

Detailed morphobiometric studies of *Bursaphelenchus xylophilus* and characterisation of other *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) associated with *Pinus pinaster* in Portugal

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Summary.- Detailed studies on *Bursaphelenchus xylophilus* are provided in this contribution. Comparative observations between field and cultured populations of this species demonstrated significant size differences: cultured specimens overall displayed larger size in all morphometric parameters. A principal component analysis (PCA) of the individuals undergoing moulting allowed their separation in four groups namely J₂-J₃, J₃-J₄, J₄-F, and J_{4M}-M; gonad length mean values of these four groups made possible to distinguish the non-moulting groups J₂, J₃, J_{4F} and adults. Seven *Bursaphelenchus* species (*B. hellenicus*, *B. leoni*, *B. pinasteri*, *B. sexdentati*, *B. teratospicularis*, *B. tusciae* and *B. xylophilus*), associated with *Pinus pinaster* in Portugal, were characterized, including biometrical measurements and ratios as well excised spicules observed under SEM; furthermore, *B. hellenicus*, *B. pinasteri*, *B. sexdentati*, *B. tusciae* and *B. xylophilus* were characterised on the basis of their ITS-RFLP profiles. *B. sexdentati* and *B. xylophilus* were the only species found in high numbers in some of the samples.

Key-words: *Bursaphelenchus* spp., *B. xylophilus*, ITS-RFLP, morphometrics, *Pinus pinaster*, Portugal.

Resumen.- En este trabajo se presentan detallados estudios sobre *Bursaphelenchus xylophilus*. Un análisis comparado de datos de poblaciones naturales y cultivadas de esta especie demostró diferencias significativas de tamaño: los ejemplares cultivados mostraron en conjunto mayor tamaño en todos los parámetros morfométricos. Un análisis de componentes principales (ACP) de los individuos en proceso de muda hizo posible su separación en cuatro grupos, a saber J₂-J₃, J₃-J₄, J_{4F}-F, y J_{4M}-M; la longitud media de la gónada de dichos cuatro grupos permitió distinguir los grupos que no se encuentran en proceso de muda: J₂, J₃, J_{4F} y J_{4M} y adultos. Siete especies de *Bursaphelenchus* (*B. hellenicus*, *B. leoni*, *B. pinasteri*, *B. sexdentati*, *B. teratospicularis*, *B. tusciae* y *B. xylophilus*), que están asociadas con *Pinus pinaster* en Portugal, fueron caracterizadas, incluyendo medidas e índices biométricos y la observación con microscopía electrónica de barrido de espículas aisladas; además, *B. hellenicus*, *B. pinasteri*, *B. sexdentati*, *B. tusciae* y *B. xylophilus* se caracterizaron sobre la base de sus perfiles ITS-RFLP. *B. sexdentati* y *B. xylophilus* fueron las únicas especies encontradas en número elevado en algunas de las muestras.

Palabras clave: *Bursaphelenchus* spp., *B. xylophilus*, ITS-RFLP, morfometría, *Pinus pinaster*, Portugal.

INTRODUCTION

Since the first report, in 1999, of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Bührer, 1934) Nickle 1970, in Portugal and in Europe (Mota *et al.*, 1999), intensive surveys have been conducted in Portuguese forests (Prolunp, 2007), as well as in other European countries. In Portugal,

B. xylophilus, has been found only associated with *Pinus pinaster* Aiton trees and with its vector *Monochamus galloprovincialis* Olivier (Sousa *et al.*, 2001). Detailed studies on the developmental stages of *B. xylophilus* were conducted and are herein presented. Morphobiometric studies of the different propagative juvenile stages provide a more accurate characterisation of this important quarantine species

and an easier differentiation. Similar works have already been done for Japanese populations of *B. xylophilus* (Mamiya, 1975; Ishibashi *et al.*, 1978).

Worldwide, there are about 90 described species of *Bursaphelenchus*, most of them associated with coniferous trees, usually mycetophagous and associated with bark beetles (Hunt, 1993; Vieira *et al.*, 2006). In Portugal, *P. pinaster* wood samples and insects associated with this pine species were analysed, and besides *B. xylophilus* nine other *Bursaphelenchus* species have been identified (Braasch, 2001; Penas *et al.*, 2004, 2006a & 2006b).

The presence of *B. pinophilus* in Portugal was reported by Braasch (2001) and *B. mucronatus*, a species more frequently found in central and northern Europe (e.g., Baujard *et al.*, 1979; McNamara & Stoen, 1988; Tomminen *et al.*, 1989), was found only once, and all the few specimens collected were used for DNA analysis (Penas *et al.*, 2004). A new species, *B. antoniae* Penas, Metge, Mota & Valadas, 2006 was found associated with insects belonging to the genus *Hylobius* (Penas *et al.*, 2006a; referred as *Bursaphelenchus* sp.) and it was thoroughly described, including detailed molecular characterisation (Penas *et al.*, 2006b). RFLP analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA, has proved to be a useful tool for *Bursaphelenchus* species differentiation (Burgermeister *et al.*, 2005). This paper is a complementary study of the earlier publications, and morphometric and molecular characterisation of the other Portuguese species is hereby presented.

MATERIALS AND METHODS

Sampling and nematode extraction: A total of 4810 maritime pine (*P. pinaster*) samples (each consisting of ca 60 g of drilled dead or fresh wood) were collected as part of the annual surveys carried out by PROLUNP (National Program for Pinewood Nematode Control), processed using a modified Baermann funnel technique for nematode extraction and preliminary identification carried out as described in Penas *et al.* (2004). For morphological and molecular studies, cultures of the different nematode populations were established and maintained in non sporulating *Botrytis cinerea* and *Monilinia fructicola*.

Morphological and biometric characterisation:

Important morphological characters such as head region shape, spicule shape, papillae number and their disposition on the male tail, shape of female tail, vulval region, and number of incisures in the lateral field were considered for species identification. For light microscope (Olympus BX51) observations, nematodes were mounted in temporary slides and photographed using an Olympus DP10 digital camera. All populations measured, with exception of *B. xylophilus* populations - Bxy₂ and Bxy₃, were collected from wood samples from naturally infested pines. Nematodes were fixed in hot FA (4:1) solution for at least 48 hours, processed by the glycerol-ethanol method (Seinhorst, 1959) and mounted in permanent slides. Specimens were measured and drawn using a camera lucida attached to an Olympus BX-51 microscope. Permanent slides of the several described species are deposited in Departamento de Protecção das Plantas, Estação Agronómica Nacional (EAN), Instituto Nacional de Recursos Biológicos (INRB), Oeiras, Portugal. Spicules of the different species were excised for SEM observations following the protocol described in Penas *et al.* (2006b).

Morphobiometric detailed studies of *B. xylophilus*:

A *B. xylophilus* population collected from naturally infested *P. pinaster* was subdivided into two groups of nematodes: one which was immediately measured (Bxy₁) while the other was inoculated and reared on a culture of non sporulating *B. cinerea* and measured later (Bxy₂ - measured after 15 days from a culture without subculturing and Bxy₃ - measured after one year in culture, subcultured every two weeks). These populations were maintained under monoxenic conditions in non sporulating *B. cinerea*, at room temperature. Morphobiometric data of Bxy₁ were compared with the data obtained of Bxy₂ and Bxy₃. Permanent slides of these nematodes are deposited in Departamento de Protecção das Plantas, EAN, INRB, Oeiras, Portugal.

Studies on *B. xylophilus* biology were conducted and the developmental stages were morphobiometrically characterised. *B. xylophilus* nematodes cultured on *B. cinerea* were extracted using a modified Baermann funnel technique. Besides adults, the pine wood nematode has four propagative juvenile stages, although the first stage (J₁) occurs still within the egg (Mamiya, 1975). *B. xylophilus* dispersal forms, third dispersal stage (J_{III})

TABLE I. ITS-RFLP profiles of *Bursaphelenchus* spp. Approximate size of the PCR product and the DNA fragment sizes after digestion with five restriction enzymes.

Species	PCR product (bp)	Restriction fragments (bp)				
		RsaI	HaeIII	MspI	Hinfl	AluI
<i>B. hellenicus</i>	1074	674	603		602	410
		295	482	732	420	392
		187	193	488	310	298
<i>B. pinasteri</i>	1080	600			296	343
		475	1080	680	221	254
				344	125	135
<i>B. sexdentati</i>	993	590	610		528	
		440	319	968	326	990
			150		248	
<i>B. tusciae</i>	953	362		577		319
		223	620	313	460	244
		163	325	127		206
<i>B. xylophilus</i>	958	508	723	582	283	453
		439	208	391	265	242
					149	156
						100

and fourth dispersal stage (J_{IV}) were collected from wood and from the tracheae of *M. galloprovincialis*, respectively. Moulting and non-moulting propagative juvenile nematodes of the second (J₂), third (J₃) and fourth stage (J₄) and the dispersal forms (J_{III} and J_{IV}) were mounted in temporary mounts and stained (1% acetic orcein for 24 hours in the case of the propagative forms and 48 hours for the dispersal forms) (Ishibashi *et al.*, 1978). Adult specimens were mounted in temporary mounts without staining. Several measurements were made including total body length, gonad length, by using a camera lucida attached to an Olympus BX-51 microscope.

To test for patterns of variation between moulting and non-moulting propagative nematodes, two principal component analyses (PCA) based on a correlation matrix with standardised data were performed. Four variables were inputted to discriminate for variations, namely body length, body diameter, gonad length and tail length, which are among the most important characters to distinguish the different juvenile stages of this species (*e.g.*, Mamiya, 1975; Ishibashi *et al.*, 1978). Correlations among components were calculated, and the results were represented in a combination of two axes (components), where the nearest points correspond to data with the highest similarity, and vice-versa (Morrison, 1976). Mean gonad length of

moulting groups was used to separate non-moulting stages, being a structure known to reorganise and rapidly grow during the moults of several nematode species (*e.g.* Yuen, 1965). Statistical analyses were carried out using the software Statistica 6 (StatSoft, Inc. 2003).

Molecular profiles: DNA isolation was carried out using nematodes collected from wood or from 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). Extracted DNA was amplified using a Biometra thermocycler, following the method of Braasch *et al.* (1999). Five µl of the PCR product was analysed in 1% agarose gel, stained in 1 µg/ml ethidium bromide solution and visualised using the Versa Doc analysis system. The amplified product was digested in a water bath at 37° C for at least three hours using 10 U of each of the five enzymes (*RsaI*, *HaeIII*, *MspI*, *Hinfl*, and *AluI*) (Amersham BioSciences®) following the manufacturer's instructions. These enzymes are known to produce species specific ITS-RFLP profiles for *Bursaphelenchus* species (Braasch *et al.*, 1999; Burgermeister *et al.*, 2005). Digestion fragments were resolved in a 2% agarose gel, stained and analysed as described above.

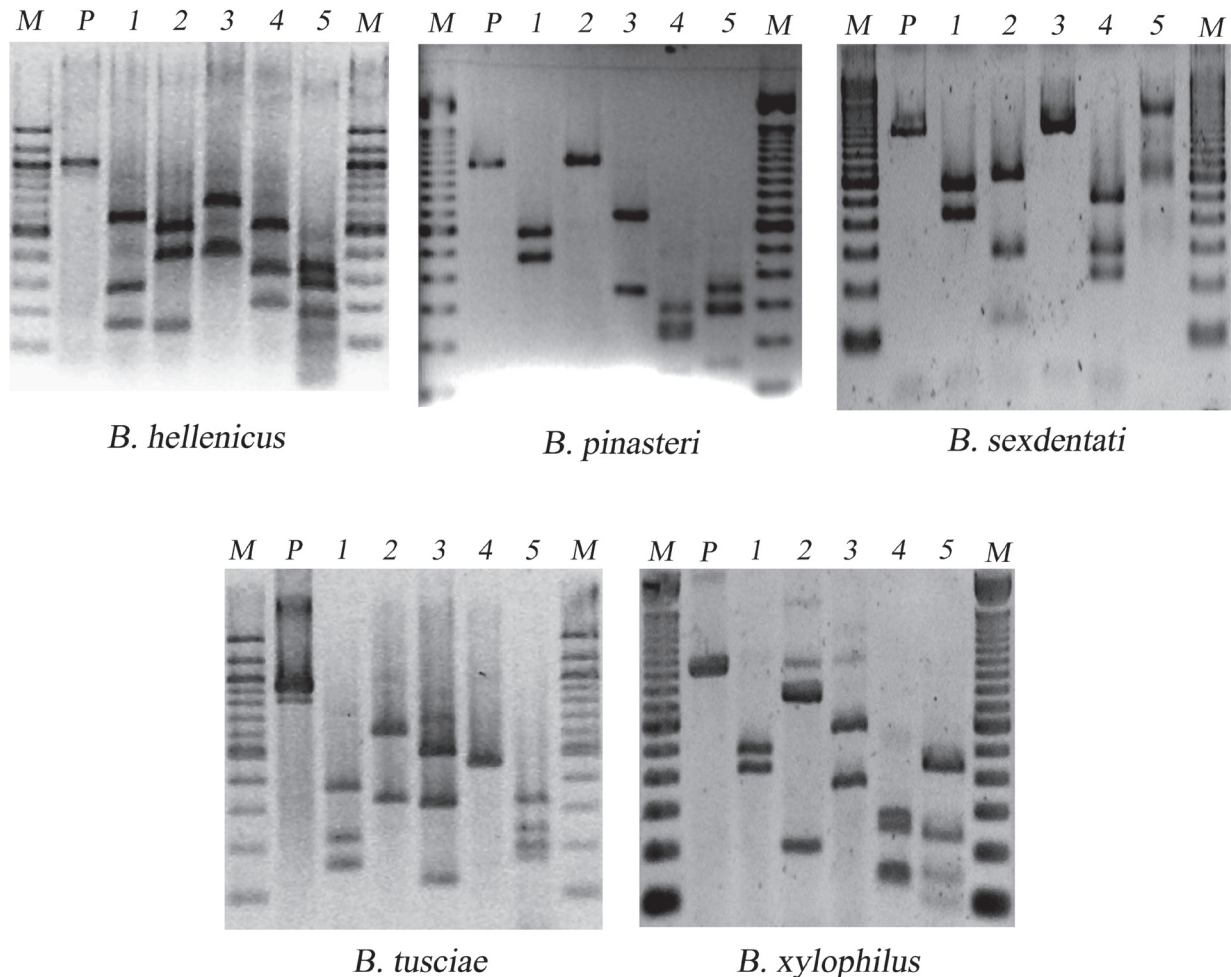


FIGURE 1. ITS-RFLP profiles of five *Bursaphelenchus* species. *Rsa*I (1), *Hae*III (2), *Msp*I (3), *Hinf*I (4) and *Alu*I (5) were the five restriction enzymes used for digestion of the amplified rDNA fragment of each species. M represents the molecular marker (100bp ladder, Invitrogen Life Technologies) and P the PCR product.

RESULTS

The studies permitted to identify the species *B. pinasteri* Baujard, 1980 (referred as *B. hofmanni* in Penas *et al.*, 2002; and as *Bursaphelenchus* sp.1 in Penas *et al.*, 2004). Besides this species, *B. xylophilus*, *B. hellenicus* Skarmoutsos, Braasch & Michalopoulou, 1998, *B. leoni* Baujard, 1980, *B. sexdentati* Rühm, 1960, *B. teratospicularis* Kakuliya & Devdariani, 1965 and *B. tusciae* Ambrogioni & Palmisano, 1998 are characterised. From the ten species occurring in Portugal, only *B. xylophilus* and *B. sexdentati*

occurred, occasionally, in high numbers (hundreds or thousands of nematodes per pine sample). All other species occurred, invariably, at less than one hundred specimens per sample.

The ITS profiles were successfully generated for *B. hellenicus*, *B. pinasteri*, *B. sexdentati*, *B. tusciae* and *B. xylophilus* (Fig. 1) (Table I). Restriction patterns of *B. leoni* and *B. teratospicularis* were not produced. It was not possible to maintain these species and the specimens extracted from the wood samples were not sufficient to obtain enough DNA for their ITS profiles. Several PCR amplifications of these species were made, however no results were obtained.

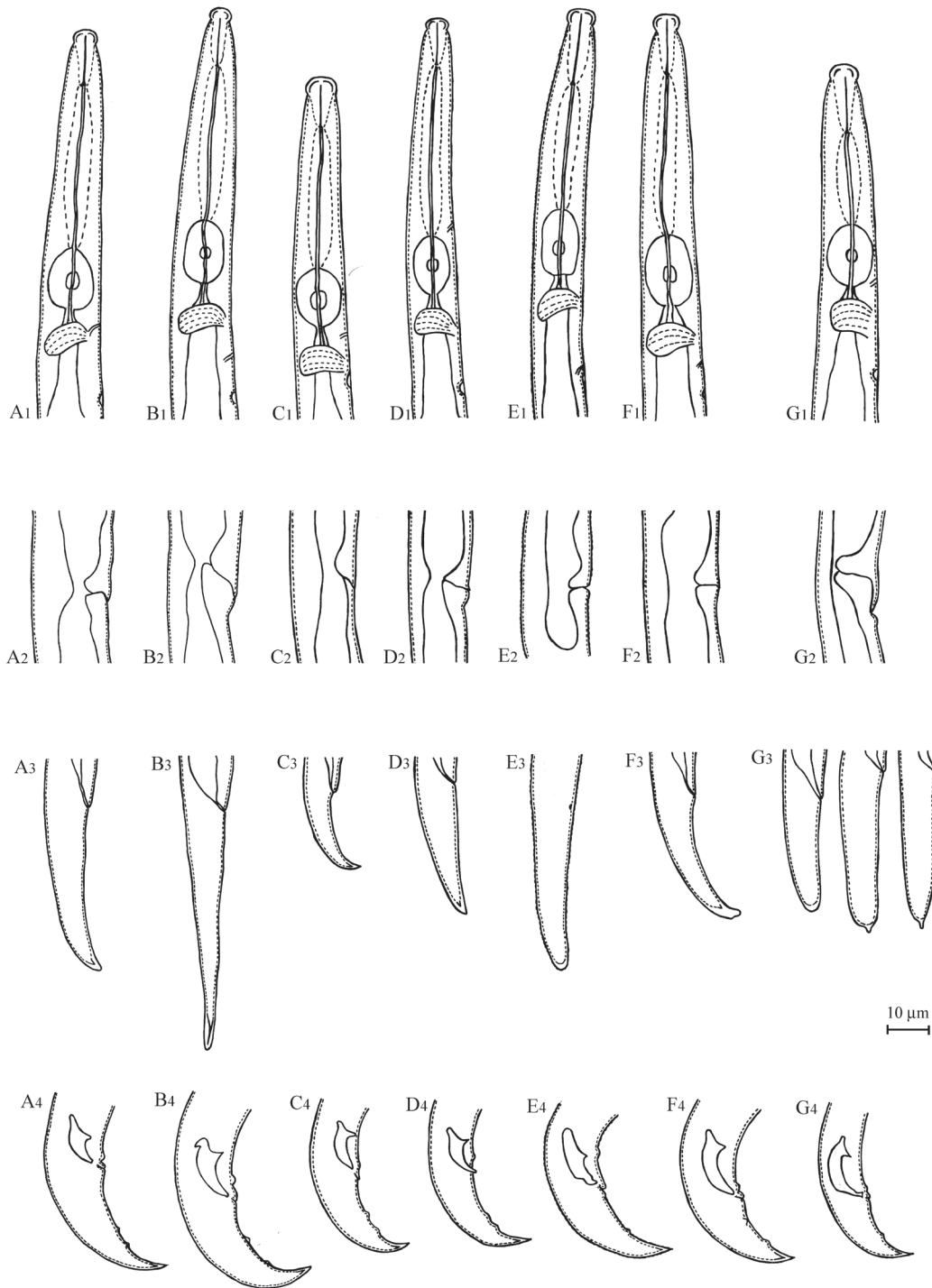


FIGURE 2. Camera lucida drawings of *Bursaphelenchus* species most important characters: anterior region (1), vulval region (2), female tail (3) and male tail (4). The species represented are: *B. hellenicus* (A), *B. leoni* (B), *B. pinasteri* (C), *B. sexdentati* (D), *B. teratospicularis* (E), *B. tusciae* (F) and *B. xylophilus* (G).

TABLE II. Measurements of *Bursaphelenchus xylophilus* (in μm and in form: mean \pm SD (range)).

Population Character	n	Bxy1 - Tróia		Bxy2 - Fungi culture (15 days)		Bxy3 - Fungi culture (one year)		Bxy4 - Tróia (mucronate form)	
		20 ♀♀	20 ♂♂	20 ♀♀	20 ♂♂	20 ♀♀	20 ♂♂	10 ♀♀	17 ♂♂
Body length		585.8 \pm 43.9 (510-663)	570.0 \pm 60.0 (449-691)	1165.6 \pm 116.4 (932-1343)	1062.3 \pm 125.3 (833-1249)	1132.0 \pm 94.3 (906-1308)	1039.0 \pm 85.9 (868-1174)	966.6 \pm 108.9 (809-1147)	853.5 \pm 78.7 (695-989)
a		41.9 \pm 4.2 (32.8-50.6)	46.0 \pm 4.5 (40.2-58.5)	53.0 \pm 4.4 (44.7-63.1)	51.2 \pm 5.7 (41.2-60.9)	45.6 \pm 2.7 (39.4-50.3)	45.7 \pm 2.2 (41.3-48.9)	53.9 \pm 3.6 (49.0-58.8)	54.3 \pm 5.5 (38.7-63.7)
b		10.1 \pm 0.5 (9.1-11.2)	9.6 \pm 0.7 (8.2-10.7)	14.9 \pm 1.2 (13.2-17.4)	13.7 \pm 1.3 (11.3-16.0)	14.7 \pm 1.2 (11.6-16.8)	13.7 \pm 0.9 (11.6-15.4)	13.3 \pm 0.9 (12.1-14.3)	12.4 \pm 0.9 (10.4-13.9)
c		25.4 \pm 2.2 (20.2-29.0)	21.6 \pm 1.6 (19.1-24.6)	29.2 \pm 3.0 (22.6-33.6)	27.3 \pm 2.1 (22.8-31.4)	28.1 \pm 3.5 (21.9-34.4)	26.8 \pm 1.9 (23.6-31.4)	24.4 \pm 2.8 (18.8-28.0)	25.3 \pm 0.2 (20.4-29.0)
c'		2.9 \pm 0.3 (2.2-3.3)	2.4 \pm 0.3 (1.8-2.9)	3.5 \pm 0.3 (2.9-4.2)	2.4 \pm 0.2 (2.0-2.8)	3.5 \pm 0.4 (2.7-4.3)	2.4 \pm 0.2 (1.9-2.8)	4.0 \pm 0.4 (3.3-4.6)	2.4 \pm 0.2 (2.1-3.1)
V		71.5 \pm 0.8 (70.1-72.9)	-	72.6 \pm 1.0 (70.4-74.5)	-	75.4 \pm 4.7 (71.5-93.4)	-	72.6 \pm 0.6 (71.5-73.5)	-
Lip region diam.		6.0 \pm 0.3 (5.5-6.5)	6.0 \pm 0.4 (5.5-6.5)	7.2 \pm 0.4 (6.5-8.0)	7.0 \pm 0.3 (6.5-7.5)	8.0 \pm 0.5 (7-9)	7.7 \pm 0.4 (7.0-8.5)	6.6 \pm 0.4 (6-7)	6.6 \pm 0.4 (6-7)
Lip constriction diam.		5.3 \pm 0.3 (5-6)	5.2 \pm 0.4 (4.5-6.0)	6.3 \pm 0.4 (5.5-7.0)	6.2 \pm 0.3 (5.5-6.5)	7.1 \pm 0.4 (6.5-8.0)	6.7 \pm 0.3 (6.0-7.0)	5.9 \pm 0.6 (5.0-6.5)	5.7 \pm 0.4 (5.5-6.5)
Lip region height		3.0 \pm 0.1 (2.5-3.0)	3.0 \pm 0.2 (2.5-3.5)	3.9 \pm 0.3 (3-4)	3.6 \pm 0.3 (3-4)	4.0 \pm 0.2 (3.5-4.5)	3.8 \pm 0.3 (3.4-4.0)	3.6 \pm 0.3 (3-4)	3.5 \pm 0.4 (3-4)
Stylet		11.2 \pm 0.8 (10.0-12.5)	11.0 \pm 1.2 (10-14)	14.6 \pm 1.3 (12-17)	14.4 \pm 1.0 (13.0-17.5)	14.4 \pm 1.2 (12-16)	14.0 \pm 0.9 (12-15)	14.7 \pm 1.8 (12-17)	14.6 \pm 1.7 (11-18)
Pharynx length		58.2 \pm 3.4 (53-64)	59.3 \pm 3.6 (55-70)	78.3 \pm 4.8 (70-91)	77.5 \pm 3.5 (70-83)	58.7 \pm 3.2 (52-63)	76.1 \pm 2.6 (70-80)	72.7 \pm 4.4 (66-81)	69.1 \pm 5.0 (61-80)
Median bulb length		14.5 \pm 0.7 (13-16)	14.6 \pm 0.9 (13-16)	18.7 \pm 1.0 (17-20)	17.5 \pm 0.6 (16-18)	18.6 \pm 0.9 (17-21)	17.7 \pm 0.8 (17-19)	16.6 \pm 0.8 (15-18)	16.2 \pm 0.8 (15-17)
Median bulb diam.		9.2 \pm 0.5 (8-10)	8.9 \pm 0.7 (8-11)	13.1 \pm 1.0 (11.0-14.5)	11.8 \pm 0.5 (11-13)	13.3 \pm 1.0 (11-15)	12.4 \pm 0.8 (10.5-14.0)	11.0 \pm 0.8 (10-12)	10.8 \pm 0.7 (10-12)
Body diam.- middle median bulb		11.8 \pm 0.8 (11-13)	11.4 \pm 1.0 (10-14)	16.4 \pm 1.2 (14-19)	15.4 \pm 1.0 (14-17)	17.2 \pm 1.2 (14-19)	15.8 \pm 1.0 (13-17)	14.1 \pm 0.8 (13.0-15.5)	13.6 \pm 0.8 (12-15)
Body diam.- base median bulb		12.0 \pm 1.0 (11-14)	11.6 \pm 1.1 (10.0-14.5)	17.0 \pm 1.1 (15.0-19.5)	15.7 \pm 1.0 (14-18)	17.8 \pm 1.2 (15.0-19.5)	16.4 \pm 1.0 (13.5-18.0)	14.4 \pm 1.0 (13-16)	13.9 \pm 0.9 (12-16)
Distance ant. end - exc. pore		51.9 \pm 3.9 (44-58)	53.3 \pm 5.2 (44-66)	62.7 \pm 9.0 (52-79)	63.0 \pm 6.7 (57-75)	72.1 \pm 7.4 (62-84)	72.3 \pm 6.6 (59-81)	79.3 \pm 6.6 (67-90)	70.8 \pm 6.0 (62-81)
Distance ant. end - hemizonid		76.2 \pm 4.6 (70-86)	75.9 \pm 5.1 (67-87)	100.4 \pm 6.4 (90-115)	101.2 \pm 5.3 (93-107)	108.5 \pm 8.2 (87-125)	107.2 \pm 6.1 (93-117)	96.4 \pm 4.5 (92-105)	93.7 \pm 5.5 (86-107)
Distance ant. end - post. ph. gl.		116.1 \pm 9.5 (103-132)	118.5 \pm 6.1 (106-133)	164.8 \pm 8.4 (154-185)	157.5 \pm 11.3 (134-173)	173.7 \pm 9.8 (159-197)	167.3 \pm 11.1 (143-186)	149.6 \pm 10.1 (135-168)	132.0 \pm 14.4 (103-155)

TABLE II. (Cont.)

Population Character	Bxy1 - Tróia		Bxy2 - Fungi culture (15 days)		Bxy3 - Fungi culture (one year)		Bxy4 - Tróia (mucronate form)		
	n	20♀	20♂	20♀	20♂	20♀	20♂	10♀	17♂
Body diam. at end of pharyngeal glands		13.0±1.0 (11.5-15.0)	12.3±1.0 (11-15)	20.2±1.5 (18-23)	18.9±1.0 (16-21)	21.7±1.7 (18.0-24.5)	20.4±1.3 (17.5-22.0)	16.4±1.6 (14-19)	15.4±1.0 (14-18)
Anterior genital branch		241.8±48.4 (152-336)	300.9±42.3 (252-409)	655.9±93.5 (443-832)	788.9±88.3 (618-901)	612.6±82.7 (453-792)	719.2±83.5 (502-837)	422.2±105.1 (295-595)	412.6±73.3 (294-573)
Posterior genital branch		113.9±8.1 (103-134)	-	173.8±10.5 (155-198)	-	162.4±12.0 (142-182)	-	164.0±14.6 (147-192)	-
Body diam. at vulva		14.4±1.2 (12.5-17.5)	-	23.2±1.5 (20.0-25.5)	-	24.1±1.4 (20.0-25.5)	-	18.6±1.9 (16-21)	-
Vulva to anus distance		142.6±16.5 (101-177)	-	277.3±29.8 (224-339)	-	256.3±21.8 (213-304)	-	225.4±26.1 (185-268)	-
Distance ant. end - vulva		418.9±32.4 (358-469)	-	846.9±88.8 (675-980)	-	852.5±81.1 (654-970)	-	701.9±80.7 (587-838)	-
G1 (%)		41.1±6.7 (27.1-54.2)	-	56.1±4.1 (47.5-63.2)	-	54.1±5.7 (43.0-64.4)	-	43.4±8.2 (34.9-57.7)	-
G2(%)		19.5±0.8 (18.5-21.1)	-	15.0±1.5 (12.6-17.5)	-	14.4±1.7 (11.4-18.9)	-	17.2±1.4 (15.2-19.3)	-
Anal / cloacal body diam.		8.2±0.7 (7-9)	11.2±1.0 (10.0-13.5)	11.5±0.8 (10.0-12.5)	16.3±1.0 (15-18)	11.8±0.8 (10-13)	16.4±0.8 (14.0-17.5)	9.9±0.7 (9-11)	13.9±0.8 (12.5-15.5)
Tail		23.2±2.4 (18-27)	26.5±2.5 (22-29)	40.0±3.7 (33-50)	38.9±3.2 (32.5-45.0)	40.8±5.2 (27-51)	38.9±3.4 (35-47)	39.8±3.7 (33-44)	33.8±3.1 (28-40)
T		-	52.9±6.9 (45.1-66.5)	-	74.4±3.5 (68.0-81.6)	-	69.1±5.0 (57.8-79.8)	-	48.5±7.9 (35.6-62.4)
Spicule (condylus to distal end)		-	17.8±1.7 (16-21)	-	26.2±1.9 ()	-	28.0±2.2 (23.5-30.0)	-	23.7±1.3 (21-26)
Spicule (rostrum to distal end)		-	11.2±0.9 (10-13)	-	15.9±1.4 (13.5-18.0)	-	17.1±1.5 (15.0-19.0)	-	14.3±0.7 (13.0-15.5)
Spicule (curved median line)		-	19.3±2.0 (16.5-24.0)	-	29.5±1.8 (26.5-32.5)	-	30.4±2.5 (25.0-33.5)	-	26.3±1.5 (23-28)
Spicule (rostrum to condylus)		-	6.4±0.8 (5.0-8.5)	-	10.7±0.9 (8.5-12.0)	-	11.4±1.2 (9.0-13.5)	-	9.6±1.0 (7.5-11.0)
Distance preclo. papilla - cloa. ap.		-	3.3±0.4 (2.5-4.0)	-	3.9±0.5 (3-5)	-	4.7±0.7 (3-6)	-	3.9±0.7 (2-5)
Distance clo. Ap. - first post. pap.		-	11.6±1.0 (10-14)	-	14.4±1.2 (12.5-17.0)	-	14.4±1.7 (11.0-17.5)	-	14.4±1.2 (12.5-16.5)

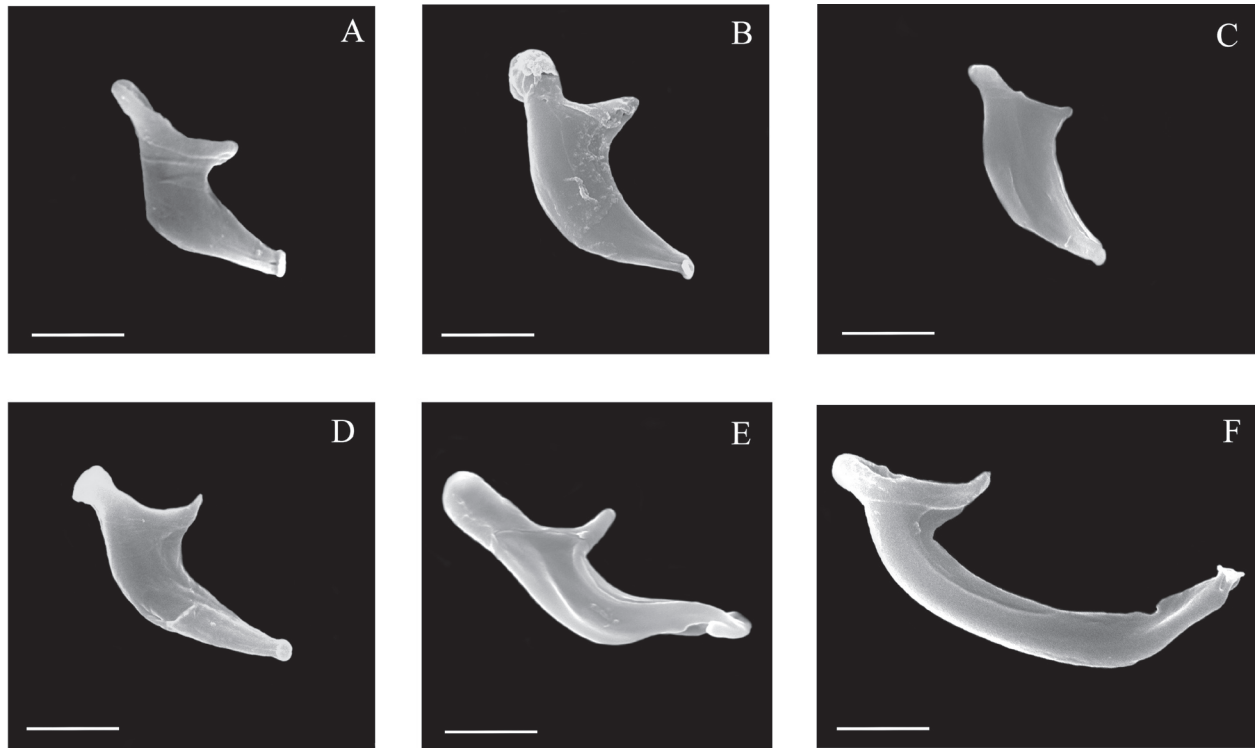


FIGURE 3. SEM pictures of excised spicules: A: *B. hellenicus*. B: *B. leoni*. C: *B. pinasteri*. D: *B. sexdentati*. E: *B. teratospicularis*. F: *B. xylophilus*. (Scale bar = 5 μ m).

***Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle 1970**
(Figs 1, 2G & 3F)

Material examined: Populations Bxy₁ and Bxy₄ were collected from wood material from Tróia (Setúbal), in the case of Bxy₁ 20 females and 20 males were measured and in Bxy₂ 10 females and 17 males. From populations collected from fungal cultures, Bxy₂ and Bxy₃, 20 females and 20 males were measured in both cases. Developmental stages characterised in Table III were collected from a fungal culture with the exception of J_{III} and J_{IV} which were collected from wood samples and from *M. galloprovincialis*, respectively.

Measurements: See Table II.

Female: When heat-relaxed, the female becomes ventrally curved with a ventral inflexion in the vulval region. Cuticle with fine transverse striations and

with four incisures in the lateral field. Head rounded, set off from the body by a distinct constriction. Stylet with small basal knobs. Excretory pore position varying from the beginning to the middle or even the end of median bulb. Hemizonid 1.0 to 1.5 body diameters behind median bulb. Vulva opening forming a 90° angle with the body line; anterior lip extended as a long flap covering vulva. Post-uterine branch extending from 60% to 80% of vulva-anus distance. Subcylindrical female tail with rounded tip (U-shaped), although sometimes presenting a short mucron.

Male: Anterior part of the body similar to female. Posterior part of the body ventrally curved with the tail strongly curled. Large and narrow spicules with a long lamina, angular in posterior third; capitulum almost parallel to shaft axis with a short, rounded condylus and a pointed rostrum; distal tip with a distinct disc-like cucullus (Fig. 3F). A total of seven caudal papillae with the following arrangement: one single ventral pre-anal papilla, one adanal pair

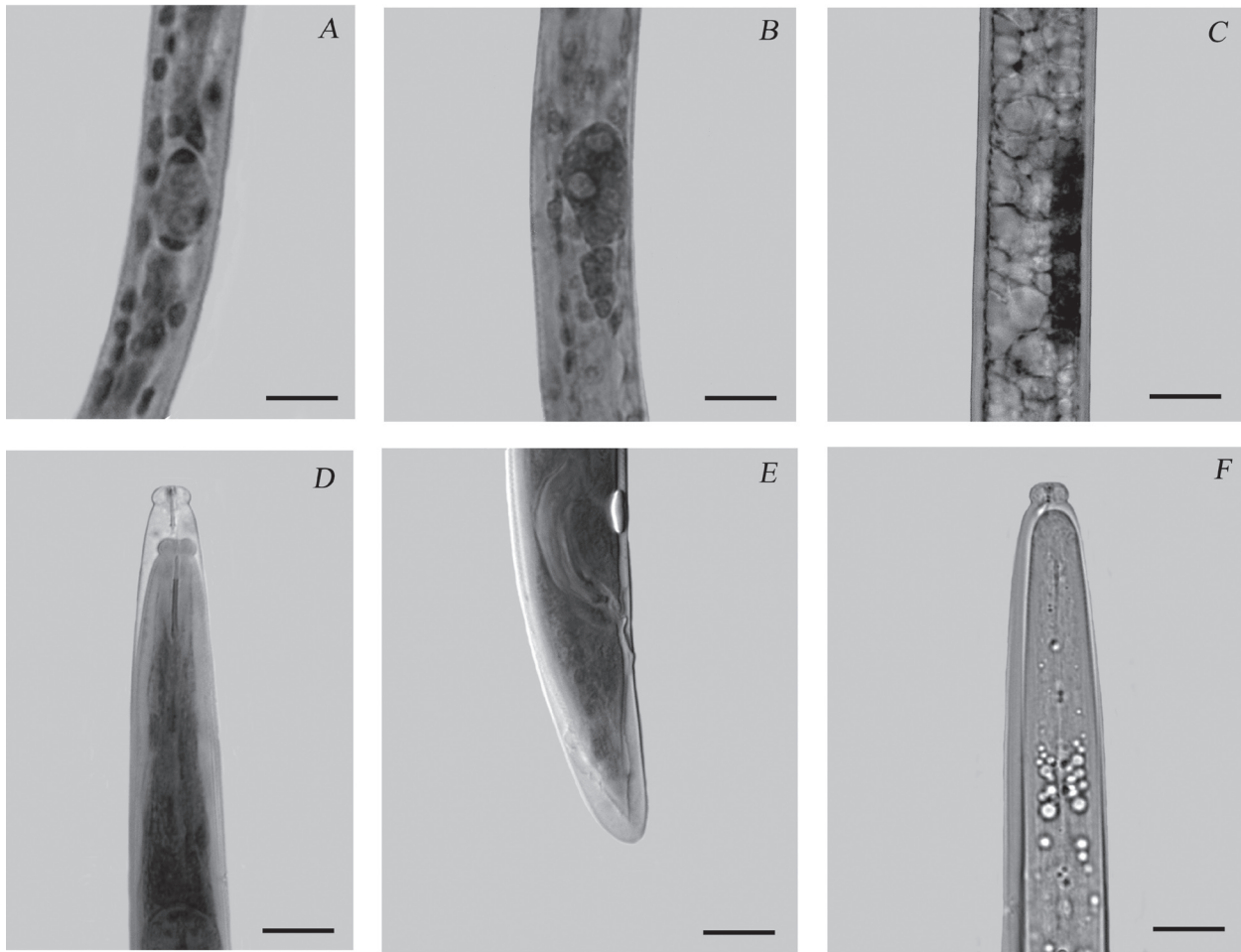


FIGURE 4. Light micrographs of *B. xylophilus* developmental stages. A: J_2 gonad. B: J_3 gonad. C: J_{III} gonad. D: Moulting from J_{4M} to male (anterior region). E: Moulting from J_{4M} to male (tail). F: Moulting from J_{III} to J_{IV} (head). (Scale bar = 10 μm).

and two contiguous post-anal pairs (at ca 40% of tail length from cloaca). Ventral view of bursa showed that it can be oval or truncate with the posterior edge straight or curved inwards.

Distribution: Maritime pine wood collected from "affected zone", Portugal.

Remarks: The *B. xylophilus* Portuguese populations share the morphological features typical of *B. xylophilus*, *B. mucronatus* and *B. fraudulentus*, species included in 'xylophilus'-group: the spicule shape, the arrangement of the seven male caudal papillae and the long vulval flap. The morphological

character that leads to the identification of most of the Portuguese populations of *B. xylophilus* is the female tail: shape, typically subcylindrical with a rounded terminus. Nevertheless, a few Portuguese populations had females with a short mucron on the tail, which required ITS-RFLP analysis for an accurate identification. Measurements of the Portuguese populations of *B. xylophilus* show that there is a great variability among different populations and sometimes even within the same population.

In Europe *B. xylophilus* is solely found in Portugal, being associated with maritime pine and confined to the so-called "affected zone". This species was only associated with the cerambycid *M. galloprovincialis*.

TABLE III. Measurements of *Bursaphelenchus xylophilus* developmental stages (in μm and in form: mean \pm SD (range)).

Character	n	J_2	J_2 - J_3	J_3	J_3 - J_4	J_4		J_F	J_M	Adult		J_{III}	J_{IV}
		20	14	34	18	29♀	15♂	20♀	23♂	14♀	19♂	16	15
Body length	254.4 \pm 22.1 (225-315)	346.1 \pm 30.1 (300-405)	438.1 \pm 46.5 (327-544)	518.0 \pm 34.3 (464-591)	634 \pm 41.4 (541-721)	592.7 \pm 49.7 (519-682)	791.4 \pm 100.2 (651-946)	708.2 \pm 96.4 (519-915)	912.2 \pm 111.9 (604-1091)	842.2 \pm 172.9 (605-1121)	670.9 \pm 41.5 (620-788)	578.8 \pm 53.4 (505-687)	
a	28.5 \pm 3.2 (24.3-34.6)	26.3 \pm 2.5 (23.6-32.4)	32.6 \pm 2.9 (27.2-38.8)	32.4 \pm 1.8 (29.0-34.9)	39.6 \pm 2.1 (35.1-42.7)	38.9 \pm 4.3 (34.6-49.7)	36.5 \pm 3.0 (31.4-41.5)	36.2 \pm 3.3 (28.8-45.8)	41.9 \pm 5.3 (33.6-51.9)	41.5 \pm 5.1 (31.5-49.7)	37.0 \pm 3.0 (32.7-43.9)	41.0 \pm 2.9 (36.1-44.8)	
c	18.8 \pm 1.5 (17.2-22.5)	19.9 \pm 2.8 (16.8-25.3)	21.3 \pm 2.2 (16.4-26.7)	21.3 \pm 1.3 (18.6-23.3)	23.7 \pm 1.5 (21.4-26.2)	25.2 \pm 1.5 (22.4-27.3)	24.7 \pm 1.8 (21.3-28.7)	26.1 \pm 2.5 (22.4-32.0)	26.6 \pm 2.4 (22.2-30.3)	21.9 \pm 2.2 (18.3-26)	23.2 \pm 1.8 (19.4-26.2)	20.0 \pm 1.8 (18.1-20.5)	
c'	2.2 \pm 0.3 (1.7-2.8)	2.1 \pm 1.3 (1.7-2.5)	2.3 \pm 0.2 (1.9-3.0)	2.3 \pm 0.2 (2.0-2.8)	2.8 \pm 0.3 (2.3-3.3)	2.2 \pm 0.2 (1.7-2.4)	2.8 \pm 0.2 (2.3-3.2)	2.1 \pm 0.2 (1.7-2.5)	3.3 (2.7-4.1)	2.5 \pm 0.1 (2.4-2.9)	2.4 \pm 0.3 (2.0-2.9)	3.3 \pm 0.3 (2.7-3.88)	
Stylet	8.8 \pm 1.2 (6.5-10)	10.7 \pm 0.6 (10-11)	11 \pm 0.7 (10-13)	11.8 \pm 0.4 (11-12)	12.3 \pm 0.6 (11-13)	12.4 \pm 0.9 (11-14)	12.9 \pm 0.8 (11-14)	12.6 \pm 0.8 (11-13.5)	13.6 \pm 0.9 (12-15)	13.3 \pm 1.0 (11-15)	11.4 \pm 0.9 (10-13)	-	
Body diam.	9.1 \pm 1.0 (7-11)	13.6 \pm 0.9 (12-15)	13.5 \pm 0.9 (12-15)	16 \pm 1.2 (14-19)	16.0 \pm 1.0 (14-18)	15.3 \pm 1.6 (12-17)	21.6 \pm 1.3 (19-23)	19.6 \pm 1.6 (17-23)	21.9 \pm 2.4 (17-25)	20.2 \pm 2.7 (15-26)	18.2 \pm 1.2 (15-20)	14.1 \pm 0.8 (13-16)	
Median bulb length	12.7 \pm 1.2 (10-15)	14.7 \pm 1.1 (13-17)	15.0 \pm 1.0 (13-18)	15.8 \pm 1.2 (14-18)	16.7 \pm 0.9 (15-18)	16.2 \pm 0.9 (15-18)	18.2 \pm 1.0 (17-20)	17.2 \pm 0.9 (16-20)	17.9 \pm 1.0 (17-20)	17.4 \pm 1.5 (15-22)	14.9 \pm 0.9 (13-16)	12.4 \pm 1.0 (11-15)	
Median bulb diam.	8.1 \pm 0.5 (7-9)	9.4 \pm 0.7 (8-10)	10.0 \pm 0.6 (9-11)	10.8 \pm 0.6 (10-12)	11.3 \pm 0.7 (10-13)	10.8 \pm 0.7 (10-12)	11.8 \pm 0.9 (10-13.5)	11.4 \pm 0.6 (10-12)	13.3 \pm 0.7 (12-14)	12.6 \pm 1.2 (11-15)	10.2 \pm 0.7 (9-11)	6.6 \pm 0.5 (6-7)	
Gonad length	11.4 \pm 1.9 (9-16)	20.1 \pm 5.9 (14-33)	33.9 \pm 6.7 (22-51)	85.1 \pm 13.6 (62-108)	160.0 \pm 21.2 (123-195)	173.1 \pm 41.4 (114-230)	312.3 \pm 61.7 (207-437)	465.7 \pm 82.9 (252-648)	537.1 \pm 91.6 (306-674)	542.4 \pm 143.4 (360-860)	31.6 \pm 4.5 (25-42)	74.0 \pm 19.4 (37-96)	
Tail length	13.6 \pm 0.8 (11-15)	17.1 \pm 2.0 (14-21)	20.6 \pm 2.3 (15-24)	24.3 \pm 1.3 (2-2-27)	27.0 \pm 2.5 (21-33)	23.2 \pm 1.8 (19-26)	32.0 \pm 3.3 (26-38)	27.2 \pm 3.4 (19-36)	34.4 \pm 4.2 (24-41)	38.9 \pm 4.5 (30-45)	28.9 \pm 2.5 (26-35)	30.0 \pm 3.4 (27-38)	
Anal/cloacal body diam.	6.3 \pm 0.8 (5-7)	8.9 \pm 1.1 (7-11)	9.0 \pm 0.8 (7-10)	10.4 \pm 0.8 (9-12)	9.7 \pm 0.7 (9-11)	10.7 \pm 0.8 (10-12)	11.5 \pm 1.1 (10-13)	13.4 \pm 1.3 (11-16)	10.5 \pm 1.1 (9-12)	15.4 \pm 2.3 (12-20)	12.3 \pm 0.6 (11-13)	8.8 \pm 0.6 (8-10)	
L/gonad length	22.3 \pm 3.4 (16.7-27.1)	19.3 \pm 6.3 (11.7-32.1)	13.4 \pm 2.9 (9.0-23.7)	6.6 \pm 0.2 (5.0-7.9)	4.0 \pm 0.6 (3.4-5.7)	3.6 \pm 0.7 (2.6-4.7)	2.6 \pm 0.4 (2.2-3.5)	1.5 \pm 1.0 (1.4-2.1)	1.7 \pm 0.2 (1.4-2.0)	1.6 \pm 0.3 (1.3-2.3)	21.8 \pm 3.0 (15.4-27.2)	8.5 \pm 2.7 (6.2-14.2)	
Gonad length/L	4.6 \pm 0.9 (3.7-6.0)	6.5 \pm 1.4 (4.0-8.5)	7.8 \pm 1.4 (4.8-11.1)	16.4 \pm 2.0 (12.7-20.1)	25.3 \pm 3.2 (17.4-29.8)	29.4 \pm 5.8 (21.3-38.3)	39.3 \pm 4.9 (28.6-46.2)	65.5 \pm 5.2 (48.6-74.2)	58.8 \pm 7.0 (50.3-74.0)	64.1 \pm 10.0 (43.3-78.3)	4.7 \pm 0.8 (3.7-6.5)	12.7 \pm 2.9 (7.0-16.0)	
* 100													

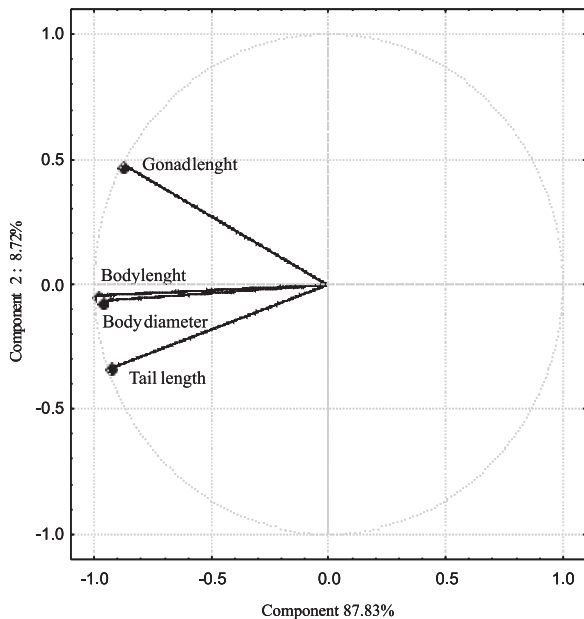


FIGURE 5. Bi-dimensional projection of four morphometric characters of moulting *B. xylophilus* based on a correlation matrix of the principal component analyses.

Populations of *B. xylophilus* are easily maintained on non sporulating *B. cinerea* and *M. fructicola*. The ITS-RFLP pattern of *B. xylophilus* (Fig. 1) is identical to the pattern of a Chinese population (Burgermeister *et al.*, 2005).

Morphobiometric detailed studies of B. xylophilus: Measurements of population Bxy₁ were compared with data of the same population maintained on fungi 15 days (Bxy₂) and one year (Bxy₃) (Table II). The data show that Bxy₁ is smaller than Bxy₂ and Bxy₃. The higher values of the different characters of populations from fungi (Bxy₂ and Bxy₃) compared to the field population (Bxy₁) could result from the more adequate conditions (e.g., temperature and food availability). After ratios analyses, body length and the anterior genital branch were found to increase more on the fungi populations than the majority of the remaining characters (e.g., body diameter, pharynx length, tail length), which might be also a direct consequence of the extremely favourable conditions to which the individuals were exposed, which resulted in bigger individuals and

intensive reproductive activity. These results suggest that biometrical data of fungi-reared nematode populations should be interpreted with some caution, namely when they are used to describe new species.

The use of 1% acetic orcein allowed detailed observation and measurement of the gonad development of the different juvenile stages, often in very good conditions (Fig. 4). A total of four biological variables, comprising total body length, body diameter, gonad length and tail length, were inputted to a PCA and tested for patterns of variation between moulting specimens, with the first two components accounting for 96.55% of the variation, thus accounting for practically all the variation observed. The first principal component was negatively correlated with the four variables inputted while the second component was positively correlated with gonad length and more negatively correlated with the tail length, being responsible for the differences between the groups J_{4M} - M and J_{4F} - F (Fig. 5). The PCA segregated the moulting individuals into four groups: nematodes moulting from second juvenile stage to third juvenile stage (J₂-J₃), from third to fourth stage (J₃-J₄), from female fourth stage to adult female (J_{4F} - F) and from male fourth stage to adult male (J_{4M} - M) (Fig. 6).

The propagative juvenile stages could be separated by the mean values of the gonad length of the four moulting groups discriminated by the first PCA. The results of the gonad length and other characters of the different developmental stages are presented on Table III. The juvenile stages previously discriminated could also be visualised on a second PCA analysis using the same variables (total body length, body diameter, gonad length and tail length), where the first two components accounted for 96.82% of the variation. Similarly to the previous analyses, the first principal component was negatively correlated with the four variables while the second component was positively correlated with gonad length (Figs 7 & 8). These studies confirmed the existence of three immature stages (J₂, J₃ and J₄), which if added to the first stage occurring within the egg (Mamiya, 1975) result in the four developmental stages that most authors describe for this species (Mamiya, 1975; Ishibashi *et al.*, 1978). The distinction of J_{4F} from J_{4M} was based on morphological observations. In J_{4F} a hyaline depression surrounded by some cells near the ventral body wall, indicates the position of the future vulva.

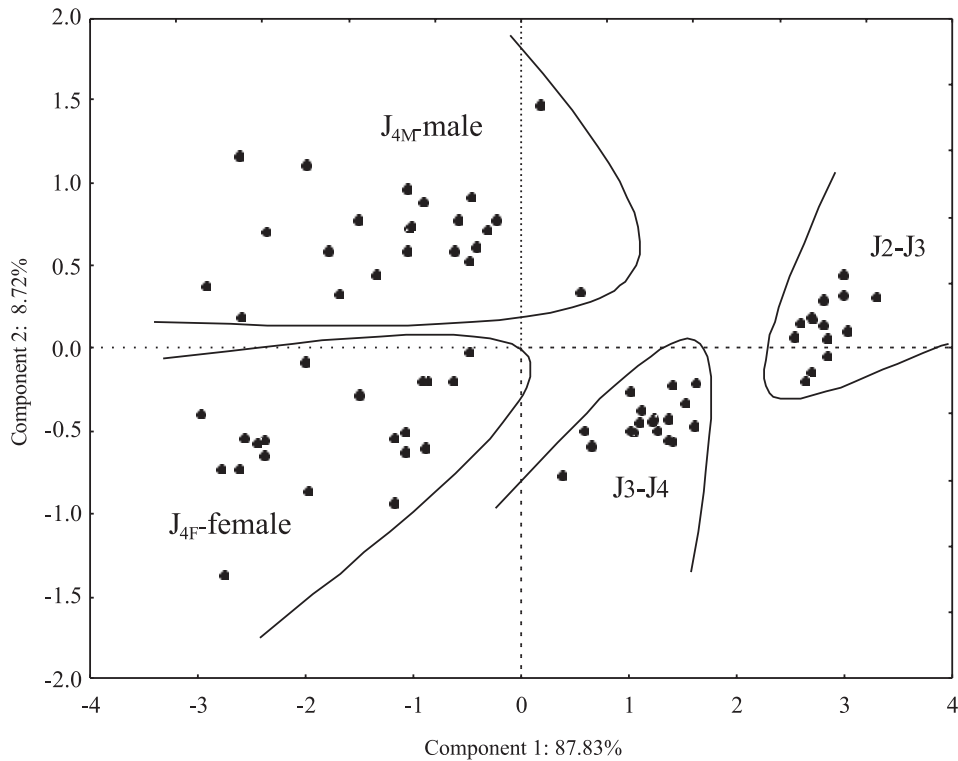


FIGURE 6. Bi-dimensional projection of 75 moulting *B. xylophilus* individuals and separation of the four groups based on a correlation matrix of the principal component analyses.

The gonad length (Table III) increased both during moulting and within juvenile stages, indicating that growth occurred throughout all the development, as previously reported for this species by Ishibashi *et al.* (1978). This is further corroborated by the observation that, when the moulting specimens are excluded, there are wide gaps in gonad length between stages. The other characters, like the total body length, showed some degree of overlapping between stages, which is also in accordance with studies of Japanese populations (Mamiya, 1975; Ishibashi *et al.*, 1978).

The dispersal forms (J_{III} and J_{IV}) can be separated based on morphological characteristics: J_{III} body content is filled with lipid droplets, with the anterior region well defined and tail with rounded terminus and J_{IV} has dome-shaped head, stylet not discernable, poorly defined median bulb and an elongate conoid tail (Fig. 9). The moultings from J_{III} to J_{IV} (Fig. 4), J_{III}

to J_4 and J_{IV} to adult were observed. Gonad length of J_{III} is very similar to J_3 , but the same is not verified for other characters like body length and tail length. The J_{IV} gonad length is less than one-half of J_4 , being closer to the values obtained for moulting J_3 - J_4 individuals (Table III). Both the J_{III} and J_3 stages had similar gonad length although the other characters differed as J_{III} displayed higher values for all, which might be a consequence of the J_{III} nematodes entering into a dispersal phase, favouring a larger size and an accumulation of lipids. In the subsequent stage, the gonad length of the J_{IV} stage was less than one-half of the propagative J_4 , which might have resulted from a suppression of the gonad development during the transport by the vector, which is in concordance with previous observations that the J_{IV} *B. xylophilus* nematodes do not feed or breed when they are being dispersed by their vector insects (e.g., Mamiya, 1984; Linit, 1990).

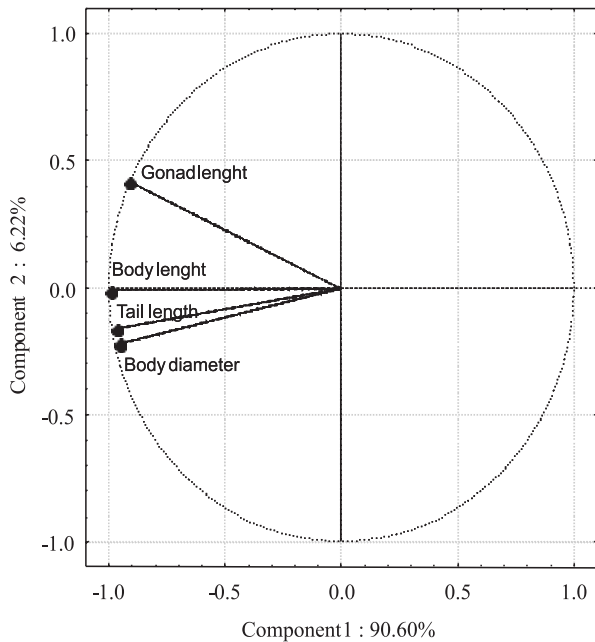


FIGURE 7. Bi-dimensional projection of four morphometric characters of *B. xylophilus* juvenile stages based on a correlation matrix of the principal component analyses.

***Bursaphelenchus hellenicus* Skarmoutsos, Braasch & Michalopoulos, 1998**
(Figs. 1, 2A & 3A)

Material examined: Thirteen females and 14 males of one population (Bhe) extracted from wood material collected in Tróia (Setúbal).

Measurements: See Table IV.

Female: Body slightly ventrally curved when heat killed. Three incisures in the lateral field (not easy to observe) and cuticle with fine transverse striations. Round shaped lip region, set off by a constriction. Stylet with small basal thickenings. Nerve ring just bellow median bulb. Excretory pore located at 0.5-1.0 body diameters behind median bulb and hemizonid at 1.0-1.5 body diameters behind median bulb. Vulva forming a 90° angle with the body surface, with anterior lip slightly extended forming a very small flap that not covers totally the vulval region. Post-uterine branch long, sometimes reaching about 60%

of vulva-anus distance. Female tail conoid, with the tip usually round but occasionally pointed; terminus often slightly ventrally curved.

Male: Anterior part of body similar to female. Posterior part of the body hook-like, curved ventrally. Small, rose thorn-shaped spicules; concave capitulum with prominent digitate rostrum and an elongate, bluntly rounded condylus; distal end with a small cucullus (Fig. 3A). A total of seven caudal papillae: a single pre-anal papilla, one adanal pair, one ventral post-anal pair at ca 46% of tail length from cloaca and a smaller, ventral pair at beginning of bursa at ca 57% of tail length from cloaca (not always visible). Male tail ending pointed with a bursa usually quadrangular with posterior edge indented (ventral view).

Distribution: Maritime pine wood collected from Santarém, Setúbal, Sines and Viseu districts, Portugal.

Remarks: Morphology and morphometrics of Portuguese populations generally agree with the original description (Skarmoutsos *et al.*, 1998): females are bigger than males with a total body length of ca 800 µm vs 713 µm, male spicules with ca 15 µm long and V-ratio of ca 73%. Vulval region, spicule shape, female tail form, number of incisures in the lateral field are the morphological characters that corroborate the identity of these Portuguese populations with the original population (Skarmoutsos *et al.*, 1998). Nevertheless, the Portuguese population shows some differences such as one more pair of post-anal papillae, just before the beginning of the bursa.

In Europe this species was already reported from Greece (Skarmoutsos *et al.*, 1998; Michalopoulos-Skarmoutsos *et al.*, 2004), Germany (Braasch *et al.*, 1999) and in intercepted *Larix*. sp. wood in Russia (Braasch *et al.*, 2001).

The original population was collected in Greece from *Pinus brutia* (Skarmoutsos *et al.*, 1998). The Portuguese populations were extracted from maritime pine, collected from different locations within the demarcated zone and from a location (Viseu) in the central region of Portugal (Penas *et al.*, 2004). Dauer juveniles of this species were found associated with insects *Tomiscus piniperdae*, *Ips sexdentatus* and *Hylurgus ligniperda* (Penas *et al.*, 2006a). *Bursaphelenchus hellenicus* can be maintained

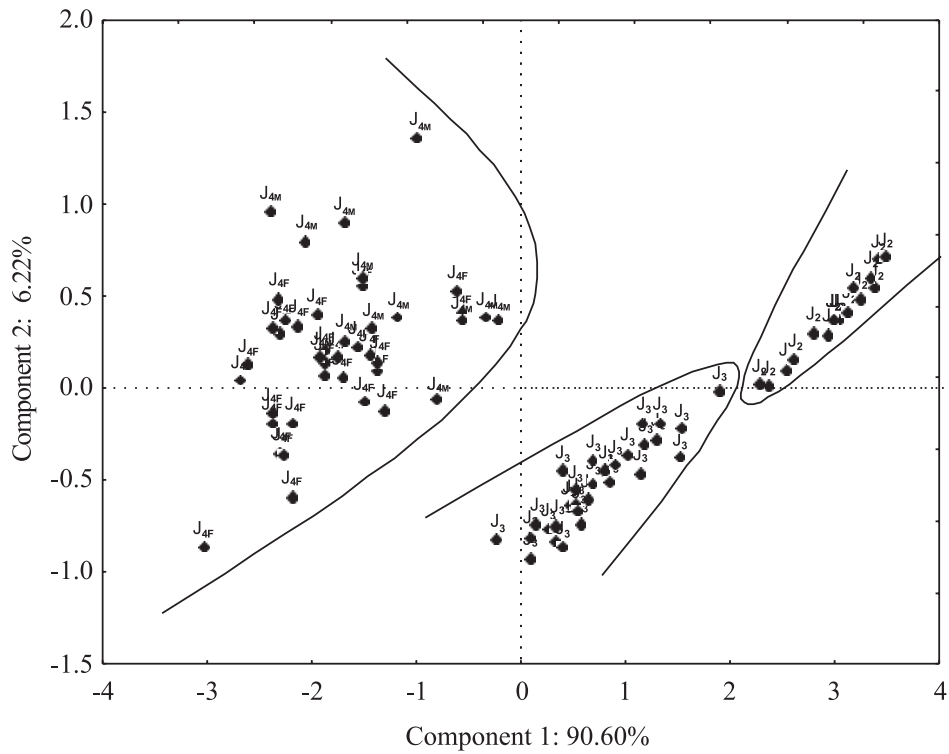


FIGURE 8. Bi-dimensional projection of 89 *B. xylophilus* individuals separated into three juvenile stages (J_2 , J_3 and J_4) and separation of the groups based on a correlation matrix of the principal component analyses.

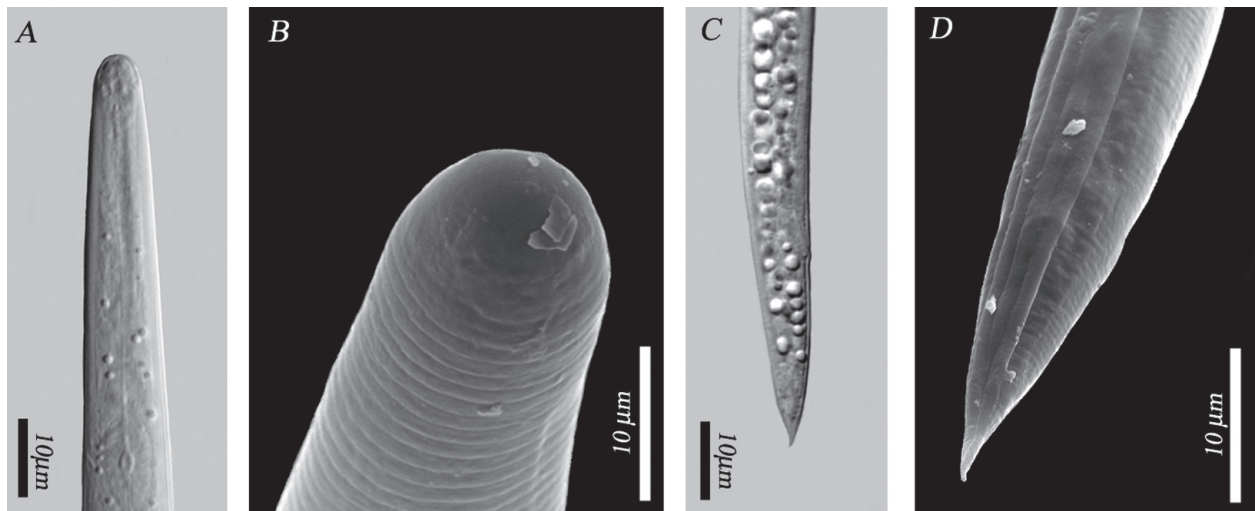


FIGURE 9. *Bursaphelenchus xylophilus* dauer juvenile (J_{IV}). A: Light micrograph (LM) of anterior region. B: Scanning electron micrograph (SEM) of anterior region. C: LM of posterior region. D: SEM of posterior region.

TABLE IV. Measurements of *Bursaphelenchus hellenicus* (in μm and in form: mean \pm SD (range)).

Population	n	Bhe - Tróia	
		13 ♀♀	14 ♂♂
Character			
Body length		800.7 \pm 70.9 (607-908)	713.7 \pm 55.2 (637-802)
a		40.2 \pm 4.0 (30.4-46.1)	39.8 \pm 2.2 (36.6-43.9)
b		10.6 \pm 0.9 (8.0-11.6)	9.6 \pm 0.6 (8.9-10.6)
c		18.8 \pm 1.6 (14.8-21.1)	19.3 \pm 1.0 (17.4-21.2)
c'		3.7 \pm 0.3 (3.2-4.3)	2.6 \pm 0.2 (2.2-2.8)
V		73.8 \pm 3.3 (71.9-84.5)	-
Lip region diam.		6.9 \pm 0.3 (6.5-7.5)	6.8 \pm 0.4 (6.0-7.5)
Lip constriction diam.		5.8 \pm 0.3 (5.5-6.5)	5.7 \pm 0.3 (5-6)
Lip region height		3.8 \pm 0.3 (3.5-4.0)	3.9 \pm 0.2 (3.5-4.0)
Stylet		13.7 \pm 0.9 (12-15)	13.5 \pm 0.9 (12-15)
Pharynx length		75.7 \pm 1.8 (73-78)	73.9 \pm 2.4 (71-78)
Median bulb length		17.2 \pm 0.9 (15-19)	16.4 \pm 0.8 (15-17)
Median bulb diam.		12.7 \pm 0.7 (12-14)	11.5 \pm 0.5 (11-12)
Body diam. at middle of median bulb		12.7 \pm 0.8 (12-14)	15.4 \pm 0.9 (14-17)
Body diam. at base of median bulb		12.7 \pm 0.7 (12-14)	15.5 \pm 0.9 (14-17)
Distance anterior end to excretory pore		83.3 \pm 5.2 (78-96)	79.8 \pm 5.5 (70-88)
Distance anterior end to hemizonid		100.3 \pm 3.9 (95-104)	96.9 \pm 4.7 (90-106)
Distance ant. end to posterior end of pharyngeal glands		157.6 \pm 7.6 (146-173)	151.4 \pm 5.5 (141-161)
Body diam. at end of pharyngeal glands		18.3 \pm 1.0 (16-19)	17.3 \pm 1.0 (16-19)
Anterior genital branch		351.5 \pm 36.9 (297-425)	480.1 \pm 62.2 (377-568)
Posterior genital branch		109.2 \pm 11.9 (91-132)	-
Body diam. at vulva		19.5 \pm 1.4 (17-22)	-
Vulva to anus distance		176.9 \pm 9.9 (164-191)	-
Distance from anterior end to vulva		589.5 \pm 39.8 (513-673)	-
G1 (%)		44.3 \pm 7.2 (36.5-63.4)	-
G2 (%)		13.7 \pm 1.6 (10.8-16.5)	-
Anal/cloacal body diam.		11.6 \pm 0.9 (10-13)	14.4 \pm 0.9 (13.0-15.5)
Tail		42.7 \pm 1.8 (41-47)	37.1 \pm 2.7 (33-41)
T		-	67.1 \pm 4.7 (57.4-74.4)
Spicule (condylus to distal end)		-	15.5 \pm 0.7 (14.5-17.0)
Spicule (rostrum to distal end)		-	7.4 \pm 0.4 (7-8)
Spicule (curved median line)		-	12.0 \pm 0.5 (11-13)
Spicule (rostrum to condylus)		-	8.7 \pm 0.8 (7-10)
Distance single precloacal papilla to cloacal aperture		-	3.9 \pm 0.5 (3-5)
Distance cloacal aperture to first pair of postcloacal papillae		-	17.3 \pm 1.2 (15.5-19.5)
Distance cloacal aperture to second pair of postcloacal papillae		-	21.1 \pm 0.6 (20.5-22.0)

in cultures of non sporulating *B. cinerea* and *M. fructicola*. The ITS-RFLP pattern obtained for the Portuguese population of *B. hellenicus* (Fig. 1) is identical to the pattern of the Greek population (Burgermeister *et al.*, 2005).

***Bursaphelenchus leoni* Baujard, 1980**
(Figs 2B & 3B)

Material examined: Three populations, Ble₁, Ble₂ and Ble₃, extracted from wood material, collected

from Grândola (Setúbal), Tróia (Setúbal) and Companhia das Lezírias (Santarém), respectively. From Ble₁ five females and six males were measured, from Ble₂ 12 females and 13 males, and from Ble₃ 17 females and 18 males.

Measurements: See Table V.

Female: Adults of *B. leoni* are characterised by having long slender bodies, with a fine transverse striations. When killed by heat the females present an inflexion at the vulval region. Lateral field with

TABLE V. Measurements of *Bursaphelenchus leoni* (in μm and in form: mean \pm SD (range)).

Population Character	Ble ₁ - Grândola		Ble ₂ - Troia		Ble ₃ - Companhia das Lezírias	
	5 ♀♀	6 ♂♂	12 ♀♀	13 ♂♂	17 ♀♀	18 ♂♂
Body length	635.8±37.9 (584-676)	587.3±61.6 (514-687)	733.1±33.9 (671-781)	680.4±52.7 (569-772)	747.7±52.7 (679-846)	690.9±47.1 (640-839)
a	37.4±2.3 (34.4-39.8)	37.3±2.5 (33.4-39.9)	39.5±3.0 (32.5-43.9)	39.3±2.8 (36.2-46.5)	40.5±2.7 (36.4-45.6)	38.3±2.4 (32.5-42.0)
b	9.4±0.4 (8.8-9.9)	8.9±0.8 (7.8-10.3)	10.9±0.8 (9.8-12.0)	10.2±0.8 (9.2-11.5)	10.3±0.8 (8.9-11.3)	9.8±0.7 (8.6-11.8)
c	10.7±0.5 (10.2-11.5)	17.3±2.2 (15.6-21.5)	11.6±0.9 (10.2-13.5)	18.3±1.2 (16.9-20.9)	12.3±1.2 (9.9-14.6)	18.3±1.1 (16.8-20.7)
c'	6.2±0.4 (5.6-6.7)	2.7±0.3 (2.3-3.0)	6.4±0.6 (5.8-7.9)	2.5±0.2 (2.1-2.8)	5.8±0.6 (4.9-7.0)	2.6±0.2 (2.4-2.9)
V	68.8±0.6 (68.3-69.8)	-	68.4±0.9 (66.7-70.0)	-	69.3±0.7 (68.1-70.2)	-
Lip region diam.	6.1±0.2 (6.0-6.5)	6.1±0.2 (6.0-6.5)	6.2±0.4 (5.5-7.0)	6.3±0.3 (6-7)	6.1±0.3 (5.5-6.5)	6.1±0.2 (6.0-6.5)
Lip constriction diam.	4.7±0.3 (4.5-5.0)	4.8±0.3 (4.5-5.0)	5.0±0.2 (4.5-5.5)	5.0±0.3 (4.5-5.5)	4.8±0.4 (4.0-5.5)	4.9±0.3 (4.5-5.5)
Lip region height	3.4±0.2 (3.0-3.5)	3.5±0.3 (3-4)	3.1±0.2 (3.0-3.5)	3.4±0.3 (3-4)	3.4±0.2 (3.0-3.5)	3.4±0.3 (3-4)
Stylet	12.7±1.2 (11-14)	12.5±0.8 (12-14)	12.5±1.2 (11.0-14.5)	12.6±1.0 (11-14)	13.3±1.1 (12-15)	12.6±0.9 (11-14)
Pharynx length	67.5±1.0 (66.0-68.5)	66.3±2.3 (64.0-70.5)	67.4±3.4 (63-74)	66.8±3.0 (62-70)	72.8±3.5 (68-79)	70.6±2.8 (66-76)
Median bulb length	15.7±0.8 (15-17)	15.3±0.9 (14.0-16.5)	16.0±1.0 (14-17)	15.2±1.0 (14-17)	17.1±0.9 (15-18)	16.3±0.9 (15-18)
Median bulb diam.	10.2±0.8 (9.5-11.5)	9.8±0.3 (9-11)	11.0±0.7 (10-12)	10.5±0.5 (10-11)	10.9±0.7 (10-12)	10.3±0.7 (9-12)
Body diam. - middle med. bulb	13.3±0.7 (13.0-14.5)	13.3±0.8 (12-14)	14.1±0.8 (13-15)	14.2±0.8 (13-16)	14.9±0.9 (14-17)	14.8±0.8 (14.0-16.5)
Body diam. - base of med. bulb	13.9±0.7 (13-15)	13.8±1.1 (12-15)	14.5±0.6 (13.5-15.5)	14.5±0.8 (13-16)	15.1±0.9 (14-17)	15.0±0.8 (14.0-16.5)
Distance ant. end - excret. pore	82.6±2.9 (78-85)	85.2±5.5 (78-93)	89.1±2.8 (84-92)	89.3±5.2 (83-96)	91.4±4.7 (84-102)	89.6±5.0 (83-99)
Distance ant. end - hemizonid	89.8±1.1 (88-91)	93.0±8.2 (83-107)	94.7±4.0 (88-100)	94.2±5.5 (81-103)	98.7±4.9 (90-106)	98.5±5.9 (92-107)
Distance ant. end to posterior end of pharyngeal glands	129.4±5.2 (121-134)	126.2±9.0 (119-143)	143.1±8.0 (131-156)	135.6±11.3 (113-155)	146.0±8.4 (125-160)	138.6±5.1 (130-147)
Body diam. at end phar. glands	15.1±0.9 (14-16)	15.2±1.3 (13-17)	16.4±1.2 (15-18)	16.3±1.2 (14-18)	17.1±0.9 (16-19)	17.1±0.9 (16-19)
Anterior genital branch	198.2±20.3 (163-213)	335.8±35.3 (300-378)	290.2±32.8 (239-348)	434.2±61.9 (305-520)	291.1±35.9 (233-364)	487.8±45.2 (419-564)
Posterior genital branch	95.3±10.2 (81-104)	-	92.7±14.4 (74-119)	-	99.8±11.6 (84-116)	-
Body diam. at vulva	16.6±1.1 (15-18)	-	18.8±1.3 (16.5-21.5)	-	18.3±1.0 (17-20)	-
Vulva to anus distance	141.0±17.5 (124-165)	-	167.9±14.1 (151-196)	-	165.5±14.5 (145-198)	-
Distance anterior end to vulva	437.0±23.7 (402-462)	-	501.2±25.1 (453-540)	-	518.4±37.8 (472-593)	-
G1 (%)	31.3±4.1 (24.5-34.3)	-	39.5±3.0 (33.4-44.6)	-	39.1±5.1 (30.0-46.4)	-
G2 (%)	14.8±0.7 (13.9-15.6)	-	12.6±1.5 (10.2-15.2)	-	13.4±1.6 (11.3-16.4)	-
Anal/ cloacal body diam.	9.6±0.7 (9.0-10.5)	12.8±0.8 (12-14)	10.0±0.8 (9-11)	14.6±0.6 (13.0-15.5)	10.6±0.7 (9.5-12.0)	14.3±0.7 (13.5-16.0)
Tail	59.4±2.2 (56-62)	34.0±2.3 (31.5-37.0)	63.6±3.8 (56-71)	37.2±2.7 (31-41)	61.1±5.2 (53-71)	37.8±2.5 (33-43)

TABLE V. (Cont.)

Population	Ble ₁ - Grândola			Ble ₂ - Tróia		Ble ₃ - Companhia das Lezírias	
	n	5 ♀♀	6 ♂♂	12 ♀♀	13 ♂♂	17 ♀♀	18 ♂♂
G1 (%)		31.3±4.1 (24.5-34.3)	-	39.5±3.0 (33.4-44.6)	-	39.1±5.1 (30.0-46.4)	-
G2 (%)		14.8±0.7 (13.9-15.6)	-	12.6±1.5 (10.2-15.2)	-	13.4±1.6 (11.3-16.4)	-
Anal/cloacal body diam.		9.6±0.7 (9.0-10.5)	12.8±0.8 (12-14)	10.0±0.8 (9-11)	14.6±0.6 (13.0-15.5)	10.6±0.7 (9.5-12.0)	14.3±0.7 (13.5-16.0)
Tail		59.4±2.2 (56-62)	34.0±2.3 (31.5-37.0)	63.6±3.8 (56-71)	37.2±2.7 (31-41)	61.1±5.2 (53-71)	37.8±2.5 (33-43)
T		-	57.6±7.4 (43.7-65.3)	-	63.6±5.6 (53.6-72.5)	-	70.7±6.3 (59.2-76.2)
Spicule (condylus to distal end)		-	16.3±1.5 (14-18)	-	18.7±0.9 (17.5-20.5)	-	18.1±0.9 (16.5-20.0)
Spicule (rostrum to distal end)		-	10.2±0.9 (9.0-11.5)	-	8.5±0.7 (7.0-9.5)	-	11.8±0.8 (10.5-13.5)
Spicule (curved median line)		-	14.0±1.1 (12-15)	-	16.3±0.7 (15.5-18.0)	-	15.9±1.0 (14.5-18.0)
Spicule (rostrum to condylus)		-	7.9±0.7 (7-9)	-	8.5±0.7 (7.0-9.5)	-	8.1±0.6 (7-9)
Distance from single precloacal papilla to cloacal aperture		-	4.8±1.1 (3.0-6.5)	-	5.8±0.8 (4.5-7.5)	-	5.4±0.6 (4.0-6.5)
Distance cloacal aperture to first pair of postcloacal papillae		-	12.8±1.2 (11.5-15.0)	-	14.5±1.1 (12-16)	-	14.2±1.1 (12.0-16.5)
Distance cloacal aperture to second pair of postcl. papillae		-	20.5±0.8 (19.5-21.5)	-	22.6±1.8 (19-25)	-	22.4±1.3 (19.5-24.0)

three incisures. Lip region rounded, well set off by deep, well defined constriction. Stylet with weakly developed basal thickenings. Excretory pore just anterior to hemizonid, at 0.5-1.0 body diameters behind median bulb. Vulva oblique, with the anterior cuticle extended, forming a very small flap not covering totally the vulval opening. Post-uterine branch long, frequently extending about 60% of vulva-anus distance. Female tail conoid, very long (ca 61.5 μ m long), with the tip varying in shape, pointed, finely rounded and sometimes slightly digitate.

Male: Anterior part of body displays similar features to female. Ventral curvature of the posterior part of the body. Spicules medium to large with pointed rostrum and well developed, posteriorly recurved condylus; although not always discernible, distal tip slightly bent (Fig. 3B). Male tail with seven caudal papillae: a single ventral pre-anal papilla, one adanal pair, one post-anal pair at ca 38% of tail length from cloaca and one small pair just before the beginning of bursa at ca 60% of tail length from cloaca. Male tail terminus pointed and bursa truncate with the posterior edge curved inwards when observed in ventral view.

Distribution: Maritime pine wood collected from Aveiro, Castelo Branco, Coimbra, Lisboa, Porto, Santarém, Setúbal, Sines, Vila Real and Viseu districts, Portugal.

Remarks: Morphological and morphometric characters of Portuguese *B. leoni* are close to the original description (Baujard, 1980). Within the same population, male and females have similar body length, although females are in general slightly bigger than males; however, high variability was observed among the Portuguese populations, with one of *B. leoni* populations (Ble₁) being considerably smaller than the others populations (Ble₂ and Ble₃). The head region, the very long conoid female tail and the typical spicule shape are the morphological characters that clearly lead to *B. leoni* identification. Besides the three pairs of papillae (one adanal pair and two post-anal pairs), *B. leoni* possesses one single ventral pre-anal papilla, not mentioned in the original description.

B. leoni is widely distributed throughout Europe (Ryss *et al.*, 2005) being a typical species from the Mediterranean countries: France (Baujard, 1980), Italy (Ambrogioni *et al.* 1994), Greece (Skarmoutsos

& Skarmoutsos, 1999), Spain (Escuer *et al.*, 2002) and Cyprus (Philis & Braasch, 1996). In Portugal, *B. leoni* was one of the most frequently found species, with a wide range of geographical dispersion throughout the country (Penas *et al.*, 2004). As in the original description, the Portuguese populations were collected from *P. pinaster*. The insect vector of this species was not found. This species was not successfully maintained in sporulating and non sporulating *B. cinerea*, or in *M. fructicola* and although probably being mycetophagous, this species might require a very specific fungus or conditions to reproduce.

***Bursaphelenchus pinasteri* Baujard, 1980**

= *B. hofmanni* (*sensu* Penas *et al.*, 2002)

= *Bursaphelenchus* sp.1 (*sensu* Penas *et al.*, 2004)

(Figs 1, 2C & 3C)

Material examined: Two populations, Bpi₁ and Bpi₂, extracted from wood material, collected from Melides (Sines), and Murta, Pousadas (Santarém), respectively. From Bpi₁ 14 females and 13 males were measured, and from Bpi₂ 9 females and 4 males.

Measurements: See Table VI.

Female: When killed by heat, female body is strongly ventrally curved acquiring a C-form. Cuticle with fine transverse striations, and three incisures in the lateral field. High, rounded lip region, separated from the rest of the body by a very weak constriction. Stylet with very small basal thickenings. Excretory pore at the end of median bulb and hemizonid one diameter behind median bulb. Vulva oblique, with the anterior lip forming a small flap. Post-uterine branch not very long, occupying ca 50% of vulva-anus distance. Female tail conoid with a pointed tip; typically, just behind anus, the tail narrows abruptly and after gradually tapering until the terminus.

Male: Anterior features similar to female. Posterior part of the body with a stronger ventral curvature than the female. Spicules with lamina not strongly curved, rostrum prominent conical or pointed, condylus rounded and distal tip with no distinct cucullus (Fig. 3C). Male tail with seven caudal papillae: a single ventral pre-anal papilla, one adanal and two post-anal pairs. Male tail terminus pointed and bursa with oval shape (ventral view).

TABLE VI. Measurements of *Bursaphelenchus pinasteri* (in μm and in form: mean \pm SD (range)).

Character	Population	Bpi ₁ - Melides		Bpi ₂ - Murta,Pousadas	
		n	14 ♀♀	13 ♂♂	9 ♀♀
Body length		597.2 \pm 53.9 (518-740)	526.2 \pm 31.8 (468-574)	693.1 \pm 30.0 (645-737)	559.5 \pm 51.3 (506-629)
a		39.5 \pm 2.7 (35.1-46.3)	39.0 \pm 1.8 (36.0-41.7)	43.5 \pm 2.2 (39.9-46.8)	44.7 \pm 3.3 (40.5-48.4)
b		9.9 \pm 0.8 (8.8-12.3)	8.9 \pm 0.4 (7.9-9.4)	10.9 \pm 0.6 (9.9-12.0)	9.4 \pm 0.5 (9-10)
c		23.1 \pm 2.3 (19.6-27.4)	19.1 \pm 1.8 (16.7-23.2)	23.2 \pm 1.9 (19.9-26.4)	19.5 \pm 1.2 (18-21)
c'		3.5 \pm 0.3 (3.0-4.1)	2.6 \pm 0.2 (2.3-3.0)	3.5 \pm 0.2 (3.1-3.8)	2.5 \pm 0.2 (2.4-2.7)
V		71.2 \pm 4.0 (57.8-74.3)	-	71.9 \pm 1.0 (70.5-73.4)	-
Lip region diam.		7.0 \pm 0.4 (6.5-8.0)	6.8 \pm 0.3 (6.5-7.0)	7.2 \pm 0.4 (7-8)	6.8 \pm 0.6 (6.0-7.5)
Lip constriction diam.		6.3 \pm 0.4 (5.5-7.0)	5.9 \pm 0.3 (5.5-6.5)	6.5 \pm 0.4 (6-7)	6.1 \pm 0.6 (5.5-7.0)
Lip region height		3.8 \pm 0.3 (3-4)	3.6 \pm 0.3 (3-4)	3.8 \pm 0.3 (3.5-4.0)	3.8 \pm 0.3 (3.5-4.0)
Stylet		11.0 \pm 0.6 (10-12)	11.0 \pm 1.0 (10-14)	12.5 \pm 1.1 (11.0-14.5)	11.3 \pm 0.5 (11-12)
Pharynx length		60.1 \pm 2.9 (54-64)	59.1 \pm 1.6 (56-62)	63.9 \pm 2.9 (57-67)	59.3 \pm 3.5 (55-63)
Median bulb length		15.3 \pm 0.9 (14-17)	14.5 \pm 0.5 (14-15)	16.7 \pm 1.1 (14.5-18.0)	14.8 \pm 1.0 (14-16)
Median bulb diam.		11.4 \pm 0.5 (11-12)	10.0 \pm 0.4 (9-11)	10.8 \pm 0.4 (10-11)	9.5 \pm 1.0 (9-11)
Body diam. at middle med. bulb		13.0 \pm 0.6 (12-14)	12.2 \pm 0.6 (11-13)	13.7 \pm 0.9 (12-15)	11.4 \pm 0.5 (11-12)
Body diam. at base median bulb		13.2 \pm 0.6 (12-14)	12.3 \pm 0.6 (11-13)	13.8 \pm 1.0 (12.0-15.5)	11.4 \pm 0.5 (11-12)
Distance ant. end - excretory pore		62.2 \pm 2.1 (58-65)	58.6 \pm 4.1 (50-64)	64.8 \pm 8.8 (57-77)	60.0 \pm 2.8 (58-62)
Distance ant. end - hemizonid		78.5 \pm 3.2 (72-83)	74.8 \pm 2.9 (70-78)	9.2 \pm 4.4 (74-84)	75.3 \pm 1.2 (74-76)
Distance ant. end - posterior end of pharyngeal glands		127.9 \pm 8.2 (108-139)	123.8 \pm 4.5 (115-131)	143.3 \pm 7.4 (127-152)	133.7 \pm 7.2 (129-142)
Body diam. at end phar. glands		14.6 \pm 0.6 (14-16)	13.3 \pm 0.7 (12-14)	14.8 \pm 1.2 (13-17)	12.3 \pm 0.6 (11.5-13.0)
Anterior genital branch		213.2 \pm 21.1 (185-257)	267.5 \pm 32.3 (219-326)	242.1 \pm 25.6 (199-282)	337.5 \pm 15.0 (316-350)
Posterior genital branch		72.2 \pm 5.1 (64-82)	-	74.9 \pm 8.2 (66-89)	-
Body diam. at vulva		14.9 \pm 0.5 (14-16)	-	14.9 \pm 1.1 (12-16)	-
Vulva to anus distance		137.9 \pm 6.9 (124-147)	-	164.2 \pm 7.9 (154-177)	-
Distance ant. end - vulva		423.9 \pm 27.0 (376-471)	-	498.4 \pm 24.8 (461-533)	-
G1 (%)		35.8 \pm 3.4 (30.3-42.3)	-	34.9 \pm 3.3 (29.4-40.5)	-
G2 (%)		12.2 \pm 1.1 (9.6-13.8)	-	10.8 \pm 1.3 (9.4-12.8)	-
Anal/cloacal body diam.		7.4 \pm 0.5 (6.5- 8.0)	10.7 \pm 0.7 (9.0-11.5)	8.7 \pm 0.7 (8-10)	11.5 \pm 0.7 (11.0-12.5)
Tail		26.0 \pm 2.5 (23-31)	27.6 \pm 2.0 (23-31)	30.0 \pm 3.0 (27-37)	28.8 \pm 2.2 (26-31)
T		-	42.6 \pm 13.7 (37.7-50.0)	-	60.5 \pm 3.3 (55.6-62.5)
Spicule (condylus to distal end)		-	13.3 \pm 0.6 (12-14)	-	12.6 \pm 0.8 (11.5-13.0)
Spicule (rostrum to distal end)		-	8.4 \pm 0.4 (7.5-9.0)	-	7.9 \pm 0.3 (7.5-8.0)
Spicule (curved median line)		-	11.8 \pm 0.5 (11.0-12.5)	-	11.5
Spicule (rostrum to condylus)		-	6.2 \pm 0.6 (5-7)	-	5.8 \pm 0.5 (5-6)
Distance from single precloacal papilla to cloacal aperture		-	3.5 \pm 0.7 (2.0-4.5)	-	3.6 \pm 0.3 (3.5-4.0)
Distance from cloacal aperture to first pair of postcloacal papillae		-	11.9 \pm 1.1 (10-14)	-	12.0 \pm 8.0 (11-13)
Distance cloacal aperture to second pair of postcl. papillae		-	15.9 \pm 1.0 (14-17)	-	15.8 \pm 1.2 (14.0-16.5)

Distribution: Maritime pine wood collected from Bragança, Évora, Lisboa, Santarém, Setúbal, Sines and Viseu, Portugal districts.

Remarks: Portuguese populations of *B. pinasteri* are overall in accordance with the original description (Baujard, 1980) *i.e.*: females are slightly bigger than males, three incisures in the lateral field, excretory pore at the end of median bulb, typical female tail

with anus forming a protuberance, male tail with one single ventral pre-anal papilla and three pairs of papillae (one adanal and two post-anal pairs) with identical disposition in the male tail as the original population.

B. pinasteri was only reported in France (Baujard, 1980), Germany (Schönfeld *et al.*, 2001) and Spain (Escuer *et al.*, 2002), always associated with a *Pinus* species and its vector was never reported. It appears

to be strongly associated with maritime pine, since it was originally described associated to this pine species (Baujard, 1980). In Portugal, this species was only collected from *P. pinaster* and, after *B. xylophilus*, was the most frequently found species in the demarcated zone (Penas *et al.*, 2004). Despite the frequency of occurrence, the insect that vectors this species was not found. *B. pinasteri* was reared on non sporulating *B. cinerea* and *M. fructicola*. The ITS-RFLP pattern of the Portuguese *B. pinasteri* (Fig.1) is similar to the one obtained for the German population (Burgermeister *et al.*, 2005).

***Bursaphelenchus sexdentati* Rühm, 1960**
(Figs 1, 2D & 3D)

Material examined: Three populations, Bse₁, Bse₂ and Bse₃, extracted from wood material, collected from Tróia (Setúbal), Companhia das Lezírias (Santarém) and Chamusca (Santarém), respectively. From Bse₁ 9 females and 6 males were measured, from Bse₂ 6 hembras and 10 machos, and from Bse₃ 20 females and 20 machos.

Measurements: See Table VII.

Female: Slightly curved with a ventral inflexion in vulval region when killed by heat. Cuticle with fine transverse striations, and lateral field with four incisures. Lips rounded, head set off by a constriction. Stylet with small basal swellings. Position of the excretory pore varying; located just before the beginning of the median bulb or immediately after the end of median bulb. Hemizonid at 1.0-1.5 body diameters behind median bulb. Vulva oblique, anterior vulval lips forming a small flap; a post-vulval swelling often present. Post-uterine branch long extending for about 65-70% of vulva-anus distance. Conoid tail with the tip varying from rounded to digitate.

Male: Anterior part of the body similar to female. Posterior region of the body with a more pronounced ventral curvature than in female. Spicules stout, with lamina gently curved, capitulum usually with a prominent pointed rostrum, a well developed rounded-squared condylus, and in the distal end a knob-like cucullus not always discernable (Fig. 3D). Male tail with seven caudal papillae: one single

ventral pre-anal pair, sub-ventral adanal and two post-anal pairs (one pair at *ca* 40% of tail length from cloaca and a second pair at *ca* 54% of tail length from cloaca). Male tail mucronate and with a bursa truncate with posterior edge curved inwards.

Distribution: Maritime pine wood collected from Coimbra, Porto, Santarém, Setúbal and Viseu districts, Portugal.

Remarks: The Portuguese population shows similarities in morphological features with the original description, but some differences can be observed in certain characters. The overall spicule shape, as well as the number of caudal papillae are similar, but Portuguese populations have smaller spicules than the specimens of original description (11-15 *vs* 19-22 μ m) and exhibit a small cucullus, not reported in the original description (Rühm, 1960). Nevertheless, *B. sexdentati* spicule measurements fall within the wider range (13-22 μ m) reported by Braasch (2001) for these species. The presence of a cucullus (knob-like appendage) was already reported for the *B. sexdentati* Italian populations (Ambrogioni & Caroppo, 1998), for the Greek population (Lange *et al.*, 2007), and together with the Portuguese population they were grouped and named as "South European type" of *B. sexdentati* (Lange *et al.*, 2007). The female tail is not rounded as the original population (Rühm, 1960) but conoid like the Italian populations (Ambrogioni & Caroppo, 1998).

This species occurs from Eastern Europe, Georgia (Kurashvili *et al.*, 1980), Lithuania and Russia (Vosilite, 1990) to Central and Western Europe, Germany (Rühm, 1960), Italy (Ambrogioni & Caroppo, 1998), Greece (Skarmoutsos & Skarmoutsos, 1999), Austria (Tomiczek, 2000), Spain (Escuer *et al.*, 2002; Escuer *et al.*, 2004) and Cyprus (Braasch & Philis, 2002). *B. sexdentati* was found frequently associated with *P. pinaster* wood in and outside the affected zone (Penas *et al.*, 2004). *B. sexdentati* original population was collected from *Ips sexdentatus* (Rühm, 1960) and in Portugal this species was extracted from maritime pine (Penas *et al.*, 2004) and found associated with *Orthotomicus erosus* and *H. ligniperda* (Penas *et al.*, 2006a). This species can be maintained in cultures of non sporulating *B. cinerea* and *M. fructicola*. Molecular analysis of Portuguese populations of *B. sexdentati* (Fig. 1) leads to an ITS-RFLP pattern

TABLE VII. Measurements of *Bursaphelenchus sexdentati* (in µm and in form: mean ± SD (range)).

Character	Bse. - Tróia			Bse. - Companhia das Lezírias			Bse. - Chamusca		
	n	9 ♀♀	6 ♂♂	n	6 ♀♀	10 ♂♂	n	20 ♀♀	20 ♂♂
Body length	999	77±81.1 (803-1032)	819.7±138.8 (645-1031)	954.2±144.9 (816-1189)	821.8±56.0 (739-924)	832.7±76.5 (741-939)	773.0±78.0 (595-911)		
a	50	5±2.1 (47.2-53.5)	51.2±3.2 (47.0-54.3)	52.3±4.5 (48.7-59.5)	51.1±4.1 (46.5-58.5)	48.3±3.6 (41.6-56.1)	55.3±4.1 (49.2-63.0)		
b	14	0±1.4 (12.4-16.5)	12.5±1.5 (10.9-14.9)	13.9±2.0 (12.3-17.5)	12.0±0.7 (10.9-14.2)	12.2±0.9 (10.5-14.0)	11.6±0.9 (10.1-13.6)		
c	27	3±2.8 (23.2-31.9)	26.1±2.8 (23.8-30.3)	23.7±1.4 (21.7-28.0)	23.7±1.4 (21.7-28.0)	23.3±1.9 (19.4-28.6)	24.3±2.7 (20.5-30.6)		
c'	3	4±0.3 (3.0-4.1)	2.5±0.1 (2.3-2.7)	3.5±0.5 (2.9-4.1)	2.7±0.2 (2.4-3.0)	3.6±0.3 (3.2-4.0)	2.8±0.3 (2.4-3.3)		
V	74	0±0.6 (73.2-75.1)	-	74.0±0.8 (72.6-74.9)	-	74.3±0.3 (72.6-86.9)	-		
Lip region diam.	6	9±0.3 (6.5-7.5)	6.4±0.2 (6.0-6.5)	6.9±0.4 (6.5-7.5)	6.8±0.3 (6.5-7.5)	6.3±0.4 (6-7)	6.1±0.4 (5.5-7.0)		
Lip constriction diam.	6	1±0.2 (6.0-6.5)	5.8±0.6 (4.5-6.0)	6.1±0.4 (5.5-6.5)	5.7±0.4 (5-6)	5.5±0.5 (4.5-6.0)	5.4±0.4 (5-6)		
Lip region height	3	6±0.4 (3-4)	3.3±0.3 (3.0-3.5)	3.4±0.2 (3.0-3.5)	3.4±0.2 (3.0-3.5)	3.4±0.4 (3-4)	3.1±0.4 (2.5-4.0)		
Stylet	14	7±0.7 (14-16)	13.6±1.4 (11-15)	13.3±1.0 (12-14)	13.6±1.3 (11-15)	12.8±1.0 (11-14)	12.4±1.1 (11.0-14.5)		
Pharynx length	67	4±4.1 (62-74)	65.3±4.2 (59-69)	68.5±2.7 (65-72)	68.3±2.4 (64.5-71.0)	68.0±3.6 (60-74)	66.3±2.5 (61-71)		
Median bulb length	17	0±1.0 (15-18)	16.5±2.2 (13-19)	16.8±1.5 (15-19)	15.5±1.0 (14-17)	15.8±1.0 (14-17)	15.5±1.0 (13.5-17.0)		
Median bulb diam.	10	9±0.6 (10-12)	10.1±1.3 (7.5-11.0)	10.7±1.0 (9-12)	9.9±0.7 (9-11)	9.8±0.6 (9-11)	9.2±0.5 (8-10)		
Body diam. at middle of med. bulb	14	3±0.6 (13.5-15.5)	13.1±1.7 (10.0-14.5)	13.8±1.1 (12-15)	13.4±1.6 (11-16)	12.6±0.7 (12-14)	11.9±1.0 (9.5-13.0)		
Body diam. at base of median bulb	14	9±0.7 (14-16)	13.1±1.8 (10-15)	14.2±1.2 (12-15)	13.5±1.6 (11-16)	12.7±0.8 (12.0-14.5)	12.2±1.0 (10.0-13.5)		
Distance anterior end - excr. pore	53	4±4.7 (46-58)	51.8±4.4 (46-56)	54.5±3.1 (50-57)	51.3±1.3 (50-53)	49.5±5.9 (36-60)	48.6±5.6 (35-61)		
Distance anterior end - hemizonitid	89	1±4.2 (81-96)	84.3±7.3 (75-92)	92.0±5.6 (87-99)	87.9±2.3 (84-90)	92.6±4.7 (84-101)	90.5±6.0 (83-98)		
Distance from anterior end to posterior end of phar. glands	150	9±21.7 (122-186)	129.7±6.1 (119-138)	149.6±14.4 (131-162)	140.8±9.9 (128-157)	144.0±14.3 (118-167)	137.9±9.7 (114-155)		
Body diam. at end phar. glands	16	6±0.5 (16-17)	15.1±2.2 (11.0-17.5)	16.4±1.9 (14.0-18.5)	14.8±1.1 (14.0-17.5)	14.6±1.1 (13-17)	13.3±1.2 (10-15)		
Anterior genital branch	398	4±64.9 (311-524)	501.5±140.9 (383-754)	497.3±95.0 (370-660)	522.0±73.7 (421-674)	321.1±67.6 (176-434)	442.8±85.0 (248-619)		
Posterior genital branch	142	1±14.1 (124-165)	-	141.3±19.6 (107-158)	-	132.9±20.8 (99-168)	-		
Body diam. at vulva	18	7±1.2 (17.0-20.5)	-	17.9±2.1 (15-20)	-	17.5±1.4 (15.5-20.0)	-		
Vulva to anus distance	211	4±19.5 (173-226)	-	212.5±30.2 (180-261)	-	187.2±20.5 (150-226)	-		
Distance from anterior end to vulva	695	6±61.7 (595-775)	-	706.7±111.7 (601-887)	-	618.5±56.0 (544-726)	-		
G1 (%)	42	9±9.2 (30.5-60.9)	-	52.0±4.4 (45.2-57.4)	-	38.4±6.6 (23.3-47.3)	-		
G2 (%)	15	1±1.0 (13.4-16.5)	-	14.9±1.8 (13.1-17.1)	-	16.0±2.4 (13.0-21.8)	-		
Anal/cloacal body diam.	10	1±0.5 (9.0-10.5)	12.8±1.2 (11.0-14.5)	9.9±1.0 (8.5-11.0)	12.8±1.3 (11.5-14.0)	10.0±0.7 (9-11)	11.6±0.6 (10-13)		
Tail	34	6±1.8 (32-37)	31.3±3.0 (27-35)	34.2±4.4 (29-41)	34.0±2.0 (31-38)	35.9±3.6 (30-44)	32.0±3.1 (27-38)		
T	-	-	61.1±11.7 (47.5-77.1)	-	60.0±6.3 (51.0-72.9)	-	57.0±8.1 (41.6-72.6)		
Spicule (condylus to distal end)	-	-	14.8±1.7 (12-17)	-	15.5±1.6 (13-18)	-	13.7±1.1 (11.5-15.0)		
Spicule (rostrum to distal end)	-	-	8.9±1.0 (7.5-10.5)	-	9.1±1.0 (7.0-10.5)	-	8.1±0.9 (6.5-9.5)		
Spicule (curved median line)	-	-	13.3±1.4 (11-15)	-	14.1±1.3 (11.5-16.0)	-	12.6±1.2 (10-15)		
Spicule (rostrum to condylus)	-	-	7.2±1.0 (5.5-8.0)	-	7.2±0.9 (6-9)	-	6.4±0.6 (5.5-7.5)		
Distance from single precloacal papilla to cloacal aperture	-	-	3.3±0.8 (2-4)	-	4.0±0.6 (3-5)	-	3.4±0.6 (2.0-4.5)		
Distance from cloacal aperture to first pair of postcloacal papillae	-	-	13.3±0.8 (12.5-14.5)	-	13.2±1.1 (10.5-14.5)	-	12.8±1.6 (10-15)		
Distance from cloacal aperture to second pair of postcloacal papillae	-	-	17.1±1.0 (16.0-18.5)	-	18.2±1.5 (15-21)	-	17.6±1.9 (14-20)		

similar to the one obtained for a German population (Burgermeister *et al.*, 2005).

***Bursaphelenchus teratospicularis* Kakulyia &
Devdariani, 1965**
(Figs 2E & 3E)

Material examined: Twenty females and 15 males of one population (Bte) extracted from wood material collected in Tróia (Setúbal).

Measurements: See Table VIII.

Female: When heat-relaxed, female body becomes slightly ventrally curved. Cuticle with very distinct transverse striation; incisures of lateral field not discernible. Head region wide and flattened in the top, separated from the body by a very weak constriction. Stylet long, well developed with small basal swellings. Large, rounded-rectangular median bulb, *ca* 18 μ m long and 10 μ m diameter. Excretory pore at 1.5-2.0 body diameters behind median bulb, and hemizonid a short distance behind the excretory pore. Vulva oblique, with no vulval flap and vulval lips slightly prominent. Very small post-uterine branch, only extending about 20% of the vulva-anus distance. Tail with U-shaped tip and with anus almost indiscernible.

Male: Anterior part of the body similar to female. Male posterior part of the body adopting a C-shape, strongly curved in the tail. Spicules straight, with a very small pointed rostrum and a long rounded condylus; distal end with no cucullus but with the tip dorsally slightly curved (not always discernible) (Fig. 3E). Male tail with two pairs of caudal papillae: one adanal pair and one post-anal pair just in front of the beginning of the bursa. Male tail ending pointed with a small bursa with the posterior edge curved inwards.

Distribution: Maritime pine wood collected from Castelo Branco, Santarém, Setúbal, Sines, Vila Real and Viseu districts, Portugal.

Remarks: Portuguese population's morphological features and measurements generally agree with the original description (Kakulyia & Devdariani, 1965) although specimens are bigger than the original

population and with a wider range of values, more like the *B. teratospicularis* Italian population (Ambrogioni & Caroppo, 1998). *B. teratospicularis* possesses some distinct features when compared to the other *Bursaphelenchus* species, such as, the different spicule shape, the head region shape and the coarse cuticle annulation.

This species is widely distributed throughout Europe: Georgia (Kakulyia & Devdariani, 1965), Italy (Ambrogioni & Caroppo, 1998), Greece (Skarmoutsos & Skarmoutsos, 1999), Germany (Schönfeld *et al.*, 2001), Cyprus (Braasch & Philis, 2002) and Spain (Escuer *et al.*, 2002). *B. teratospicularis* Portuguese populations were collected from maritime pine (Penas *et al.*, 2004) and some specimens were found in one cocoon-like structure associated with the insect *O. erosus* (Penas *et al.*, 2006a). This *Bursaphelenchus* species is very similar to other nematodes belonging to the genus *Ektaphelenchus*, found in similar cocoon-like structures associated to *O. erosus* in Portugal; the differentiation was based in the bursa presence in the ending of the male tail in case of *Bursaphelenchus* specimens (Penas *et al.*, 2006a). The original population of *B. teratospicularis* was also found associated with an insect of the genus *Orthotomicus* (Kakulyia & Devdariani, 1965). This species was not successfully maintained in fungal cultures.

Bursaphelenchus tusciae
Ambrogioni & Palmisano, 1998
(Figs 1 & 2F)

Material examined: Two populations, Btu₁ and Btu₂, extracted from wood material, collected from Santarém and Tróia (Setúbal), respectively. From Btu₁ two females and six males were measured, and from Btu₂ five females and six males.

Measurements: See Table IX.

Female: When heat-killed become ventrally bent with the tail forming a hook. Cuticle with fine transverse striations; lateral field with three incisures. Lip region high, narrow, with round lips, set off by deep constriction. Stylet with small basal thickenings. Excretory pore located one body diameter behind median bulb, and hemizonid about 0.5 body diameters behind the excretory pore. Very

TABLE VIII. Measurements of *Bursaphelenchus teratospicularis* (in μm and in form: mean \pm SD (range)).

Population	n	Bte - Pegões	
		20 ♀♀	15 ♂♂
Character			
Body length		690.6 \pm 68.0 (597-931)	571.1 \pm 38.4 (497.0-648.0)
a		38.8 \pm 1.5 (34.4-40.9)	35.6 \pm 2.6 (29.2-39.4)
b		12.0 \pm 1.1 (10.3-15.3)	8.2 \pm 0.7 (6.8-9.2)
c		15.6 \pm 1.2 (14.0-18.8)	18.1 \pm 1.6 (13.8-20.7)
c'		3.7 \pm 0.3 (3.2-4.3)	2.3 \pm 0.2 (1.9-2.8)
V		78.8 \pm 0.7 (77.6-79.9)	-
Lip region diam.		7.5 \pm 0.5 (7-9)	7.4 \pm 0.5 (6.5-8.0)
Lip constriction diam.		6.8 \pm 0.3 (6.0-7.5)	6.5 \pm 0.4 (6-7)
Lip region height		3.6 \pm 0.4 (3-4)	3.8 \pm 0.4 (3-4)
Stylet		6.8 \pm 0.3 (6.0-7.5)	18.1 \pm 2.1 (15-22)
Pharynx length		76.2 \pm 4.7 (65-82)	69.7 \pm 4.4 (62-75)
Median bulb length		18.6 \pm 0.9 (17-21)	17.1 \pm 0.7 (16-18)
Median bulb diam.		10.2 \pm 0.7 (9-12)	9.7 \pm 0.7 (8-11)
Body diam. at middle of median bulb		14.1 \pm 0.8 (13.0-16.5)	13.4 \pm 1.0 (12-15)
Body diam. at base of median bulb		14.1 \pm 0.8 (13.0-16.5)	13.6 \pm 0.9 (12-15)
Distance from anterior end to excretory pore		107.4 \pm 10.9 (90-138)	92.5 \pm 5.3 (84-99)
Distance from anterior end to hemizonid		110.0 \pm 9.9 (94-137)	100.5 \pm 5.3 (92.0-108.0)
Distance ant. end - post. end of phar. glands		225.6 \pm 13.5 (202-250)	190.3 \pm 12.5 (159-210)
Body diam. at end of pharyngeal glands		17.4 \pm 1.6 (15-23)	15.7 \pm 0.9 (14-17)
Anterior genital branch		273.3 \pm 53.7 (201-406)	269.3 \pm 25.1 (224-302)
Posterior genital branch		19.8 \pm 2.3 (15-23)	-
Body diam. at vulva		17.8 \pm 1.5 (16.5-23.0)	-
Vulva to anus distance		105.8 \pm 10.7 (90-132)	-
Distance from anterior end to vulva		544.0 \pm 55.1 (477-741)	-
G1 (%)		39.4 \pm 5.7 (31.2-52.1)	-
G2 (%)		2.9 \pm 0.4 (2.4-3.5)	-
Anal/ cloacal body diam.		17.8 \pm 1.5 (16.5-23.0)	13.7 \pm 0.8 (12-15)
Tail		45.3 \pm 5.5 (36-58)	37.1 \pm 2.7 (27-38)
T		-	47.3 \pm 5.0 (40.6-55.5)
Spicule (condylus to distal end)		-	17.7 \pm 0.8 (16.5-19.0)
Spicule (rostrum to distal end)		-	7.9 \pm 0.5 (7-9)
Spicule (curved median line)		-	14.3 \pm 1.2 (13.0-16.5)
Spicule (rostrum to condylus)		-	10.1 \pm 0.5 (9-11)
Distance from single precloacal papilla to cloacal aperture		-	9.1 \pm 1.6 (6.5-13.5)
Distance from cloacal aperture to postcloacal papillae pair		-	12.6 \pm 2.1 (9-15)

small vulval flap formed by a small extension of the anterior lip. Post-uterine branch long, extending about 60% of vulva-anus distance. Female tail very long, terminus usually curved and hook-like with rounded or slightly digitate tip.

Male: Anterior part of the body similar to female. The male body becomes J-shaped when heat relaxed. Spicules straight and long; condylus small, rounded and posteriorly recurved; rostrum prominent and usually pointed; and cucullus absent. Male tail with seven caudal papillae: a single ventral pre-anal papilla, one adanal pair and two post-anal pairs (one

pair at *ca* 33% of tail length from cloaca and another pair at *ca* 54%, just in front of the beginning of bursa). Bursa quadrangular, with posterior edge indented in ventral view.

Distribution: Maritime pine wood collected from Coimbra, Faro, Santarém, Setúbal, Sines and Viseu districts, Portugal.

Remarks: Morphologically, the Portuguese population of *B. tusciae* is very similar to the original description, showing three incisures in the lateral

TABLE IX. Measurements of *Bursaphelenchus tusciae* (in μm and in form: mean \pm SD (range)).

Population	n	Btu ₁ - Santarém		Btu ₂ - Tróia	
		2 ♀♀	6 ♂♂	5 ♀♀	6 ♂♂
Character					
Body length	633, 629	655 \pm 66.4 (594-773)	768 \pm 74.4 (676-871)	724 \pm 47.1 (674-814)	
a	42.2, 40.6	43.9 \pm 3.0 (39.6-48.3)	37.3 \pm 1.1 (35.7-38.7)	39.1 \pm 2.4 (35.4-42.1)	
b	11.3, 12.1	9.0 \pm 0.5 (8.5-9.7)	14.2 \pm 0.8 (13.0-14.8)	9.7 \pm 0.2 (9.4-10.1)	
c	16.7, 20.3	24.3 \pm 1.6 (22.6-26.5)	18.7 \pm 0.8 (16.9-21.9)	24.4 \pm 3.1 (21.5-29.1)	
c'	4.0, 3.4	2.2 \pm 0.1 (2.0-2.3)	4.0 \pm 0.5 (3.4-4.4)	2.3 \pm 0.3 (1.9-2.7)	
V	72.8, 73.8	-	72.5 \pm 0.7 (72.0-75.5)	-	
Lip region diam.	6.5, 6.0	6.2 \pm 0.3 (6.0-6.5)	6.8 \pm 0.4 (6-7)	6.5 \pm 0.4 (6-7)	
Lip constriction diam.	5.5, 5.0	5.4 \pm 0.4 (5-6)	5.8 \pm 0.4 (5-6)	5.5 \pm 0.4 (5-6)	
Lip region height	3.5, 3.0	3.4 \pm 0.2 (3.0-3.5)	3.6 \pm 0.2 (3.5-4.0)	3.7 \pm 0.3 (3.5-4.0)	
Stylet	12.0, 11.5	12.8 \pm 0.8 (12-14)	15.2 \pm 0.8 (14-16)	13.8 \pm 0.8 (13-15)	
Pharynx length	71, 68	72.5 \pm 4.1 (69-80)	74.8 \pm 3.9 (69-79)	74.8 \pm 5.1 (70-84)	
Median bulb length	15, 16	15.5 \pm 1.0 (14-17)	18.2 \pm 1.3 (17-20)	18.7 \pm 2.4 (16-23)	
Median bulb diam.	10, 10	10.2 \pm 0.7 (9-11)	11.7 \pm 0.4 (11.5-12.0)	11.9 \pm 1.3 (10.0-13.5)	
Body diam. at middle of median bulb	13, 12	12.2 \pm 0.8 (11-13)	15.6 \pm 1.1 (14-17)	15.3 \pm 1.4 (13-17)	
Body diam. at base of median bulb	13, 13	12.7 \pm 0.5 (12-13)	16.6 \pm 1.1 (15-18)	15.6 \pm 1.6 (13-18)	
Distance anterior end to excretory pore	83, -	84.2 \pm 5.1 (79-94)	88.8 \pm 4.1 (85-94)	90.3 \pm 5.8 (84-99)	
Distance from anterior end to hemizonid	93, 90	93.5 \pm 6.9 (88-107)	100.4 \pm 5.9 (95-110)	98.7 \pm 5.8 (95-110)	
Distance from anterior end to posterior end of pharyngeal glands	126, 127	134.7 \pm 10.7 (121-149)	142.0 \pm 16.3 (117-155)	144.0 \pm 12.1 (130-161)	
Body diam. at end of pharyngeal glands	14, 13	13.8 \pm 0.4 (13-14)	18.9 \pm 1.1 (17.5-20.0)	17.9 \pm 1.7 (14.5-19.0)	
Anterior genital branch	257, 224	134.7 \pm 10.7 (121-149)	336.8 \pm 42.9 (282-401)	309.2 \pm 60.6 (235-420)	
Posterior genital branch	83, 86	-	100.5 \pm 23.9 (76-122)	-	
Body diam. at vulva	16, 15	-	20.2 \pm 1.7 (18.5-22.0)	-	
Vulva to anus distance	132, 133	-	165 \pm 14.1 (144-183)	-	
Distance from anterior end to vulva	461, 464	-	561.4 \pm 60.4 (490-636)	-	
G1 (%)	40.6, 35.6	-	42.4 \pm 7.2 (34.9-53.5)	-	
G2(%)	13.1, 13.7	-	13.0 \pm 3.4 (9.6-16.3)	-	
Anal/ cloacal body diam.	9.5, 9.0	12.2 \pm 0.8 (11-13)	10.7 \pm 0.4 (10-11)	13.1 \pm 1.1 (12-15)	
Tail	38, 31	26.9 \pm 1.8 (25-30)	39.6 \pm 5.0 (35-48)	29.9 \pm 3.0 (24.5-33.0)	
T	-	55.8 \pm 3.0 (51.2-59.9)	-	42.9 \pm 9.1 (32.6-59.0)	
Spicule (condylus to distal end)	-	12.0 \pm 1.2 (10.0-13.5)	-	19.4 \pm 1.1 (18-21)	
Spicule (rostrum to distal end)	-	18.7 \pm 1.5 (16-20)	-	12.8 \pm 0.5 (12.0-13.5)	
Spicule (curved median line)	-	18.2 \pm 1.8 (16-20)	-	17.8 \pm 1.4 (16.0-19.5)	
Spicule (rostrum to condylus)	-	7.4 \pm 0.6 (7.0-8.5)	-	7.6 \pm 0.9 (6.5-9.0)	
Distance from single precloacal papilla to cloacal aperture	-	3.3 \pm 0.4 (3-4)	-	3.3 \pm 0.8 (2.5-4.5)	
Distance from cloacal aperture to first pair of postcloacal papillae	-	8.9 \pm 1.9 (6-11)	-	10.0 \pm 2.6 (6.5-13.5)	
Distance from cloacal aperture to second pair of postcloacal papillae	-	15.3 \pm 1.5 (13.0-16.5)	-	16.2 \pm 1.8 (13.5-18.0)	

field, the same excretory pore position, seven caudal papillae with similar arrangement, identical spicule shape, and the typical female tail. Measurements of both populations show that the original Italian population is bigger than the Portuguese.

B. tusciae was only found in Italy (Ambrogioni & Palmisano, 1998), associated with dead wood of *P. pinea*; and in Germany in wood of *P. sylvestris* (Schönfeld *et al.*, 2001). In Portugal, this species

was found for the first time in maritime pine and associated with the insect *H. ligniperda* (Penas *et al.*, 2004, 2006a). *B. tusciae* is not a frequent nematode species, but it was reported in different regions throughout Portugal (Penas *et al.*, 2004, 2006a). This species was maintained in non sporulating *B. cinerea* and *M. fructicola* for a short time. *Bursaphelenchus tusciae* ITS-RFLP pattern obtained (Fig. 1) is similar to that published for an Italian population (Burgermeister *et al.*, 2005).

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