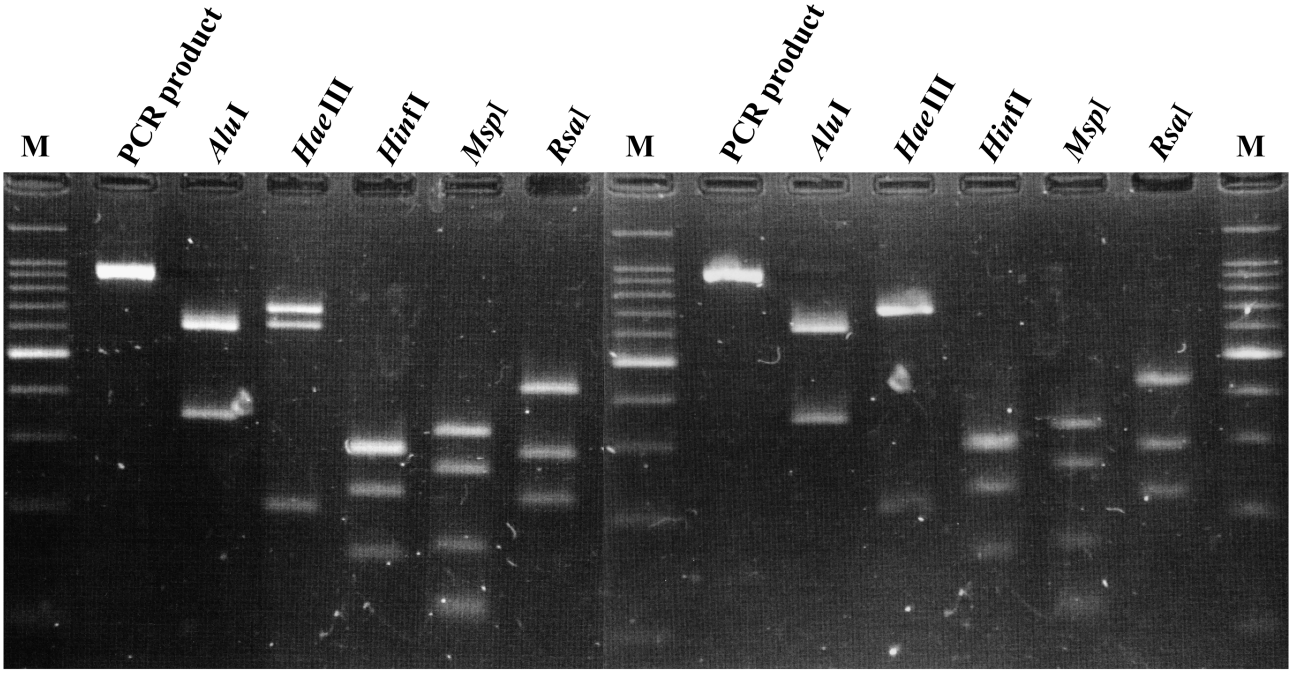

Fig. 2. *ITS-RFLP profiles of two Japanese isolates of* Bursaphelenchus doui. *M = 100 bps ladder.*

Table 2. *ITS-RFLP profiles for original description, two Japanese populations and a Chinese population of* Bursaphelenchus doui. *Profiles cited as: actual size (approximate size calculated from DNA size marker). The profiles are categorised as types I-III. ITS-RFLP types I and III are distinguished from each other by the strength of the '300 bp' band of* Hin*fI fragments, which consists of two (283 bp and 292 bp) bands in types I and II, but of a single (283 bp) band in type III.*

Enzyme	Restriction site	Original description[1)]	Japan (Tokyo: NK204)	Japan (Shizuoka: NK217)	Taiwanese population (Ne26/04)[2)]
PCR product	–	(1000)	981	981	981
*Alu*I	AG/CT	(360), (620)	365, 616	365, 616	365, 616
*Hae*III	GG/CC	(80), (210), (650)	53*, 83*, 205, 640, 723	53*, 205, 723	53*, 83*, 205, 640
*Hin*fI	G/ANTC	(170), (240), (300)	24*, 154, 228, 283, 292	24*, 154, 228, 283, 292	24*, 83*, 154, 228, 209, 283
*Msp*I	C/CGG	(120), (170), (280), (330)	110, 114, 165, 264, 328	110, 114, 165, 264, 328	110, 114, 165, 264, 328
*Rsa*I	GT/AC	(240), (310), (450)	22*, 228, 296, 435	22*, 228, 296, 435	22*, 228, 296, 435
ITS-RFLP category		I	I, II	II	III

[1)] Braasch *et al.* (2005). Actual size is not given.
[2)] Calculated from a DNA sequence deposited in the GenBank database with accession number AM157743.
Underlined: sequence variation within an individual.
[*] Not clearly seen.

within and between populations of *B. mucronatus*, *B. naujaci*, *B. glochis*, *B. pinophilus* and *B. sinensis*. However, there was no clear morphometrical variation amongst *B. doui* isolates, *i.e.*, ranges of most morphometric values overlapped amongst the three populations (see Table 1). Our material was obtained from 2-week-old *B. cinerea* cultures on 2%MEA, but culture conditions, *e.g.*, nutrient condition and media additives, might affect nematode

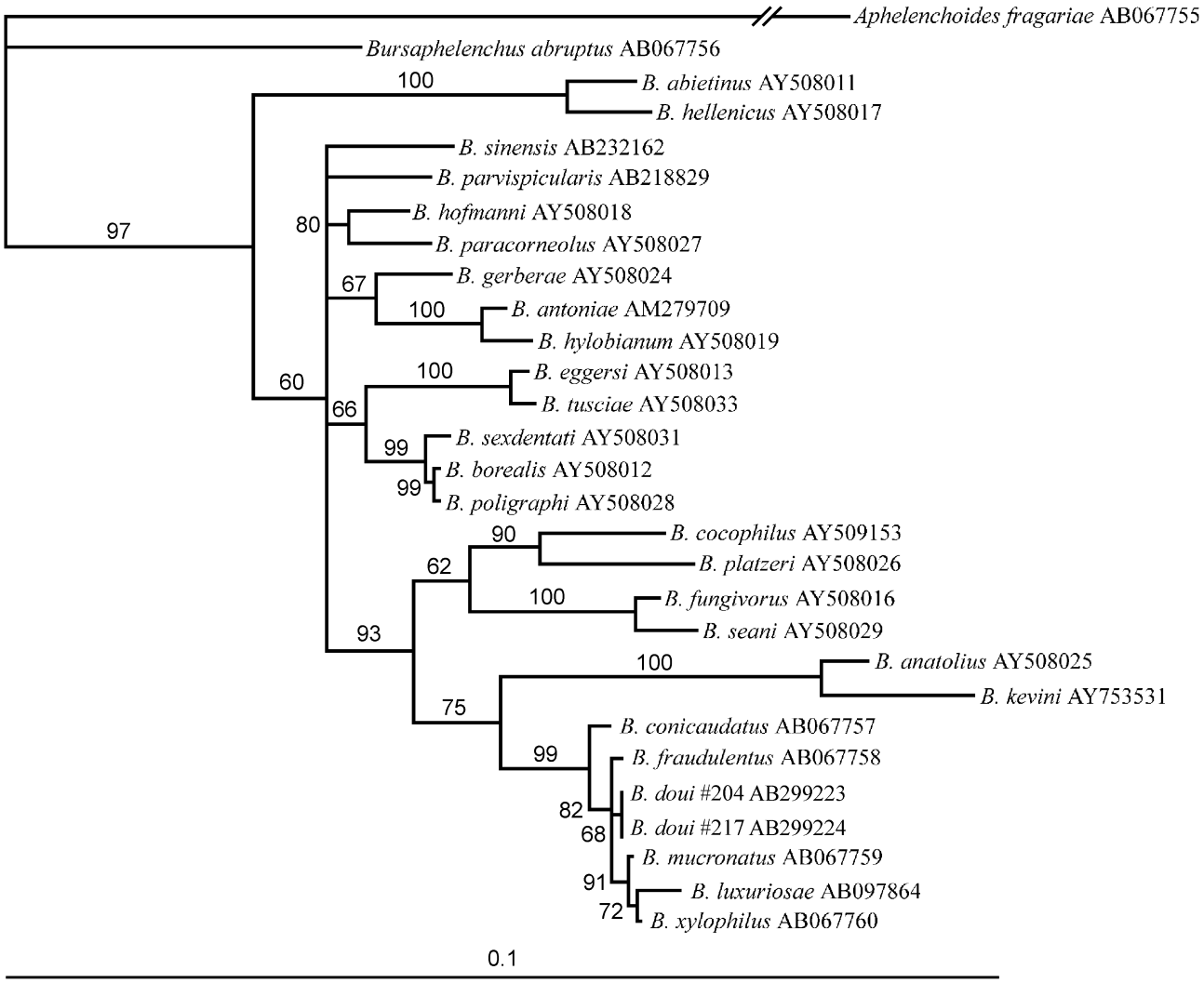

Fig. 3. *Molecular phylogenetic relationship of 27* Bursaphelenchus *species based on partial 18S rDNA. Phylogenetic trees were generated by maximum likelihood analysis with 1000 bootstrap replications.* Aphelenchoides fragariae *served as outgroup species.*

morphometrics as reported for *B. seani* by Giblin and Kaya (1984).

When comparing molecular sequences, 14 and three substitution sites were found in 981 bp of ITS and 742 bp of D2D3, respectively. In *B. mucronatus*, a close relative of *B. doui*, 11 substitution sites were found among seven D2D3 LSU sequences (GenBank accession numbers AY508086, AY508087, AY508088, AY508089, AY508090, AY508091 and DQ364688) and 30 substitution sites and five deletion/insertions were found amongst six ITS sequences (GenBank accession numbers U93554, DQ841162, AY347916, AY347915, AY347914 and AM179514). Differences in the molecular sequence amongst *B. mucronatus* isolates partially reflect geographic origin (see Ye *et al.*, 2007). In addition, Kanzaki and Futai (2002b) reported mitochondrial sequence variation within *B. conicaudatus* populations derived from geographical isolates. Thus, molecular variation amongst *B. doui* populations may reflect some kind of geographical variation. *Bursaphelenchus doui* is probably distributed in Asian countries as the species has been found in Taiwan, South Korea, China and Japan (Braasch *et al.*, 2005; Gu *et al.*, 2006). Further sampling and molecular analysis may reveal the precise distributional range and geographical variation of *B. doui*.

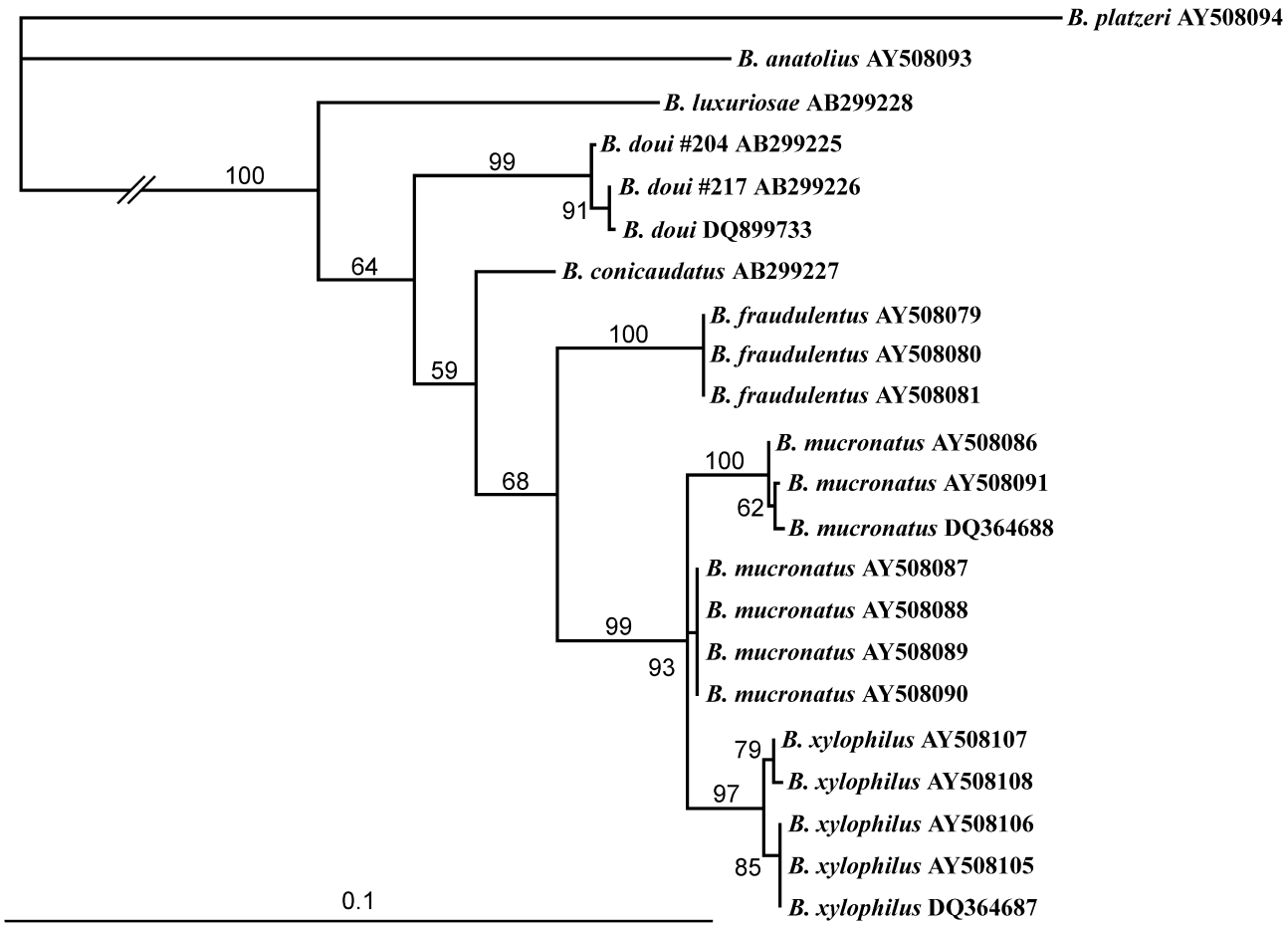

Fig. 4. *Molecular phylogenetic relationship of six* xylophilus*-group nematodes based on D2/D3 LSU rDNA. Phylogenetic trees were generated by maximum likelihood analysis with 1000 bootstrap replications.* Bursaphelenchus anatolius *and* B. platzeri*, sister taxa of the* xylophilus*-group, served as outgroup species.*

Table 3. *Intraspecific sequence variations in* Bursaphelenchus doui*; ITS region.*

Isolate code	Substitution sites from the 5′ end													
	71	89	108	111	124	143	148	569	594	610	641	698	730	878
NK204	T	C	A	A	A	T	A	A	G	G	C/T	G	T	G
NK217	C	C	G	C	T	C	T	G	G	G	T	G	T	G
NE26/04*	C	A	G	C	T	C	T	A	A	A	C	C	C	A

* Taiwanese population.

The vector beetle of *B. doui* was identified as *M. subfasciatus*, a species of longhorn beetle. The developmental stage of the *B. doui* dauer, *i.e.*, third- or fourth-stage dauer, was not determined as all dauers recovered were used to establish the culture. However, vector usage of *B. doui*, *i.e.*, dauer juveniles that entered *Monochamus* tracheae, seems similar to those of the other *xylophilus*-group species, namely, *B. baujardi*, *B. conicaudatus*, *B. crenati*, *B. eroshenkii*, *B. fraudulentus*, *B. kolymensis**,

* Suspected to be a junior synonym of *B. mucronatus* (Braasch *et al.*, 2005).

Table 4. *Intraspecific sequence variations in* Bursaphelenchus doui*; D2D3 LSU.*

Isolate code	Substitution sites from 5′ end		
	109	137	323
NK204	C	T	T
NK217	T	C	T
DQ899733*	T	C	C

SSU sequences of NK204 and NK217 were identical to each other.
* Strain name and original locality were not given in the database.

B. luxuriosae, *B. mucronatus*, *B. singaporensis* and *B. xylophilus* (see Ryss *et al.*, 2005). Usually, the fourth-stage dispersal (dauer) juveniles of the *xylophilus*-group enter the tracheal system and are vectored by longhorn beetles belonging to the tribe Lamiini, although the vector insects for *B. baujardi*, *B. crenati*, *B. eroshenkii*, *B. fraudulentus*** and *B. singaporensis* have not been identified (Kanzaki & Futai, 2003a; Walia *et al.*, 2003; Gu *et al.*, 2005; Ryss *et al.*, 2005; Penas *et al.*, 2006). The relationship between *B. doui* and *M. subfasciatus* supports the hypothesis that the *xylophilus*-group-Lamiini relationship began with the ancestor of the *xylophilus*-group and has been maintained during subsequent speciation (Kanzaki & Futai, 2003b). However, at present, only five (six if *B. kolymensis* is included) instances of *xylophilus*-group-Lamiini relationships have been clarified, namely: *B. xylophilus-Monochamus* spp.; *B. mucronatus-Monochamus* spp.; *B. conicaudatus-Psacothea hilaris*; *B. luxuriosae-Acalolepta luxuriosa*; and *B. doui-M. subfasciatus* (reviewed in Kanzaki & Futai, 2003a, b; Ryss *et al.*, 2005; Penas *et al.*, 2006). Further field surveys on Lamiini beetles and molecular phylogenetic analysis on both nematodes and beetles may be needed to confirm the co-speciation/co-evolution hypothesis.

Bursaphelenchus doui described in the present study was isolated from coniferous packaging materials (Braasch *et al.*, 2005), and also isolated from *P. densiflora*. However, we assume that the potential risk of *B. doui* for pine species is relatively low as *B. doui* did not produce any significant wilting symptom on Japanese black pine, a species highly susceptible to pine wilt. Kanzaki and Futai (2006) reported that *B. mucronatus*, which is generally considered to be avirulent, could kill shaded (weak light condition) Japanese red pine seedlings and cause 65% mortality. Braasch (2000) reported that the mortality of *P. sylvestris* inoculated with *B. mucronatus* was increased by extreme drought and/or high temperature. Thus, additional pathogenicity tests under various conditions may be needed to evaluate precisely the potential risk of *B. doui*.

Monochamus subfasciatus, the vector of *B. doui*, is an euryphagous species that usually feeds and oviposits on broad-leaved trees, although it has sometimes been reported on conifers as well (Kojima & Nakamura, 1986; Kusama & Takakuwa, 1995). In the present study, the tree species from which the vector beetle emerged was not identified, although the euryphagy of *M. subfasciatus* suggests that *B. doui* is usually an inhabitant of dead broad-leaved trees.

** Rühm (1956) isolated *B. fraudulentus* from frass of *Cerambyx scopolii* and *Trypophloeus granulatus*. As it was not isolated from a beetle, the natural vector of the nematode is still unknown.

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References

BOLLA, R.I. & BOSCHERT, M. (1993). Pinewood nematode species complex: Interbreeding potential and chromosome number. *Journal of Nematology* 25, 227-238.

BRAASCH, H. (2000). Influence of temperature and water supply on mortality of 3-year-old pines inoculated with *Bursaphelenchus xylophilus* and *B. mucronatus*. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 52, 244-249.

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.

BRZESKI, M.W. & BAUJARD, P. (1997). Morphology and morphometrics of *Bursaphelenchus* (Nematoda: Aphelenchoididae) species from pine wood of Poland. *Annales Zoologici* 47, 305-319.

BURGERMEISTER, W., METGE, K., BRAASCH, H. & BUCHBACH, E. (2005). ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution. *Russian Journal of Nematology* 13, 29-42.

CHEN, X., GU, J. & ZHANG, J. (2005). [Nematodes detected from imported packing wood in Ningbo port.] *Journal of Zhejiang Forestry Science and Technology* 25, 59-62.

GIBLIN, R.M. & KAYA, H.K. (1984). Host, temperature and media additive effects on the growth of *Bursaphelenchus seani*. *Revue de Nématologie* 7, 13-17.

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., BRAASCH, H., BURGERMEISTER, W. & ZHANG, J. (2006). Records of *Bursaphelenchus* spp. intercepted in imported packaging wood at Ningbo, China. *Forest Pathology* 36, 323-333.

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.

IWAHORI, H., TSUDA, K., KANZAKI, N., IZUI, K. & FUTAI, K. (1998). PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundamental and Applied Nematology* 21, 655-666.

KANZAKI, N. (2006). [Taxonomy and systematics of *Bursaphelenchus* nematodes.] *Journal of the Japanese Forestry Society* 88, 392-406.

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

KANZAKI, N. & FUTAI, K. (2002b). Phylogenetic analysis of the phoretic association between *Bursaphelenchus conicaudatus* (Nematoda: Aphelenchoididae) and *Psacothea hilaris* (Coleoptera: Cerambycidae). *Nematology* 4, 759-771.

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

KANZAKI, N. & FUTAI, K. (2003b). Application of molecular phylogenetic analysis to the evolution and co-speciation of entomophilic nematodes. *Russian Journal of Nematology* 11, 107-117.

KANZAKI, N. & FUTAI, K. (2006). Is *Bursaphelenchus mucronatus* a weak pathogen to the Japanese red pine? *Nematology* 8, 485-489.

KANZAKI, N. & FUTAI, K. (2007). Isolation of *Bursaphelenchus sinensis* (Nematoda: Parasitaphelenchidae) from dead Japanese black pine, *Pinus thunbergii* Parl. in Japan. *Journal of Nematode Morphology and Systematics* 9, 129-136.

KOJIMA, K. & NAKAMURA, S. (1986). [*Food plants of cerambycid beetles (Cerambycidae, Coleoptera) in Japan.*] Hiroshima, Japan, Hioba Society of Natural History, 336 pp.

KUSAMA, K. & TAKAKUWA, M. (1995). [Subfamily Lamiinae (part.).] In: The Japanese Society of Coleopterology (Ed.). [*The longhorn-beetles of Japan in color (reprint).*] Tokyo, Japan, Kodansha, pp. 352-493.

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.

PENAS, A.C., BRAVO, M.A., NAVES, P., BONIFÁCIO, L., SOUSA, E. & MOTA, M.M. (2006). Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) and other nematode genera associated with insects from *Pinus pinaster* in Portugal. *Annals of Applied Biology* 148, 121-131.

RYSS, A., VIEIRA, P., MOTA, M.M. & KULINICH, O. (2005). A synopsis of the genus *Bursaphelenchus* Fuchs (Nematoda: Aphehelenchida: Aphelenchoididae) with a key to species. *Nematology* 7, 393-458.

WALIA, K.K., NEGI, S., BAJAJ, H.K. & KLIA, 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.