Annals of Applied Biology ISSN 0003-4746

RESEARCH ARTICLE

Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) and other nematode genera associated with insects from *Pinus pinaster* in Portugal

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Keywords
Bursaphelenchus spp.; insects; *Pinus pinaster*; Portugal; vectors.

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Received: 30 June 2005; revised version accepted: 23 January 2006.

doi:10.1111/j.1744-7348.2006.00042.x

Abstract

Insects associated with maritime pine, *Pinus pinaster*, in Portugal were collected and screened for the presence of *Bursaphelenchus* species. Nematodes were identified using Internal Transcribed Spacers-Restriction Fragment Length Polymorphism (ITS-RFLP) analysis of dauer juveniles and morphological identification of adults that developed from dauer juveniles on fungal cultures or on cultures in pine wood segments at 26°C. Several associations are described: *Bursaphelenchus teratospicularis* and *Bursaphelenchus sexdentati* are associated with *Orthotomicus erosus*; *Bursaphelenchus tusciae*, *B. sexdentati* and/or *Bursaphelenchus pinophilus* with *Hylurgus ligniperda* and *Bursaphelenchus hellenicus* with *Tomicus piniperda*, *Ips sexdentatus* and *H. ligniperda*. An unidentified *Bursaphelenchus* species is vectored by *Hylobius* sp. The previously reported association of *Bursaphelenchus xylophilus* with *Monochamus galloprovincialis* was confirmed. The association of *Bursaphelenchus leoni* with *Pityogenes* sp. is not definitively established and needs further studies for clarification. Other nematode genera besides *Bursaphelenchus* were found to be associated with the insects sampled, including two different species of *Ektaphelenchus*, *Parasitorhabditis* sp., *Parasitaphelenchus* sp., *Contortylenchus* sp. and other unidentified nematodes. The *Ektaphelenchus* species found in *O. erosus* is morphologically similar to *B. teratospicularis* found in the same insect; adults of both the species are found in cocoon-like structures under the elytra of the insects.

Introduction

Approximately one third of the nematodes belonging to the order Aphelenchida Siddiqi, 1980 are associated with insects (Poinar, 1983). These nematodes establish a variety of associations with the insects, which may be described as commensalism, e.g. phoresy (to the benefit of the nematode but not affecting the insect), mutualism (both the organisms benefit) or parasitism (nematodes benefit at the expense of the insect) (Giblin-Davis, 2004).

Most *Bursaphelenchus* Fuchs, 1937 species are mycetophagous, feeding on fungi in the galleries of bark beetles and thus benefit from being transported by the insects from an area of diminished resources to a more favourable locality (phoresy) (Giblin, 1985). However, *Bursaphelenchus xylophilus* (Steiner & Bührer, 1934) Nickle, 1970 (the causal agent of pine wilt disease) and *Bursaphelenchus cocophilus* (Cobb, 1919) Baujard, 1989 (the causal agent of red ring disease of coconut) are, potentially, cases of mutualism between nematode and insect populations (Giblin-Davis, 2004). *Bursaphelenchus xylophilus* is transported from host to host by their vectors, *Monochamus* spp. (for a complete review on this subject, see Ryss *et al.*, 2005) and *B. cocophilus* by *Rhynchophorus palmarum* (Dean, 1979; Griffith, 1987), damaging their host plants and therefore creating new breeding sites suitable for vector reproduction and proliferation

(Mamiya, 1983; Giblin, 1985; Giblin-Davis, 1993). These two nematode species have developed the ability to parasitise plants, being capable of feeding on plant host epithelial cells and probably evolving from phoretically transmitted mycetophagous ancestors (Giblin-Davis *et al.*, 2003).

Although not all associations between *Bursaphelenchus* species and insects are known, most of the species are vectored by insects belonging to the families Scolytidae, Cerambycidae and Curculionidae (Ryss *et al.*, 2005).

To survive environmental stresses during transportation (e.g. starvation and/or desiccation), *Bursaphelenchus* spp., like other nematodes, have a specialised dispersal juvenile form, called the dauer juvenile. The dauer juvenile is morphologically and physiologically distinct from the other juvenile stages and is resistant to adverse conditions, allowing the nematode to invade and survive in the insect vector until it reaches a new host plant (Fuchs, 1915; Poinar, 1983). Dauer juveniles have a dome-shaped head, a vestigial stylet (or may lack this structure), a degenerate oesophagus and oesophageal glands, a poorly delimited median bulb and a subcylindrical tail with digitate terminus. These morphological features, coupled with the fact that the body is filled with stored lipids, allow easy differentiation of the dauer stage (Hunt, 1993). Dauer juveniles can be the third juvenile stage in some species (e.g. *Bursaphelenchus seani*, *Bursaphelenchus kevini*, *B. cocophilus*) (Giblin & Kaya, 1983; Giblin *et al.*; 1984, Gerber *et al.*, 1989) or the fourth stage juvenile in others (e.g. *B. xylophilus*, *Bursaphelenchus mucronatus*, *Bursaphelenchus conicaudatus*) (Mamiya, 1975; Mamiya & Enda, 1979; Kanzaki & Futai, 2001).

Bursaphelenchus spp. can be ectophoretic, carried from tree to tree on the insect body, in the tracheae or beneath the wings or elytra of the beetle vector, or may be endophoretic and carried in the reproductive tract or haemocoel of the vector. For example, *Bursaphelenchus abietinus* was found under the wings of *Pityokteines spinidens*, *P. curvidens* and *P. vorontzowi* (Braasch & Schmutzenhofer, 2000), and *Bursaphelenchus hylobianum* in the haemocoel of *Hylobius albosparsus* (Korenchenko, 1980).

Since the first report in Portugal and in Europe of the quarantine pathogen *B. xylophilus* (Mota *et al.*, 1999), intensive annual surveys under a National Programme of Pinewood Nematode Control [Programa Nacional de Luta contra o Nemátode do Pinheiro (PROLUNP)—http://www.dgf.min-agricultura.pt/prolunp/html/home-final.htm] have been carried out to monitor the spread of *B. xylophilus* and to determine its dispersal and its vectors. *B. xylophilus* is confined to the Setúbal peninsula and the surrounding areas—the affected zone, and *Monochamus galloprovincialis* Olivier was found to be its vector (Sousa *et al.*, 2001). Besides *B. xylophilus*, other *Bursaphelenchus* species in Portugal have been found to be associated with *Pinus pinaster* Aiton (Sousa *et al.*, 2002; Penas *et al.*, 2004).

The purpose of this study was to identify the insect vectors of *Bursaphelenchus* spp. associated with *P. pinaster* in Portugal and to identify other nematode genera that are associated with the vectors. This will help determine whether these *Bursaphelenchus* species represent a potential risk to the forests.

Materials and methods

Insect sampling and nematode isolation

Bark- and wood-boring insects belonging mainly to the families Cerambycidae, Scolytidae, Buprestidae and Curculionidae (Coleoptera) were captured between 1999 and 2004 from six different localities in Portugal (Fig. 1) and were screened for the presence of *Bursaphelenchus* species.

Three methods were used to obtain the adult insects. The first method consisted of collecting the insects from *P. pinaster* trees displaying symptoms of decline (between 1999 and 2004), mainly from the affected zone and also from the north-western Portugal. Pine trees were cut and divided into logs. Some of the logs were debarked and all the adult insects were collected, while the remaining logs were kept in boxes at room temperature to allow insect emergence. Because of the presence of *B. xylophilus*, a more intensive survey was required in the area affected by this species and two additional methods were used for insect collection: trap trees and flight traps. Trap trees (2003–04) consisted of healthy trees that were felled and divided into logs, sprayed with 70% ethanol to attract insects and left for 1 month in the field. After 1 month, some of the logs were caged and left at room temperature to allow insect emergence, while the remaining were debarked to collect the insects. Flight traps (2003–04) were installed on *P. pinaster* trees and baited with terpentine and ethanol.

All the adult insects collected were identified in the laboratory to species or genus level. To determine the presence of nematodes, the insects were placed individually in a Syracuse dish in a small amount of water. First, the elytra and the wings were opened and observed and then the insect was crushed and left in water for few hours at room temperature. All the nematodes resembling *Bursaphelenchus* dauer juveniles were collected for species identification.

Molecular identification

Whenever possible, dauer juvenile identification was made using ITS-RFLP analysis as morphological and biometrical identification of these juvenile forms to the species level is extremely challenging.

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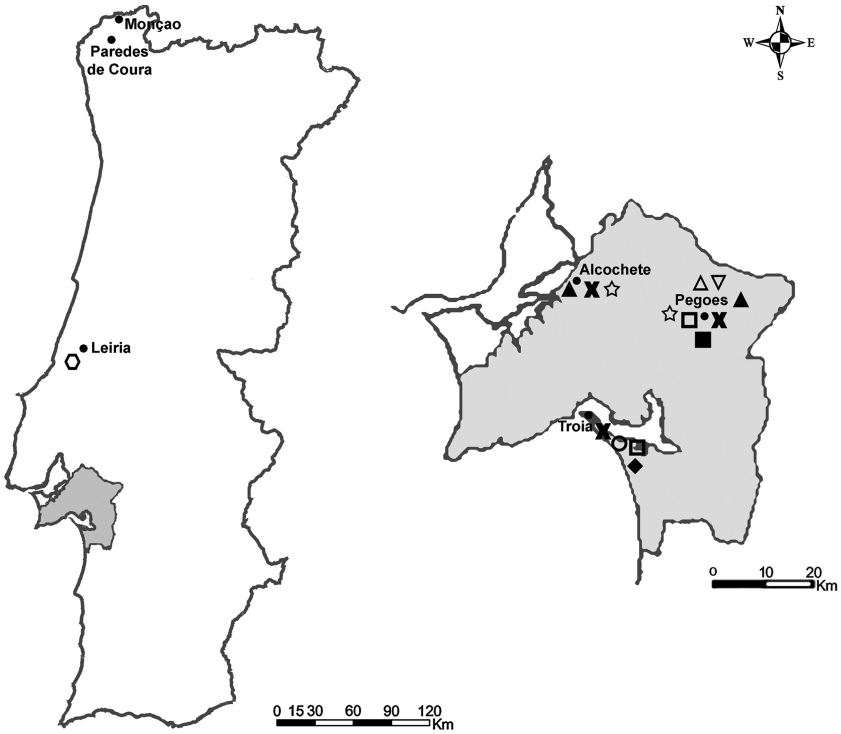

Figure 1 Sampling sites (●) and distribution of *Bursaphelenchus*–insect associations. ▭: region affected by *B. xylophilus*. Associations: *Bursaphelenchus* sp.–*Hylobius* sp. (⟨⟩); *B. xylophilus–M. galloprovincialis* (**x**); *B. teratospicularis–O. erosus* (◯); *B. sexdentati–O. erosus* (▭); *B. sexdentati*/*B. pinophilus–H. ligniperda* (■); *B. hellenicus–T. piniperda* (△); *B. hellenicus–I. sexdentatus* (▽); *B. hellenicus–H. ligniperda* (▲); *B. tusciae–H. ligniperda* (☆) and *B. leoni–H. ligniperda* (◆).

ITS-RFLP identification of dauer juveniles was made using at least five nematodes collected from the same insect. The procedure used for DNA extraction and PCR was as described in the study by Penas *et al.* (2004). Amplification of Internal Transcribed Spacer (ITS) regions of rDNA was performed using primers F194 and P5368 as described by Ferris *et al.* (1993) and Vrain (1993), respectively. Following PCR, 5 µL of the amplified sample was analysed in a 1% agarose gel and DNA fragments were visualised by staining in 1 µg mL^{-1} of ethidium bromide. 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, *Hin*fI and *Alu*I) (Amersham BioSiences®, Uppsala, Sweden) following the manufacturer's instructions. These five restriction enzymes are known to generate species-specific ITS-RFLP profiles (Burgermeister *et al.*, 2005). The restriction fragments were resolved by electrophoresis in a 2% agarose gel, stained with ethidium bromide and analysed as described above.

Morphological identification

For species morphological identification, three different methods were used to obtain adults from the collected dauer juveniles: inoculation and incubation of juveniles in branches of *P. pinaster* and/or in fungal cultures (*Monilinia fructicola* or nonsporulating *Botrytis cinerea*) and incubation of dauer juveniles in water. For inoculation of dauer juveniles on wood, *P. pinaster* branches were cut into segments approximately 13 cm in length and 3–4 cm in diameter and sealed at both ends with paraffin. The juveniles were suspended in 0.2 mL of water and inoculated with a syringe into an orifice made in the middle of the wood segment. The orifices were sealed with paraffin and the segments were enclosed in the sealed plastic bags and stored at 26 ± 2°C for 4 weeks. Some segments of the same branches were kept under the same conditions, without inoculation and used as control to ensure absence of previous infestation with *Bursaphelenchus* spp.. After this period, the segments were cut into 3-mm discs and the nematodes were extracted using the Baermann funnel technique for 48 h. The second method to allow the dauer juveniles to develop into adult stage used fungal cultures. The juveniles were suspended in 0.1 mL of water, inoculated with a micropipette into a Petri dish containing a fungal culture and incubated at 26°C for 3 weeks. The nematodes were extracted using a modified Baermann funnel

technique for 24 h. For the last method, the dauer juveniles were maintained in water at 26°C and their development was observed daily. This last method allowed the developmental stage of dauer juveniles (third or fourth) to be determined by observation of the developmental stage obtained after the first molt. The adult nematodes obtained from all methods were identified in temporary mounts using an Olympus BX51 (Hamburg, Germany) light microscope and scanning electron microscope (SEM) observations. For SEM studies, the nematodes were prepared as described by Eisenback (1985) and observed in a JEOL 35 SEM. Several characteristics were used for *Bursaphelenchus* species identification including spicule shape, vulval region, number and disposition of caudal papillae, female tail and number of lateral lines. The identification method used depended on the abundance and condition of the juveniles obtained from each insect.

Other nematode genera were mounted on temporary slides, observed and identified under the light microscope.

Results

Bursaphelenchus spp.—insect associations

A total of 3294 insects belonging to 22 different species were collected and screened for the presence of *Bursaphelenchus* nematodes during this survey (Table 1); 2400 insects emerged from symptomatic *P. pinaster* trees, 668

Table 1 Insects screened for the presence of *Bursaphelenchus* species between 1999 and 2004

Family/Species	Observed Insects (*n*)	Insects with *Bursaphelenchus* Dauer Juveniles (%)	*Bursaphelenchus* Species Associated
Coleoptera			
Cerambycidae			
Monochamus galloprovincialis[a]	541	20	*Bursaphelenchus xylophilus*
Arhopalus ferus	26	0	—
Arhopalus syriacus	80	0	—
Pogonocherus perroudi	55	0	—
Acanthocinus griseus	78	0	—
Spondylis buprestoides	13	0	—
Rhagium inquisitor	3	0	—
Ergates faber	1	0	—
Scolytidae			
Orthotomicus erosus	899	19	*Bursaphelenchus teratospicularis* *Bursaphelenchus sexdentati*
Tomicus piniperda	168	24	*Bursaphelenchus hellenicus*
Ips sexdentatus	300	19	*B. hellenicus*
Hylurgus ligniperda	557	5	*Bursaphelenchus tusciae* *B. hellenicus* *Bursaphelenchus sexdentati* and/or *Bursaphelenchus pinophilus*[b]
Pityogenes sp.	175	2	*Bursaphelenchus leoni*[c]
Hylastes sp.	34	0	—
Buprestidae			
Crysobothris solieri	50	0	
Calcophora mariana	11	0	—
Phaenops cyanea	3	0	—
Curculionidae			
Pissodes castaneus	177	2	—
Eremotes porcatus	50	2	—
Hylobius sp.	62	14	*Bursaphelenchus* sp.
Elateridae			
Unidentified sp.	10	0	—
Hymenoptera			
Siricidae			
Sirex noctilio	1	0	—
Total	3294	13	—

[a]Only collected from the affected zone.
[b]Only differentiated by molecular methods.
[c]Association not definitively established.

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insects were obtained from trap trees and 226 insects were captured in flight traps. Most of the *Bursaphelenchus* spp. were found to be associated with insects of the family Scolytidae. Nine insect species were found carrying *Bursaphelenchus* dauer juveniles (Table 1; Fig. 1).

Bursaphelenchus xylophilus was only found associated with *M. galloprovincialis*. No other nematodes were found in the *M. galloprovincialis* specimens observed. *Bursaphelenchus teratospicularis* Kakuliya & Devdariani, 1965 and *Bursaphelenchus sexdentati* Rühm, 1960 were found associated with *Orthotomicus erosus* Wollaston (Figs 2 and 3); *B. sexdentati* and/or *Bursaphelenchus pinophilus* Brzeski & Baujard, 1997 with *Hylurgus ligniperda* F.; an unidentified *Bursaphelenchus* sp. with *Hylobius* sp. (Fig. 4); *Bursaphelenchus tusciae* Ambrogioni & Palmisano, 1998 with *H. ligniperda* and *Bursaphelenchus hellenicus* Braasch & Michalopoulou, 1998 with *Tomicus piniperda* L., *Ips sexdentatus* Boern and *H. ligniperda* (Fig. 5). *Bursaphelenchus leoni* Baujard, 1980 was only found once, a single male in the sawdust attached to one *Pityogenes* sp.

Dauer location in the insects

With the exception of *B. xylophilus,* which was found packed in the tracheae of its vector, and *Bursaphelenchus* sp., that was found within the insect body, all other *Bursaphelenchus* dauer juveniles were found under the elytra and wings, between the folds of the insect body and on the insect's hairs (Fig. 6).

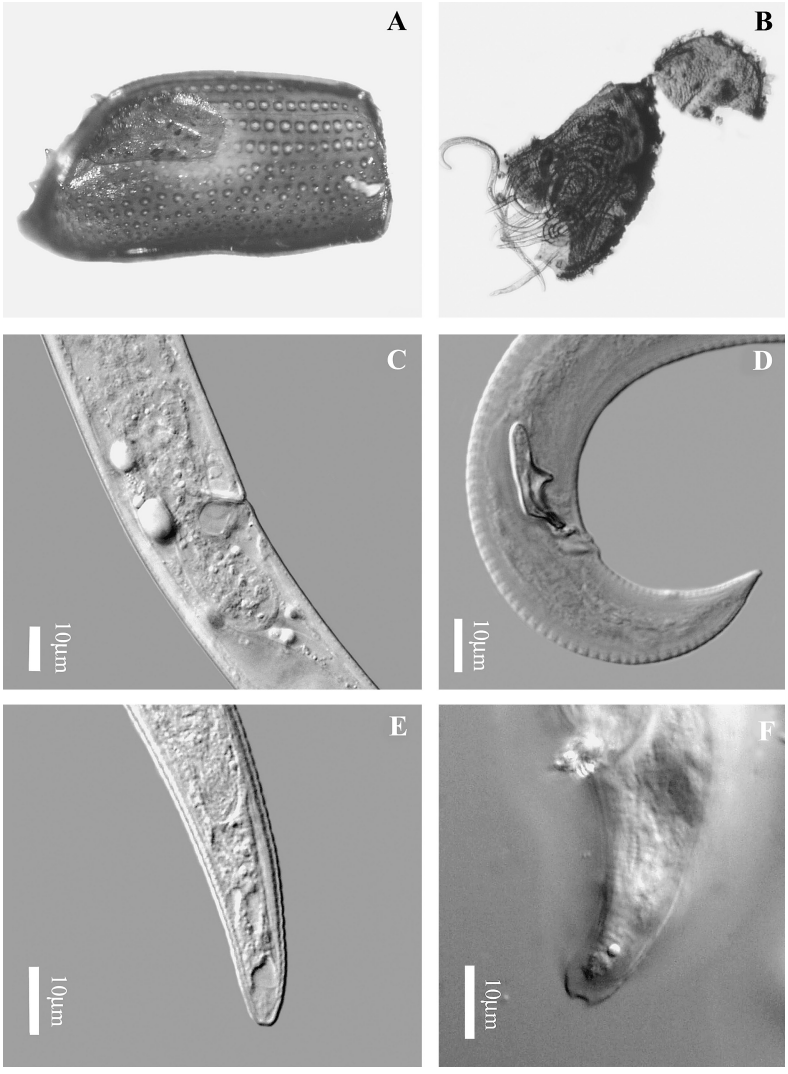

Figure 2 Adults of *Bursaphelenchus teratospicularis* associated with *Orthotomicus erosus*. (a) light micrograph (LM) of cocoon under the elytra, (b) cocoon with adults inside (LM), (c) vulval region (LM), (d) male tail (LM), (e) female tail (LM), (f) bursa on male tail tip (LM).

Dauer description and abundance

Bursaphelenchus spp. dauer juveniles found associated with these insects were morphologically very similar, with a dome-shaped head, an indistinct stylet, a poorly defined median bulb and anterior lip region and an elongated conoid tail, with the terminus mucronate, digitate or pointed. With the exception of *B. xylophilus*, which had a body length of 500–600 μm, all the collected species had a similar body length (400–500 μm). These juveniles displayed slow movements and survived for a few days in water.

The number of *Bursaphelenchus* dauer juveniles was low in most cases, usually between 10–100 per insect, only exceptionally reaching some hundreds of nematodes. However, *B. xylophilus* was occasionally found in high numbers (thousands) in the tracheal system of its vector.

Molecular identification

ITS-RFLP analysis of the dauer juveniles found in *O. erosus* originated a restriction pattern similar to *B. sexdentati*, after comparison with reference patterns established by Burgermeister *et al.* (2005) (Fig. 3). Dauer juveniles collected from other *O. erosus*, *H. ligniperda* and *I. sexdentatus* specimens were analysed using this method, but no successful results were obtained.

Morphological identification

With the exception of the *B. leoni–Pityogenes* sp. association, the diagnosis of the remaining associations was made based on the morphological identification of the adults obtained from successful inoculations of dauer juveniles on segments of pine branches or on the fungal cultures. No *Bursaphelenchus* species were collected from the pine branches used as controls.

Dauer juvenile development in water was successfully observed for *B. xylophilus* and *B. hellenicus*. *B. xylophilus* dauer juveniles molted directly to the adult stage and dauer of *B. hellenicus* molted first to the fourth juvenile stage (propagative form) and only later to the adult stage. In the case of *B. xylophilus*, about 25–50% of the dauer juveniles developed to the adult stage (females and males) after 72 h at 26°C. *B. hellenicus* dauer juveniles molted to the fourth juvenile stage after 120 h, and subsequently, very few reached the adult stages after 240 h (Fig. 5). In two *H. ligniperda* individuals, adults of *B. hellenicus* were found mixed with the dauer juveniles.

Adults of *B. teratospicularis* were found in one cocoon-like structure beneath the elytra of one *O. erosus*; this cocoon contained 25 adults comprised of 3 males and 22 females.

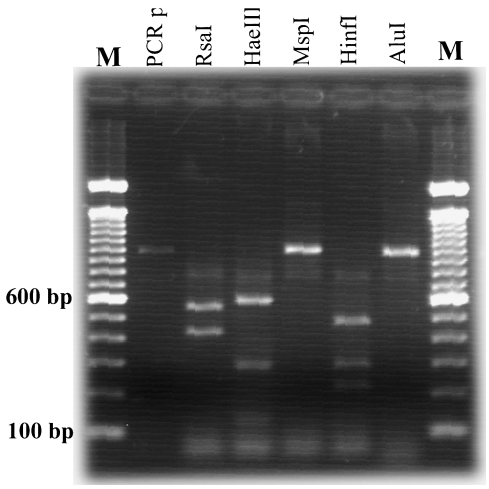

Figure 3 ITS-RFLP pattern of dauer juveniles of *Bursaphelenchus sexdentati* associated with *Orthotomicus erosus*.

In addition to the nematodes described above, *Bursaphelenchus* dauer juveniles were collected from *O. erosus*, *T. piniperdae*, *I. sexdentatus*, *H. ligniperda*, *Pityogenes* sp., *Pissodes castaneus* and *Eremotes porcatus*, which could not be identified.

Other nematode genera associations with insects

Other nematode genera were found associated with 9 of the 22 insect species observed (Table 2; Fig. 7). *O. erosus* and *H. ligniperda* carried two different *Ektaphelenchus*

Table 2 Other nematode genera associated with the 3294 insects observed

Nematode Genus	Insect Infested with Nematodes (%)
Ektaphelenchus sp.A	*Orthotomicus erosus* (3)
Ektaphelenchus sp.B	*Hylurgus ligniperda* (7)
Parasitaphelenchus spp.	*Ips sexdentatus* (23)
	H. ligniperda (5)
Parasitorhabditis spp.	*O. erosus* (20)
	Tomicus piniperda (1)
	I. sexdentatus (33)
	H. ligniperda (10)
Contorthylenchus spp.	*O. erosus* (22)
	I. sexdentatus (9)
	H. ligniperda (5)
Unidentified	*O. erosus* (16)
	T. piniperda (9)
	I. Sexdentatus (42)
	H. ligniperda (38)
	Pissodes castaneus (6)
	Eremotes porcatus (10)
	Hylobius sp. (26)

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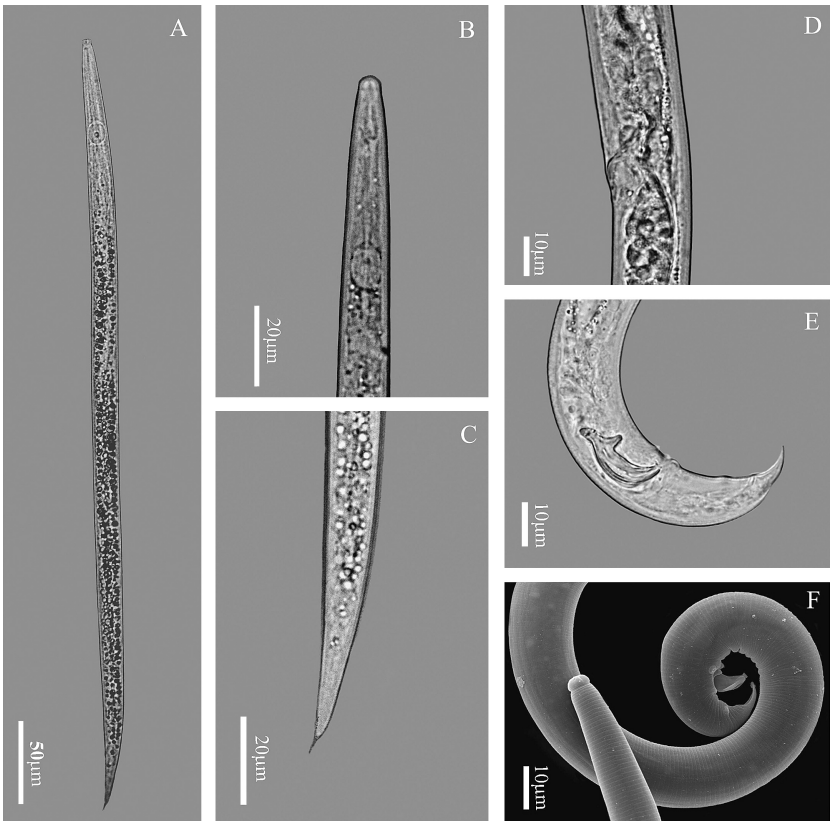

Figure 4 *Bursaphelenchus* sp. associated with *Hylobius* sp. Dauer juveniles isolated from the insect (a–c). (a) light micrograph (LM) of dauer juvenile, (b) anterior region (LM), (c) tail (LM). Adults obtained from pine branches inoculated with dauer juveniles (d–f), (d) vulval region (LM), (e) male tail (LM), (f) scanning electron micrograph of male.

species in cocoon-like structures under the elytra. In both cases, only immature, inseminated females were found, varying in numbers from 10 to 50 per insect (Fig. 7a–7d). Juveniles of the genus *Parasitorhabditis* (Fig. 7g), internal parasites of bark beetles (Massey, 1974), were found in the gut of *O. erosus*, *T. piniperdae*, *I. sexdentatus* and *H. ligniperda*; these juveniles moved quickly and constantly in water and are characterised by their distinct cuticle annulations in the anterior region, very slender body and an elongated conoid tail that was pointed to the extent that it was almost filiform. Other juveniles, of the genus *Parasitaphelenchus* (Fig. 7e), were found in the haemocoel of *I. sexdentatus* and *H. ligniperda*. These juveniles appeared in low numbers (<100) and were slender and of medium size, with the body narrowing sharply towards the head and less sharply towards the tail. The lip region of these nematodes was rounded, continuous with the body contour and had a typical hook projection, while the tail was rounded, narrowing and had a short sharp mucron. *O. erosus*, *I. sexdentatus* and *H. ligniperda* were found associated with nematodes of the genus *Contortylenchus* (Fig. 7f). Hundreds of eggs and juveniles of this genus were present in the body haemocoel, and several females (>10 per beetle) were seen to occur together in the insect body. No males were recovered from the body cavity of the insects. Juveniles of different sizes were present, which had a very thin cuticle. These juveniles collapsed rapidly when removed from the insect to the water as a result of the differential pressure. Other nematode genera were found associated with the collected insects, but their identification was not possible (Fig. 7h and Fig. 7i).

Discussion

This study corroborates preliminary results for Portugal (Sousa *et al.*, 2002) and also confirms that one insect species can vector several *Bursaphelenchus* species (e.g. *O. erosus* and *H. ligniperda*; Table 1). Likewise, it was shown that the same *Bursaphelenchus* species can have different insect vectors (e.g. *B. hellenicus*; Table 1), confirming previous observations for this genus (Braasch, 2001; Ryss *et al.*, 2005). This suggests a nonspecialised relationship between *Bursaphelenchus* spp. and their

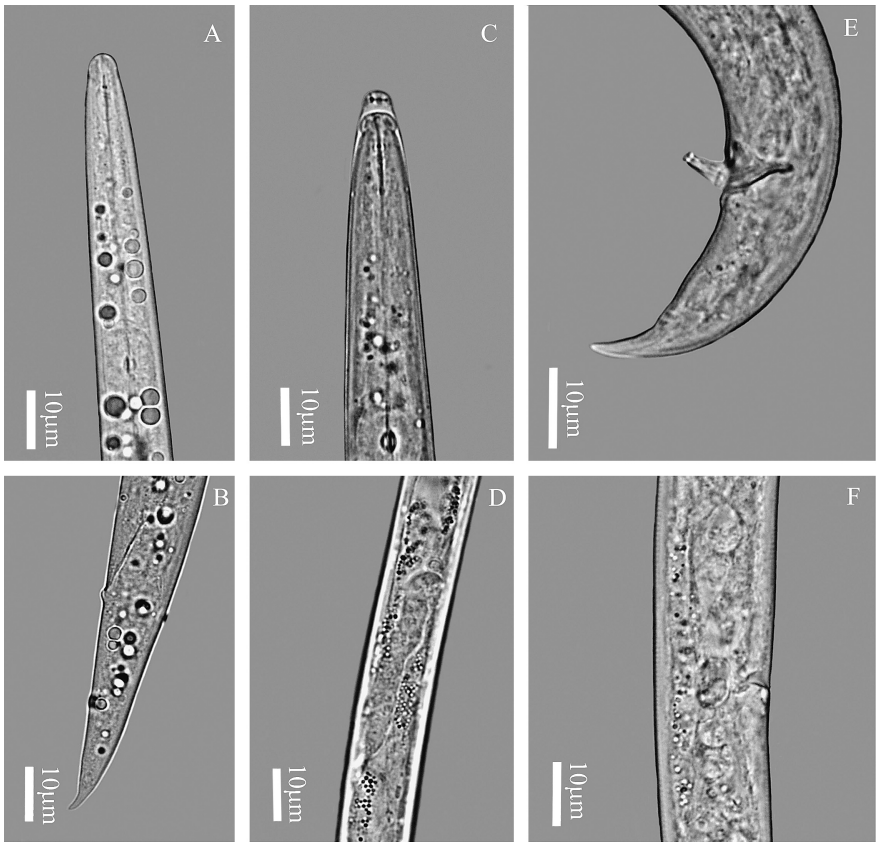

Figure 5 *Bursaphelenchus hellenicus* associated with *Hylurgus ligniperda*. (a) light micrograph (LM) of dauer juvenile anterior region, (b) tail of dauer juvenile (LM), (c) molt of dauer juvenile to fourth propagative juvenile (LM), (d) gonad of fourth juvenile stage (LM), (e) male tail (LM), (f) vulval region (LM).

vectors. However, in Portugal, *B. xylophilus* may have a close and specialised relationship with *M. galloprovincialis* as this nematode species was not found in any other insect and *M. galloprovincialis* was not associated with any nematode species other than *B. xylophilus*. An association between *B. mucronatus* and *M. galloprovincialis* has been reported in several European countries (Magnusson & Schroeder, 1989; Tomminen *et al.*, 1989; Palmisano *et al.*, 1992; Braasch *et al.*, 1999). However, this association was not found in Portugal, despite the high number of *Monochamus* observed. This may be because that the observed *Monochamus* originated from the affected zone where *B. xylophilus* occurrence may have suppressed *B. mucronatus* dispersion. Moreover, even

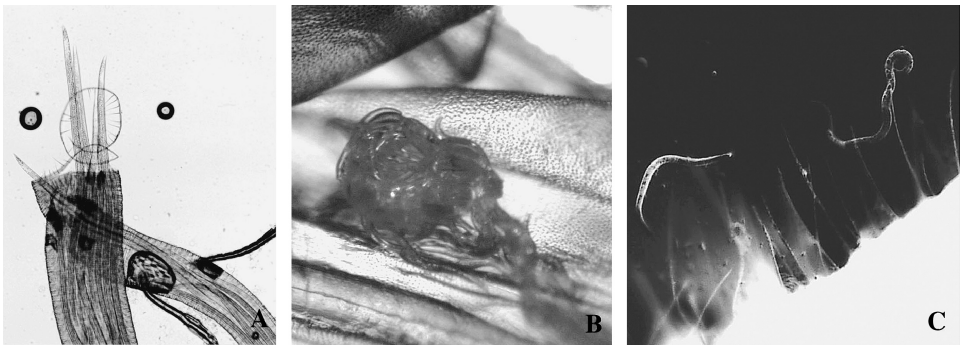

Figure 6 (a) *Bursaphelenchus xylophilus* inside the tracheae of *Monochamus galloprovincialis*, (b) *Bursaphelenchus* dauer juveniles on *Ips sexdentatus* wings, (c) *Bursaphelenchus* dauer juveniles on *Ips sexdentatus* hair.

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outwith the affected zone, *B. mucronatus* does not occur frequently, being only found in one sample (Penas *et al.*, 2004). Curiously, one of the species, *Bursaphelenchus* sp.1 (Penas *et al.*, 2004), most frequently found in maritime pine in Portugal in a previous study was not found in this study. It is possible that this species is vectorised by an unsurveyed insect belonging to a distinct family of xylophagous Coleoptera, like Bostrychidae or Anobiidae, or even by insects from a different order, as some *Bursaphelenchus* spp. are associated with Hymenoptera and Lepidoptera (Ryss *et al.*, 2005).

Some of the associations described have been previously reported in other countries. The association between *B. teratospicularis* and insects belonging to the genus *Orthotomicus* has been previously reported (Kakuliya & Devdariani, 1965); *B. hellenicus* has formerly been associated with *T. piniperda* (Braasch *et al.*, 2000). The following phoretic associations: *B. hellenicus–I. sexdentatus*, *B. hellenicus–H. ligniperda*, *B. sexdentati–O. erosus*, *B. sexdentati–H. ligniperda* and *B. tusciae–H. ligniperda* have never been previously reported. *Bursaphelenchus* sp., which is associated with *Hylobius* sp., was previously identified as *B. hylobianum* Korenchenko, 1980 (Penas *et al.*, 2004), but further molecular and biological studies contradict this identification.

The presence of the adults of *B. hellenicus* found in two *H. ligniperda* specimens together with the dauer juveniles of the same species might be explained by the high temperature and moisture that the insects were exposed to during transport from the field to the laboratory.

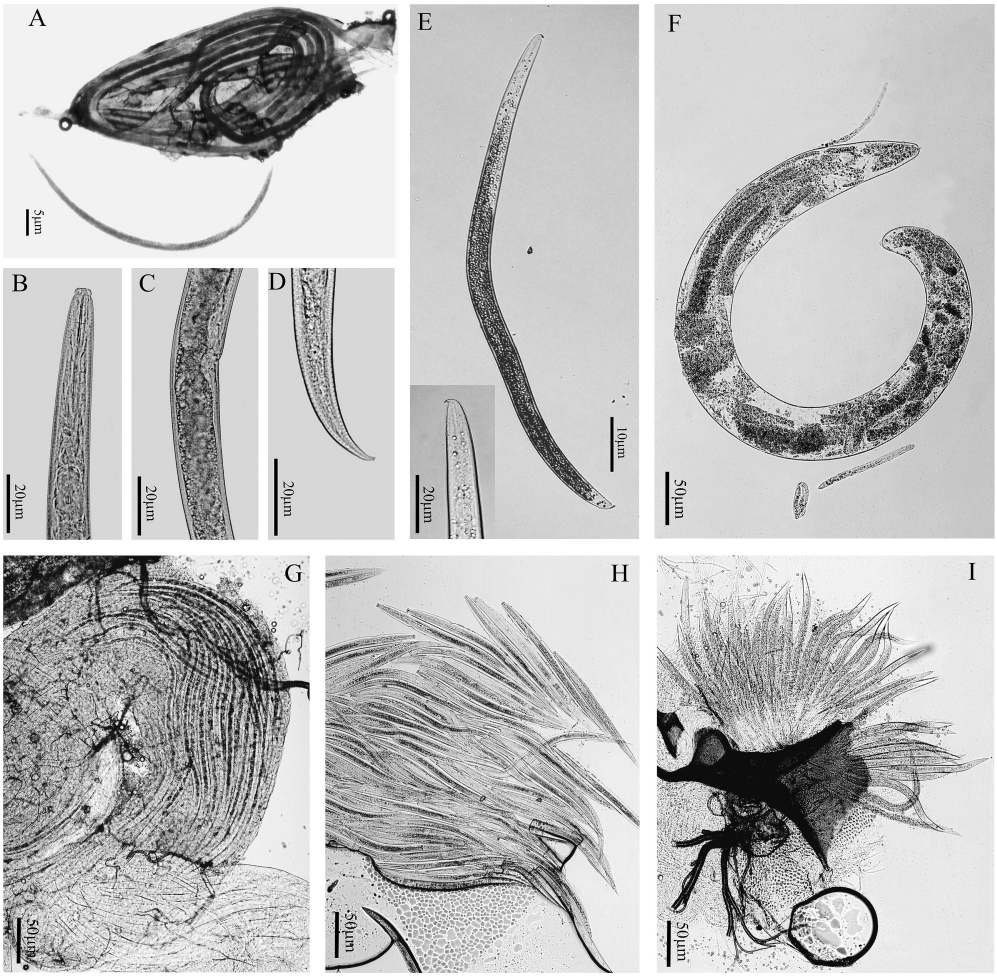

Figure 7 Other nematodes genera associated with the insects observed. *Ektaphelenchus* sp.2 females associated with *Hylurgus ligniperda* (a–d). (a) light micrograph (LM) of cocoon with females inside, (b) anterior region (LM), (c) vulval region (LM), (d) female tail (LM), (e) *Parasitaphelenchus* sp. (LM); inset: head (LM), (f) *Contortylenchus* sp. (LM), (g) *Parasitorhabditis* sp. within insect gut (LM), (h, i) unidentified nematode genera (LM).

However, we have shown that two moults are required for this nematode to reach the adult stage and that the dauer juvenile stage is the third stage.

The hypothesis that *Pityogenes* sp. is a vector of *B. leoni* requires confirmation because only one male was observed in the sawdust attached to the insect. *B. leoni* is difficult to multiply and to maintain under controlled conditions, and even if *B. leoni* dauer juveniles were obtained, it would be difficult to obtain adults for identification. This nematode may therefore be underrepresented in our analysis.

Given this, and similar problems in obtaining adult nematodes from dauer juveniles, we aimed to use molecular methods for identification from dauer stages wherever possible. However, ITS-RFLP patterns of dauer juveniles were difficult to obtain. It is possible that the high lipid content of this survival stage made obtaining DNA from the few nematodes available in a form suitable for PCR amplification problematic.

Bursaphelenchus teratospicularis may have an affinity with nematodes belonging to the genus *Ektaphelenchus*. Adults of this species were found in a cocoon-like structure under the elytra of *O. erosus*. Some *Ektaphelenchus* species have been reported as being transported in similar structures under the insect elytra (Thorne, 1935; Rühm, 1956; Massey, 1974). Morphologically, *B. teratospicularis* shares many characteristics with *Ektaphelenchus*. Both are characterised by a slender, medium-sized, ventrally arcuate body; a coarsely annulated cuticle; flattened cephalic region; wide and distinctly offset stylet with a long wide lumen; cylindrical procorpus joining a large, prominent, rounded-rectangular median bulb; vulva with lips not protuberant; intestine ending in a blind sac and an anus that is very difficult to discern (Hunt, 1993). Furthermore, *Ektaphelenchus* cocoons are described as usually containing only immature females and sporadically males (Massey, 1974). Of the *B. teratospicularis* adults found in *O. erosus*, 22 were females and 3 males. The presence of a terminal bursa on the male tail tip of these nematodes, absent in the genus *Ektaphelenchus* (Hunt, 1993), was the specific and diagnostic character used to include it in the genus *Bursaphelenchus*. Because of the similarities between these species to nematodes in the genus *Ektaphelenchus* in morphology and cocoon-forming habit, a more detailed and precise study will be required to clarify their taxonomic status.

This work is a contribution to the knowledge on the distribution and biology of the genus *Bursaphelenchus* in Portugal and Europe. More knowledge on the biology of *Bursaphelenchus* species and about the interactions with their vectors is needed. With new *Bursaphelenchus* species being described from Europe, studies on these relationships as well as on the biology of vectors (e.g. number of generations per year, maturation feeding and feeding habits) will allow predictions of possible risk caused by these species to European forests.

Acknowledgements

This research was supported by grant 11 189/98 of the Portuguese PRAXIS XXI project, Fundação Ciência e Tecnologia (FCT) and PARLE D project (Instituto Nacional de Investigação Agrária-INIA and FCT) and is now currently being supported by the European Union fifth Framework, project QLK5-CT-2002-00672—PHRAME (Development of improved pest risk analysis techniques for quarantine pests, using pinewood nematode, *B. xylophilus*, in Portugal as a model system). This paper is a portion of the PhD dissertation of the first author, University of Évora, who is supported by a doctoral scholarship from FCT (BD 8920/2002). The authors kindly thank Prof. Esperança Pina, Faculdade de Ciências Biomédicas, Universidade Nova de Lisboa, for the use of the SEM; Mr Octávio Chaveiro, Estação Agronómica Nacional, for technical assistance and Prof. John Webster and Dr John Jones for reviewing this paper.

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