Morphological and molecular characterisation of mucronate isolates ('M' form) of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae)

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Summary. Morphological and molecular characters of five isolates of the mucronate ('M') form of *Bursaphelenchus xylophilus* were compared with the round-tailed ('R') form of *B. xylophilus* and with *B. mucronatus* (European type and East Asian type). The spicules of these species (types or forms) are similar. The 'M' form of *B. xylophilus* is distinguished from the 'R' form of *B. xylophilus* by a distinct mucro at the female tail end. It differs from the European type of *B. mucronatus* by slightly shorter female tail mucro and position of excretory pore. It is distinguished from the East Asian type of *B. mucronatus* by female tail shape and shorter female tail mucro. The conventional five restriction endonucleases (*Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I) used for obtaining ITS-RFLP patterns of *Bursaphelenchus* species cannot distinguish the 'M' and 'R' forms of *B. xylophilus*, but the two forms can be differentiated by use of two additional restriction endonucleases (*Hpy*188 I and *Hha* I). The molecular phylogenetic analysis based on the sequences of D2D3 LSU rDNA, ITS1/2 region and mtCOI revealed that the 'M' form of *B. xylophilus* is genetically closest to the 'R' form of *B. xylophilus*, and that their sequence divergence is small.

Key words: *Bursaphelenchus mucronatus*, *Bursaphelenchus fraudulentus*, D2D3, mtCOI, ITS-RFLP, molecular taxonomy, morphology.

The shape of the female tail end is an important taxonomic feature in the *xylophilus* group of the genus *Bursaphelenchus* (Braasch *et al.*, 2009). The presence or absence of a mucro at the female tail end has been a most essential criterion to distinguish the dangerous pine pest *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 from the related *B. fraudulentus* (Rühm, 1956) Goodey, 1960 and the widely distributed *B. mucronatus* Mamiya & Enda, 1979. In the original description of *B. xylophilus* (Steiner & Buhrer, 1934), only round-tailed females were mentioned. Nickle *et al*. (1981) wrote in their redescription of *B. xylophilus*: "Tail subcylindrical, usually with broadly rounded terminus". However, Mamiya & Kiyohara (1972) figured in their description of *B. lignicolus* (syn. *B. xylophilus*) from Japan beside round-tailed females also female tails with a small mucro and wrote: "Tail subcylindric with broadly rounded or digitate terminus". Since the report of a mucronate ('M') form of *B. xylophilus* detected from balsam fir (*Abies balsamea*) in Minnesota and Wisconsin, USA (Wingfield *et al*., 1983), uncertainty in morphological distinction of *B. xylophilus* from related species became evident. Moreover, a certain percentage of round-tailed *B. xylophilus* becomes mucronate during multiplication in trees (Braasch, 1996).

In inoculation experiments with round-tailed *B. xylophilus* (isolate US 15) on young Scots pines, nematodes were re-extracted from trees after three months, and 108 re-extracted females were studied. Among these, only 35% were round-tailed, 8% had conical tails, 17% had a distinct mucro (up to 4-5

μm), whereas 40% had a very small mucro of 1 μm length. After multiplying on *Botryotinia fuckeliana*, the 'R' form of *B. xylophilus* became again round-tailed. The 'M' form of *B. xylophilus* (US 10) and several isolates of *B. mucronatus* investigated in inoculation experiments did not change their tail shape after living for 3 months in host trees or in culture. Zhao & Yang (2005) also reported that a *B. xylophilus* isolate without mucro was inoculated onto Chinese pine (*Pinus tabulaeformis*), and 85% of the females re-isolated from the dead trees had a mucro. The mucro disappeared after inoculation on *Pestalotia* sp. Zheng *et al*. (2007) reported that an 'R' form isolate of *B. xylophilus* detected from a pine tree in Ningbo, China had a distinct mucro up to 2.9 μm (mean 1.7 μm), but the mucro disappeared after culturing on *B. fuckeliana*. Fonseca *et al*. (2008) conducted morpho-biometrical studies on 12 *B. xylophilus* isolates collected from Portugal and maintained on *Botryotinia fuckeliana*, found a wide variation in the female tails, from round, digitate to mucronate. All isolates had mucronate tailed females ranging from 30-60%, but females with a round tail always existed. Slightly mucronate females of 'R' form isolates of *B. xylophilus* have been repeatedly observed (Mamiya & Kiyohara, 1972; Chen *et al*., 1986; Mota *et al*., 1999). It should be noted, however, that isolates of the 'R' form of *B. xylophilus* always maintain at least a portion of round-tailed females.

Since the first report of a mucronate form (all females with a mucro) of *B. xylophilus* by Wingfield *et al*. (1983), this phenomenom has also been reported from Canada, Japan (Bolla & Boschert, 1993) and China (Ma *et al*., 1996), and it was also detected in *Pinus thunbergii*. However, the mucronate C2 isolate from Canada (Quebec) was subsequently identified as *B. mucronatus* by molecular methods (Harmey & Harmey, 1993; Braasch *et al*. 1995). A *B. xylophilus* population found in packaging wood transported from Taiwan to Nanjing, China (provided by Prof. M. Lin, Nanjing) also exhibited the 'M' form (Braasch, 2008). Baujard *et al*. (1979) reported on an unexplained *Pinus pinaster* decline and the occurrence of a mucronate *B. xylophilus* (*B. lignicolus*) in a forest in France. It became pathogenic only during the particularly dry summer of 1976, when trees were under severe stress. However, De Guiran & Boulbria (1986) identified isolates from this region as *B. mucronatus*. De Guiran and Bruguier (1989) found that the French 'M' form isolate could produce normal fertile hybrids with *B. mucronatus* (a Japanese isolate) as well as with *B. xylophilus* (also a Japanese isolate), but the same isolate did not produce fertile hybrids when mated with an 'M' form isolate of *B. xylophilus* from Minnesota.

Dwinell and Nickle (1989) considered the 'M' form isolates to be closely related to *B. mucronatus*, but Bolla and Boschert (1993) confirmed that interbreeding occurred between some 'M' and 'R' form isolates of *B. xylophilus*, whereas interbreeding of *B. xylophilus* and *B. mucronatus* was rare. Harmey and Harmey (1993) considered the 'M' form of *B. xylophilus* to be clearly related to the 'R' form of *B. xylophilus* by DNA fingerprinting method. The ITS-RFLP patterns of the two forms of *B. xylophilus* obtained with the standard digestion enzymes were identical (Hoyer *et al*., 1998; Li *et al*., 2009), but different from those of *B. mucronatus* and *B. fraudulentus* (Burgermeister *et al*., 2009).

The 'M' form of *B. xylophilus* from balsam fir was not pathogenic to Scots or red pine seedlings in the USA, whereas it was pathogenic to glasshouse-grown balsam fir seedlings (Wingfield *et al*., 1983). In a glasshouse study, Panesar *et al*. (1989) found that Scots pine in Finland was highly susceptible to a British Columbia 'M' form isolate and moderately susceptible to two Quebec 'M' form isolates, but mortality of pines occurred more rapidly following inoculation with 'R' form nematodes than with 'M' form nematodes. Braasch (2000) also observed that an 'M' form isolate of *B. xylophilus* (US10, Minnesota) can kill 3-year-old Scots pines in climate chambers at 25°C, but 3 weeks later than the 'R' form.

The pathogenicity may be linked with morphology because, up to now, no 'M' form of *B. xylophilus* has been found pathogenic on unstressed pines or other coniferous trees under natural conditions (De Guiran & Bruguier, 1989). So, it is not only of theoretical interest, but also of practical importance to distinguish the two forms of *B. xylophilus*. The present paper reports on morphological and molecular differences between the two forms of *B. xylophilus* and differentiation of the 'M' form from *B. mucronatus* and *B. fraudulentus*.

MATERIAL AND METHODS

Culturing and morphological observations. Five cultures of the 'M' form of *B. xylophilus* kept on *Botryotinia fuckeliana*/PDA and several *B. xylophilus*, *B. mucronatus* and *B. fraudulentus* cultures or isolates detected from packaging wood were studied (see Table 1). An 'R' form isolate of *B. xylophilus* (isolate 4049) detected in packaging wood with a 'Heat Treatment' mark of 'US', was examined for the presence of a mucro before and

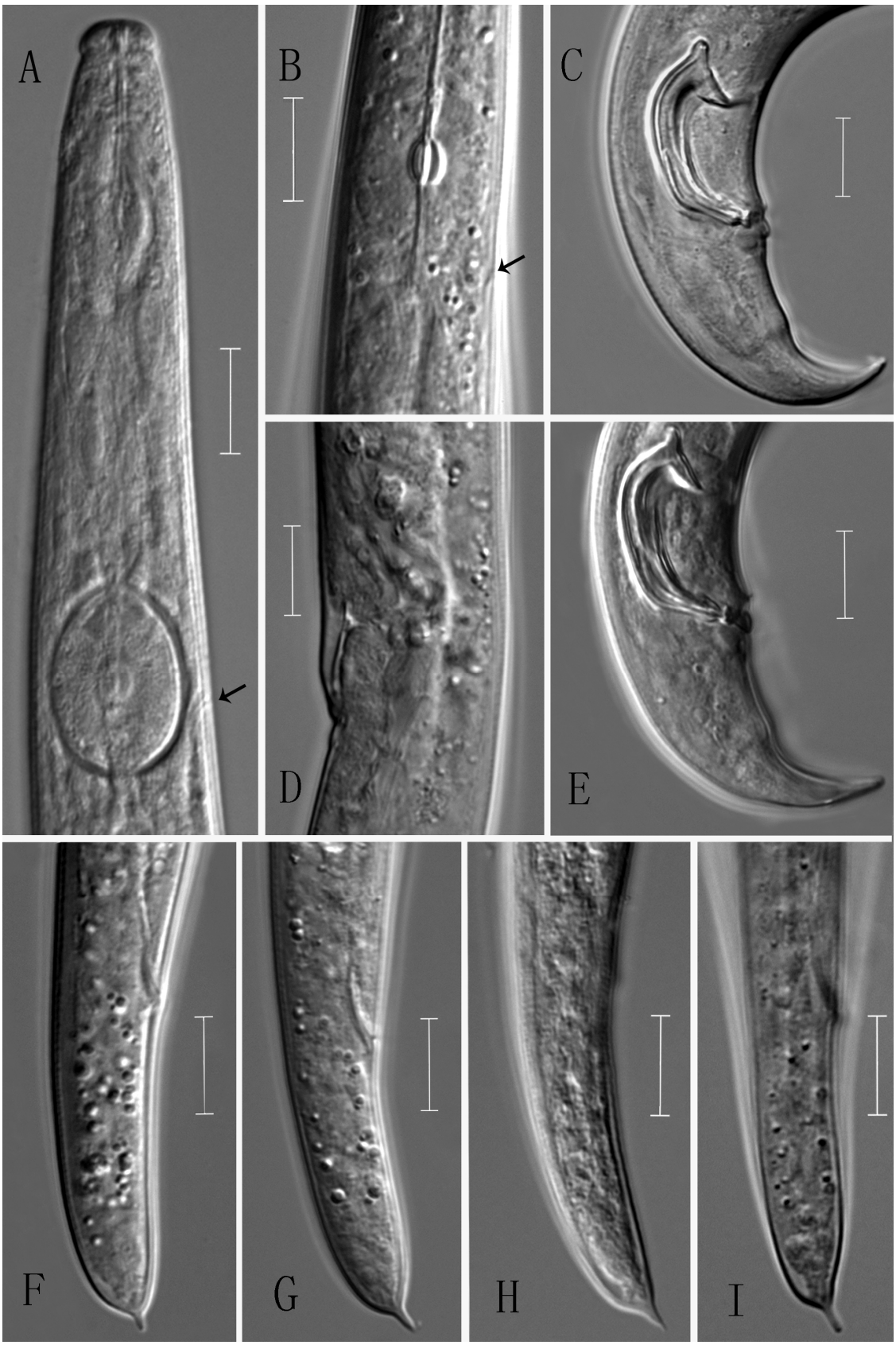

Fig. 1. Light photomicrographs of the 'M' form *Bursaphelenchus xylophilus* (isolate CA) A, B: Head region (shows excretory pore position); C, E: Male tail; D: Vulva region; F- I: Female tails. (Scale bars = 10 µm).

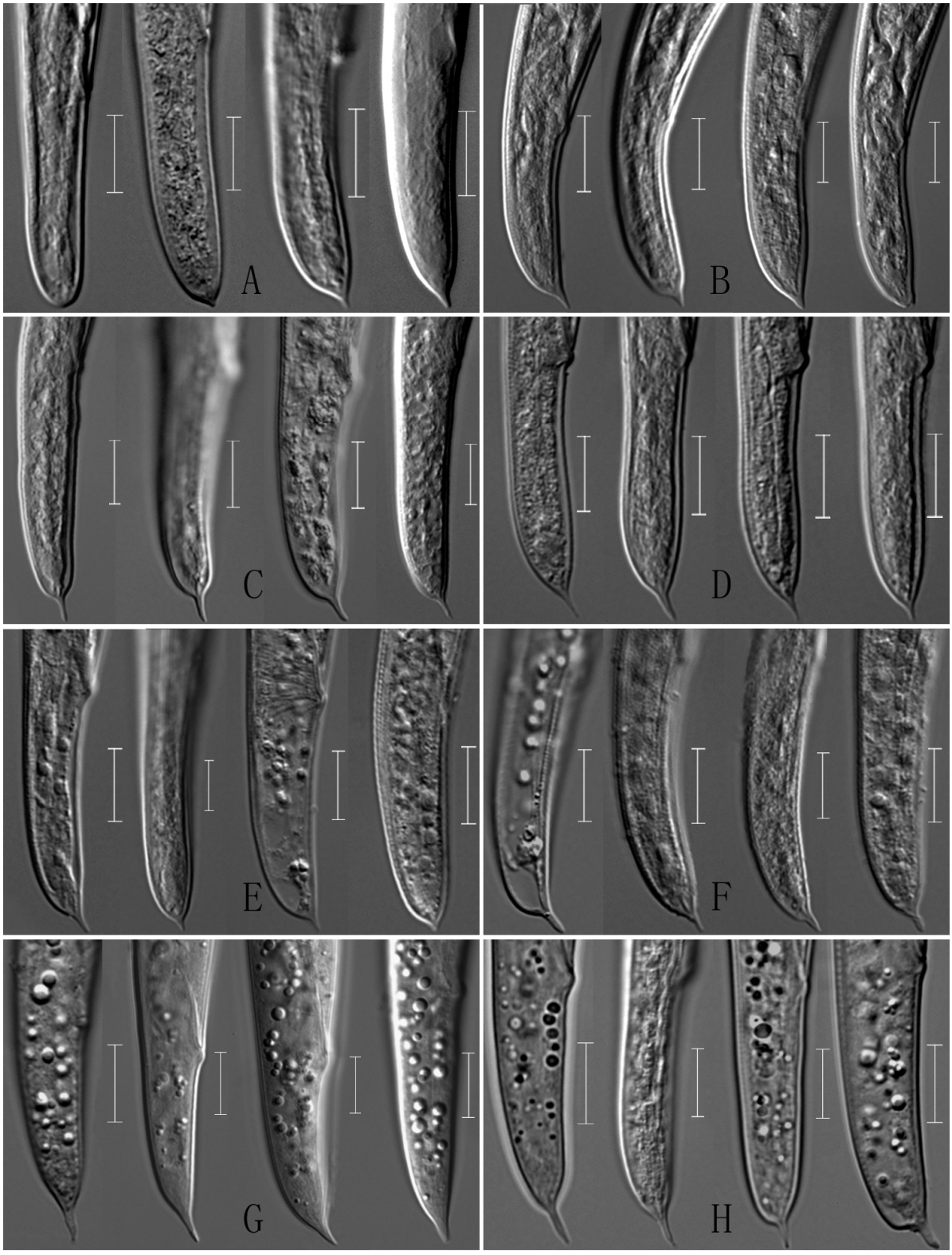

Fig. 2. Light microphotographs of female tail shape. A:'R' form *Bursaphelenchus xylophilus*; B: *B. fraudulentus*; C: 'M' form *B. xylophilus* (isolate US10); D: 'M' form *B. xylophilus* (isolate TWRC); E: 'M' form *B. xylophilus* (isolate BC); F: 'M' form *B. xylophilus* (isolate Q52A); G: East Asian type of *B. mucronatus*; H: European type of *B. mucronatus*. (Scale bars = 10 μm.).

Table 1. *Bursaphelenchus xylophilus, B. mucronatus* and *B. fraudulentus* isolates used for study.

Species[a]	Isolate number	Locality	Plant host[b]	GenBank accession No.		
				ITS	28S	mtCOI
B. xylophilus (R)	121AD	USA	*Pinus* packaging wood	JF317234	JF317244	JF317257
B. xylophilus (R)	4049	USA	*Pinus* packaging wood	JF317232	–	JF317256
B. xylophilus (R)	30697	USA	*Pinus* packaging wood	–	–	JF317255
B. xylophilus (R)	39906	Mexico	*Pinus* packaging wood	JF317233	JF317245	JF317253 JF317254
B. xylophilus (M)	US10	Minnesota, USA	*Abies balsamea*	GU206790	JF317243	JF317251
B. xylophilus (M)	Q52A	Quebec, Canada	Mixed spruce and fir chips	JF317230	JF317241	JF317252
B. xylophilus (M)	BC	British Columbia, Canada	Coniferous wood chips	JF317231	JF317240	JF317248
B. xylophilus (M)	CA	Canada	Packaging wood	JF317229	JF317239 EU295503[c]	JF317249
B. xylophilus (M)	TWRC	Taiwan, China	Packaging wood	AM179515	JF317242	JF317250
B. mucronatus (E)	38624	Ukraine	*Pinus* packaging wood	–	–	JF317258
B. mucronatus (E)	860A	Spain	*Pinus* packaging wood	–	–	JF317262
B. mucronatus (E)	4228	Brazil	*Pinus* packaging wood	–	–	JF317259
B. mucronatus (E)	5459	Israel	*Pinus* packaging wood	JF317237	–	–
B. mucronatus (E)	XSNB	China	*Pinus massoniana*	-	–	–
B. mucronatus (E)	53106	Ukraine	*Pinus* packaging wood	JF317238	–	–
B. mucronatus (EA)	424B	Japan	*Pinus* packaging wood	JF317235	JF317246	JF317260
B. mucronatus (EA)	39571	Korea	*Pinus* packaging wood	JF317236	JF317247	JF317261
B. fraudulentus	Ne 4b / 08	Poland	*Quercus robur*	–	–	–

[a] After species name in brackets: 'R' represents for 'R' form, 'M' represents for 'M' form, 'E' represents for European type", 'EA' represents for 'East Asian type'

[b] Because packaging wood is a circulating product and repeatedly used, its real origin remains unsure.

[c] According to Li *et al*., 2009.

after culturing on *B. fuckeliana* and *Pestalotiopsi*s sp. The light micrographs were made with a Zeiss Imager Z1 microscope equipped with a Zeiss AxioCam MRm CCD camera. All isolates were identified by morphological and molecular methods (if all the females had a distinct mucro, but the standard RFLP pattern was similar to *B. xylophilus*, the isolate was identified as the 'M' form of *B. xylophilus*; if round-tailed females were found, the isolate was identified as the 'R' form of *B. xylophilus*).

Molecular profiles. Single nematodes were washed in ddH_2O under a dissecting microscope and transferred to a 200 µl Eppendorf tube (containing 8 µl ddH_2O and 1 µl 10×PCR buffer) using a small picking needle. The tube was stored at -70°C for at least 30 min, after which it was heated at 85°C for 2 min. Subsequently, 1 µl proteinase K (1 mg ml^{-1}) was added into the tube, and it was incubated at 56°C for 30 min and 95°C for 10 min. DNA samples were stored at -20°C until they were used as PCR template.

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, the D2D3 LSU region of rDNA and the partial mtCOI gene, respectively. Primers for amplification of SSU were forward primer K4f (5'-ATG CAT GTC TAA GTG GAG TAT TA -3') and reverse primer K1r (5'- TTC ACC TAC GGC TAC CTT GTT ACG ACT -3') (Penas *et al*., 2006). Primers for amplification of ITS1/2 were forward primer F194 (5'- CGT AAC AAG GTA GCT GTA G -3') (Ferris *et al*., 1993) and reverse primer 5368r (5'- TTT CAC TCG CCG TTA CTA AGG -3') (Vrain, 1993). Primers for amplification of D2/D3 LSU were forward primer D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and reverse primer D3Br (5'-TCG GAA GGA ACC AGC TAC TA-3') (De Ley *et al*., 1999). Primers for amplification of mtCOI were forward primer COI-F1 (5'- CCT ACT ATG ATT GGT GGT TTT GGT AAT TG -3') and reverse primer COI-R2 (5'- GTA GCA GCA GTA AAA TAA GCA CG -3') (Kanzaki and Futai, 2002). PCR conditions were as described by Li *et al*. (2008) and Ye *et al*. (2007). PCR products were separated on 1% agarose gels and visualized by staining with ethidium bromide. Most of the ITS1/2, LSU and mtCOI PCR products were directly sequenced in both directions. Cloning procedures were applied only for those PCR products that did not yield high quality sequences. All SSU PCR

products were cloned before sequencing (by Invitrogen, Shanghai, China). The final sequences were then deposited into GenBank database under accession numbers shown in Table 1.

To obtain 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 seven restriction endonucleases *Rsa* I, *Hae* III, *Msp* I, *Hinf* I, *Alu* I, *Hpy*188 I and *Hha* 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 partial LSU, ITS1/2 and mtCOI sequences (the partial SSU sequences of different *B. xylophilus* isolates are almost identical and not suitable for further analysis) were analysed and aligned using the program ClustalW implemented in MEGA version 4.0 (Tamura *et al*., 2007). Phylogenetic trees were generated with the Neighbor Joining (NJ) method using the Tajima-Nei distance option. Bootstrapping analysis was performed with 1000 replicates.

RESULTS

Measurements. Measurements of two mucronate isolates of *B. xylophilus* were compared with each isolate of the European type of *B. mucronatus* and *B. fraudulentus*, which are shown in Table 2.

Measurements of the excretory pore position and mucro length of five isolates of the 'M' form of *B. xylophilus* and related species are shown in Table 3.

Description of the 'M' form of *Bursaphelenchus xylophilus* (Fig. 1)

Male. Body J-shaped when heat-killed. Cuticle weakly annulated, lateral field with four incisures (*i.e*. three ridges). Lip region offset. Stylet with small basal thickenings. Procorpus cylindrical. Median bulb strongly developed, oval, with valve plates situated centrally or slightly posteriorly. Pharyngeal gland lobe slender and well developed, about four body diameters long, overlapping intestine dorsally. Nerve ring located *ca* 3-8 μm posterior to median bulb. Excretory pore located posterior to median bulb or at the position of median bulb, but never anterior to it (Table 3). Hemizonid located posterior to excretory pore. Testis single, spermatocytes arranged in two rows. Cloaca opening lips slightly protruding. Spicules arcuate, condylus slightly offset from the dorsal spicule line, ventral rostrum elongate, sharply pointed; distal ends of spicules with distinct cucullus. Tail strongly recurved, terminus pointed, talon-like in lateral view. Seven caudal papillae present: a single median papilla just preanal, an adanal pair just before the anus, two post-anal pairs just anterior to the beginning of bursal flap and close to each other. Bursa long, oval or spade-shaped.

Female. Body slightly ventrally arcuate when heat-relaxed. Cuticle and lip region similar to male. Ovary outstretched, developing oocytes in two rows. Vulva with prominent cuticular flap. Spermatheca elongate-oval or irregular, sometimes containing round sperms. Postuterine sac elongate, a little more than half vulva to anus distance. Tail subcylindrical, slightly ventrally curved, tail terminus with an offset mucro, on average 2.2-3.0 μm long (1.5-4.2 μm) (Table 3 and Fig. 1 & 2).

Morphological relationships. The 'M' form of *B. xylophilus* is morphologically most similar to the European type of *B. mucronatus*. It is distinguished from it by slightly shorter mucro on female tail (mean 2.2-3.0 μm *vs* 3.0-5.0 μm) (Fig. 3), position of excretory pore behind median bulb (isolates US10 and TWRC) or at the level of median bulb (isolates BA, CA and Q52A), but never anterior to median bulb, whereas the excretory pore of the European *B. mucronatus* is situated at the level of median bulb or anterior to it (Braasch, 2008) or slightly posterior to median bulb (isolate 860A) depending on different provenances.

The mucronate form of *B. xylophilus* is distinguished from the East Asian type of *B. mucronatus* by the female tail shape (mucro well offset from the sub-cylindrical tail *vs* mucro as continuation of the conical tail) (Fig. 2) and the length of the mucro on the female tail (mean 2.2-3.0 μm *vs* 4.4 μm). The position of excretory pore, which is usually behind the median bulb in the East Asian type of *B. mucronatus* (Braasch, 2008) as in the original description (Mamiya & Enda, 1979), however, exceptionally at level or anterior to median bulb (isolate 424B), is not always suitable for identification.

The 'M' form of *B. xylophilus* is also morphologically similar to the 'R' form of *B. xylophilus* except the female tail terminus. The female tail terminus of round-tailed *B. xylophilus* is broadly rounded and usually without mucro, although several females may possess a small mucro less than 2 μm long or even up to 3.4 μm in a few females. In isolate 4049, about one half of the females detected from packaging wood had a round tail, and the other half showed a very small mucro about 0.5-1 μm long. After culturing on *B. fuckeliana* for one month, more than half of females showed a mucro of about 1-2 μm length, whereas *ca* 10% of the females had a mucro of 2-2.4 μm, and the remaining specimens had a mucro less than 0.5 μm long or no mucro. However, after culturing them

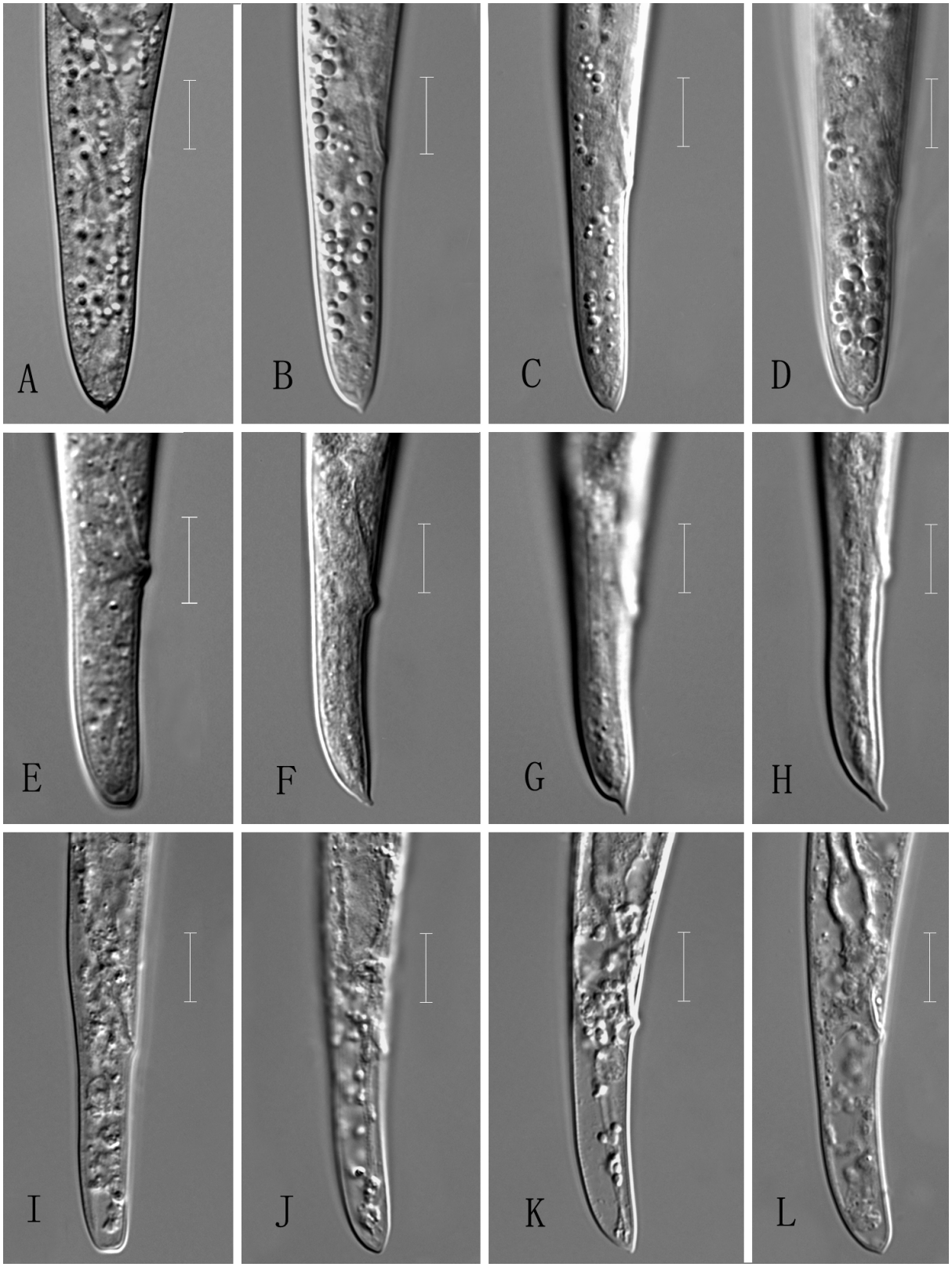

Fig. 3. Light microphotographs of female tails of 'R' form of *Bursaphelenchus xylophilus* (isolate 4049) in different situations. A-D: Detected from the packaging wood; E-H: After culturing on *B. fuckeliana*; I-L: After culturing on *Pestalotiopsis* sp. (Scale bars = 10 μm.)

Table 2. Measurements of two isolates of the 'M' form of *Bursaphelenchus xylophilus* and each one isolate of the European type of *B. mucronatus* and *B. fraudulentus*. All measurements are in µm and in the form: mean±s.d. (range).

	B. xylophilus (isolate US10, 'M' form)		*B. xylophilus* (isolate CA, 'M' form)		*B. mucronatus* (isolate XSNB, 'EA' type)		*B. fraudulentus* (isolate Ne 4b / 08)	
	Male	Female	Male	Female	Male	Female	Male	Female
n	15	15	15	15	15	15	15	15
L	826±64.7 (692-921)	862±73.8 (700-972)	851±45.8 (780-944)	1,001±50.5 (908-1,096)	769±67.4 (667-884)	914±129.2 (721-1,125)	742±39.0 (685-795)	811±37.6 (725-854)
a	44.5±4.6 (36.9-55.2)	40.0±5.2 (32.5-46.6)	41.4±3.8 (35.6-50.1)	41.5±4.3 (34.1-48.8)	46.1±3.9 (37.0-52.8)	46.5±3.8 (40.6-55.1)	36.5±2.0 (32.4-38.7)	33.5±2.8 (28.6-37.4)
b	11.3±0.8 (10.0-12.5)	11.9±1.2 (10.6-14.9)	12.9±0.9 (11.1-13.9)	12.8±0.6 (11.9-13.9)	10.9±0.8 (9.5-12.1)	12.5±1.5 (10.2-15.2)	9.8±0.6 (9.1-11.2)	10.5±0.6 (9.3-11.3)
b'	5.9±0.3 (5.4-6.4)	6.0±0.8 (4.3-7.2)	7.3±0.5 (6.6-8.0)	7.3±0.5 (6.3-8.3)	5.7±0.7 (4.7-7.4)	6.4±0.5 (5.4-7.1)	6.0±0.2 (5.7-6.6)	6.3±0.3 (5.8-6.9)
c	23.0±1.7 (20.2-25.5)	24.5±2.8 (21.3-31.2)	28.4±1.9 (25.6-32.4)	33.0±1.9 (29.3-36.0)	24.1±1.9 (21.2-27.5)	28.2±2.2 (24.4-31.8)	24.6±1.5 (21.3-26.0)	30.6±3.5 (22.7-35.0)
c'	2.4±0.1 (2.2-2.6)	3.5±0.3 (3.1-4.2)	2.1±0.1 (1.9-2.3)	3.0±0.2 (2.8-3.2)	2.4±0.2 (2.1-2.8)	3.4±0.3 (3.1-4.4)	1.9±0.1 (1.7-2.2)	2.6±0.3 (2.3-3.1)
V or T	52.0±9.4 (44.8-76.9)	71.2±1.2 (69.3-74.9)	52.1±3.5 (42.7-56.6)	74.4±0.9 (72.2-75.2)	56.3±5.8 (46.9-69.1)	73.0±1.1 (71.2-74.8)	50.1±3.2 (43.5-54.6)	75.9±0.7 (74.8-77.1)
Max body diam	18.7±2.0 (14.9-23.3)	21.9±3.2 (17.7-30.0)	20.7±2.0 (17.3-24.1)	24.3±2.8 (19.6-28.6)	16.7±1.1 (14.8-18.3)	19.7±2.4 (15.8-24.1)	20.4±1.5 (18.5-23.7)	24.3±1.4 (22.1-26.9)
Lip diam	7.4±0.4 (6.7-8.0)	7.3±0.5 (6.3-8.6)	7.4±0.4 (6.5-8.0)	7.7±0.5 (6.5-8.3)	7.1±0.3 (6.7-7.5)	7.7±0.4 (6.8-8.3)	7.9±0.2 (7.6-8.2)	8.0±0.2 (7.7-8.3)
Lip height	3.3±0.3 (2.7-3.8)	3.3±0.1 (3.0-3.4)	3.3±0.2 (3.0-3.7)	3.2±0.4 (2.5-3.9)	3.1±0.4 (2.6-3.7)	3.1±0.3 (2.6-3.6)	3.2±0.2 (2.9-3.5)	3.2±0.2 (2.9-3.6)
Stylet length	15.2±0.6 (13.7-16.2)	15.0±0.4 (14.3-15.5)	14.6±0.7 (13.7-16.1)	15.0±0.6 (13.7-15.9)	14.0±0.6 (13.1-15.2)	14.5±0.7 (13.3-15.6)	14.8±0.5 (14.2-15.8)	14.9±0.4 (14.4-15.8)
Median bulb length	16.9±1.1 (13.9-18.0)	17.6±1.1 (15.8-20.0)	16.7±0.5 (16.0-17.7)	17.8±0.8 (16.1-19.0)	16.8±0.8 (15.5-18.2)	18.3±1.2 (16.7-20.0)	17.4±0.4 (16.9-17.9)	17.6±0.4 (16.9-18.5)
Median bulb diam	11.2±0.9 (10.0-13.7)	11.2±1.2 (10.1-14.4)	11.1±0.5 (10.1-11.7)	12.5±1.0 (10.9-14.4)	11.0±0.4 (10.0-11.9)	12.6±1.2 (10.7-14.7)	12.6±0.4 (12.2-13.3)	12.8±0.4 (12.4-13.5)
Excretory pore position	82.4±3.8 (74.7-89.0)	82.1±3.5 (75.6-87.7)	73.8±5.7 (65.5-83.4)	69.8±2.6 (66.1-71.6)	62.1±8.5 (50.1-80.6)	61.7±7.3 (51.2-74.0)	53.8±2.2 (50.0-57.0)	53.9±5.3 (48.3-68.7)
Spicule(chord)	23.5±1.2 (21.5-25.5)	–	23.7±1.1 (21.2-25.0)	–	22.5±1.4 (20.1-24.9)	–	24.9±1.0 (22.7-26.0)	–
Spicule(curved median line)	26.5±1.4 (24.0-28.9)	–	27.4±1.3 (25.4-28.8)	–	27.0±2.1 (22.3-29.5)	–	27.3±1.3 (25.0-29.8)	–
Ovary or testis length	429.2±83.0 (327.6-633.0)	390.7±77.8 (261.4-520.0)	442.5±34.5 (376.0-495.0)	368.5±32.0 (320.0-431.4)	431.6±47.6 (354.8-540.0)	399.2±100.7 (277.1-586.6)	380.9±31.2 (321.0-426.0)	305.0±83.4 (53.0-383.0)
Post-uterine sac length	–	139.9±22.0 (111.2-182.0)	–	182.9±15.1 (160.0-217.7)	–	142.1±17.1 (118.4-171.8)	–	108.6±5.7 (100.0-116.0)
Post-uterine sac length/ Vulva to anus distance (%)	–	64.3±12.5 (41.6-82.9)	–	71.6±4.5 (62.5-80.4)	–	68.3±7.5 (49.9-79.8)	–	65.8±2.7 (62.1-70.8)
Tail length	35.9±2.4 (32.7-40.8)	35.4±3.4 (31.2-44.6)	30.0±1.5 (27.3-33.5)	30.5±1.6 (27.4-32.3)	32.0±2.7 (27.3-36.7)	32.4±3.1 (27.0-37.4)	30.3±3.0 (27.0-36.0)	26.8±2.4 (23.6-32.0)
Anal body diam	15.2±1.1 (14.1-17.5)	10.1±0.8 (9.2-11.7)	14.5±0.6 (13.5-15.7)	10.4±0.4 (9.9-11.2)	13.5±0.9 (12.6-15.2)	9.6±1.0 (8.3-11.3)	16.2±0.8 (15.0-17.1)	10.5±0.6 (9.6-11.3)
Mucro length	–	3.0±0.6 (2.1-4.2)	–	2.6±0.5 (1.7-3.4)	–	4.3±0.7 (3.2-5.4)	–	1.6±0.8 (0-2.8)

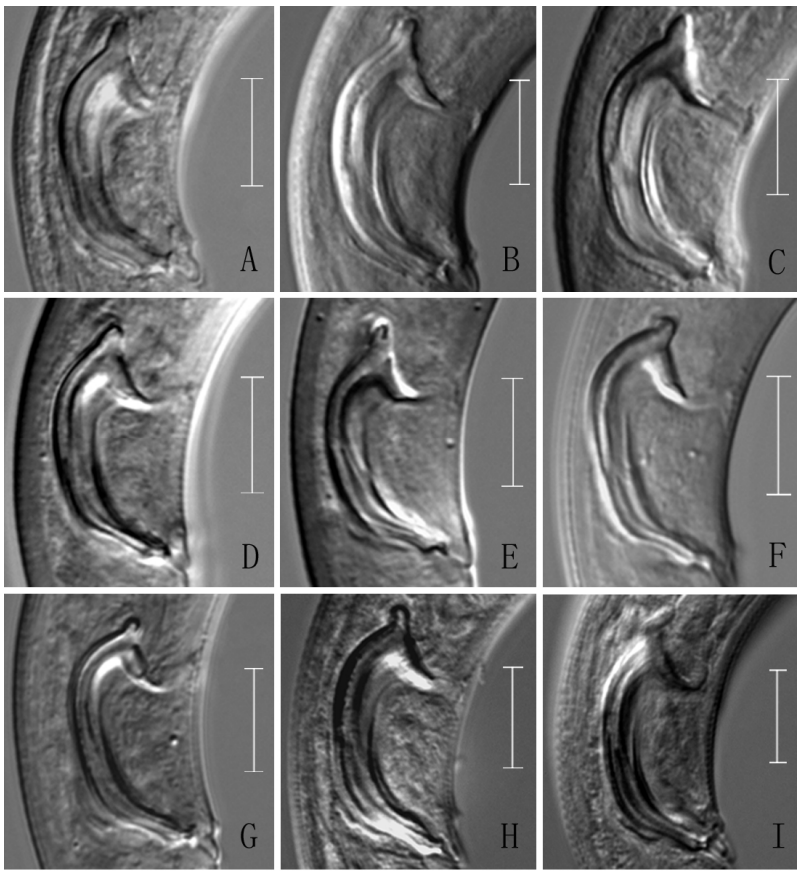

Fig. 4. Light microphotographs of spicule shape. A: *Bursaphelenchus xylophilus* 'R' form; B, C: *B. xylophilus* 'M' form; D, E: East Asian type of *B. mucronatus*; F, G: European type of *B. mucronatus*; H, I: *B. fraudulentus*. (Scale bars = 10 µm.).

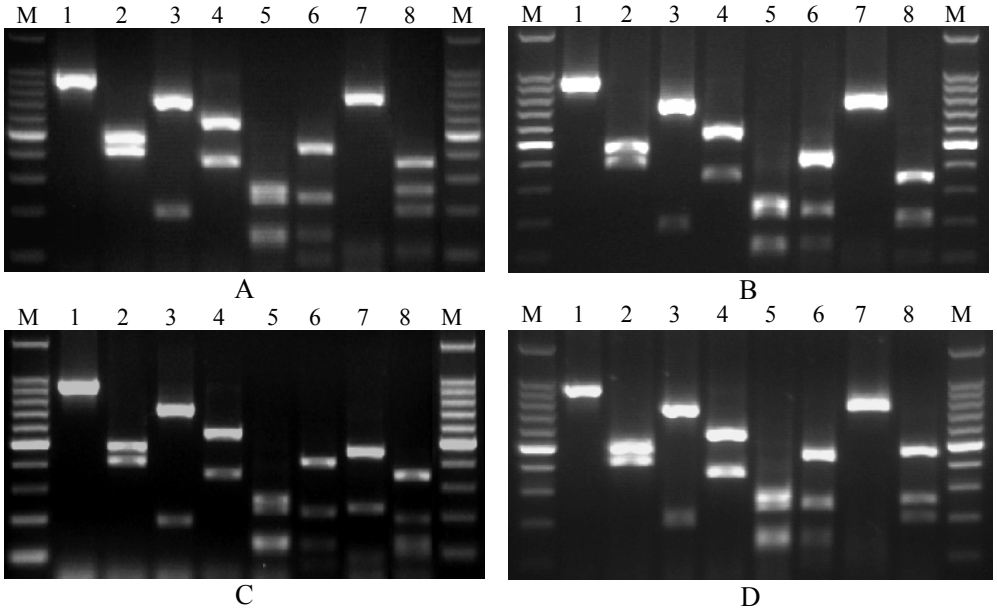

Fig. 5. RFLP patterns. A: 'R' form *Bursaphelenchus xylophilus* (for isolates 121AD, 4049 and 30697); 'R' form *B. xylophilus* (for isolate 39906); C:'M' form *B. xylophilus* (for isolates US10 and Q52A); D: 'M' form *B. xylophilus* (for isolates CA, BC and TWRC). M = Molecular size marker (100 bp ladder); Lane 1: rDNA amplification product; Lanes 2-8: Digestion products obtained with *Rsa*I, *Hae*III, *Msp*I, *Hinf*I, *Alu*I, *Hpy*188I and *Hha*I . Sizes of PCR product and its restriction fragments are shown in Table 4.

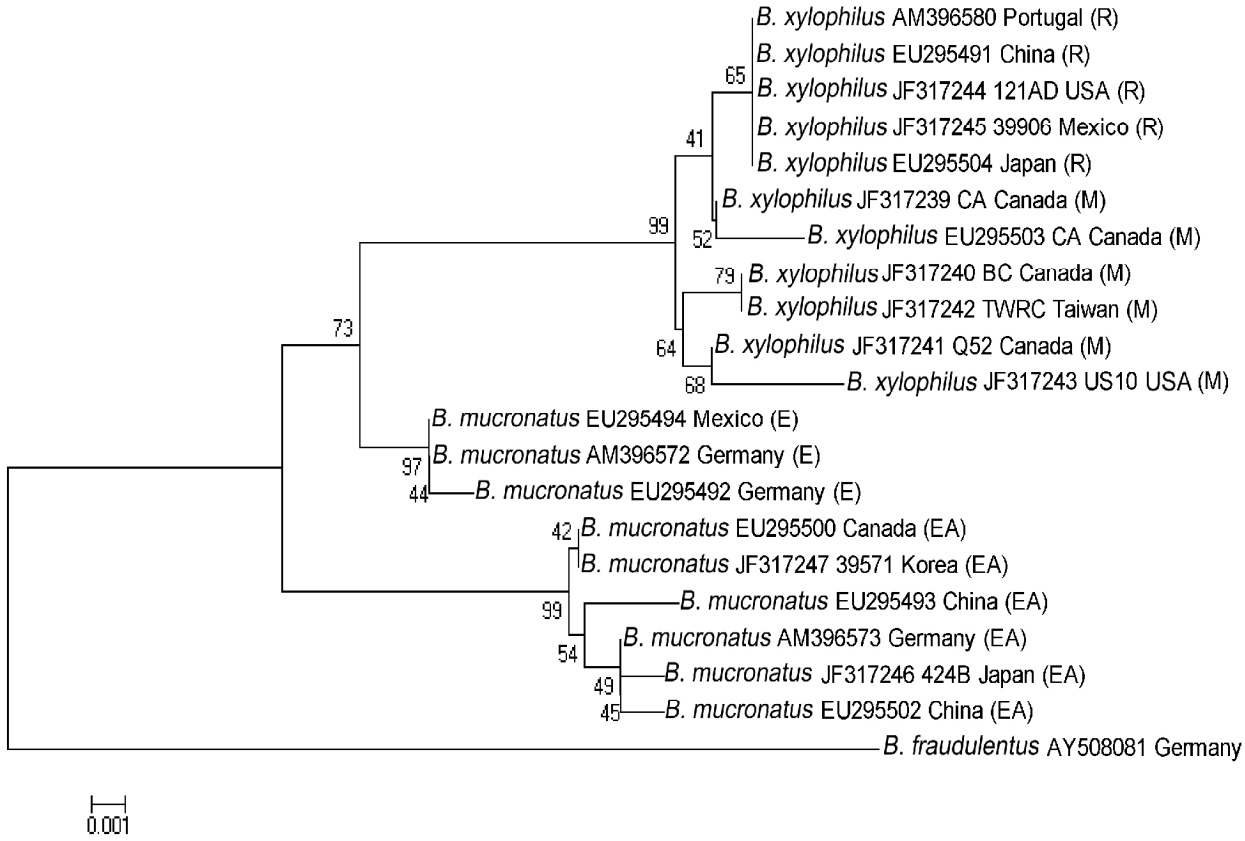

Fig. 6. Molecular phylogenetic status of *Bursaphelenchus xylophilus* and *B. mucronatus* based on partial 28S sequences. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

Table 3. Measurements of excretory pore position and mucro length of 'M' form of *B. xylophilus* compared with some related species. All measurements are in µm and in the form: mean±s.d. (range).

Isolates	Excretory pore to the posterior margin of median bulb[a]		Excretory pore to hemizonid		Mucro length
	Female (n=15)	Male (n=15)	Female (n=15)	Male (n=15)	Female (n=15)
B. xylophilus (30697, R)	9.8±3.0 (3.3-13.6)	10.0±2.4 (6.7-13.6)	12.1±3.7 (9.0-23.0)	11.5±3.4 (8.5-21.0)	(0-2)[b]
B. xylophilus (4049, R)	-1.3±7.4 (-13.0-14.0)	2.9±8.0 (-12.7-13.0)	16.2±5.1 (8.0-23.6)	15.8±6.3 (6.2-30.9)	0.9±0.6 (0-2.3)
B. xylophilus (US10, M)	12.1±3.6 (7.0-18.9)	11.8±2.2 (8.6-14.8)	11.9±3.5 (6.5-17.7)	11.5±2.3 (8.4-14.7)	3.0±0.6 (2.1-4.2)
B. xylophilus (Q52, M)	6.8±9.6 (-5.9-23.1)	-2.4±4.4 (-9.6-4.8)	20.7±5.3 (14.0-30.3)	23.7±4.4 (16.9-30.8)	2.7±0.5 (2.1-3.7)
B. xylophilus (BC, M)	-7.3±3.4 (-14.1-0)	-4.1±7.9 (-16.0-9.8)	30.7±6.3 (19.7-40.3)	32.9±7.1 (21.1-44.3)	2.2±0.4 (1.5-3.1)
B. xylophilus (CA, M)	-6.2±5.1 (-13.7-0)	-6.1±4.8 (-13.3-0)	25.2±5.5 (17.3-37.0)	24.3±4.4 (17.3-32.0)	2.6±0.5 (1.7-3.4)
B. xylophilus (TWRC, M)	9.7±4.2 (2.1-16.1)	5.2±2.7 (0-11.3)	18.3±3.8 (12.7-24.7)	21.9±6.1 (13.5-36.7)	2.5±0.4 (1.8-3.4)
B. mucronatus (31399, E)	-13.3±4.1 -(20.5-5.8)	-16.2±1.4 (-18.0-0)	33.8±4.5 (24.0-42.2)	34.1±5.1 (21.0-40.0)	4.4±0.4 (3.6-5.2)
B. mucronatus (860A, E)	5.9±3.9 (-3.2-10.6)	6.5±3.6 (0-15.4)	17.3±3.9 (7.0-20.0)	18.3±2.3 (12.9-21.0)	4.0±0.8 (2.6-5.6)
B. mucronatus (424B, EA)	-5.9±6.8 (-16.9-2.1)	-14.9±11.0 (-29.4-2.8)	25.3±6.0 (17.4-39.4)	32.7±11.7 (16.2-48.0)	4.4±0.4 (3.7-5.1)

[a]When the excretory pore is anterior to the posterior margin of median bulb, '-' is added before the number.
[b]Most females have a broadly rounded tail terminus without mucro, but several females have a mucro less than 2 µm.

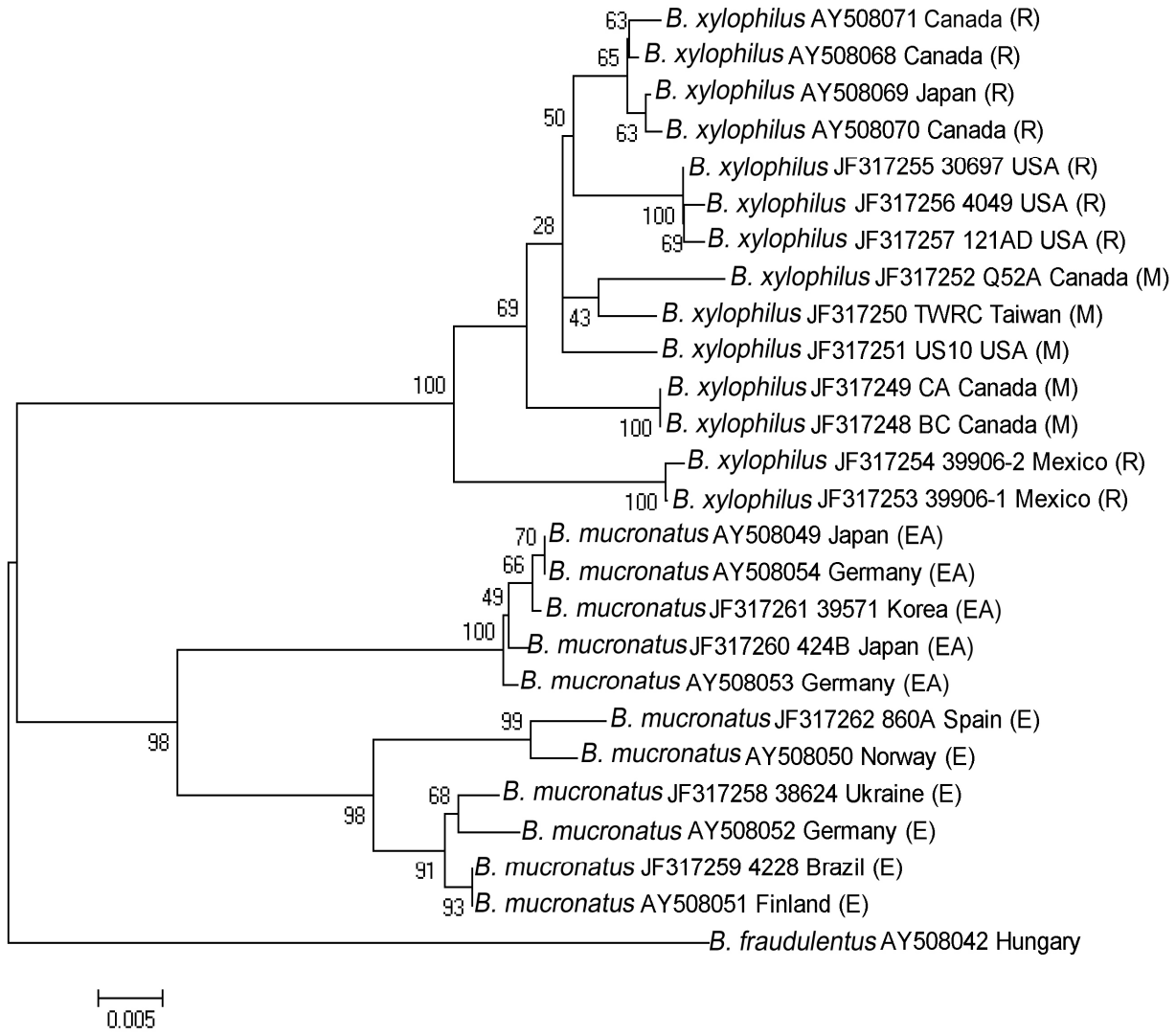

Fig. 7. Molecular phylogenetic status of *Bursaphelenchus xylophilus* and *B. mucronatus* based on ITS1/2 sequences. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

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on *Pestalotiopsis* sp., apart from some round-tailed females, most females had a bluntly pointed tail terminus (Fig. 3). On the other hand, in the mucronate form of *B. xylophilus*, all females have a mucro of about 2-3 µm length on their tail terminus. The spicules of both forms of *B. xylophilus* and both forms of *B. mucronatus* are similar (Fig. 4).

The 'M' form of *B. xylophilus* is also similar to *B. fraudulentus*, which is distinguished from *B. xylophilus* and *B. mucronatus* by stouter body (mean a=36.5 and 33.5 for males and females, respectively *vs* a>40), shorter female tail (mean length 26.8 µm, c'=2.6 *vs* mean length more than 30 µm, c'>3.0) with a short mucro having a wide base, and also the cucullus of the spicules is not distinctly expanded as in the other species of the *xylophilus* group (Fig. 4 H, I). Carletti *et al*. (2005) even found an Italian *B. fraudulentus* populations with mean a=30-33, whereas mean c' was also 2.6 for females, and the excretory pore was always behind the median bulb.

Molecular profiles and phylogenetic status. The ITS-RFLP profiles of rDNA are shown in Figure 5 and Table 4. With the conventional five enzymes *Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I (Burgermeister *et al.*, 2009), the ITS-RFLP patterns of the two forms of *B. xylophilus* are identical. By means of the additional enzymes *Hpy*188 I and *Hha* I, the 'M' form and 'R' form of *B. xylophilus* can be differentiated, but 'M' and 'R' form isolates also show fragment differences between isolates of the same form (Fig. 5). As indicated in Table 4, differentiation between the 'R' form isolates and the two 'M' form isolates US10 and Q52 A is based only on restriction

fragments obtained with *Hpy*188 I, whereas differentiation between the 'R' form isolates and the three 'M' form isolates BC, CA and TWRC relies only on fragments obtained with *Hha* I. Smaller differences in *Hha* I fragments are also seen between the 'R' form isolate 39906 on one hand and the three 'R' form isolates 121AC, 4049 and 30697 on the other hand.

The molecular phylogenetic status of the two forms of *B. xylophilus* and *B. mucronatus* is shown in Figures 6, 7 and 8. In the 28S tree, the 'R' form isolates create a distinct group. The 'M' form isolates cluster into two groups, one containing isolate CA from Canada, separated at low bootstrap value (41) from the 'R' form isolates. The other group containing four 'M' form isolates is separated at a high bootstrap value (99). However, sequence divergence between most 'M' and 'R' form isolates represented by branch lengths within the entire *B. xylophilus* cluster (Figs 6-8) is very low.

In contrast, the European and East Asian type isolates of *B. mucronatus* form two distinct groups which are separated at high sequence divergence from each other.

In the COI tree, the 'M' isolates form two groups, one containing US10, Q52A and TWRC, the other group containing isolates CA and BC. Most of the 'R' form isolates form a distinct group, but isolate 39906 from Mexico is distinctly separated (COI sequence from another single nematode of 39906 confirmed that the sequences are correct). Sequence divergence between 'R' and 'M' isolates are low to medium. In this tree, too, the European and East Asian type isolates of *B. mucronatus* form two distinct groups well separated at high sequence divergence.

In the ITS tree, the large *B. xylophilus* cluster shows separation of 'R' form isolates (upper part) and 'M' form isolates (lower part). The 'R' subcluster and the 'M' subcluster are separated at a high bootstrap value (84). However, most genetic distances and several bootstrap values within the *B. xylophilus* cluster are very low. Therefore, separation between the 'R' and 'M' form isolates does not appear convincing. At the bottom of the *B. xylophilus* cluster, two 'M' form isolates (US10 and Q52A) are separated at slightly higher sequence divergence and bootstrap value 100 from the other 'M' and 'R' form isolates. This tree also shows that the 'R' form isolates of *B. xylophilus* from China, Portugal, Japan and Mexico form a separate group, and USA isolates and the Japanese avirulent isolate another group. As in the other two trees, the European and East Asian type isolates of *B. mucronatus* form two distinct and well separated groups.

DISCUSSION

The 'R' form of *B. xylophilus* can be distinguished rather easily from other species of the *xylophilus* group (Braasch, 2008) and also from other *Bursaphelenchus* species, whereas identification of the 'M' form of *B. xylophilus* is more difficult. Females in mucronate populations generally show a mucro on the female tail end. A protuberance or a very small mucro can also be present in a fraction of females of round-tailed populations, in particular immediately after extraction from wood. The mucro character of the 'R' form of *B. xylophilus* is not always stable, it depends on different hosts and environmental situations. However, round-tailed females are usually also present in those populations, and their percentage decreases after multiplication on fungi in cultures. So for the 'R' form of *B. xylophilus*, presence and length of a mucro is just phenotypic variation depending on environmental condition and is not genetically determined. However, the 'M' form of *B. xylophilus* does not change its mucro shape even after culturing for many years.

The presence and size of a mucro also plays an important role in morphological distinction of mucronate *B. xylophilus* from *B. mucronatus* (Fig. 2, Table 3). The East Asian type of *B. mucronatus* is characterised by a relatively long mucro as a continuation of a conical female tail, whereas the tail shape of the mucronate *B. xylophilus* is subcylindric like in the European type of *B. mucronatus* and in round-tailed *B. xylophilus*. The mucronate form of *B. xylophilus* and the European type of *B. mucronatus* can be distinguished by length of the mucro (mean 2.2-3.0 µm *vs* 3-5 µm). However, European *B. mucronatus* with a very short mucro were recorded in eastern Germany (pers. observation by Helen Braasch). The mucronate form of *B. xylophilus* and the European type of *B. mucronatus* are indeed very similar, and it is almost impossible to make a sound identification without support of molecular methods. *Bursaphelenchus fraudulentus* also has a very short mucro (Table 2) and can hardly be distinguished from the mucronate *B. xylophilus* based on this character.

The position of the excretory pore also seems to be a valuable character for species identification (Braasch, 2008; Braasch *et al*., 2009). To a limited degree, it may also be useful for differentiation of morphologically distinct forms within a species. Braasch (2008) found the excretory pore position usually behind the median bulb in the East Asian type of *B. mucronatus,* but at the level or anterior to median bulb in the European type of *B. mucronatus.*

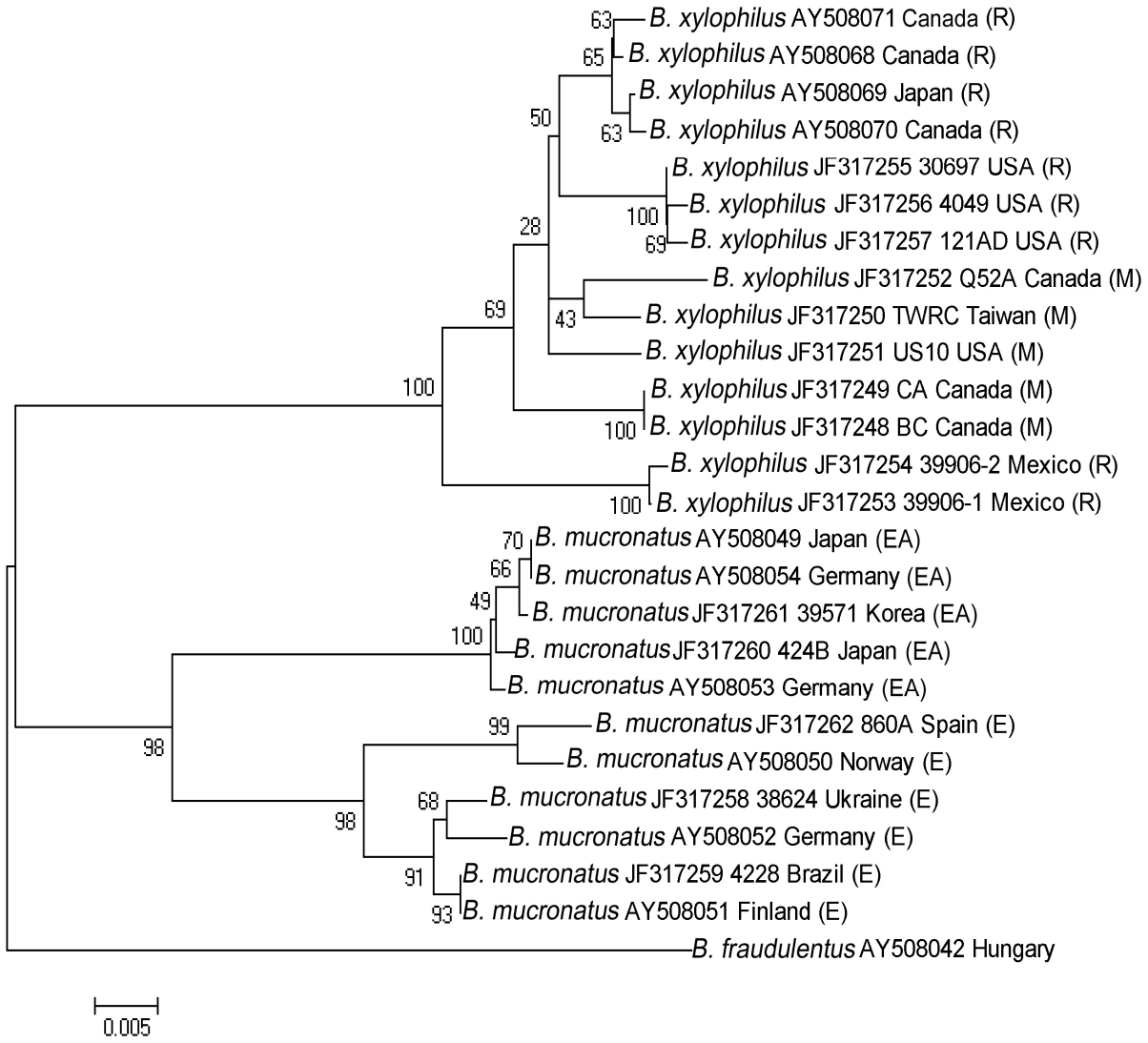

Fig. 8. Molecular phylogenetic status of *Bursaphelenchus xylophilus* and *B. mucronatus* based on partial mtCOI sequences. Numbers at branching points are bootstrap values obtained using 1000 repetitions. Scale bar: substitutions/site.

In our present study, we found that the excretory pore position in the investigated isolates was not a stable character within the two types of *B. mucronatus* (Table 3). The excretory pore of the European *B. mucronatus* was situated at the level of median bulb or anterior to it (Braasch, 2008) or slightly posterior to median bulb (isolate 860A), whereas the East Asian type had it just behind the median bulb, but exceptionally also at or in front of it. The position of excretory pore of the mucronate *B. xylophilus* is behind median bulb (isolates US10 and TWRC) or at the level of median bulb (isolates BA, CA and Q52A), but never anterior to median bulb. It was similarly located in round-tailed type of *B. xylophilus*. Consequently, this character does not seem to be very useful for differentiation, except in the case, when the mucronate *B. xylophilus* is being compared with the European type of *B. mucronatus* and many individuals are available. Thus, we determined that the excretory pore position in relation to the posterior margin of the median bulb was not a good trait since it is a movable organ, which can be highly variable even in the same individual based on feeding or fixation.

Molecular methods, for instance ITS-RFLP analysis were very helpful in distinguishing species with a very high degree of morphological similarity (Burgermeister *et al*., 2009). However, the conventional five restriction

Table 4. Sizes of PCR products and DNA restriction fragments obtained in ITS-RFLP analysis and calculated on sequencing results of the ITS1/2 regions.

Bursaphelenchus species	PCR product (bp)	Restriction fragments (bp)[1]						
		Rsa I	*Hae* III	*Msp* I	*Hinf* I	*Alu* I	*Hpy188*I	*Hha* I
The 'R' form *B. xylophilus* (for isolates 121AD, 4049 and 30697)	925	483 420 22	728 197	562 363	263 232 142 139 125 24	433 256 142 96	743 110 72	357 262 200 106
The 'R' form *B. xylophilus* (for isolate 39906)	922	483 417 22	725 197	562 360	263 232 141 139 122 25	432 238 143 96 13	741 110 71	357 223 200 107 35
The 'M' form *B. xylophilus* US10	926	486 417 23	728 198	566 360	266 232 142 138 123 25	437 237 142 96 14	492 252 110 72	358 200 140 121 107
The 'M' form *B. xylophilus* Q52A	926	487 417 22	728 198	566 360	266 232 142 139 122 25	437 241 139 96 13	492 252 110 72	357 201 140 121 107
The 'M' form *B. xylophilus* BC	926	484 420 22	728 198	563 363	263 232 142 139 125 25	433 241 143 96 13	744 110 72	467 258 201
The 'M' form *B. xylophilus* CA	924	483 419 22	727 197	562 362	263 232 141 139 124 25	432 240 143 96 13	743 110 71	466 258 200
The 'M' form *B. xylophilus* TWRC	925	484 419 22	727 198	563 362	263 232 142 139 124 25	433 240 143 96 13	743 110 72	466 258 201

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

endonucleases (*Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I) used for ITS-RFLP analysis of *Bursaphelenchus* spp. cannot distinguish the 'M' and 'R' forms of *B. xylophilus*. These two morphotypes can be differentiated by use of two additional endonucleases (*Hpy*188 I and *Hha* I). However, differentiation of the two forms is based on very few restriction fragments due to the presence or absence of a single restriction site in the 'M' or 'R' form, respectively. Moreover, other fragment differences of similar scale were also revealed between isolates of the same form.

In the molecular trees, a separation of 'M' and 'R' forms of *B. xylophilus* was visible, but most sequence divergence between the 'M' and 'R' form isolates represented by branch lengths within the entire *B. xylophilus* cluster (Figs 6-8) was very low. Thus, convincing evidence for the hypothesis that the 'R' and 'M' forms represent two genetically distinct forms of *B. xylophilus* is lacking. However,

the European and East Asian type isolates of *B. mucronatus* form two well separated groups and they are possible to be identified as subspecies.

Other authors have also done molecular analysis on both forms of *B. xylophilus* and *B. mucronatus*, though they did not mention whether the isolates studied were the 'M' or 'R' form. Beckenbach *et al*. (1992) sequenced the heat shock 70A gene of 19 populations of *B. xylophilus* and *B. mucronatus*. Their results indicated that they could be divided into five types within *B. xylophilus* and four types within *B. mucronatus*. Sequence type 1 (including isolates Q52A and Q1426, both from Quebec, Canada) and type 2 (including isolate mm, collected from a *Monochamus* sp. beetle in British Columbia, Canada) could be separated from other *B. xylophilus* groups. Iwahori *et al*. (1998) examined 15 isolates of *B. xylophilus* and *B. mucronatus* based on ITS PCR-RFLP data, which revealed a significant sequence divergence between *B. xylophilus* and *B. mucronatus*. Among the *B. xylophilus* isolates, the Canadian isolates (including BC, Q52A, St.J and FIDS) formed a separate group. Restriction fragments obtained with *Hha* I by Iwahori *et al*. (1998) confirmed that the Canadian isolates belong to the 'M' form of *B. xylophilus*. Beckenbach *et al*. (1999) analysed the ITS sequences of 11 *B. xylophilus* and *B. mucronatus* isolates and found that the two species are distinct genetic entities and significant divergence exists within each species. Within the *B. xylophilus* clade, isolates Q1426 and mm were placed basal to the other five isolates of *B. xylophilus*.

Species type or form is not dealt within the International Code of the Zoological Nomenclature. The subspecies concept has not been applied in *Bursaphelenchus* taxonomy. A widely accepted definition for 'subspecies' is that of Mayr & Ashlock (1991): "A subspecies is an aggregate of phenotypically similar populations of a species inhabiting a geographic subdivision of the range of that species and differing taxonomically from other populations of that species." An important criterion of the subspecies definition is geographical, biological or physiological isolation, whereas interbreeding may be possible in contact regions of two subspecies. Although the morphological characters of a subspecies should be stable, mixed forms may exist after interbreeding. Bolla & Boschert (1993) confirmed that interbreeding occurred between some 'M' and 'R' forms of *B. xylophilus*, whereas interbreeding of *B. xylophilus* and *B. mucronatus* was rare. Our results show that the five isolates of the 'M' form of *B. xylophilus* have fixed molecular and morphological characters; however, the criterion of isolation is not fulfilled. The 'M' form of *B. xylophilus* is mainly found in North America, like the round-tailed *B. xylophilus*. Both forms were initially found in different host trees: *Pinus* sp. for the 'R' form, *Abies* sp. and *Picea* sp. for the 'M' form. However, according to later information, the 'M' form can also develop in *Pinus* (Dwinell and Nickle, 1989; Panesar *et al*., 1989; Ma *et al*., 1996; Braasch, 2000). Wingfield and Blanchette (1983) reported that the 'M' form of *B. xylophilus* is phoretic in *M. marmorator* and *M. scutellatus*. Balsam fir is the exclusive host of *M. marmorator*, whereas *M. scutellatus* is found on many conifers including species of *Pinus*, *Abies*, *Larix* and *Picea*. The 'M' form of *B. xylophilus* from balsam fir may therefore not be restricted to balsam fir (Wingfield & Blanchette, 1983). Due to relatively small sequence divergence of the 'R' and 'M' forms of *B. xylophilus* and the lack of a clear isolation of the two morphoforms, we feel that a subspecies designation for these forms is unwarranted.

ACKNOWLEDGEMENT

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Jianfeng Gu, Jiangling Wang and H. Braasch, W. Burgermeister, T. Schröder. Морфологическая и молекулярная характеристика изолятов *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) с мукро (М-форм).

Резюме. Проведено сравнение морфологических и молекулярных особенностей пяти имеющих мукро изолятов *Bursaphelenchus xylophilus,* (т.н. М-форм), с имеющими закругленную оконечность хвостового конца формами (т.н. R–формами) *B. xylophilus* и с *B. mucronatus* европейского и восточно-азиатского типов. Спикулы этих видов и форм сходны. М-формы *B. xylophilus* отличаются от R–форм *B. xylophilus* наличием хорошо различимого мукро на оконечности хвоста самок. От европейского типа *B. mucronatus* эти формы отличаются несколько более коротким хвостовым мукро самок и положением экскреторной поры. От восточно-азиатских *B. mucronatus* М-формы отличаются формой хвостового конца самок и коротким мукро. Часто используемые для проведения анализа длин рестрикционных фрагментов (RFLP) по ITS-участку ДНК видов *Bursaphelenchus* ферменты-эндонуклеазы *Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I не позволяют разграничить М– и R–формы *B. xylophilus*, однако эти формы легко дифференцируются с использованием ферментов *Hpy*188I и *Hha* I. Молекулярно-филогенетический анализ последовательностей D2D3 LSU и ITS участка рибосомальной ДНК, а также митохондриального домена COI показал, что М-формы *B. xylophilus* близки к R–формам этого вида, а различия между ними малы.