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Bursaphelenchus antoniae sp. n. (Nematoda: Parasitaphelenchidae) associated with *Hylobius* sp. from *Pinus pinaster* in Portugal

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Summary – *Bursaphelenchus antoniae* sp. n. is described and illustrated. Dauer juveniles were isolated from the body of the large pine weevil, *Hylobius* sp., collected from maritime pine (*Pinus pinaster*) stumps, in Portugal. *Bursaphelenchus antoniae* sp. n. was reared and maintained in *P. pinaster* wood segments and on Petri dish cultures of the fungi *Botrytis cinerea* and *Monilinia fructicola*. The new species is characterised by a relatively small body length of *ca* 583 μm (females) and 578 μm (males), a lateral field with two incisures, presence of a small vulval flap and a conoid female tail with a rounded or pointed terminus. Males have stout spicules with a disc-like cucullus and seven caudal papillae arranged as a single midventral precloacal papilla, one precloacal pair and two postcloacal pairs. In the character of the lateral field, *B. antoniae* sp. n. comes close to *B. abietinus*, *B. rainulfi* and *B. hylobianum*, whilst spicule characters place it within the *piniperdae*-group *sensu* Ryss *et al.* Morphologically, *B. antoniae* sp. n. is closest to *B. hylobianum*; the spicules of these two species having flattened, wing-like, alae on the distal third of the lamina. *Bursaphelenchus antoniae* sp. n. is distinguished from *B. hylobianum* on the arrangement of the caudal papillae (two *vs* three pairs). ITS-RFLP profiles and the failure to hybridise support the separation of the two species. Phylogenetic analysis of the new species, based on the 18S rDNA sequence, supports the inclusion of this new species in the *B. hylobianum*-group *sensu* Braasch. Sequence analysis of the 28S rDNA D2/D3 domain did not place the new species in a definite group.

Keywords – cross-breeding, ITS-RFLP, morphology, morphometrics, phylogeny, sequence analysis, taxonomy.

During intensive annual surveys for the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, in Portugal, dauer juveniles of a new *Bursaphelenchus* species were found associated with the large pine weevil, *Hylobius* sp. (Coleoptera: Curculionidae) in maritime pine, *Pinus pinaster* Aiton. This new species was previously identified in Penas *et al.* (2004) as *Bursaphelenchus hylobianum* (Korenchenko, 1980) Hunt, 1993. The close resemblance between *B. antoniae* sp. n. and *B. hylobianum* was obvious, both species being found associated with the same insect host genus and displaying similar morphological features. However, further detailed studies, *i.e.*, morphological, biometrical and molecular (ITS-RFLP pattern and rDNA sequencing analysis) studies, as well as cross-breeding experiments, have shown this population to be an undescribed species. The new species is herein described as *Bursaphelenchus antoniae* sp. n.

Materials and methods

NEMATODE ISOLATION AND CULTURE

Sixty-two large pine weevils were collected from *P. pinaster* trees from Leiria, North-western Portugal, on October, 2000. These insects were placed individually in a Syracuse dish in a small amount of water and analysed under a stereo microscope (Olympus SZX12)

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for the presence of *Bursaphelenchus* species. First, the elytra and wings were opened and observed, and then the insects were crushed and left in water for 2-3 h at room temperature. *Bursaphelenchus antoniae* sp. n. dauer juveniles were isolated from the inside of the insect body (nine infested insects) and occurred in low numbers (50-100 nematodes per insect). The dauer juveniles collected were inoculated into five *P. pinaster* branch sections of *ca* 13 cm in length and 3-4 cm in diam. The segments were sealed at both ends with paraffin. A small hole was made in the middle of each section, the suspension of dauer juveniles was then inoculated with a syringe and the hole sealed with paraffin. The branches were enclosed in plastic bags and incubated at 26°C for 3 weeks. The branches were cut into thin sections of *ca* 0.5 cm thickness using an electric saw, and the nematodes were extracted using a modified Baermann funnel technique for 48 h.

Nematodes were maintained in fungal cultures for future studies. The fungi used were strains of non-sporulating forms of *Botrytis cinerea* and *Monilinia fructicola*.

One population of *B. hylobianum* (Russian population: RU-DE 16(w)) from Federal Biological Research Centre for Agricultural and Forestry, Department of National and International Plant Health nematode collection was maintained on *M. fructicola* culture and used for comparative studies with *B. antoniae* sp. n.

Morphobiometric observations

For morphological observations, nematodes collected from the pine branches cultures were mounted in water and heat-relaxed on temporary slides, observed with an Olympus BX-51 light microscope, and photographed with an Olympus DP-10 digital camera.

Type adults were fixed in hot F.A. (4:1) solution for at least 48 h, processed to glycerin using the Seinhorst (1959) method, mounted on permanent slides and measured. The nematodes were measured and drawn using a *camera lucida* attached to an Olympus BX-51 microscope.

For scanning electron microscope (SEM) observations, the specimens were prepared as described by Eisenback (1985) and viewed and photographed using a JEOL 35 scanning electron microscope. *Bursaphelenchus antoniae* sp. n. and *B. hylobianum* spicules were excised for SEM observations. The method used was modified after Eisenback (1985). Live males were transferred, to a drop of a solution composed of lactic acid (45%) + acetic acid (45%) + Rotring Brilliant Ultramarine Blue Ink (120 : 4 : 0.1), briefly heated over an alcohol lamp and left for 1-2 h in the solution. Then, under a stereo microscope (Olympus SZX12), the spicules were carefully cleaned and separated from the attached tissues using a cactus thorn and transferred to a drop of 2% formalin on a coverslip. The formalin was removed with a fine pipette and the coverslip with the spicule was attached to a stub with double-sided adhesive tape. After coating with gold, the spicules were observed and photographed using a JEOL 35 scanning electron microscope.

ITS-RFLP profiles

DNA isolation was carried out using nematodes collected from the fungal cultures. Specimens were hand-picked and transferred to an Eppendorf tube with a small drop of sterilised water. The procedure used for DNA extraction and preparation for PCR was as described in Penas *et al*. (2004).

DNA amplification for ITS-RFLP profiles was conducted using a Biometra Thermocycler, following the method of Braasch *et al*. (1999). After PCR, 5 μl of the amplified product was analysed in a 1% agarose gel. DNA fragments were visualised by staining in 1 μg/ml ethidium bromide solution and data analysis was performed using the Versa doc analysis system. Amplified DNA was digested for at least 3 h at 37°C using 10 U of each of the five enzymes (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I, and *Alu*I) (Amersham BioSciences®, Little Chalfont, UK) following the manufacturer's instructions. Species-specific ITS-RFLP profiles for *Bursaphelenchus* were generated using these five restriction enzymes (Burgermeister *et al*., 2005). The products of digestion were resolved in a 2% agarose gel, stained with 1 μg/ml ethidium bromide solution and analysed as described above.

Sequencing

PCR for sequencing was carried out employing a 50 μl reaction volume. The reaction mixture contained 2 units *Taq* DNA polymerase (Fermentas, Hanover, MD, USA), 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.01% Tween 20 (PCR buffer, Fermentas), 0.1 mM each dNTP, 2-10 μl of DNA template and 0.6 μM forward and 0.6 μM reverse primer (Roth). The primers for 18S amplification were forward primer K4f 5′-ATG CAT GTC TAA GTG GAG TAT TA-3′, K5f 5′-ATA CCG GTG CAT GGA ATA ATG GA-3′ and reverse primer K1r 5′-TTC ACC TAC GGC TAC CTT GTT ACG ACT-3′. The 28S D2/D3 domain region was amplified with

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′. Sequencing PCR was performed with a Biometra T1 thermal cycler (Biometra, Göttingen, Germany). The PCR program consisted of an initial denaturation for 2 min, 30 s at 96°C, 35 cycles with 1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min extension at 72°C and a final extension for 6 min at 72°C. After completion of the PCR, small aliquots of the samples were separated electrophoretically using a 1.8% agarose gel and 0.5×TBE buffer.

Amplicons were concentrated and desalted using Microcon YM-100 centrifugal filter devices (Millipore, Billerica, MA, USA) to accomplish sequencing products of good quality. Working steps were performed following the manufacturer instructions. Additionally, the membrane was washed with 50 μl ddH_2O. Small aliquots of each final sample were applied to 1.8% agarose gel and 0.5×TBE buffer to estimate the concentration of desalted DNA. Gels were stained with ethidium bromide (1 μg/ml) and visualised with a UV transilluminator.

According to the instructions of the sequencing company (MWG Biotech, Ebersberg, Germany), 20 ng/100 bp of PCR amplicon were air dried and sent to the company together with primers to use their Value Read Service. Each fragment was sequenced in both directions using the appropriated PCR primers for 18S rDNA and 28S D2/D3 domain to obtain overlapping sequences of the forward and reverse DNA strand.

The sequence data of *B. antoniae* sp. n. were compared to sequences of *Bursaphelenchus* species from different groups (*abietinus*-, *eggersi*-, *fungivorus*-, *hylobianum*-, *sexdentati*- and *xylophilus*-group) deposited in the GenBank database under accession numbers AM279709 PT (18S rDNA) and AM279710 PT (28S D2D3). *Aphelenchoides besseyi* was used as an outgroup. Alignments were calculated with ClustalW and phylogenetic trees were generated by neighbour-joining (NJ) and maximum parsimony (MP) algorithms in Mega 3.1 (Kumar *et al.*, 2004).

Cross-breeding tests

Cross-breeding tests between *B. antoniae* sp. n. and *B. hylobianum* were carried out under controlled conditions. Reciprocal mating between J4-adult female moults and males were performed. Under an Olympus SZX12 stereo microscope J4-adult female moults and adult males were hand-picked individually from fungal cultures under an Olympus SZX12 stereomicroscope. Ten 3 cm diam. Petri dishes were prepared: five were inoculated with *B. cinerea* and the others with *M. fructicola*. Each of these ten Petri dishes was inoculated with 25 J4-adult females and 25 males (five dishes with *B. antoniae* sp. n. ♀× *B. hylobianum* ♂ and five dishes with *B. antoniae* sp. n. ♂× *B. hylobianum* ♀). As controls, two Petri dish cultures of *M. fructicola* were inoculated with 25 specimens of both sexes of each species. All dishes were incubated at 20°C and after 3 weeks the nematodes were extracted using a modified Baermann funnel technique and counted.

***Bursaphelenchus antoniae** sp. n.**

=*B. hylobianum* in Penas *et al.*, 2004
=*Bursaphelenchus* sp. in Penas *et al.*, 2006
(Figs 1-5)

Measurements

See Table 1.

Description

Male

Displaying all features of Aphelenchoidoidea according to Hunt (1993). Body slender, cylindrical. Distal part of body curved and hook-like ventrally when heat-relaxed. Cuticle with fine annulations. Lateral field with two distinct incisures (*i.e.*, one ridge). Lip region high (*ca* 3.1 μm), rounded, offset by constriction. Stylet with small basal thickenings. Median bulb elongate-oval, *ca* 13.8 μm long and *ca* 9.1 μm diam. with a length-diam. ratio of *ca* 1.5. Excretory pore located 1.0-1.5 body diam. posterior to median bulb. Hemizonid located 5-6 μm posterior to excretory pore. Pharyngeal glands dorso-lateral, overlapping intestine for 2-3 body diam. Testis monorchic, usually anteriorly outstretched, occasionally reflexed; occupying *ca* half of body length, cells initially arranged in single row and then in two rows. Spicules paired, robust, rosethorn-shaped, strongly curved; rostrum prominent, not sharply pointed; condylus rounded and well-developed, distal end with disc-like cucullus (not always discernible). Distal third of dorsal limb of spicules laterally expanded forming flattened, wing-like, alae. Tail arcuate, terminus pointed; bursa usually truncate with posterior margin indented in some specimens. Seven caudal papillae arranged as a single midventral precloacal

* The species is named in honour of Maria Antónia Bravo for her contributions to Nematology and for her support during the first author's Ph.D. studies.

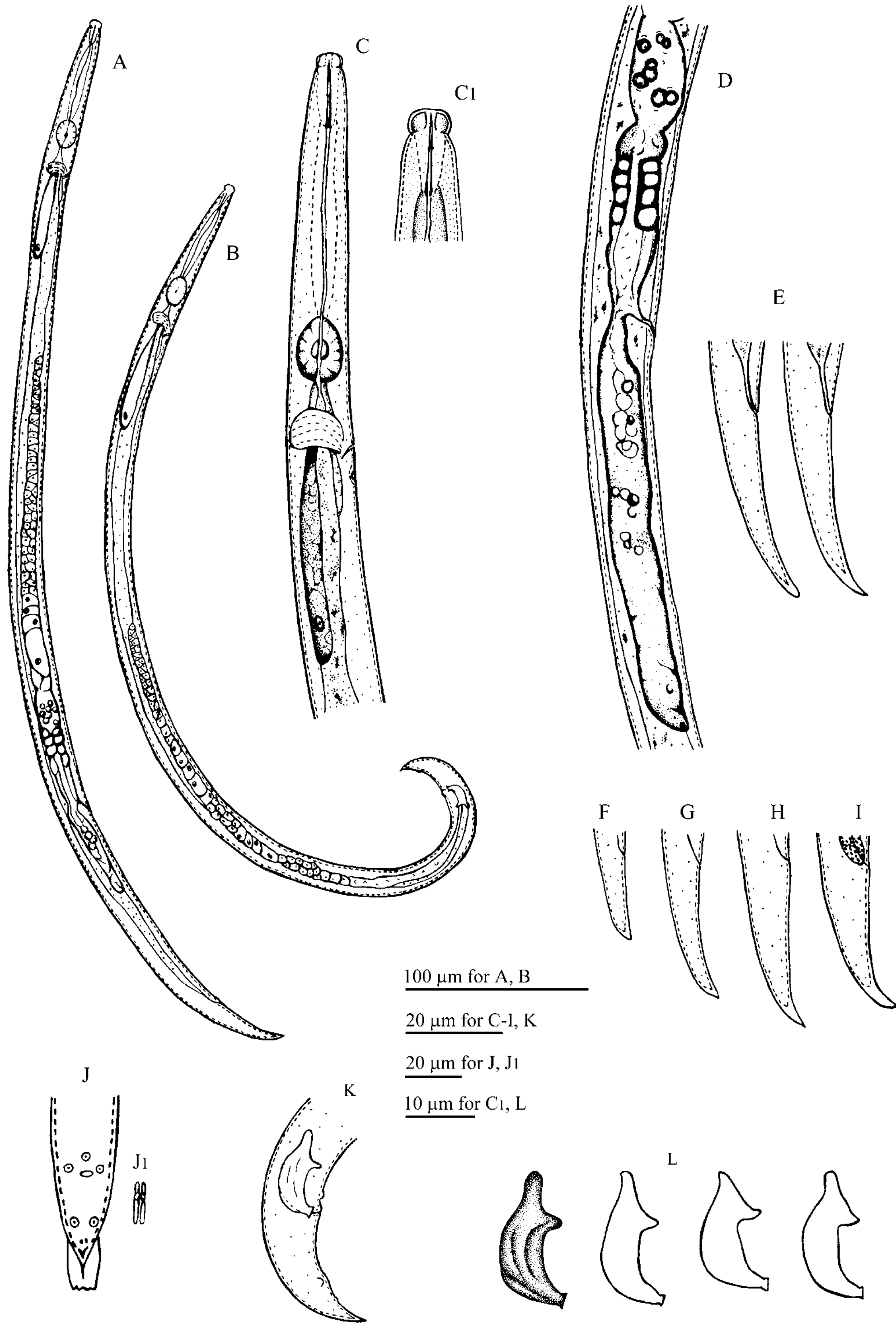

Fig. 1. Bursaphelenchus antoniae *sp. n. A: Entire female; B: Entire male; C: Female anterior region; C1: Head; D: Female vulval region; E: Female tails; F: Tail of J2; G: Tail of J3; H: Tail of J4 (female); I: Tail of J4 (male); J: Ventral view of male tail showing papillal disposition; J1: Spicules, ventral view; K: Male tail; L: Variation in male spicule shape.*

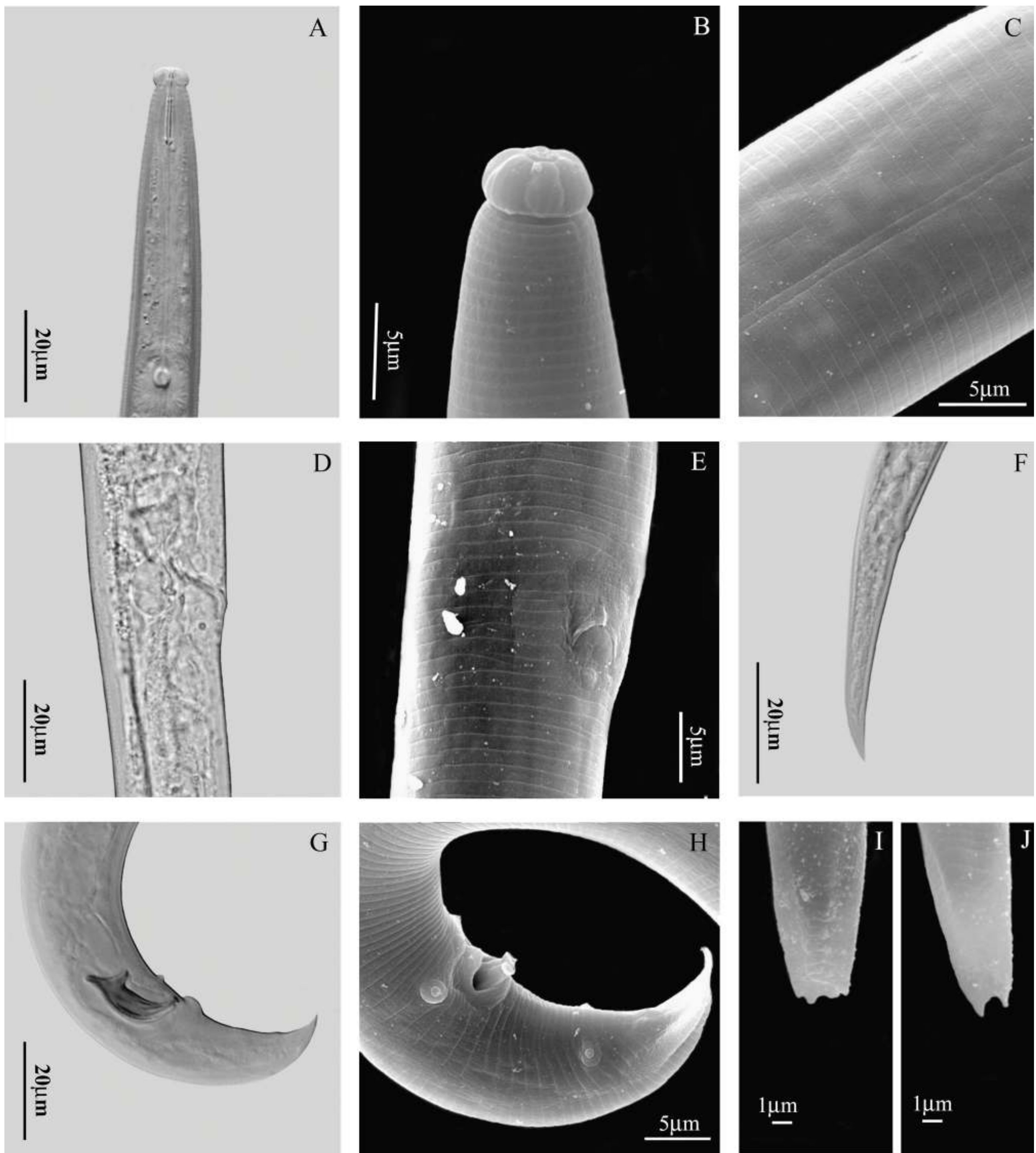

Fig. 2. Bursaphelenchus antoniae *sp. n. A: Light micrograph (LM) of anterior region; B: Scanning electron micrograph (SEM) of head; C: SEM of lateral field; D: LM of vulval region; E: SEM of vulval region; F: LM of female tail; G: LM of male tail; H: SEM of male tail; I, J: SEM of bursa.*

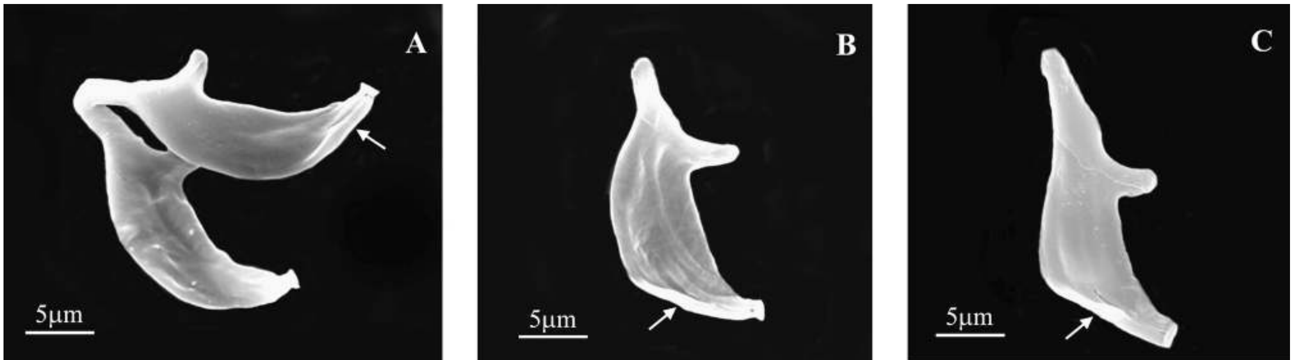

Fig. 3. *Excised spicules of* Bursaphelenchus antoniae *sp. n. (A, B) and* B. hylobianum *(C). Note the flattened wing-like structure in the distal third of the dorsal limb (arrows).*

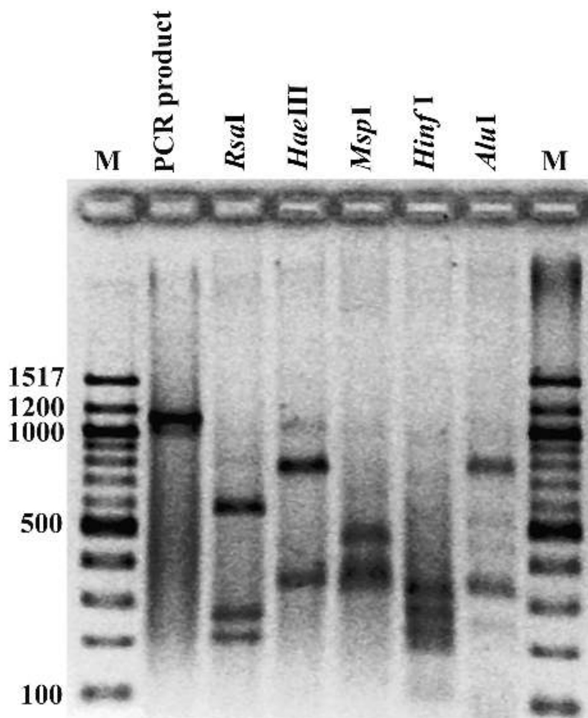

Fig. 4. *ITS-RFLP pattern of* Bursaphelenchus antoniae *sp. n.*

papilla, one precloacal pair and two postcloacal pairs (one pair at 42% of tail length from cloacal aperture and other pair just before bursa at 60% of tail length from cloacal aperture).

Female

Body slightly ventrally curved when heat-relaxed. Genital tract monoprodelphic, outstretched, cells initially arranged in single row and thereafter with two rows. Spermatheca differentiated, roundish irregular rectangle, filled with rounded sperm. Quadricollumela visible. Postuterine branch extending for *ca* 60% of vulva-anus distance, often containing sperm. Vulva inclined anteriad at *ca* 45° to body axis. Vulva with anterior lip slightly extended to form a small, distinct flap. Female tail medium length, conoid, gradually tapering to bluntly rounded or acute terminus.

Dauer juvenile

Body 400-440 μm long. Head dome-shaped, lips not defined, stylet just visible. Median bulb not well defined but recognisable in most cases. Pharynx and pharyngeal glands degenerate. Body filled with granular lipid material. Tail conical, terminus pointed.

Juvenile stages

Juveniles with conical tail: J3 and J4 (female), tail slightly ventrally curved; J4 male tail also ventrally curved. Developing gonad visible in posterior region of J4 male.

TYPE LOCALITY AND HABITAT

The new species was isolated from inside the body of the large pine weevil, *Hylobius* sp., (Coleoptera: Curculionidae). The insects emerged from *P. pinaster* (maritime pine) stumps collected from a pine wood at Leiria, north-west Portugal.

TYPE MATERIAL

Holotype male, six female paratypes and five male paratypes, deposited in the Departamento Protecção das Plantas, Estação Agronómica Nacional, Oeiras, Portugal. Other paratypes: one slide with five males and one

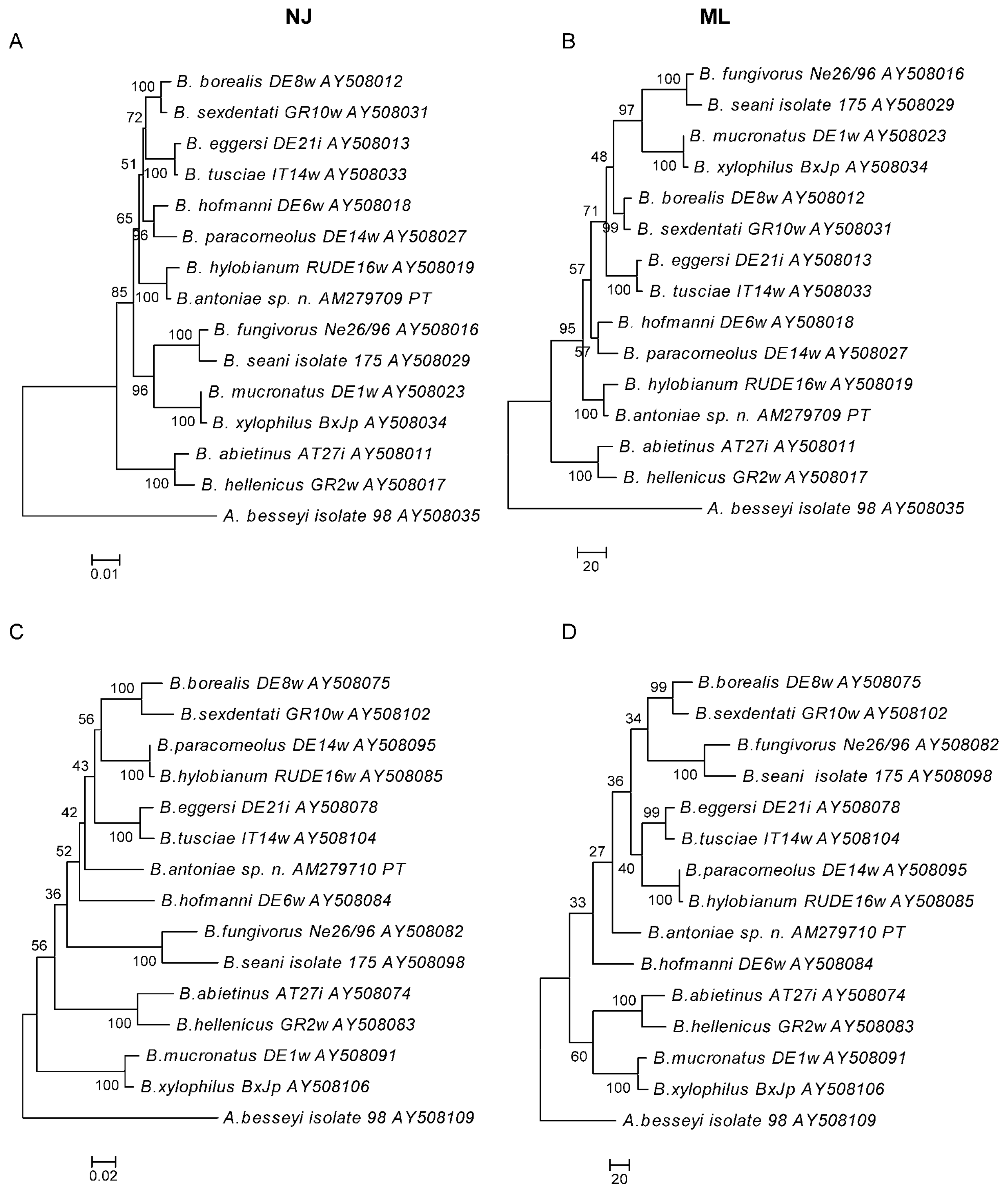

Fig. 5. *Phylogenetic relationships of* Bursaphelenchus antoniae *sp. n. and 13* Bursaphelenchus *species.* Aphelenchoides besseyi *is the outgroup. The global sequence alignments for tree constructions were calculated for 18S rDNA (A, B) and 28S D2/D3 domain (C, D) sequences by neighbour-joining (NJ) and maximum parsimony (MP) algorithms. Bootstrap values (%) are given for each node.*

Table 1. *Morphometrics of* Bursaphelenchus antoniae *sp. n. All measurements are in μm and in the form: mean ± standard deviation (range).*

	Male		Female
	Holotype	Paratypes	Paratypes
n	–	20	21
L	574	578 ± 64 (476-660)	584 ± 54 (512-709)
a	39.6	39.8 ± 3.1 (34.2-44.0)	36.6 ± 2.6 (32.0-42.1)
b	8.2	8.1 ± 0.5 (7.2-9.2)	8.5 ± 0.6 (7.7-10.0)
c	19.1	18.7 ± 1.6 (15.5-21.3)	14.6 ± 0.9 (12.8-16.3)
c′	2.3	2.4 ± 0.2 (2.1-2.9)	4.5 ± 0.4 (3.9-5.1)
V	–	–	70.8 ± 0.9 (69.1-72.2)
Lip region diam.	6	5.9 ± 0.2 (5.5-6.0)	6.1 ± 0.4 (5.5-7.0)
Lip constriction diam.	5	5.0 ± 0.2 (4.5-5.5)	5.2 ± 0.5 (4.5-6.5)
Lip region height	3	3.1 ± 0.2 (3.0-3.5)	3.1 ± 0.2 (3.0-3.5)
Stylet	13	12.1 ± 1.0 (11-14)	12.0 ± 1.0 (10-14)
Pharynx length	70	71.0 ± 5.7 (59-80)	68.8 ± 2.9 (63-74)
Median bulb length	15	13.8 ± 0.7 (12-15)	14.2 ± 0.7 (13.5-16.0)
Median bulb diam.	9	9.1 ± 0.5 (8-10)	9.8 ± 0.8 (8-12)
Median bulb length / median bulb diam.	1.7	1.5 ± 0.1 (1.4-1.6)	1.5 ± 0.1 (1.3-1.8)
Body diam. at middle of median bulb	12	11.8 ± 1.0 (10-14)	12.6 ± 0.7 (11-14)
Body diam. at base of median bulb	12	12.2 ± 1.0 (10-14)	13.0 ± 0.7 (12.0-14.5)
Distance from anterior end to excretory pore	84	81.8 ± 6.4 (71-91)	80.0 ± 6.2 (71-93)
Distance from anterior end to hemizonid	89	87.0 ± 5.6 (78-95)	85.6 ± 4.9 (75-95)
Distance from anterior end to posterior end of pharyngeal glands	140	143.1 ± 19.3 (120-177)	135.3 ± 17.0 (116-177)
Body diam. at end of pharyngeal glands	14	13.8 ± 1.3 (11-16)	14.4 ± 1.0 (13-17)
Anterior genital branch	301	343.8 ± 54.5 (228-426)	207.1 ± 26.2 (161-261)
Posterior genital branch	–	–	81.6 ± 9.9 (64-100)
Body diam. at vulva	–	–	16.4 ± 1.4 (14-20)
Vulva to anus distance	–	–	130.6 ± 14.2 (108-160)
Distance from anterior end to vulva	–	–	412.8 ± 39.4 (354-503)
G1 (%)	–	–	35.4 ± 2.8 (31.4-41.9)
G2 (%)	–	–	14.0 ± 1.5 (12.1-18.2)
Anal/cloacal body diam.	13	12.8 ± 1.1 (10.5-14.5)	9.0 ± 0.8 (8-11)
Tail	30	30.9 ± 2.0 (27-34)	40.1 ± 2.9 (34-46)
T	52.4	59.4 ± 5.9 (47.9-66.0)	–
Spicule (condylus to distal end)	18.5	17.9 ± 1.4 (15-20)	–
Spicule (rostrum to distal end)	11	10.4 ± 0.8 (9-11.5)	–
Spicule (curved median line)	17.5	17.3 ± 1.6 (14-19.5)	–
Spicule (rostrum to condylus)	8.5	8.6 ± 1.0 (6.5-10)	–
Distance from single precloacal papilla to cloacal aperture	4	4.2 ± 0.4 (3.5-5)	–
Distance from cloacal aperture to first pair of postcloacal papillae	15	13.0 ± 1.0 (11-15.5)	–
Distance from cloacal aperture to second pair of postcloacal papillae	19.5	18.7 ± 1.3 (16-21.5)	–

slide with five females in USDA Nematode Collection, Beltsville, MD, USA; one slide with five males and one slide with five females in Plant-Pathogen Interactions Division, Rothamsted Research, Harpenden, UK; one slide with five males and one slide with five females in Kyoto University, Environmental Mycoscience Laboratory Collection, Kyoto, Japan. All specimens were collected from inoculated *P. pinaster* branch segments.

Diagnosis and relationships

Bursaphelenchus antoniae sp. n. is characterised by the relatively short body in both sexes, the presence of two lines or incisures in the lateral field and by the robust and strongly curved spicules. The spicule lamina is angular distally, the rostrum digitate, and the condylus is rounded. Females have a very small vulval flap formed by a small extension of the cuticle of the anterior lip, and a conical tail that gradually tapers to an almost straight or slightly recurved, pointed or rounded terminus.

Based on these morphological characters, the new species appears close to *B. abietinus* Braasch & Schmutzenhofer, 2000, *B. rainulfi* Braasch & Burgermeister, 2002 and *B. hylobianum* (following Braasch's (2001) classification).

According to the key to species groups of Ryss *et al*. (2005), the new species belongs to the *piniperdae*-group. This group is characterised by the stout and hook-like spicule with dorsal and ventral limbs joined at the narrowed tip; elongate capitulum, rostrum and condylus well-developed and separate; rostrum located more anteriorly and condylus not recurved anteriorly; dorsal contour of lamina anteriorly smoothly curved but angular at midpoint; small cucullus present.

Bursaphelenchus antoniae sp. n. has much larger and stouter spicules than *B. abietinus* (17 *vs* 13 μm); the new species has only one precloacal pair and a single precloacal caudal papilla while *B. abietinus* has two precloacal pairs.

Comparing with *B. rainulfi*, *B. antoniae* sp. n. differs in the position of the excretory pore (in *B. rainulfi* it is located in the posterior region of the median bulb) and in spicule shape and length (13 *vs* 17 μm).

The new species seems closest to *B. hylobianum* because of spicule shape, both species sharing a characteristic structure (Fig. 3) on the spicules, *i.e*., a flattened, wing-like, alae laterally expanded in the distal third of the dorsal limb. This feature has not been reported for any other *Bursaphelenchus* species. Furthermore, the new species, was found associated with weevils of the genus *Hylobius* (Penas *et al*., 2004, 2006) as reported for *B. hylobianum* (Korenchenko, 1980). However, despite these similarities *B. antoniae* sp. n. is distinguishable from *B. hylobianum* by several morphological characters. *Bursaphelenchus antoniae* sp. n. has two postcloacal pairs of papillae whereas the original description of *B. hylobianum* reported three postcloacal pairs, although according to Braasch and Braasch-Bidasak (2002) *B. hylobianum* has only one postcloacal pair. The spicules of *B. antoniae* sp. n. resemble *B. hylobianum* spicules in shape but have a shorter condylus and a well-defined, disc-like cucullus; *B. hylobianum* was described as having cucullus by Braasch and Braasch-Bidasak (2002), although the original description does not refer to the presence of a cucullus on the distal tip of the spicules (Korenchenko, 1980).

Table 2. *ITS-RFLP profiles of* Bursaphelenchus antoniae *sp. n.*

	ITS-PCR product	*Rsa*I	*Hae*III	*Msp*I	*Hinf*I	*Alu*I
Fragment sizes (~bp)	1150	610	790	490	340	790
		290	360	370	290	340
		230			250	
					220	

ITS-RFLP profiles

Despite the morphological similarities between *B. antoniae* sp. n. and *B. hylobianum*, the ITS-RFLP pattern of *B. antoniae* sp. n. is different from that of *B. hylobianum* (Braasch & Burgermeister, 2002) (Fig. 4; Table 2). The restriction fragment pattern obtained for *Hae*III was similar, but differed in the other four enzymes used.

Sequence analysis

The total length of the aligned rDNA for *Bursaphelenchus* species varies from 1636 to 1646 bases in the 18S rDNA and 678 to 695 bases in the 28S D2/D3 region. Excluding the outgroup, the global sequence alignments of the 18S rDNA sequences examined have 1672 sites, of which 1448 are conserved, 199 variable and 158 parsimony-informative. Sequence alignments of the 28S D2/D3 region have 731 sites, of which 429 are conserved, 284 variable and 234 parsimony-informative. The phylogenetic analysis using NJ and MP methods yielded trees with different topologies for both rDNA regions (Fig. 5). Based on the 18S rDNA sequences, *B. antoniae* sp. n. is located within the *hylobianum*-group. These results are highly supported by 100% bootstrap values. The analysis of the 28S rDNA D2/D3 domain did not group *B. antoniae* sp. n. with species of a specific group, bootstrap values being low.

The phylogenetic analysis supports the conclusion from the other studies that *B. antoniae* sp. n. is a new species and close to *B. hylobianum*.

Table 3. *Final number of nematodes resulting from interspecific hybridisation between* Bursaphelenchus hylobianum *(Bh) and* B. antoniae *sp. n. (Ba) and controls.*

Mating combinations	Fungi	Final number of nematodes		Breeding
		B. hylobianum	*B. antoniae*	
♀Bh ×♂Ba	*M. fructicola*	7♀♀	6♂♂	No
♀Bh ×♂Ba	*M. fructicola*	4♀♀	6♂♂	No
♀Bh ×♂Ba	*M. fructicola*	–	6♂♂	No
♀Bh ×♂Ba	*B. cinerea*	2♀♀	2♂♂	No
♀Bh ×♂Ba	*B. cinerea*	3♀♀	3♂♂	No
♀Ba ×♂Bh	*M. fructicola*	3♂♂	2♀♀	No
♀Ba ×♂Bh	*M. fructicola*	7♂♂	2♀♀	No
♀Ba ×♂Bh	*B. cinerea*	7♂♂	5♀♀	No
♀Ba ×♂Bh	*B. cinerea*	–	6♀♀	No
♀Ba ×♂Bh	*B. cinerea*	2♂♂	1♀	No
♀Ba ×♂Ba	*M. fructicola*	–	32100*	Yes
♀Bh ×♂Bh	*M. fructicola*	12650*	–	Yes

[*] Total number of specimens of all propagative developmental stages.

Cross-breeding tests

Results of cross-breeding tests between *B. antoniae* sp. n. and *B. hylobianum*, plus controls, are shown in Table 3. No hybridisation occurred between the two species. No juveniles or eggs were observed from any of the fungal cultures inoculated with females of one species and males of the other one, although a few adults of each of the two species survived after 3 weeks. Control cultures reproduced successfully, reaching high population levels after the same incubation time.

Bionomics

Bursaphelenchus antoniae sp. n. dauer juveniles were found associated with *Hylobius* sp. suggesting that this species is vectored by this insect. This insect is associated with pine stumps and is not very common in Portugal. *Bursaphelenchus antoniae* sp. n. was never collected from wood, although it multiplies and can be maintained on branches of *P. pinaster* under controlled conditions. Furthermore, this species can be maintained on fungal cultures of *B. cinerea* and *M. fructicola*.

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