MORPHOBIOMETRIC AND MOLECULAR CHARACTERIZATION OF *BURSAPHELENCHUS* FUCHS, 1937 (NEMATODA: APHELENCHOIDIDAE) SPECIES ASSOCIATED WITH *PINUS PINASTER* AITON IN PORTUGAL

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ABSTRACT

The genus *Bursaphelenchus* comprises almost 100 species mainly from the northern hemisphere, with conifers as the most important hosts. Among the various nematode species, the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is the casual agent of pine wilt disease (PWD), and the most important forest pest for pines worldwide, classified as an A1 quarantine organism within the European Union. In 1999 this nematode was detected for the first time in Portugal and Europe associated with maritime pine, *Pinus pinaster*. Following detection, a national program denominated "Programa Nacional de Luta contra o Nemátodo da Madeira do Pinheiro" (PROLUNP) was created to, among other objectives, determine the distribution of the PWN and its associated vector(s) and host(s), and therefore intensive surveys covering the entire country were conducted with thousands of wood samples and suspected insects being analyzed. This thesis presents the listing, distribution, frequency and the insects associated with *Bursaphelenchus* species found associated with maritime pine in Portugal, identifying and characterizing the various species by morphological, biometrical and molecular biology (ITS-RFLP and rDNA sequencing analysis) techniques. To achieve the objectives, a total of 4813 maritime pine wood samples and 3294 insects from 22 species and six families were individually analyzed. A total of nine *Bursaphelenchus* species were found, namely: *B. antoniae*, *B. hellenicus*, *B. leoni*, *B. mucronatus*, *B. pinasteri*, *B. sexdentati*, *B. teratospicularis*, *B. tusciae* and *B. xylophilus*, all of them (with the exception of *B. xylophilus*) being new records for Portugal. Some of the species appear to have a widespread distribution, such as *B. leoni*, *B. teratospicularis* and *B. tusciae* while others were very rarely found and apparently have a localized distribution range within the country, namely *B. antoniae* and *B. mucronatus*. The majority of the species is characteristic of the Mediterranean region and can also be found in countries such as Spain, Italy and Greece, reflecting the affinity of our fauna with those locations. The association of *B. hellenicus* and *B. tusciae* with maritime pine is here reported for the first time. Six of the *Bursaphelenchus* species were also found associated with insects, mainly from the family Scolytidae (Coleoptera). Some of these interactions were described for the first

time, namely: *B. hellenicus* with both *Ips sexdentatus* and *Hylurgus ligniperda, B. sexdentati* with both *H. ligniperda* and *Orthotomicus erosus* and *B. tusciae* with *H. ligniperda*. The exclusive association of *B. xylophilus* with the cerambycid *Monochamus galloprovincialis* was also confirmed. The nematode´s *dauer* juveniles were usually found in low numbers in the insect vectors (*ca* 10-100 per insect), although for *B. xylophilus* a few thousand specimens per insect were sometimes found. The location of the *dauer* juveniles differed according to the species, although they were more common under the elytra and wings of the adult insects. A species new to science was detected and formally described as *B. antoniae*, associated with *Hylobius* sp. (Coleoptera; Curculionidae) beetles. Morphologically, this new species is very similar to *B. hylobianum*, although it's distinct ITS-RFLP molecular pattern (with only the enzyme HaeIII producing comparable restriction bands) and the failure of hybridization supported the two species as distinct entities. Additional phylogenetic analysis of the 18S rDNA sequence further supported the taxonomical proximity of *B. antoniae* with *B. hylobianum*. Concerning the PWN, detailed studies on the development and morphology of *B. xylophilus* were conducted, and comparative measurements of field-collected and laboratory-maintained populations demonstrated that nematodes from the second group displayed larger size in all morphometric parameters, which could derive from more adequate conditions of nourishment and/or temperature. Taxonomical studies on the development stages of *B. xylophilus* confirmed the existence of four propagative juvenile stages (J_1, J_2, J_3 and J_4), an adult stage with both sexes and two dispersal stages (J_{III} e J_{IV}), with the measurements of the gonad length allowing the separation of the propagative stages. It is hoped that the acquired knowledge will be useful on future surveys of nematodes of the *Bursaphelenchus* genus collected from either wood material or insect vectors, and facilitate the correct distinction and identification of the various species which are now known to occur.

RESUMO ALARGADO

O género *Bursaphelenchus* compreende quase 100 espécies, distribuídas sobretudo nos países do hemisfério norte do globo terrestre. Embora algumas espécies já tenham sido detectadas em plantas herbáceas, os hospedeiros vegetais mais comuns deste género são as coníferas, particularmente pinheiros.

O nemátode da madeira do pinheiro (NMP), *Bursaphelenchus xylophilus*, é considerado a espécie mais importante deste género uma vez que é o agente causal da doença da murchidão dos pinheiros ("pine wilt disease"). Originário dos Estados Unidos, onde não causa grande impacte, o NMP foi introduzido em alguns países da Ásia (China, Japão, Coreia e Taiwan) e mais recentemente na Europa (Portugal). Nestas regiões é responsável pela destruição de milhares de hectares de coníferas, assumindo uma elevada importância económica. Em Portugal, depois da sua detecção em 1999, associado a *Pinus pinaster*, foi implementado um programa nacional "Programa Nacional de Luta contra o Nemátodo da Madeira do Pinheiro" (PROLUNP) que permitiu determinar a área afectada pela praga (a sul do rio Tejo, península de Setúbal) bem como definir e implementar estratégias de controlo e prevenção da disseminação do NMP a outras zonas de Portugal. Recentemente, em Junho de 2008, foi confirmada a presença de *B. xylophilus* em outras regiões de Portugal levando as autoridades oficiais a definir todo o território continental como zona afectada e de restrição.

As prospecções intensivas realizadas nos últimos anos incluíram a recolha e análise de milhares de amostras de madeira de pinheiro bem como de insectos associados ao pinheiro bravo conduzindo à identificação de várias espécies de *Bursaphelenchus*. Assim, os estudos conduzidos neste trabalho tiveram como objectivos efectuar uma caracterização morfológica, biométrica e molecular das espécies associadas a *P. pinaster* em Portugal bem como a sua distribuição geográfica e abundância. Os estudos biométricos foram realizados com populações extraídas directamente do meio natural. Foi ainda realizada uma pesquisa que permitiu identificar os insectos a que estão associadas essas espécies, os seus possíveis vectores. Foram analisadas no total 4813 amostras de *P. pinaster* e 3294 insectos (22 espécies pertencentes a seis famílias diferentes). Foram identificadas um total de nove

espécies: *B. antoniae* n. sp., *B. hellenicus*, *B. leoni*, *B. mucronatus*, *B. pinasteri*, *B. sexdentati*, *B. teratospicularis*, *B. tusciae* e *B. xylophilus*.

Foram realizados estudos morfológicos e biométricos de todas as espécies com excepção de *B. mucronatus*; o reduzido número de exemplares encontrados em apenas uma amostra foram utilizados para efectuar o diagnóstico molecular desta espécie (ITS-RFLP). Apesar de ter havido, sempre que possível, a confirmação molecular, na maioria dos casos a caracterização morfológica e biométrica permitiu a correcta identificação das espécies. Contudo, foi imprescindível a análise molecular em algumas amostras, nomeadamente para a identificação de *B. xylophilus* e *B. sexdentati*; dada a grande semelhança entre *B. xylophilus* e *B. mucronatus* e tendo sido encontradas algumas populações de *B. xylophilus* que possuíam fêmeas com cauda mucronada, foi necessária a realização da confirmação molecular.

Com excepção de *B. xylophilus,* todas as outras espécies foram reportadas pela primeira vez em Portugal. Juntamente com *B. xylophilus*, *B. pinasteri* foi a espécie encontrada nas amostras de madeira de pinheiro com maior frequência. Algumas destas espécies como *B. leoni*, *B. teratospicularis* e *B. tusciae* foram reportadas em diferentes localidades do norte, centro e sul de Portugal, apresentando uma vasta distribuição geográfica; este resultado está em consonância com a forte associação destas espécies a climas mediterrânicos tal como acontece em Espanha, França, Itália e Grécia. Em oposição, espécies como *B. antoniae* e *B. mucronatus* foram encontradas apenas numa ocasião na região centro (Leiria) e norte (Figueira da Foz) do país, respectivamente. *Bursaphelenchus mucronatus* é igualmente pouco frequente em Espanha onde ocorre sobretudo na região norte, na Galiza. Esta espécie preferirá climas mais frios, ocorrendo com uma maior frequência nas regiões de latitude norte; esta análise é corroborada pela presença constante em países como Alemanha, Finlândia, França, Noruega, Rússia e Suécia. A nível mundial são descritas neste trabalho pela primeira vez as associações das espécies *B. hellenicus* e *B. tusciae* ao hospedeiro vegetal *P. pinaster*.

A realização deste estudo permitiu ainda descrever uma nova espécie de *Bursaphelenchus*, *B. antoniae*, associada ao insecto *Hylobius* sp. Apesar de numa primeira fase dos estudos ter sido identificada como *B. hylobianum*, dadas as semelhanças morfológicas e biométricas entre as espécies, trabalhos posteriores

baseados em análise molecular (ITS-RFLP *pattern*) bem como em testes de hibridação permitiram distingui-las. A análise filogenética da sequência 18S do rDNA de um grupo de 13 espécies de *Bursaphelenchus* onde estavam incluídas uma população de *B. antoniae* e *B. hylobianum* corroborou a hipótese de serem espécies distintas mas com grande afinidade. *Bursaphelenchus antoniae* nunca foi extraído da madeira de *P. pinaster*, no entanto, foi possível iniciar e manter uma cultura desta população em segmentos de pinheiro bravo.

De entre as nove espécies de *Bursaphelenchus* encontradas, seis foram associadas a insectos pertencentes às famílias Cerambycidae, Curculionidade e Scolytidae. Os juvenis "*dauer*"das várias espécies foram encontrados em diferentes zonas do corpo do insecto: debaixo das asas e élitros, nos pêlos ou entre os segmentos do corpo. No caso de *B. antoniae* e *B. xylophilus* os juvenis *dauer* foram encontrados no interior do corpo do insecto e nas traqueias, respectivamente. Com excepção de *B. xylophilus*, no qual em alguns casos foram encontrados milhares de juvenis *dauer* nas traqueias dos insectos, nas outras associações registaram-se sempre um pequeno número de juvenis *dauer* por insecto (entre 10 e 100). Confirmou-se a exclusividade da associação entre o cerambicídio *Monochamus galloprovincialis* e *B. xylophilus*. Foram ainda reportadas as associações de *B. teratospicularis* e *B. sexdentati* com *Orthotomicus erosus*, *B. hellenicus* com *Tomicus piniperda*, *B. hellenicus* com *Ips sexdentatus*, *B. hellenicus*, *B. tusciae* e *B. sexdentati* com *Hylurgus ligniperda* e *B. antoniae* com *Hylobius* sp. Das interacções identificadas algumas foram descritas pela primeira vez em todo o mundo nomeadamente: *B. hellenicus* associado a *I. sexdentatus* e *H. ligniperda*, *B. sexdentati* com *H. ligniperda* e *O. erosus* e, finalmente *B. tusciae* com *H. ligniperda*. Fica por confirmar em definitivo a associação de *B. leoni* a *Pytiogenes* sp.. De referir ainda que apesar de *B. pinasteri* ter sido encontrado num grande número de amostras de madeira de pinheiro não foi possível identificar o insecto a que está associado. Eventualmente esta espécie de *Bursaphelenchus* poderá estar associada a uma diferente família ou até ordem de insectos que não foi prospectada.

Relativamente a *B. xylophilus*, e dada a sua grande importância económica no país, foram realizados estudos mais detalhados acerca das suas características morfológicas e biométricas. Uma população de *B. xylophilus* (Tróia) foi recolhida do

seu meio natural sendo que parte foi fixada para posterior estudo biométrico e outra parte foi mantida em culturas de fungo *Botrytis cinerea*. Efectuaram-se medições comparativas entre a população colhida directamente da madeira de pinheiro e as mantidas em fungo. Observou-se que os especímenes cultivados em fungo apresentavam para todos os parâmetros dimensões bastante superiores. Uma vez que se constatam diferenças importantes nestes valores, dever-se-á ter muito cuidado na utilização de dados biométricos obtidos a partir de indivíduos mantidos em culturas de fungo nas descrições de espécies novas. Foram ainda realizados estudos morfológicos e biométricos dos diferentes estados propagativos e dispersivos da espécie. Os resultados confirmaram os estudos já efectuados no Japão que determinam a existência de três estádios juvenis propagativos fora do ovo, J_2, J_3 e J_4. O comprimento total do corpo, o comprimento da cauda, o diâmetro do corpo e o comprimento da gónada permitiram a separação entre estes estados juvenis.

Os estudos realizados nesta tese contribuíram para um maior conhecimento da biodiversidade em Portugal mais especificamente da nematofauna do género *Bursaphelenchus* associada a *P. pinaster* e quais os seus insectos vectores. Os estudos apresentados demonstram a fundamental complementaridade entre os métodos morfobiométricos e moleculares para uma consistente identificação das espécies de *Bursaphelenchus*.

No futuro, e considerando os novos desenvolvimentos que indicam uma propagação do NMP ao resto do país que levarão a prospecções mais intensas e a um maior número de amostras analisadas, estes estudos deverão ser tidos em conta para uma eficiente e rigorosa análise da presença ou não do *B. xylophilus* nessas amostras.

Uma vez que o NMP foi apenas reportado em pinheiro bravo, este trabalho focou-se nas espécies de *Bursaphelenchus* associados a *P. pinaster*; será de todo o interesse prospectar e alargar estes estudos pelo menos às outras duas espécies de pinheiros mais comuns em Portugal, *P. pinea* e *P. halepensis*, também de grande importância económica.

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CONTENTS

CHAPTER I. INTRODUCTION

I.1 Foreword

There are less than four billion hectares of forest in the world, covering about 30 per cent of the total land area. Some of the most important forest-related industries are located in the temperate zone and are mainly dependent of local forest resources, exploiting primary and derived forest products such as pulp, paper, lumber and chemical substances obtained from both natural and planted forests. The European forests (excluding the Russian federation) cover approximately 193 million hectares, an area which has been steadily increasing over the years (FAO, 2007). In Portugal, forests cover approximately 3 412,3 x 10^3 ha (DGRF, 2007), and the sector is responsible for 113 thousand direct jobs (2% of the active population), with each forest hectare producing an annual mean income of 344 euros, the highest value of any Mediterranean country (DGRF, 2006).

There are many diverse threats to forests worldwide, the most important being climate change, desertification, pollution, fires, deforestation and attacks by pests and pathogens (FAO, 2007). Many different organisms can be considered as forest pests and pathogens, namely fungi, bacteria, insects and nematodes, which are sometimes important causes of tree decay, tree mortality and wood depreciation. Among the different organisms, nematodes have been generally acknowledged as important tree pathogens in the last three decades (Sutherland & Webster, 1993).

A wide variety of plant parasitic nematodes such as *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Xiphinema americanum* Cobb, 1913 and *Trichodorus californicus* Allen, 1957 have been found associated with forest trees in temperate–zone climates (Sutherland & Webster, 1993). These nematodes may be responsible for enormous economic losses not only by their direct impact on the host plants but also by the surplus economic costs and negative consequences to the environment due to the necessity to implement control methods. Chemical substances used as nematicides are usually expensive, can have toxic effects to other organisms and to the environment and can leave persistent residues after application. Other options to control these pests include crop rotation, use of resistant strains of host plants and implementation of biological control programs (Powers, 1992).

Besides the negative impact of native pests and pathogens, there is nowadays a widespread problem of unintentional introduction of new and invasive species to forest ecosystems. Invasive species can be of all taxonomic groups from bacteria to mammals, and are second only to habitat destruction as a threat to global biodiversity (Mooney & Hofgaard, 1999). Awareness of the impact of forest invasive species has become heightened in recent years, as forest management activities, trade, tourism and land-use changes have facilitated several well-succeeded harmful and undesired introductions. Besides the direct and indirect impacts on native ecosystems, invasive species are responsible for enormous economic losses; in the USA, where there are presently over 50 000 non-native species, $ 120 billion dollars are spent every year in prevention and damage control (Pimentel *et al.*, 2004).

In an effort to control the spread of invasive pests, new phytosanitary measures for treating wood packaging material in international trade were recently defined for Europe, and have been or are currently being adopted by the European Union and several European countries (FAO, 2007). Nevertheless, despite the implementation of this and other similar measures, new examples of successful biological invasions continue to be described from all over the world and also from Europe and according to the latest reports there are now over 109 exotic woody phytophagous insects that have successfully invaded and established viable populations in Europe (Vanhanen, 2008).

Among nematodes, the pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, 1970 is considered to be the most important and serious conifer forest pest worldwide (Sutherland & Webster, 1993). This organism is the casual agent of pine wilt disease, and over the years it has been introduced due to human activities into several new countries such as Japan, China, Taiwan, South Korea and Portugal, where it causes significant tree mortality (Anonymous, 2006; Evans *et al.*, 2006; Mota & Vieira, 2008).

Preventing and reducing the harmful effects of invasive species such as the PWN requires an approach that incorporates biological, ecological and social sciences, economics, policy analysis and engineering (FAO, 2007). The implementation of efficient and rigorous quarantine regulations can prevent further introductions and spread of damaging nematodes, although this can only be achieved if the quarantine services have specialized

technicians able to execute an accurate and reliable identification of nematode species (Burrows, 1990), which need to be rigorously distinguished from similar taxa not classified as quarantine pests. Furthermore, development of risk analysis models requires knowing potential invasive pathways, adequate hosts and possible vectors. In the case of nematodes of the *Bursaphelenchus* Fuchs, 1937 genus, a profound knowledge on the local abundance and diversity of native species, their associated hosts and vectors and the identification of important characters for taxonomic and molecular species-characterization is required in order to avoid further unwanted introductions such as happened with *B. xylophilus* in the past.

I.2 The genus *Bursaphelenchus*

I.2.1 Classification and Taxonomy

The genus *Bursaphelenchus* was established in 1937 by Fuchs, grouping nematodes previously included in the genus *Aphelenchoides* Fischer, 1894. Over the years, other genera were synonymized with *Bursaphelenchus,* namely *Rhadinaphelenchus* J. B. Goodey, 1960, *Deviburs aphelenchus* Kakulia, 1967, *Huntaphelenchoides* Nickle, 1970, *Omemeea* Massey, 1971a, *Teragramia* Massey, 1974 and *Ipsaphelenchus* Lieutier & Laumond, 1978 (Hunt, 2008).

The taxonomic classification of this genus has not always been consentient as different authors include it either in the order Tylenchida Thorne, 1949 or the order Aphelenchida Siddiqi, 1980, along with the remaining aphelench nematodes. Nevertheless, the majority of the authors classify *Bursaphelenchus* nematodes in the order Tylenchida and suborder Aphelenchina Geraert, 1966 (Nickle, 1970; Andrassy, 1976; Luc *et al.*, 1987; Maggenti *et al.*, 1987; Nickle & Hooper, 1991).

An important taxonomic review of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 was presented by Nickle in 1970, and according to this author the order Tylenchida could be divided into two suborders, the Tylenchina Thorne, 1949 and the Aphelenchina, with phytoparasitic nematodes present in both although this biological trait evolved to a higher level in the first suborder (Nickle, 1970). In his review, Nickle included the genus

Bursaphelechus in the order Tylenchida, suborder Aphelenchina and family Aphelenchoididae (Skarbilovich, 1947) Paramonov, 1953 (Table I).

A decade later, Siddiqi discussed the presence of a protrusible stylet as the major characteristic common to the Tylenchina and Aphelenchina, and how it could have been originated from convergent evolution (Siddiqi, 1980). Using morphological, ethological, ecological, biochemical, cytogenetic and biogeographic data, this author discussed the evolutionary origin and the phylogeny of the two suborders, and concluded they had different origins and followed different evolutionary pathways. This author proposed the elevation of the Aphelenchina to the order Aphelenchida, where the genus *Bursaphelenchus* would be included (Siddiqi, 1980) (Table I).

Another eminent nematologist, Dr. Hunt, published in 1993 a book called "Aphelenchida, Longidoridae and Trichodoridae: their systematic and bionomics", where he reviewed the classifications of the genus *Bursaphelenchus* according to different authors and considered Siddiqi's classification to be the most adequate. According to both authors, nematodes included in the Aphelenchida have sufficient morphological (e.g.: spicule shape, caudal papillae or rays, position of the dorsal oesophageal gland orifice, strongly offset median bulb) and biological specific characters which allow to differentiate them from the Tylenchida and therefore constitute a distinct order (Siddiqi, 1980; Hunt, 1993). Nevertheless, Hunt considered Siddiqi's distinction of the two groups using phylogenetic arguments, without incorporating paleontological data to be excessive and speculative (Hunt, 1993).

In a more recent report where he produced a checklist of the Aphelenchoidea, Hunt (2008) changed the classification he adopted in 1993 and proposes the order Aphelenchida and suborder Aphelenchina to be rejected with these nematodes to be included in the suborder Tylenchina and superfamily Aphelenchoidea (Hunt, 2008) (Table I). These changes in the higher taxa took into account new molecular data presented over the last decades, following the proposals of De Ley and Blaxter (2002).

As seen in Table I, the genus *Bursaphelenchus* is included by Nickle (1970) in the family Aphelenchoididae along with 16 other genera, while Siddiqi (1980) includes it in the Parasitaphelenchidae Rühm, 1956 (with another genus) and Hunt (2008) in the Aphelenchoididae, which contains 25 genera. Despite the different classifications, the

absence or presence of a bursa-like flap of cuticle surrounding the terminal region of the male tail is the most important diagnostic feature to classify families or subfamilies for different authors, along with the association or endoparasitism of insects (Hunt, 1993; Ryss *et al.*, 2005).

Table I – Taxonomic classification of genus *Bursaphelenchus* according to different authors.

	Nickle, 1970	**Siddiqi, 1980**	**Hunt, 2008**
Order	**Tylenchida**	**Aphelenchida**	**Rhabditida** Chitwood, 1933
Suborder	**Aphelenchina**	**Aphelenchina**	**Tylenchina**
Superfamily	**Aphelenchoidea**	**Aphelenchoidoidea** (Skarbilovich, 1947) Siddiqi, 1980	**Aphelenchoidea**
Family	**Aphelenchoididae**	**Parasitaphelenchidae**	**Aphelenchoididae**
Subfamily	-	**Bursaphelenchinae** Paramonov, 1964	**Parasitaphelenchinae** Rühm, 1956
Genus	***Bursaphelenchus***	***Bursaphelenchus***	***Bursaphelenchus***

Although there are significant differences in the upper categories of the classification, this genus has characteristics and features that groups it and distinguishes it from the related genera. While in the species of *Bursaphelenchus* the third or fourth stage *dauer* juvenile are usually ectophoretic of insects, in the related genus *Parasitaphelenchus* Fuchs, 1929 [(which is also included in the Parasitaphelenchinae subfamily by Hunt (2008)] the fourth stage *dauer* juvenile is endophoretic in the insect haemocoel (Hunt, 1993). Furthermore, *Bursaphelenchus* males have separate spicules and strongly curved tail, while *Parasitaphelenchus* males have partially fused spicules and not so strongly curved tails (Hunt, 1993).

Besides *Parasitaphelenchus,* other genera similar to *Bursaphelenchus* are *Laimaphelenchus* Fuchs, 1937, *Aphelenchoides* and *Tylaphelenchus* Rühm, 1956 (Hunt, 2008), which can be distinguished by the absence of a bursa-like flap in the male tail. Other related genera such as *Ektaphelenchus* Fuchs, 1937, *Cryptaphelenchus* Fuchs, 1937, and *Ektaphelenchoides* Baujard, 1984 can also be separated from the genus *Bursaphelenchus* by

their indistinct and non-functional or even absent rectum and anus and the intestine which extends into the tail as a blind diverticulum (Hunt, 1993).

The type species of the genus *Bursaphelenchus* is *Bursaphelenchus piniperdae piniperdae* Fuchs, 1937. Nematodes of this genus are characterized by their short to medium length (0.3 – 1.7 mm), with females vermiform-like while males tend to acquire a ventrally arched tail with a terminal cuticular bursa-like flap of variable shape. Individuals have fine annulated cuticle, with a lateral field with none to five incisures and a cephalic region without oral disc and usually offset by a constriction. The stylet generally has small basal thickenings and is less than 30 μm long. There is a strong median bulb, oval to quadrangular, oesophageal glands overlapping the intestine and a functional anus and rectum. Males have two or more pairs of caudal papillae, one adanal and one to four pairs postanal. The spicules are paired and usually separated, with lengths that vary from 10 to 30 μm (measured along the median line); in shape they can be hook-like, sometimes linear, but never strongly curved and with capitulum having a rostrum usually prominent and pointed while the condylus is often well developed varying in shape, from bluntly rounded, pointed or recurved posteriorly; in some species the distal end has a structure called "cucullus" that can be a rounded swelling or a disc-like structure. Females have a posterior vulva generally situated at 70-80% of the body length; depending on the species, the anterior lip of the vulva extends as a flap which may cover the vulval opening. There is a postuterine sac with three to six times the width of the body diameter. The female's tail is subconoid with a terminus that can vary from fine to broadly rounded, truncate, mucronate or pointed (Hunt, 1993; Braasch, 2001; Ryss *et al.*, 2005). Some of the above features cannot be seen in the dispersal juveniles, the stage transported ectophoretically by the insect vector(s). Such specialized nematodes have between 300 - 600 μm length, body filled with granular lipid reserves, high dome-shaped head, lips not defined, oval median bulb not well developed, stylet not always discernable and a pointed tail (Hunt, 1993).

Based on morphological and molecular criteria, nematologists have clustered the various *Bursaphelenchus* spp. into groups of species with similar characteristics using different criteria. In 1982, Tarjan & Aragon proposed, for the first time, spicule morphology as the primary diagnostic feature for *Bursaphelenchus* species separation. Following this study, Giblin & Kaya (1983), separated *Bursaphelenchus* species into different groups based

on spicule shape and Yin *et al.* (1988) using also spicule features proposed a key to separate and identify the species. In her work with the European species, Braasch (2001), differentiated the various groups based mainly on the number of incisures in the lateral field but also on characters such as the number and position of the male caudal papillae, shape of the spicule and the presence and size of the female vulval flap. According to this author, there are four distinct major groups with either two, three, four or six incisures in the lateral field, although the groups with three and four incisures are further divided into sub-groups according to the other characters referred above. Therefore, Braasch considers a total of nine groups denominated *B. abietinus* group (with two incisures), *B. eggersi*, *B. leoni* and *B. hofmanni* groups (with three incisures), *B. xylophilus*, *B. sexdentati* and *B. fungivorus* groups (with four incisures) and *B. idius* group (with six incisures). The ninth group is formed by two species [*B. teratospicularis* Kakuliya & Devdariani, 1965 and *B. cryphali* (Fuchs, 1930) Rühm, 1956] with unknown number of incisures in the lateral field, although with other coherent morphological characteristics (see Braasch, 2001 for a description of the groups).

Other authors (Ryss *et al.*, 2005) suggested the sole use of the spicule structure (i.e. shape of the rostrum, shape of the condylus) for grouping species, creating six groups within the genus: the *hunti*-group, *aberrans*-group, *eidmanni*-group, *borealis*-group, *xylophilus*-group and *piniperdae*-group.

Besides these morphological characters, molecular studies were made to infer the phylogenetic relationships between *Bursaphelenchus* species and form groups of species. Metge *et al.* (2006), sequenced the ITS1, 5.8S and ITS2 of rDNA of 17 species, which permit the separation of species into two main branches forming the *xylophilus* group and *fungivorus* group. Other phylogenetic analysis of the 18S small subunit ribossomal (SSU), D2D3 domain 28S large subunit ribossomal (LSU) rDNA sequence and partial mitochondrial cytochrome oxidase subunit I (mtCOI) was made with 20 *Bursaphelenchus* species, and the sequencing formed six groups denominated *xylophilus* group, *hunti* group, *sexdentati* group, *hylobianum* group, *eggersi* group and *abietinus* group (Ye *et al.*, 2007). Overall, the various morphological and molecular studies diverge when grouping the species although, molecular studies support with some consistency the morphological variation within the genus *Bursaphelenchus*. Further studies are required to clarify the association and the similarities of the different *Bursaphelenchus* species.

I.2.2 Geographic distribution and species diversity

The genus *Bursaphelenchus* has a widespread distribution, with most of the species found in the northern hemisphere (Ryss *et al.*, 2005; Hunt, 2008). South of the equator there are records solely for *B. cocophilus* (Cobb, 1919) Baujard 1989 in South America (Schuiling & Van Dinther, 1981; Griffith & Koshy, 1990; Araújo *et al.*, 1998), *B. africanus* Braasch, Gu, Burgermeister, Brandstetter & Metge, 2007 found in China but in packaging wood from South Africa, *B. leoni* Baujard, 1980 in South Africa (Braasch *et al.*, 1998) and a *Bursaphelenchus* sp. from Australia (Ridley *et al.*, 2001), although the last two records probably correspond to species introduced along with their pine host and vectors.

The number of recognized species varies according to different authors, with Ryss *et al.* reporting 75 species in 2005, while just three years later, Hunt (2008) reported 94 species, which reflects the recent description of several new species each year (e.g.: Schönfeld *et al.*, 2006; Akbulut *et al.*, 2007; Hazir *et al.*, 2007; Kanzaki *et al.*, 2007; Zhuo *et al.*, 2007; Li *et al.*, 2008; Sriwati *et al.*, 2008) from both North America, Asia and Europe, a consequence of the economic importance of this genus and the attention given by many researchers around the world. Since 1999, the report of *B. xylophilus* for the first time in Portugal and Europe (Mota *et al.*, 1999), 29 new species have been described.

I.2.3 General bio-ecology

The majority of the *Bursaphelenchus* species are exclusively mycetophagous, feeding on the fungi found on galleries of bark beetles of the family Scolytidae Latreille. The only known exceptions are *B. xylophilus* and *B. cocophilus*, which although probably evolved from mycetophagous ancestors, are now considered to be partially or obligate phytophagous (Giblin-Davis *et al.*, 2003). In fact, *B. xylophilus* can feed on living plant tissue during part of its life cycle and is therefore partially phytophagous, while *B. cocophilus* appears not to feed at all on fungi and is therefore considered to be an obligate phytophagous (Giblin-Davis, 1993). The phytophagous life cycle of these two species is responsible for their classification as pathogenic organisms subjected to quarantine measures in several countries worldwide, as *B. cocophilus* is the causal agent of the red ring disease, one of the most important diseases of coconuts (*Cocos nucifera* L.) and other palms, while *B. xylophilus* is the casual

agent of the pine wilt disease, being an important mortality agent of some species of the genus *Pinus* L. (Hunt, 1993; Giblin-Davis *et al.*, 2003; Ryss *et al.*, 2005; Mota & Vieira, 2008).

Other species such as *Bursaphelenchus mucronatus* Mamiya & Enda, 1979, *Bursaphelenchus sexdentati* Rühm, 1960 and *Bursaphelenchus fungivorus* Franklin & Hooper, 1962 have also been sporadically reported to kill trees and/or seedlings (e.g.: Mamiya, 1999; Yang *et al.*, 1988; Braasch *et al.*, 1998), although due to methodological constrains of the inoculation experiments conducted on young seedlings, the effective pathogenicity of such species on healthy adult trees in nature needs to be further confirmed with new studies (McNamara, 2003; Arias *et al.*, 2005).

Although a few species can be found in herbaceous plants, the most common hosts for the majority of the *Bursaphelenchus* species are trees such as pines and other conifers; however, there are records of many other hosts from such diverse families as Araliaceae Juss., Areaceae Gray, Betulaceae Gray, Cupressaceae Richard ex Bartling, Fagaceae Dumortier, Juglandaceae Richard ex Kunth, Moraceae Link, Oleaceae Hoffmag & Link, Rosaceae Juss., Rubiaceae Juss., Salicaceae Mirb. and Ulmaceae Mirb. (Ryss *et al.*, 2005).

The various *Bursaphelenchus* species are vectored from one plant host to another by different insect species, the most frequent vectors being beetles from the order Coleoptera L. and the families Scolytidae (e.g.: Fuchs, 1937; Rühm, 1956; Kakuliya & Devdariani, 1965; Massey, 1974; Lieutier & Laumond, 1978; Baujard, 1980; Thong *et al.*, 1983; Braasch & Schmutzenhofer, 2000; Braasch, 2001; Arias *et al.*, 2005; Kanzaki *et al.*, 2007), the Cerambycidae Latreille (e.g.: Mamiya & Enda, 1972; Linit *et al.*, 1983; Kobayashi *et al.*, 1984; Giblin-Davis, 1993; Kishi, 1995; Kanzaki & Futai, 2003; Kanzaki *et al.*, 2008; Ryss, 2008; Togashi *et al.*, 2008; Vincent *et al.*, 2008) and Curculionidae Latreille (e.g.: Massey, 1971; Schuiling & Van Dinther, 1981; Araújo *et al.*, 1998; Giblin-Davis *et al.*, 2006a).

Along with these more frequent associations, beetles from other families such as the Nitidulidae Latreille, Antophoridae Dahlbom and Halictidae Thomson have also been found carrying *Bursaphelenchus* spp. (Giblin *et al.*, 1984; Giblin-Davis *et al.*, 1993, 2006b). Besides the Coleoptera, there are also records for *Bursaphelenchus*-insect associations on other insect orders, namely the Hymenoptera L. (Giblin & Kaya, 1983; Giblin *et al.*, 1984; Giblin-Davis *et al.*, 1993, 2005; Hazir *et al.*, 2007) and butterflies (Lepidoptera L.) of the family Sesiidae Boisduval (Rühm, 1956). Recent and detailed reviews of the *Bursaphelenchus*-host-

vectors interactions have been made by Braasch (2001) and Ryss *et al.* (2005), and although new vector-nematode associations continue to be described each year, for many of the species the insect vectors are still not known.

I.3 The pinewood nematode *Bursaphelenchus xylophilus*

I.3.1 Taxonomy

The pinewood nematode (PWN) was first described in 1934 by Steiner & Buhrer from the United States and named *Aphelenchoides xylophilus* Steiner & Buhrer, 1934. The species was transferred to the genus *Bursaphelenchus* by Nickle in 1970. Two years later, in 1972, Mamiya and Kiyohara described in Japan *Bursaphelenchus lignicolus* Mamiya & Kiyohara, 1972. A few years later Nickle *et al.* (1981) reviewed the two species and proposed a synonymy of both with *B. xylophilus* which remains today as the valid name.

Bursaphelenchus xylophilus can be morphologically distinguished from similar species by the existence of a distinct vulval flap and a rounded tail terminus in the females and by the characteristic shape of the male spicules (Mamiya, 1984) (Figure 1). The specific identification can be difficult by the existence of two distinct morphological forms of the PWN, denominated the "r" and the "m" forms, which can be separated by the shape of the female tail (Dwinell, 1997). De Guiran and Bruguier (1989) proposed the existence of a supra-species complex named "*xylophilus*", distinguishing between *B.* (suprasp. *xylophilus*) *xylophilus* (containing females with a rounded tail, the "r" form) from *B.* (suprasp. *xylophilus*) *mucronatus* (containing females having a mucronate tail, the "m" form). The taxonomical separation of *B. xylophilus* and the closely species *B. mucronatus* has been debatable among researchers, with *B. mucronatus* being characterized by the presence of a mucronate tail in females (Mamiya & Enda, 1979). Webster *et al.* (1990) grouped the morphological forms of *B. xylophilus* and *B. mucronatus* into the pinewood nematode species complex (PWNSC), similarly to Rutherford *et al.* (1990) who considered that *B. xylophilus* and *B. mucronatus* belong to a species complex in which members can exchange genetic material through intermediate forms. Using different molecular biology techniques these species can be differentiated (e.g.: Abad *et al.*, 1991; Burgermeister *et al.*, 2005). Despite some taxonomic controversy, the validity of both species as separate entities is

nowadays accepted by most modern authors (Nickle *et al.*, 1981; Webster *et al.*, 1990; Beckenbach *et al.*, 1992; Riga & Webster, 1992; Braasch, 2001; Giblin-Davis *et al.*, 2003; Ryss *et al.*, 2005; Hunt, 2008).

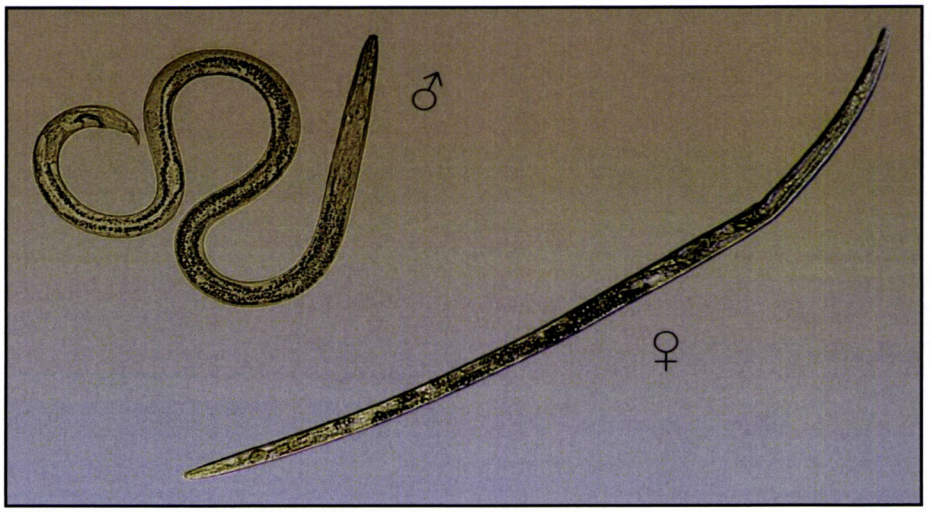

Figure 1 – *Bursaphelenchus xylophilus*, male and female. (Amp.: 100x)

I.3.2 Geographic distribution and hosts

The PWN is considered to be a native to North America, where it can be found in Canada and the USA (Dropkin & Foudin, 1979; Robbins, 1982; Bowers *et al.*, 1992; Sutherland & Peterson, 1999), with an additional report from Mexico (Dwinell, 1993). Being a native species, the PWN usually does not cause wilt in North American conifers, as both the nematode and its hosts share a common history of co-evolution which allowed the trees to become resistant or to tolerate this organism (Kiyohara & Bolla, 1990). Therefore, and with the exception of some exotic pine plantations (Evans *et al.*, 1996), in North America this nematode only colonizes conifers weakened by other biotic or abiotic agents, with Jack pine (*Pinus banksiana* Lamb.), shortleaf pine (*Pinus echinata* (Mill)) and slash pine (*Pinus elliottii* Engelm) being the most susceptible of the native pine species (Dropkin *et al.*, 1981; Burnes *et al.*, 1985; Dwinell, 1985). Besides pines, the PWN can also be found in other conifers of the genera *Cedrus* Trew, *Larix* Philip Miller and *Picea* Link (Wingfield *et al.*, 1982a, 1982b; Dwinell, 1997).

Over the years, human activity has promoted the introduction of the PWN into non-native areas outside of North America as a consequence of international trade and the global flow of untreated forest products, such as raw logs and unmanufactured wood (Evans

et al., 1996; Bergdahl, 1999; Webster, 2004; Mota & Vieira, 2008). In such new regions, *B. xylophilus* becomes an extremely aggressive and important mortality agent for some of the native pines, such as Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*Pinus densiflora* Sieb. & Zucc.) in Japan, where since its accidental introduction in the first years of the XXth century it has been responsible for very high mortality of these susceptible pines each year (Mamiya, 1983, 1984, 1988; Kobayashi, 1988; Kishi, 1995).

Wood trade continues to disperse *B. xylophilus* and the PWN eventually was introduced into China (Cheng *et al.*, 1983; Yang & Qouli, 1989; Yang, 2004), Taiwan (Tzean & Tang, 1985) and South Korea (Yi *et al.*, 1989; La *et al.*, 1999). In Europe, the PWN was reported in 1979 from south-western France by Baujard *et al.* (1979), although subsequent studies found that this nematode population was more similar in morphology to *B. mucronatus* (De Guiran & Boulbria, 1986). Nevertheless, in 1999 *B. xylophilus* was found associated with dead *Pinus pinaster* Aiton (maritime pines) from the Pegões region in Portugal, in what was the first confirmed record of this species in a European country (Mota *et al.*, 1999) (Figure 2).

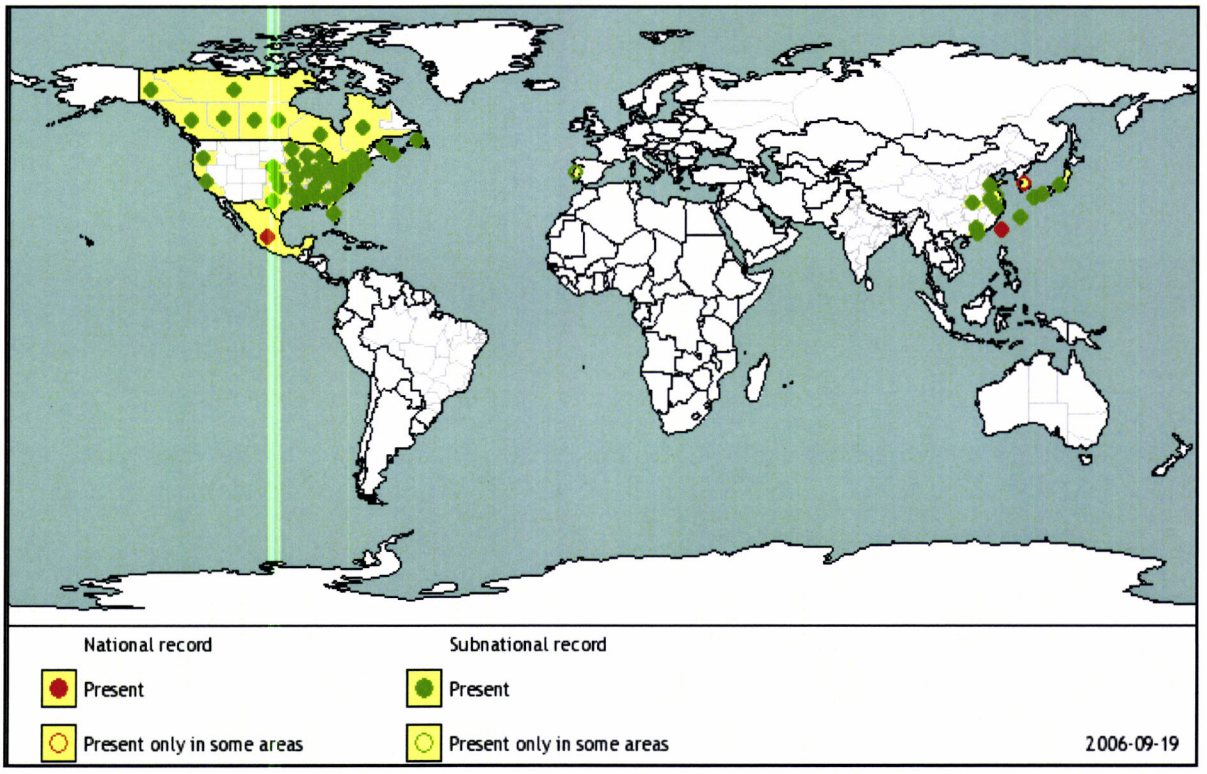

Figure 2 – Current world distribution of *Bursaphelenchus xylophilus* (adapted from OEPP/EPPO, 2008).

I.3.3 The pinewood nematode in Portugal

Following detection of the PWN in Portugal in 1999, a national program named "Programa Nacional de Luta contra o Nemátodo da Madeira do Pinheiro" (PROLUNP) was created, with the following objectives: to prevent the spread of the nematode; to perform nationwide survey of its distribution; and to implement measures to control the nematode (Anonymous, 2008). As both the pinewood nematode and its insect vectors are classified as quarantine organisms by the European community (Annex II, Part A – Section I of Directive 77/93/EEC) and EPPO, the detection of *B. xylophilus* in any European country implies strong limitations and restrictions to the transport and commercialization of non-treated pinewood and pine-derived materials from nematode-infested areas, such as southern Portugal (OEPP/EPPO, 1986, 2008; Evans *et al.*, 1996).

Shortly after the detection, systematic surveys on high-risk areas (near ports, airports, main roads, sawmills, etc) of continental Portugal found the PWN to be confined to the Setúbal peninsula, south of Lisbon and the Tejo River, with its only host being the maritime pine *P. pinaster* (Anonymous, 2006).

Maritime pine is Portugal's most important forest species as it occurs on approximately $710{,}6x10^3$ha and supports an economically important and diverse industry of wood materials and paper products (DGRF, 2007). The species is widespread in the Setúbal peninsula and adjacent areas, and the regions affected by the PWN were denominated "affected" zone, with a neighbouring "restriction zone" where severe phytossanitary constrains regarding the cut, processing and transport of the dead wood and other pine material were implemented, accordingly to the classification of *B. xylophilus* as a quarantine organism (Anonymous, 2008).

As would be expected, the strong restriction measures implemented produced significant impacts in the timber industry which reflected negatively not only in the affected area but also in the national wood industry as a whole (Mota & Vieira, 2008). Furthermore, and over the years, the PWN has been causing death and destruction of over 700 thousand maritime pine trees since its detection in 1999 (Anonymous, 2006), with additional economic, environmental and social negative impacts.

As a result of periodic surveys conducted by PROLUNP, a second focus of the PWN was detected in the spring of 2008 on maritime pine trees outside the original affected zone of

Setúbal peninsula, this time on the districts of Arganil and Lousã in the center of Portugal, which resulted in the definition of a new affected and restriction zone subjected to similar phytossanitary procedures (MADRP, 2008a, 2008b). More recently, in 27th of June of 2008, an official publication from Ministério da Agricultura, Desenvolvimento Rural e das Pescas, Diário da República, 1ª Série, nº123, Portaria nº 553-B/2008 (MADRP, 2008c), confirmed the presence of PWN in some locations outside the previous restriction zones and defines the entire national territory as affected and restriction zone.

Until recently, no consensus has emerged on the possible pathway of the PWN introduction in Portugal. This is partly due to a scarceness of studies using different sources of isolates from the affected area in the country. Several hypotheses have been put forward to explain this introduction, such as from endemic areas where the nematode naturally occurs (North America), or non-endemic areas where the nematode behaves as an exotic pest (Asia). These hypotheses were recently tested using isolates from different geographic areas of the world (Metge & Burgermeister, 2006); as well as a large number of isolates representative of the affected area in the Setúbal Península (Vieira *et al.*, 2007). In these studies the Portuguese PWN isolates reveal a high genetic similarity within isolates from Asia, and the low level of genetic diversity within the Portuguese isolates strongly suggests the occurrence of a single introduction of this species in Portugal (Vieira *et al.*, 2007).

I.3.4 Bio-ecology

Once *B. xylophilus* is introduced into a healthy tree it diffuses through the resin canals and begins to feed on the living parenchyma and epithelial cells during its phytophagous phase (Mamiya, 1984; Kishi, 1995). Inside the host tree, the nematode completes its life cycle, which is composed of four propagative juveniles and one adult stage (Ishibashi & Kondo 1977; Mamiya 1984), including a first juvenile stage (J_1) completed inside the egg, meaning that the nematodes hatch as second-stage juveniles (J_2) and pass through three moults prior to becoming adults (Mamiya, 1975).

Under favorable temperatures (aprox. 25°C), the PWN can complete its life cycle from egg to adult in four to five days (Ishibashi & Kondo, 1977; Mamiya, 1984), and afterwards each female can lay between 80 and 150 eggs during an oviposition period of up to 28-days (Mamiya, 1975; Kishi, 1995).

Besides its rapid development and high reproductive potential, *B. xylophilus* can develop through two distinct pathways which are designated as "reproductive" or "dispersal" life cycles, the first two juveniles stages (named J_1 and J_2) being common to both. When developmental conditions are adequate the nematode develops through the reproductive pathway, moults to the third (J_3) and fourth (J_4) juveniles stages and finally to the adult stage. On the other hand, when environmental conditions are unfavorable (which may be due to excessively low or high moisture content or to the absence of adequate food), the nematode switches to its dispersal pathway, with the third and fourth stage juveniles now being referred to as J_{III} and J_{IV}. As the third stage juveniles have lipid contents in their body, this allows them to withstand long periods under unfavorable conditions, which is particularly useful for locations with harsh winters (Mamiya, 1984; Warren & Linit, 1993).

While feeding on healthy parenchyma and epithelial cells, and after being introduced into a host tree, the nematodes induce tracheid cavitation, which reduces and eventually stops the water flow through the hosts' tracheids. Without transpiration, the tree's needles progressively acquire a yellowish to brown coloration, and with the tree's death different species of fungi appear in the wood (Malek & Appleby, 1984; Mamiya, 1984; Kishi, 1995) (Figure 3). The contamination of the dead tree with fungi causes the PWN to switch to its mycetophagous cycle (Mamiya, 1983, 1984).

Figure 3 – Pine trees killed with *Bursaphelenchus xylophilus* (Tróia).

After being infected with the pinewood nematode, the majority of susceptible trees die within three to six weeks in the United States (Malek & Appleby, 1984) and five to eight weeks in Japan (Mamiya, 1984), depending on the ambient temperature and precipitation. Ambient temperature is extremely important in regulating wilt expression, as pine wilt disease usually occurs with higher severity in regions with mean air temperatures exceeding 20ºC for several consecutive weeks (Rutherford & Webster, 1987; Rutherford *et al.*, 1990; Evans *et al.*, 1996).

To be dispersed from an infected tree into a healthy one, the PWN needs to be actively transported by a vector insect which emerges from the dead tree carrying with *B. xylophilus* (Linit, 1988; Kishi, 1995). Before the vector's emergence, the nematodes begin to gather around its pupal chambers in the spring and eventually molt into J_{IV} juveniles, which is a specialized non-feeding and dispersive stage also known as *dauer* juveniles or *dauer* larvae (Mamiya, 1983, 1984). The *dauer* juveniles enter into the spiracles of the insect's callow adult in the pupal chamber (Mamiya, 1972; Mamiya & Enda, 1972; Linit *et al.*, 1983; Wingfield & Blanchette, 1983), in numbers which vary from a few hundred to as high as many thousands nematodes in the body of each insect (Linit *et al.*, 1983; Kobayashi *et al.*, 1984; Malek & Appleby, 1984; Sousa *et al.*, 2001, 2002). The *dauer* juveniles are transported mainly inside the trachea of the insect vector (Mamiya, 1984). The nematode load carried by the insects is dependent on environmental conditions in the wood and the pupal chamber, being influenced by the nematode's abundance, wood water content (Mamiya, 1984; Togashi, 1989; Warren & Linit, 1992) and fungi species (Wingfield & Blanchette, 1983; Warren *et al.*, 1995; Maehara & Futai, 1996, 1997, 2002), and the vector's timing of emergence (Kobayashi *et al.*, 1984; Mamiya, 1984; Kishi, 1995).

Worldwide, the most important vectors of the PWN are beetles belonging to the genus *Monochamus* Dejean (Linit, 1988; Kishi, 1995), which belong to the order Coleoptera and family Cerambycidae. The most important vector in North America is *Monochamus carolinensis* (Olivier) (Linit, 1988), *Monochamus alternatus* Hope being the main vector in Northeast Asia (Kobayashi *et al.*, 1984; Mamiya, 1984; Kishi, 1995). In Portugal, the sole vector is also a *Monochamus* species, namely the native pine sawyer *M. galloprovincialis* Olivier (Sousa *et al.*, 2001, 2002). Although other Coleoptera have sporadically been found to carry *B. xylophilus* in their bodies, the low frequency and nematode loads suggest that

they are not efficient PWN vectors (Wingfield & Blanchette, 1983; Kobayashi *et al.*, 1984; Linit, 1988) (Figure 4).

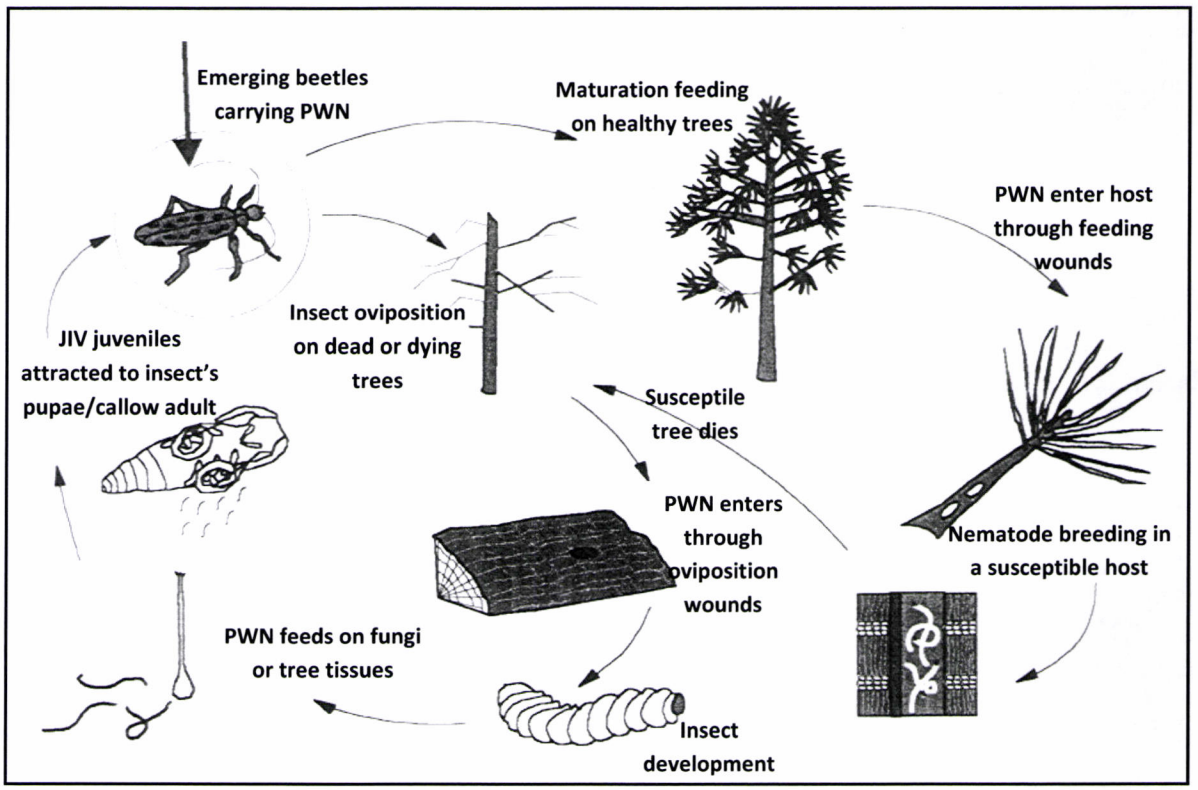

Figure 4 – Schematic representation of the general interactions between the insect vector, the PWN and the tree host (adapted from Evans et al., 1996).

The transmission of *B. xylophilus* to a new tree host can occur by maturation feeding or the oviposition activity of the adult *Monochamus* (Linit, 1988; Kishi, 1995). Transmission through maturation feeding occurs after emergence of the beetles, as the insects fly to feed on the branches of healthy pine trees and infect them with the nematode when the J_{IV} juvenile leave their body and enter the host through its feeding wounds (Mamiya & Enda, 1972; Mamiya, 1984; Wingfield & Blanchette, 1983; Linit, 1990). The transmission through oviposition takes place when the female insects lay its eggs on dead or weakened trees, with the nematodes again leaving the insect's body and enter the new host through the oviposition wounds (Wingfield, 1983; Wingfield & Blanchette, 1983; Linit, 1990). Both transmission pathways have been intensively studied over the years for *M. carolinensis* in North America and *M. alternatus* in Southeast Asia, and have also recently been described for *M. galloprovincialis* in Portugal, with transmission through the maturation feeding appearing to be more frequent and efficient than through oviposition (Naves *et al.*, 2007a, 2007b).

I.4 Classic and modern Taxonomy

Many plant parasitic nematodes represent a serious threat to forests and agriculture. Precise identification of an undetermined species found damaging a vegetable crop or intercepted in an untreated wood package is often required, and an accurate, reliable and quick diagnosis is needed for the implementation of an efficient control and a management strategy against nematode problems (Burrows, 1990). Additionally, the misidentification of quarantine species can have enormous economic and ecological implications (Van Halteren, 1995), and addresses the needs for accurate identifications and the taxonomic problems of this particular animal group.

In comparison to other groups of plant pests, the identification of nematodes has always been particularly difficult since these micro-organisms present very few conspicuous morphological characters for diagnosis (Vrain & McNamara, 1994; De Ley & Blaxter, 2002). Moreover, in some circumstances the difficulty is greater when it is necessary to identify the juveniles. Identification can be achieved by classical methods, with time consuming observations and measurements under a light microscope (LM) (Burrows, 1990). Despite its difficulty and being time consuming, classical identifications based on morphological characters is an extremely important approach, at least at an early phase, to identify and characterize nematode problems and to answer specific questions. Nevertheless, an inadequate use of the LM or identifications performed by inexperienced taxonomists can lead to misidentifications; furthermore, sometimes species simply cannot be precisely identified due to the low resolution achieved by the light microscope, not sufficient to distinguish some morphological characters important for separating lower taxonomic levels. Since such difficult characters can be ambiguous, obscure or display inter- and intra-specific variability, this can lead to ambiguous diagnostics (Curran *et al.*, 1985; Coomans, 2002).

Despite its importance, morphological observations do not have, in some cases, sufficient discriminatory power. The use of biochemical and molecular characters, based on differences in proteins, lipids, carbohydrates and nucleic acids, has become an important complement to the traditional morphological studies and has been used for over 30 years on nematode identification (Curran, 1992; Fox & Atkinson, 1986).

Using standardized basic methods applicable to almost any taxa, molecular tools can provide useful diagnostic data. Besides being a successful instrument for taxonomic identification, molecular techniques can also be applied to phylogenetic studies, discriminating the evolutionary relationships between different taxa. Reliable species identification and phylogenetic relationships between isolates within the genus *Bursaphelenchus* has been achieved using different molecular techniques developed and applied by various nematologists. Studies using molecular techniques have focused on DNA probe analysis (Bolla *et al.*, 1998; Abad *et al.*, 1991; Harmey & Harmey, 1993), DNA sequencing (Beckenbach *et al.*, 1992; Iwahori *et al.*, 1998; Metge *et al.*, 2006; Lange *et al.*, 2007; Ye *et al.*, 2007), RAPD-PCR (Irdani *et al.*, 1995; Metge & Burgermeister, 2006; Vieira *et al.*, 2007) and PCR-RFLP (Iwahori *et al.*, 1998; Burgermeister *et al.*, 2005; Lange *et al.*, 2007).

Until recently, the majority of studies on the relationships within the genus *Bursaphelenchus* were made with morphological characters, such as the morphology of the male spicule, the female tail and the number of incisures in the lateral field (e.g.: Massey, 1974; Giblin-Davis, 1993; Hunt, 1993; Braasch, 2001; Ryss *et al.*, 2005). A more recent study (Metge *et al.*, 2006) attempted to infere the phylogenetic relationships between 17 *Bursaphelenchus* species using molecular techniques to characterize the ITS1, 5.8S and ITS2 sequences of rDNA. Other studies were made on gene sequences to verify and establish groups within some of the *Bursaphelenchus* species (Lange *et al.*, 2007; Ye *et al.*, 2007). The results obtained with the diverse molecular techniques were very much in accordance with the species-groups previously determined by other authors (Braasch, 2001; Ryss *et al.*, 2005) using solely morphological observations (Ye *et al.*, 2007), which reflects the interest of using both methods for nematode identification and studies on taxonomic relationships.

An example of using these complementary methods is the identification of the pinewood nematode *B. xylophilus*, which is integrated in a group (the "xylophilus" group) of very similar species such as *B. mucronatus*. Morphologically, these two species can only be securely distinguished when females with rounded tails are present, indicating the species as *B. xylophilus* (Braasch, 2001). Nevertheless, wood samples can sometimes just contain females with mucronate tails, or no females at all and therefore males and/or juveniles. In such cases, molecular studies are required for a precise diagnosis of the species present.

It's reasonable to assume that only a combination of classical and modern methods can provide a more accurate and reliable knowledge on species identification and taxonomic relationships. Although molecular techniques and related bioinformatic software are becoming more sophisticated each year and turning into a widespread scientific tool, classical identification based on morphological characters and measurements will always be required and should not be excluded from studies on nematode taxonomy.

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CHAPTER II. OBJECTIVES AND THESIS ORGANIZATION

II.1 Aims of this thesis

The taxonomy and relationships of an economically important genus such as *Bursaphelenchus* has been subject to periodic revisions (e.g.: Nickle, 1970; Tarjan & Aragon, 1982; Braasch, 2001; Ryss *et al.*, 2005; Hunt, 2008), requiring solid and updated national and regional information and knowledge which frequently does not exist. Such was the case for the genus *Bursaphelenchus* in the Iberian Peninsula and, more specifically, in Portugal, which had never been studied before the detection of *B. xylophilus* in 1999 and, consequently, no species of this genus had been recorded from the country.

The detection of the pinewood nematode in Portugal (Mota *et al.*, 1999) had serious social and economic implications as this is an EPPO A1 list quarantine pest (Evans *et al.*, 1996), which means that most countries have established severe restrictions to wood exported from regions affected by this organism (Bergdahl, 1988; Halik & Bergdahl, 1994). The high phenotypic plasticity of this and other similar nematodes of the genus *Bursaphelenchus*, their enhanced capacity to survive for long periods under unfavorable wood conditions and their ability to disperse by insect vectors help to explain the importance of this nematode genus and the successful introductions of several species into new countries and geographic regions which have occurred. In fact, many *Bursaphelenchus* species, including the PWN, are routinely intercepted in imported packaging and wood material in countries such as Austria (Tomiczek *et al.*, 2003), Finland (Tomminen, 1991) and Germany (Braasch *et al.*, 2001), among others. The successful interception of these and other nematode species prior to their introduction requires an accurate and rigorous identification and diagnosis of the various species, which can be achieved by morphological and/or molecular studies, along with a complete knowledge of their life cycles and the identification of possible vector insects.

Furthermore, the detection of the pinewood nematode in Portugal in 1999 in a limited and restricted area south of Lisbon suggests that this quarantine organism was recently introduced into Europe. Although the pine sawyer *M. galloprovincialis* has been found to be the nematode's vector in Portugal (Sousa *et al.*, 2001, 2002), the

survey and identification of other potential vectors from the Scolitydae and Cerambycidade families is an essential task for the implementation of any successful control strategy, which is yet to be done.

In view of these requirements, the main objectives of the present research project have been to study the diversity of the *Bursaphelenchus* fauna associated with maritime pine in Portugal, to characterize species distribution, their abundance and the taxonomic relationships using both morphological and molecular data, along with the study of the association of the various nematode species with their insect vectors and particularly possible vectors of the PWN, these being studies which had never been conducted on Portugal.

II.2 Thesis structure

This research work comprehends five chapters, with a listing of the bibliographic references presented at the end of each chapter. The results are presented in Chapter III in the form of four scientific papers (corresponding to sub-chapters), following the cronologicall order in which they were submitted and accepted for publication. These four sub-chapters should be addressed not as hermetic studies but as interdependent and integrated units, with the studies of the last papers already taking into consideration the results of the previous ones.

All papers have been published in peer-reviewed international journals, and each follows the journal's specific guidelines and therefore has diverse formattings and presentations, although they all generally consist of an introduction, description of material and methods, results, discussion of the results and the cited literature. Studies were partially or fully supported by grants from the project 11 189/98 of the Portuguese PRAXIS XXI (Fundação para a Ciência e Tecnologia), the PARLE D project (Instituto Nacional de Recursos Biológicos and Fundação para a Ciência e Tecnologia), the European Union 5th Framework project QLK5-CT-2002-00672-PHRAME (*Development of improved pest risk analysis techniques for quarantine pests, using pinewood nematode*, Bursaphelenchus xylophilus, *in Portugal as a model system*), and by a doctoral scholarship from the FCT (BD 8920/2002).

Chapter I reviews the current literature on the genus *Bursaphelenchus*, namely its historical and current taxonomy, morphological characterization of the genus, taxonomical relationships of the species, geographic distribution, species diversity and general bio-ecology of the genus, with special attention given to the pinewood nematode *B. xylophilus* following its detection in Portugal. A brief review of the importance of the morphological and more recent bio-molecular characters and techniques and their importance to general nematode taxonomy and more specifically to the genus *Bursaphelenchus* is also reviewed.

In **Chapter II** the main objectives of this thesis and structure are described.

Chapter III presents studies on *Bursaphelenchus* species identification, diversity, distribution, abundance, taxonomy and association with insect vectors on maritime pine in Portugal, and is divided into four sub-chapters:

Sub-chapter III.1 studies the diversity of *Bursaphelenchus* species associated with maritime pine within and outside the demarcated zone of the PWN, describing and characterizing with morphological (LM and SEM) and molecular techniques (ITS-RFLP) the various species, presenting the most important morphological characters for diagnostic and proposing a new staining method for the spicules.

Sub-chapter III.2 addresses the association of the species of the *Bursaphelenchus* and other genera with bark and wood boring insects collected from maritime pine, using internal transcribed spacers-restriction fragment length polymorphism (ITS-RFLP) analysis of *dauer* juveniles and adults and morphological characterization of adults developed from *dauer* juveniles to identify the nematodes.

Sub-chapter III.3 reports the description of a *Bursaphelenchus* species new to science which was detected in Portugal during previous studies, and is for the first time described and illustrated. The paper also discusses the most important morphological characters of this new species and its taxonomic position and relationships within the *Bursaphelenchus* genus.

Sub-chapter III.4 presents a detailed morphobiometric analysis of *B. xylophilus* along with a morphological characterization (biometrics, ratios and spicules) of seven other *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) associated with

Pinus pinaster in Portugal. Aditionally, five of these species are also characterized using ITS-RFLP techniques, and their molecular profiles are presented.

In **chapters IV** and **V** results of the four publications are summarized and reviewed in a discussion referring the most important observations and conclusions suggesting future lines of research.

Finally, in **appendix** are presented other manuscripts related to the subject of this thesis co-published by the author.

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CHAPTER III. Characterization of *Bursaphelenchus* spp. from Portugal and associated insects (original publications by chronological order)

Sub - chapter III.1

Penas, A. C., Correia, P., Bravo M. A., Mota, M. & Tenreiro, R. (2004). Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) associated with maritime pine in Portugal. *Nematology*, 6: 437-453.

Nematology, 2004, Vol. 6(3), 437-453

Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) associated with maritime pine in Portugal

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Summary – Species of *Bursaphelenchus* associated with maritime pine, *Pinus pinaster*, from Portugal – within and outside the quarantine restricted demarcated zone of *B. xylophilus* – are described and characterised both morphologically (LM and SEM) and with the use of molecular biology (ITS-RFLP). A new staining method for spicules is proposed. Species include *B. hellenicus*, *B. hylobianum*, *B. leoni*, *B. pinophilus*, *B. sexdentati*, *B. tusciae*, *B. teratospicularis*, *B. xylophilus* and *Bursaphelenchus* sp. 1. *Bursaphelenchus hylobianum* was collected from the insect *Hylobius* sp. The most frequent species in the demarcated zone, besides *B. xylophilus*, was *Bursaphelenchus* sp. 1. Morphological characterisation is compared with the original descriptions and discussed. The differentiation between *B. pinophilus* and *B. sexdentati* is not clear in the literature and is discussed. Since differentiation of *B. xylophilus* (mucronate form) from *B. mucronatus*, and *B. pinophilus* from *B. sexdentati*, as well as their juvenile forms, is almost im possible on the basis of morphological features, a molecular approach based on ITS-RFLPs was used. Ribosomal DNA containing the 5.8S gene, the internal transcribed spacer region 1 and 2, and partial regions of 18S and 28S gene were amplified by PCR. Restriction profiles of the amplified products generated species-specific differences, leading to the unambiguous identification of isolates belonging to *B. xylophilus*, *B. mucronatus*, *B. sexdentati*, *B. tusciae* and *B. hylobianum*.

Keywords – diagnostics, identification, ITS-RFLP, morphology, *Pinus pinaster*, survey.

During a survey conducted within a national Praxis XXI research project no. 11 189/98, 'Survey and study of *Bursaphelenchus* and other nematode species associated with cerambycid insects in pine trees in Portugal', the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle 1970, was detected in March of 1999, associated with maritime pine (*Pinus pinaster* Aiton). This represented the first report of this important pest and pathogen in Portugal and Europe (Mota *et al.*, 1999).

Official authorities implemented an intensive survey of the region where the nematode was detected and later expanded it to the rest of the country within a National Programme of Pinewood Nematode Control (PROLUNP, Programa Nacional de Luta contra o Nemátode do Pinheiro – http://www.dgf.min-agricultura.pt/prolunp/html/home-final.htm). As a result, this A1 quarantine organism was confirmed as being restricted within an area in the Setúbal region to the southeast of Lisbon. This infested area has been precisely delimited by annual surveys and a buffer zone, about 20 km wide, was established and included in a demarcated zone subject to restrictive quarantine measures. Since then, in addition to an intensive annual survey of the demarcated zone, the survey programme has also been carried out in the rest of the country (Mota & Vieira, 2003).

About 25 species of *Bursaphelenchus* associated with conifers have been reported in Europe (Braasch, 2001; Mota & Vieira, 2004). Some of these species, such as *B. mucronatus* Mamiya & Enda, 1979 and *B. xylophilus*, display similar morphological features which may con-

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found precise identification, a crucial step in establishing quarantine measures.

Morphology is an essential component of nematode differentiation and, in many cases, provides a rapid and unambiguous species diagnosis. However, this identification is highly dependent on the experience and interpretative skills of the researcher. In some groups of nematodes, morphological characters are only a first approach and require confirmation by more accurate methods. Differences in nucleic acid sequences, as revealed by means of molecular biology techniques such as ITS-RFLP (Hoyer *et al.*, 1998; Braasch *et al.*, 1999), may help to characterise each species and complement its morphological description. Furthermore, DNA-based methods provide an attractive solution to problems associated with morphological identification, since they do not rely on the expression of the genome and are independent of environmental influence or developmental stage.

The purpose of this research was to survey and identify the *Bursaphelenchus* species associated with maritime pine in Portugal by means of morphological and molecular analysis (PCR-ITS RFLP).

Materials and methods

During the annual surveys carried out by PROLUNP, a total of 4810 samples of pine wood material from trees displaying symptoms of pine wilt disease, corresponding to a small proportion of all samples, were examined for *Bursaphelenchus* species in the nematology laboratory of EAN. Nematodes were extracted using a modified Baermann funnel technique. In addition, 62 specimens of *Hylobius* sp., collected from *Pinus pinaster*, were crushed in a Syracuse dish and examined under a stereoscopic microscope for possible *Bursaphelenchus* dauer juveniles. These were inoculated into 15 cm pine branch segments and *Botrytis cinerea* Petri dishes, and incubated for 1 month at 26°C.

Nematode identification was based on observations of the main morphological characters, particularly vulval flap, shape of spicules, female tail (Braasch, 2001) and head shape using light (LM) and scanning electron microscopy (SEM). For spicule observation, a new staining method was tested with the use of Rotring® Brilliant Ultramarine Blue ink (stock solution = one 0.5 ml cartridge in 20 ml of acetic acid; prepare a final solution by diluting 1 ml of stock solution in 10 ml of lactophenol). Live nematodes were transferred to a drop of this solution and heated briefly over an alcohol lamp. This staining method was applied to *B. hellenicus* Skarmoutsos, Braasch & Michalopoulou, 1998 and *B. sexdentati* which have inconspicuous spicules. For SEM studies, nematodes were fixed in a mixture of 4% gluteraldehyde/2% formaldehyde for several days, post-fixed in 2% OsO_4 overnight, dehydrated in an ethanol series, critical point dried and sputter coated with gold (Eisenback, 1985). Observations were made with a Jeol 35 SEM.

After extraction from wood, aliquots of one to ten nematodes were stored for DNA extraction. Nematodes were heated at 95°C for 5 min, homogenised on a glass slide with a micro pestle (Eppendorf®) and DNA obtained using the DNeasy Tissue Kit (Quiagen®). This procedure was applied to different nematode life stages, namely adult, dauer juvenile, propagative juvenile and resistant juvenile.

The ITS regions of rDNA were amplified using primers F194 and P5368 as described by Ferris *et al.* (1993) and Vrain (1993), respectively. All polymerase chain reactions were performed in a final volume of 50 μl using 10 ng/μl of template DNA, 1 μM of each primer, 0.2 μM of dNTPs (Invitrogene®), 2 U of *Taq* DNA polymerase (Invitrogene®), 1× Reaction Buffer (Invitrogene®) and 1.25 mM of MgCl2 (Invitrogene®). The reaction mixture was overlaid with sterile mineral oil to prevent evaporation during PCR cycling. A Stratagene® Robocycler was used for amplification and the reaction consisted of one denaturation step at 94°C for 1 min, 35 cycles with denaturation at 94°C for 1 min, annealing at 51°C for 1 min, polymerisation at 72°C for 2 min and a final extension step at 72°C for 5 min. After PCR, 5 μl of amplified product was analysed by electrophoresis in a 1% agarose gel. Data analysis was performed using the Kodak® 1D 2.0 system and 100 bp DNA Ladder (Invitrogene®) as a molecular size marker.

Restriction analysis of ITS regions was performed with *Alu*I, *Hae*III and *Rsa*I restriction endonucleases (Invitrogene®), using an aliquot of 4 μl of the PCR product and 10 U of each enzyme, according to the manufacturer's instructions. Fragments were resolved by electrophoresis in a 2% agarose gel and data were analysed as described above.

Results

The geographic distribution of *Bursaphelenchus* species in Portugal is presented in Table 1 and Figure 1. Morphological identification was based on original and other descriptions. The species are illustrated by light and scan-

Table 1. *Origin and location of* Bursaphelenchus *species in Portugal.*

No.	Species	Host	Location
1	*B. hellenicus*	*Pinus pinaster*	Samora Correia, Santarém Alcácer do Sal, Setúbal Melides, Sines Castro Daire, Viseu
2	*B. hylobianum*	*Hylobius* sp.	Leiria
3	*B. leoni*	*P. pinaster*	Ovar, Aveiro Sertã, C. Branco Oliveira Hospital, Coimbra Oeiras, Lisboa Marco de Canaveses, Porto Ferreira Zêzere, Santarém Rio Maior, Santarém S. Correia, Santarém Aiana, Setúbal Alcácer do Sal, Setúbal Azeitão, Setúbal Casal do Marco, Setúbal Grândola, Setúbal Santana, Setúbal Melides, Sines Sabrosa, Vila Real Castro Daire, Viseu Nelas, Viseu Mangualde, Viseu
4	*B. mucronatus*	*P. pinaster*	Figueira da Foz, Coimbra
5	*B. sexdentati*	*P. pinaster*	Figueira da Foz, Coimbra Póvoa do Varzim, Porto S. Correia, Santarém Grândola, Setúbal Mangualde, Viseu
5a	*Bursaphe-lenchus* spp.*	*P. pinaster*	Aveiro, Aveiro Caminha, Braga Fafe, Braga Paredes de Coura, Braga Sertã, C. Branco O. Hospital, Coimbra Guadalupe, Évora Vendas Novas, Évora Faro, Faro Pinhel, Guarda Pedrógão Grande, Leiria Loures, Lisboa Oeiras, Lisboa Rio Maior, Santarém Salvaterra Magos, Santarém Canha, Setúbal Carvalhal, Setúbal Montijo, Setúbal Melides, Sines Sabrosa, Vila Real Vila Real, Vila Real Castro Daire, Viseu Mangualde, Viseu Moimenta da Beira, Viseu Nelas, Viseu
6	*B. teratospi-cularis*	*P. pinaster*	Sertã, C. Branco Sertã, C. Branco Biscainho, Santarém Azeitão, Setúbal Grândola, Setúbal Marateca, Setúbal Melides, Sines Santo André, Sines Sabrosa, Vila Real Mangualde, Viseu
7	*B. tusciae*	*P. pinaster*	Figueira da Foz, Coimbra Faro, Faro Coruche, Santarém F. Salvaterra, Santarém Grândola, Setúbal Melides, Sines Castro Daire, Viseu Mangualde, Viseu
8	*B. xylophilus*	*P. pinaster*	Infested zone
9	*Bursaphe-lenchus* sp. 1	*P. pinaster*	Vila Flor, Bragança Cabrela, Évora Ota, Lisboa Biscainho, Santarém Coruche, Santarém F. Salvaterra, Santarém S. Magos, Santarém Samora Correia, Santarém S. do Mato, Santarém S. Estêvão, Santarém Amora, Setúbal Alcácer do Sal, Setúbal Carvalhal, Setúbal Comporta, Setúbal Corroios, Setúbal C. Caparica, Setúbal Feijó, Setúbal Grândola, Setúbal Santiago Cacém, Setúbal S. Martinho, Setúbal Melides, Sines Castro Daire, Viseu

* *B. pinophilus* or *B. sexdentati* (subject to confirmation; see Discussion for more details).

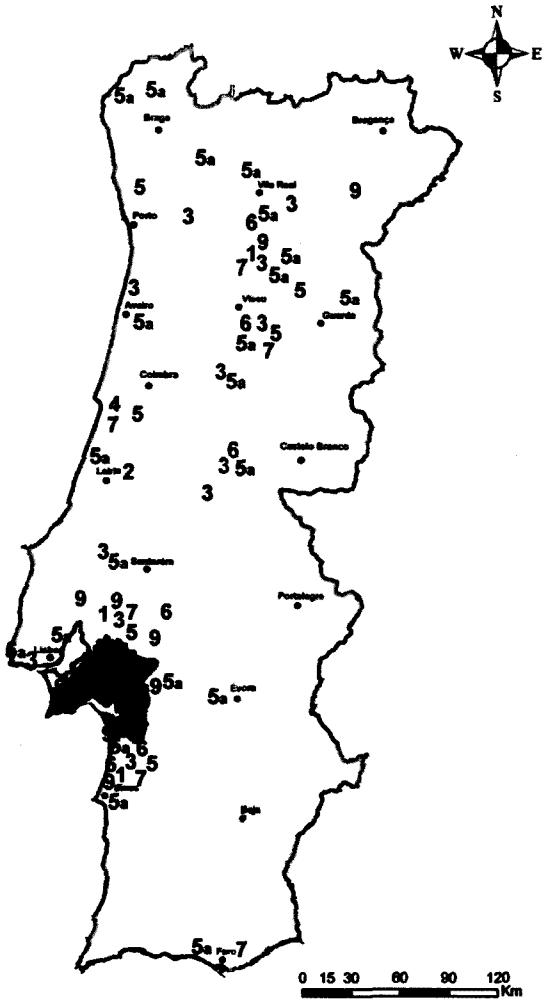

Fig. 1. *Occurrence of* Bursaphelenchus *species in continental Portugal. 1:* B. hellenicus*; 2:* B. hylobianum*; 3:* B. leoni*; 4:* B. mucronatus*; 5:* B. sexdentati*; 5a:* Bursaphelenchus *spp.; 6:* B. teratospicularis*; 7:* B. tusciae*; 8:* B. xylophilus*; 9:* Bursaphelenchus *sp. 1 (see Table 1 for details of locations).*

ning electron microscopy (Figs 2-10). Besides *B. xylophilus* (Fig. 9), other *Bursaphelenchus* species were collected and identified from pine wood, namely: *B. hellenicus* (Fig. 2); *B. leoni* Baujard, 1980 (Fig. 4); *B. mucronatus* (Fig. 5); *B. teratospicularis* Kakuliya & Devdariani, 1965 (Fig. 7); *B. tusciae* Ambrogioni & Palmisano, 1998 (Fig. 8); *B. sexdentati* Rühm, 1960 (Fig. 6) and *Bursaphelenchus* sp. 1 (Fig. 10). The presence of *B. pinophilus* Brzeski & Baujard, 1997 in Portugal was previously confirmed by Braasch (2001). ITS-RFLP analysis of some populations suspected to be *B. sexdentati* and/or *B. pinophilus* produced the characteristic pattern for *B. sexdentati*. However, it was not always possible to distinguish clearly between *B. pinophilus* and *B. sexdentati*, the nematodes in such cases being referred to as *Bursaphelenchus* spp. One of the species found, *Bursaphelenchus* sp. 1, was very similar to *B. pinasteri* Baujard, 1980, although it exhibits some characters, such as head and spicule shape and number of incisures in lateral fields, identical to *B. hofmanni* Braasch, 1998. Morphological characters used to diagnose the different *Bursaphelenchus* species are listed and compared in Table 2.

The staining method with blue ink allowed the spicule shape of *B. hellenicus* and *B. sexdentati* to be clearly seen (Figs 2, 6).

The number of samples in which each *Bursaphelenchus* species was found is presented in Table 3. Populations levels were usually low. Apart from *B. xylophilus*, the most frequent species in the Demarcated Zone was *Bursaphelenchus* sp. 1, while *B. mucronatus* was only found in one sample.

Nine *Hylobius* sp. contained dauer juveniles of *Bursaphelenchus* sp. under the elytra. Adult nematodes collected from *B. cinerea* plates and pine branches, previously inoculated with these dauer juveniles, were identified as *B. hylobianum* Korenchenko, 1980 (Fig. 3). As in the original description from Russia (Korenchenko, 1980), this nematode was found in Portugal associated with *Hylobius* beetles.

In addition to morphological observations, ITS-RFLP analysis was employed for confirmation and differentiation of *B. xylophilus, B. mucronatus, B. sexdentati, B. tusciae* and *B. hylobianum*. Amplification of ITS regions yielded a single DNA fragment of 950 bp for *B. xylophilus, B. mucronatus* and *B. tusciae*, of 1100 bp for *B. sexdentati* and of 1150 bp for *B. hylobianum*. Subsequent analysis of ITS regions with *Alu*I, *Hae*III and *Rsa*I endonucleases produced characteristic restriction profiles that clearly differentiated these five *Bursaphelenchus* species (Fig. 11). All collected mucronate forms of *B. xylophilus* individuals displayed the characteristic restriction pattern of this species, thereby allowing for their differentiation from *B. mucronatus*. Similarly, all propagative and resistant juveniles displayed the specific *B. xylophilus* pattern. Specific patterns were also obtained for dauer juveniles of both *B. xylophilus* and *B hylobianum*.

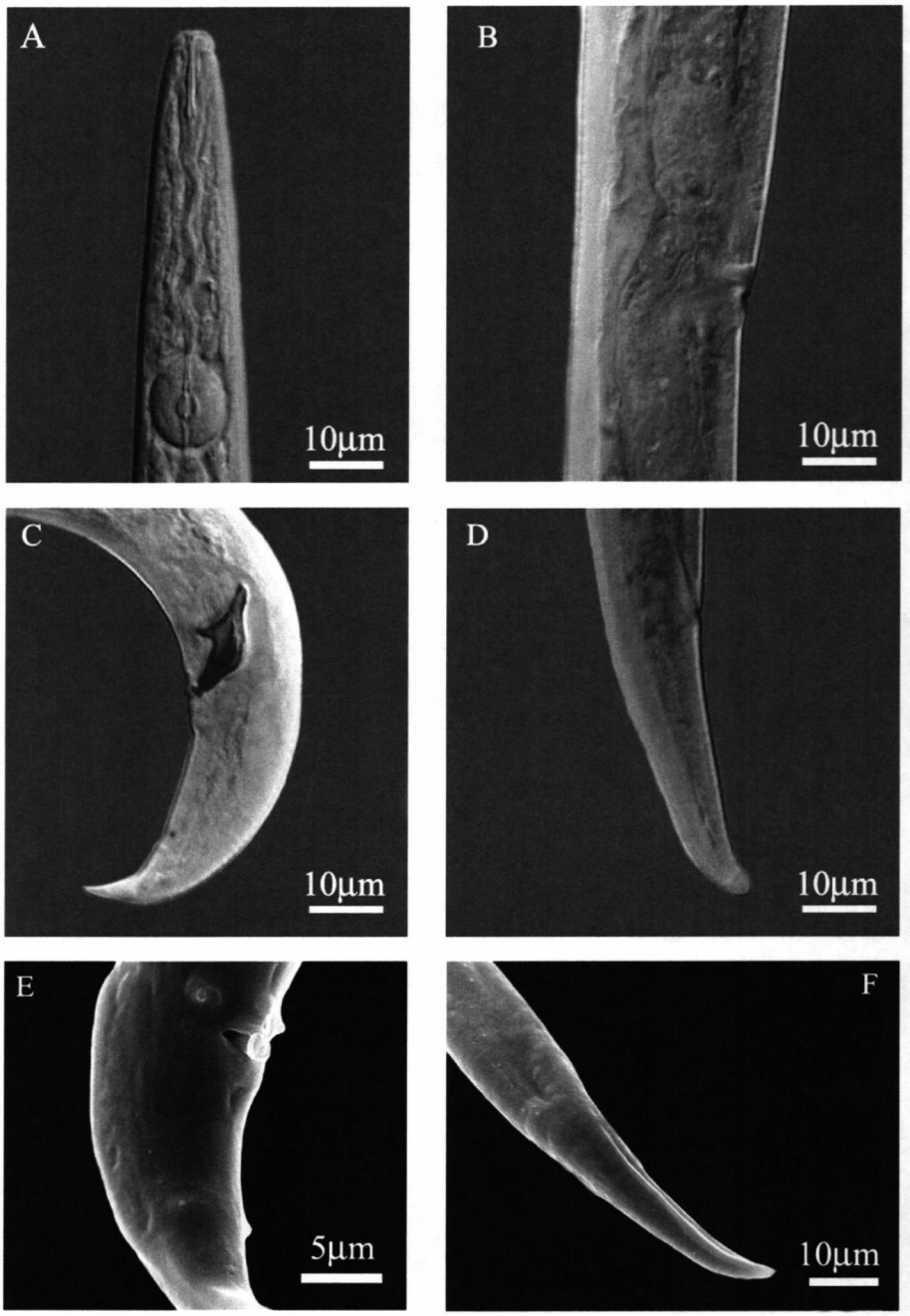

Fig. 2. Bursaphelenchus hellenicus. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail (stained spicule); D: LM of female tail; E: Scanning electron micrograph (SEM) of male tail showing caudal papillae and protracted cucullus of spicules; F: SEM of female tail.*

Table 2. *Diagnostic morphological characters for species of* Bursaphelenchus *occurring in Portugal.*

Species	Cuticle annulation	No. of lateral incisures	Lip region	Vulva	Female tail	Spicule	Male caudal papillae
B. hellenicus	Fine	3	High, rounded, set off by constriction	Anterior lip slightly extended to form very small flap	Conoid with more a less rounded terminus, slightly ventrally bent	Small, rosethorn-shaped; rostrum prominent, blunt; apex well developed, elongate, bluntly rounded; distinct disc-like cucullus	Single pre-anal papilla; one pair adanal; one ventral post-anal and smaller, ventral, pair at beginning of bursa (not always visible)
B. hylobianum	Fine	2	High, rounded, set off by constriction	Anterior lip slightly extended to form small, distinct, flap	Conoid, gradually tapering with terminus bluntly rounded to acute	Robust, rosethorn-shaped, strongly curved; prominent rostrum not sharply pointed; apex well developed; disc-like cucullus (not always distinct)	Single ventral preanal papilla; one pair adanal; one pair post-anal and one small pair at beginning of bursa
B. leoni	Fine	3	Rounded, well set off by deep constriction	Anterior lip extended to form short flap; area posterior to flap often swollen	Very long, conoid, terminus variable in shape, finely rounded, sometimes slightly digitate or with a slight constriction	Medium to large with distinct, pointed, rostrum; apex well developed and dorsally hooked; distal end with slight hook-like process (not always distinct)	Single ventral pre-anal papilla; one pair adanal; one pair post-anal and one small pair at beginning of bursa
B. mucronatus	Fine	4	Rounded, set off by constriction	Anterior lip forming long flap overlapping vulva	Subcylindrical, rounded, with very long mucron	Large, limb long and strongly curved with transverse bar of capitulum almost parallel to shaft axis; apex bluntly rounded, rostrum prominent and pointed; distinct disc-like cucullus	Single ventral pre-anal papilla, one pair adanal and two contiguous post-anal pairs
B. sexdentati Bursaphelenchus spp.*	Fine	4	Rounded, set off by constriction	Anterior lip slightly extended to form small flap; post vulval swelling often present	Conoid, gradually tapering, with variable rounded or slightly digitate terminus	Rosethorn-shaped, medium size; rostrum prominent, pointed; apex well developed, broadly rounded-squared; distinct knob-like cucullus	Single ventral pre-anal papilla; one pair subventral adanal; two post-anal pairs

Table 2. *(Continued).*

Species	Cuticle annulation	No. of lateral incisures	Lip region	Vulva	Female tail	Spicule	Male caudal papillae
B. teratospicularis	Coarse	Not known	Flattened, slightly set off by weak constriction	Vulval lips only protruding very slightly; flap absent	Conical, with broadly rounded terminus	Mitten-shaped with pointed rostrum; apex well developed, bluntly rounded often forming a dorsally directed curve to shaft; distal tip dorsally curved, no cucullus	Adanal pair; one post-anal subventral pair at beginning of bursa
B. tusciae	Fine	3	Rounded, set off by distinct constriction	Anterior lip slightly extended to form small flap	Very long, conoid, often ventrally bent with variable terminus (rounded or slightly digitate, usually curved and hook-like)	Relatively long, centre of capitulum depressed; rostrum prominent, pointed; apex small, rounded (often dorsally bent like a small hook); no cucullus	Single ventral pre-anal papilla; one pair pre-anal; one pair ventral post-anal; one small ventral pair at beginning of bursa
B. xylophilus	Fine	4	Rounded, set off by constriction	Anterior lip forming long flap overlapping vulva	Subcylindrical with rounded terminus occasionally bearing short mucron	Large, limb long and strongly curved with transverse bar of capitulum almost parallel to shaft axis; apex bluntly rounded; rostrum prominent and pointed; distinct disc-like cucullus	Single ventral pre-anal papilla; one pair adanal and two contiguous post-anal pairs
Bursaphelenchus sp. 1	Fine	3	High, rounded, slightly set off by weak constriction	Anterior lip forming a small flap	Conoid, narrowing abruptly just behind anus and gradually tapering to pointed terminus	Rosethorn-shaped not strongly curved; rostrum prominent, more or less pointed; apex rounded, almost as long as rostrum; distinct cucullus absent	Single ventral pre-anal papilla; two subventral pairs, one adanal; one post-anal near tail terminus

* *B. pinophilus* or *B. sexdentati* (subject to confirmation; see Discussion for more details).

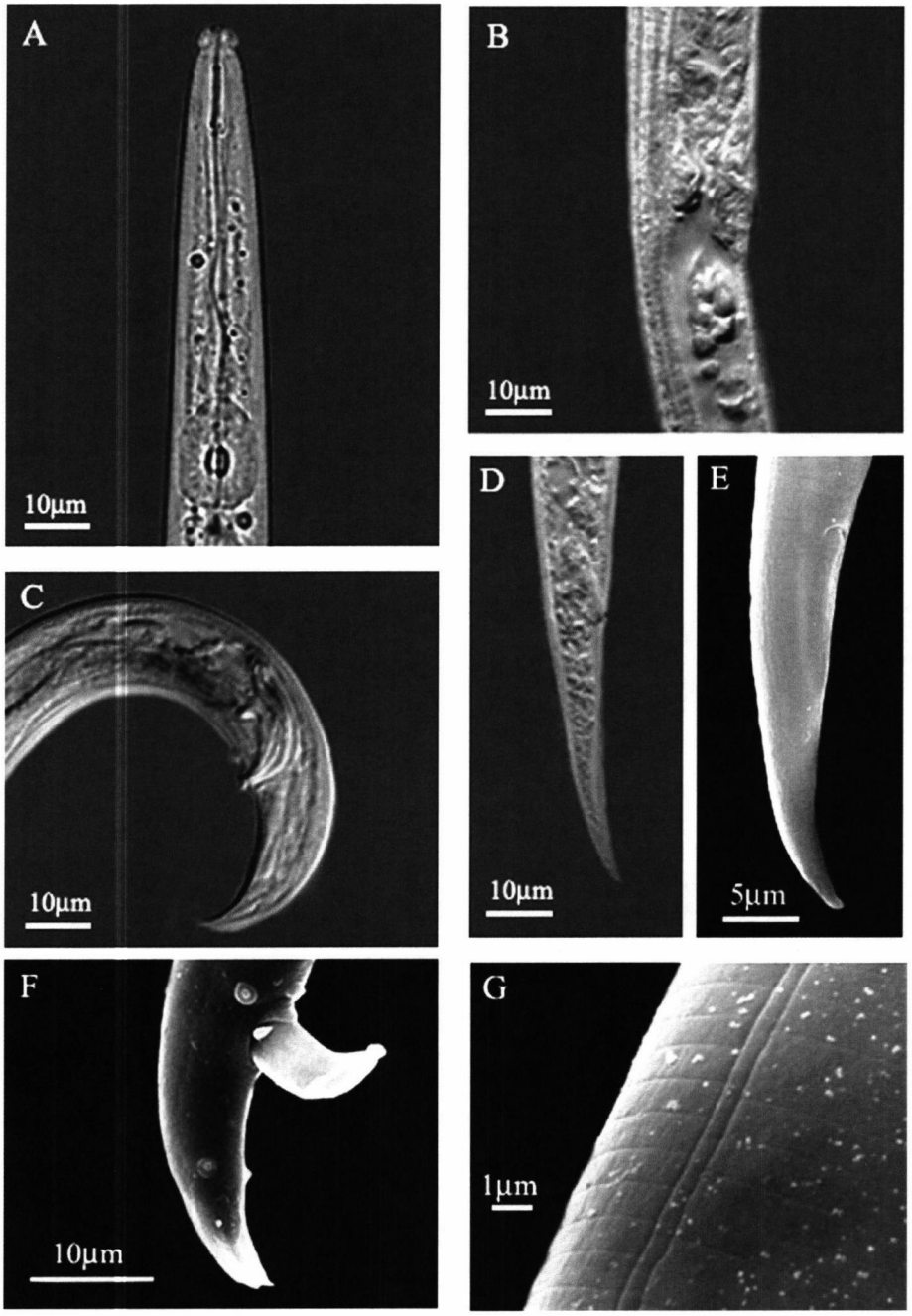

Fig. 3. Bursaphelenchus hylobianum. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; D: LM of female tail; E: Scanning electron micrograph (SEM) of female tail; F: SEM of male tail with protracted spicule and caudal papillae; G: SEM of lateral field.*

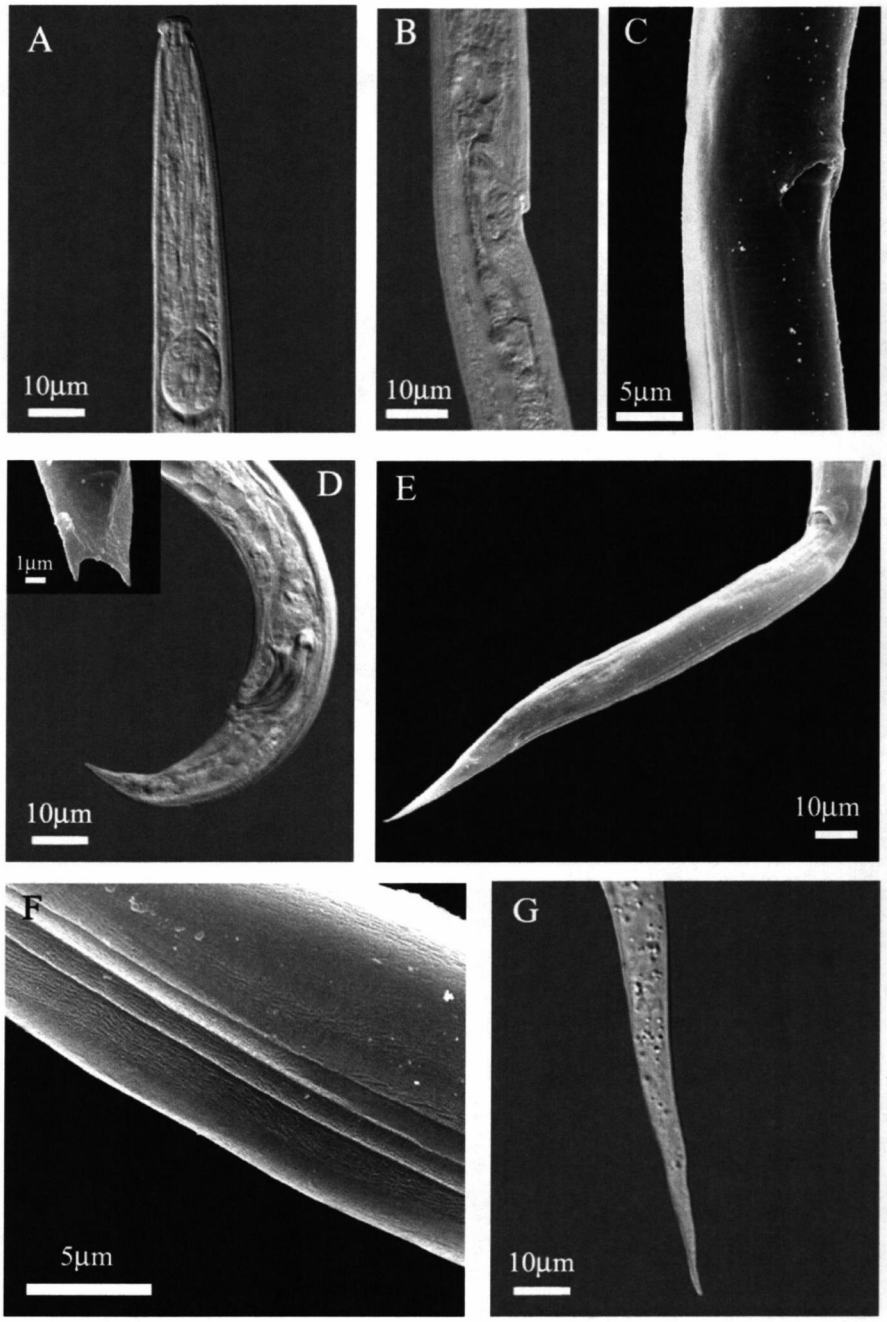

Fig. 4. Bursaphelenchus leoni. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: Scanning electron micrograph (SEM) of vulval region; D: LM of male tail; inset: SEM of bursa; E: SEM of female tail; F: SEM of lateral field; G: LM of female tail.*

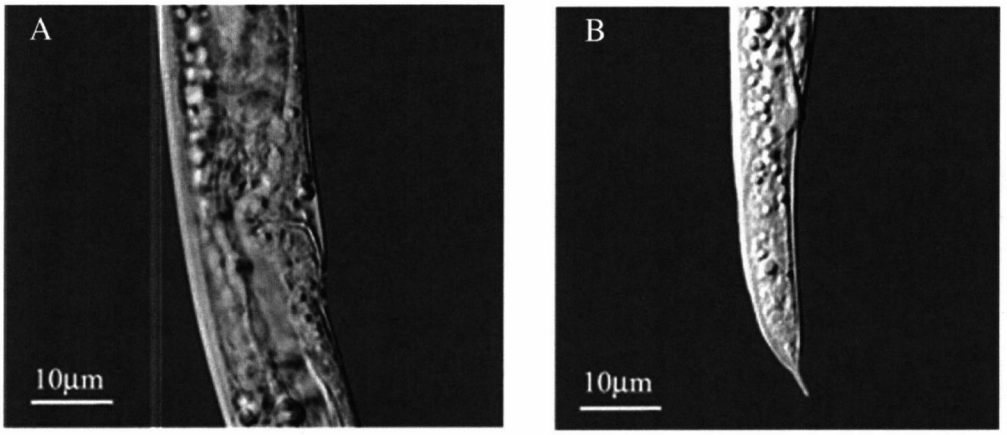

Fig. 5. Bursaphelenchus mucronatus. *A: Light micrograph (LM) of vulval region; B: LM of female tail.*

Table 3. *Number of wood samples containing species of* Bursaphelenchus *within the National Survey programme and the Demarcated Zone.*

Bursaphelenchus species	National Survey (1999-2002) 1129 samples	Demarcated Zone (1999-2003) 3681 samples
B. hellenicus	1	4
B. hylobianum	0	0
B. leoni	30	15
B. mucronatus	1	0
B. sexdentati	5	–
Bursaphelenchus spp.*	24	17
B. teratospiculatis	4	11
B. tusciae	7	10
B. xylophilus	0	1422**
Bursaphelenchus sp. 1	3	134

* *B. pinophilus* or *B. sexdentati* (subject to confirmation; see Discussion for more details).
** Results of a partial survey; for more detailed information see: http://www.dgf.min-agricultura.pt/prolunp/html/home-final.htm

Discussion

The greater number of *Bursaphelenchus* species found in northern and central Portugal reflect the higher density of maritime pine forest from those regions whereas the large number of species within the demarcated zone simply reflects a more intensive survey.

Tail and head shape, appearance of the vulval region and particularly the shape and comparative size of the spicules were sufficient for the separation of most species. The presence of round-tailed females with a well developed vulval flap, together with males having the typical spicule shape, provided a definitive morphological identification for *B. xylophilus*.

Differentiation based solely on morphological characters is not reliable for some species. Some Portuguese *B. xylophilus* populations have mucronate-tailed females, a feature which may result in confusion of this species with *B. mucronatus*. ITS-RFLP analysis, however, discriminates the mucronate form of *B. xylophilus* from *B. mucronatus* (Tarès *et al.*, 1992).

Concerning the differentiation of the species *B. sexdentati* and *B. pinophilus*, Rühm (1960), in the original description of *B. sexdentati*, did not mention the presence of a cucullus. Brzeski and Baujard (1997) did not diagnose *B. pinophilus* in comparison to *B. sexdentati*, although Braasch (2001) distinguished the two species on the basis of the presence of a cucullus in *B. pinophilus*. However, using SEM techniques, Ambrogioni and Caroppo (1998) showed *B. sexdentati* to have a distinct cucullus. Although, most of our populations exhibited spicules with a distinct cucullus, something which would seem to indicate that they belong to *B. pinophilus*, ITS-RFLP analysis of these same populations produced the characteristic pattern for *B. sexdentati*. In addition to clarifying morphological identification of nematodes, this molecular method also facilitates the identification of juvenile stages of *Bursaphelenchus* species, thereby eliminating the need for

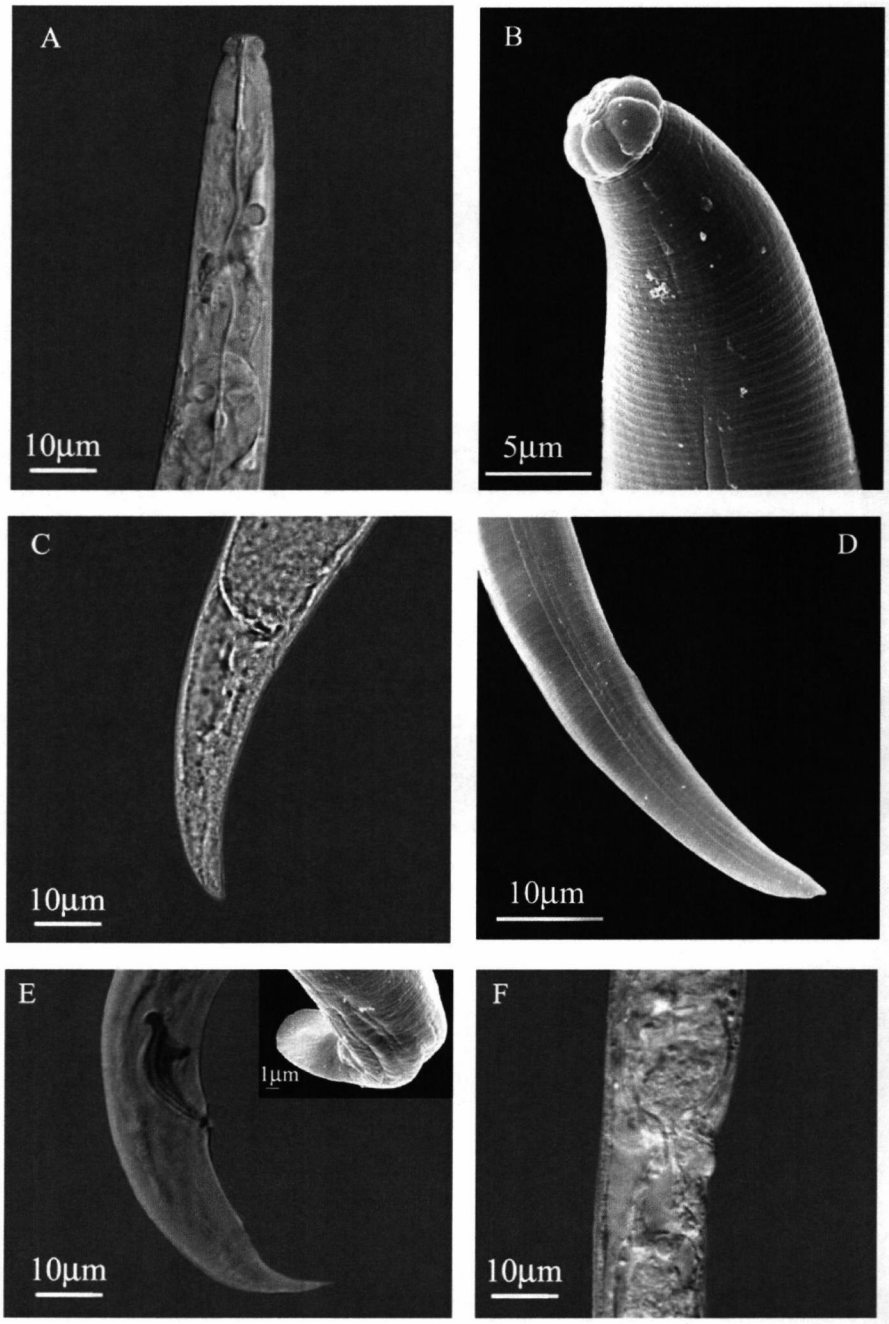

Fig. 6. Bursaphelenchus sexdentati. *A: Light micrograph (LM) of anterior region; B: Scanning electron micrograph (SEM) of anterior region; C: LM of female tail; D: SEM of female tail; E: LM of mail tail (stained spicule); inset: SEM of bursa; F: LM of vulval region.*

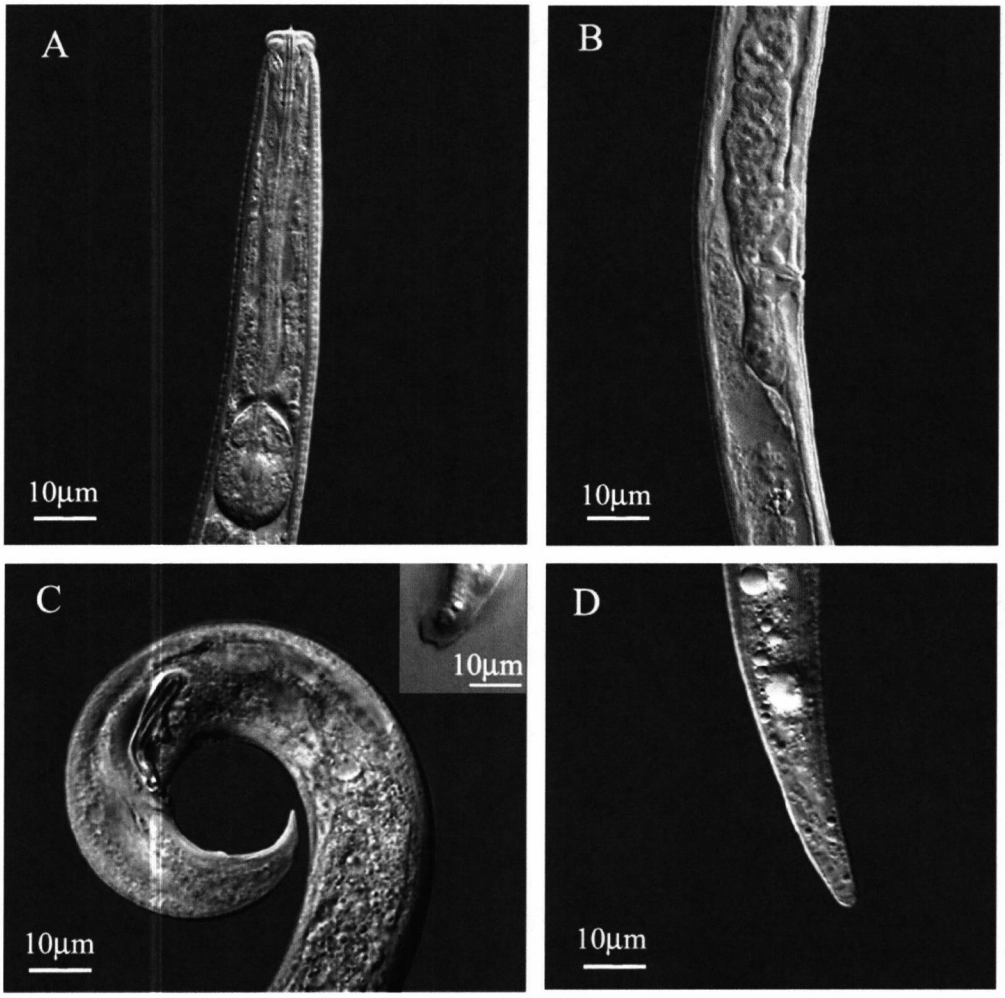

Fig. 7. Bursaphelenchus teratospicularis. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; inset: LM of bursa; D: LM of female tail.*

culturing to the adult stages and increasing both the speed and reliability of the diagnosis. Furthermore, considering the abundance of rDNA in the genome, this method can be effectively applied to individual nematodes, a significant advantage.

As confirmed by the results of this investigation, ITS-RFLP analysis is a more direct approach for identification of *Bursaphelenchus* species, since specific patterns of restriction fragments have been obtained for each of the five species studied. Although preliminary species determination can be reached by estimation of the number and size of the restriction fragments obtained (Braasch *et al.*, 2001), the use of one reference sample for each species under consideration is strongly recommended. In fact, apart from some inaccuracy that may occur in the estimation of fragment size under different electrophoretic conditions, unoptimised restriction conditions may lead to partial or incomplete digestions making it difficult for correct species allocation of otherwise unidentified isolates.

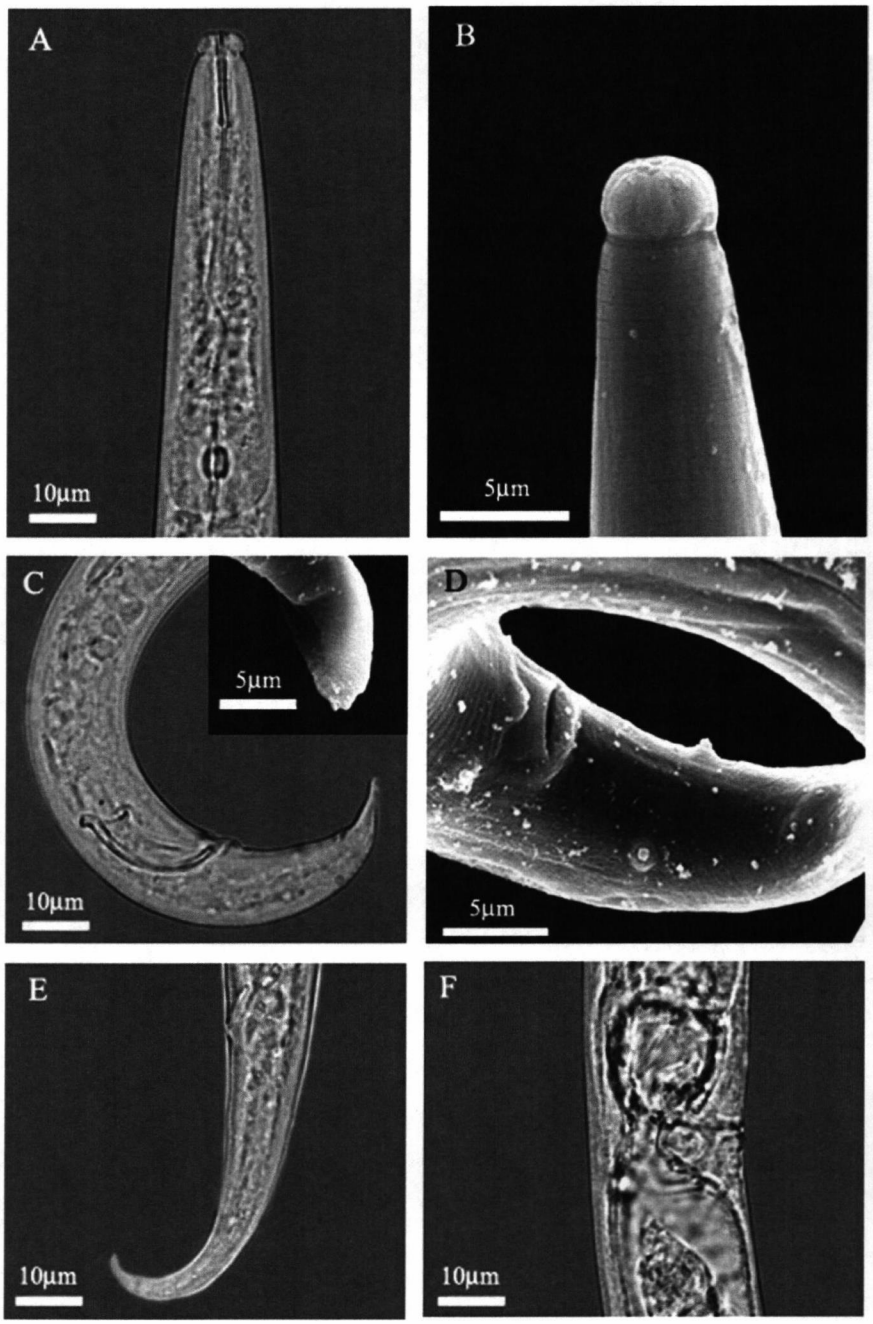

Fig. 8. Bursaphelenchus tusciae. *A: Light micrograph (LM) of anterior region; B: Scanning electron micrograph (SEM) of anterior region; C: LM of male tail; inset: SEM of bursa; D: SEM of male tail showing caudal papillae; E: LM of female tail; F: LM of vulval region.*

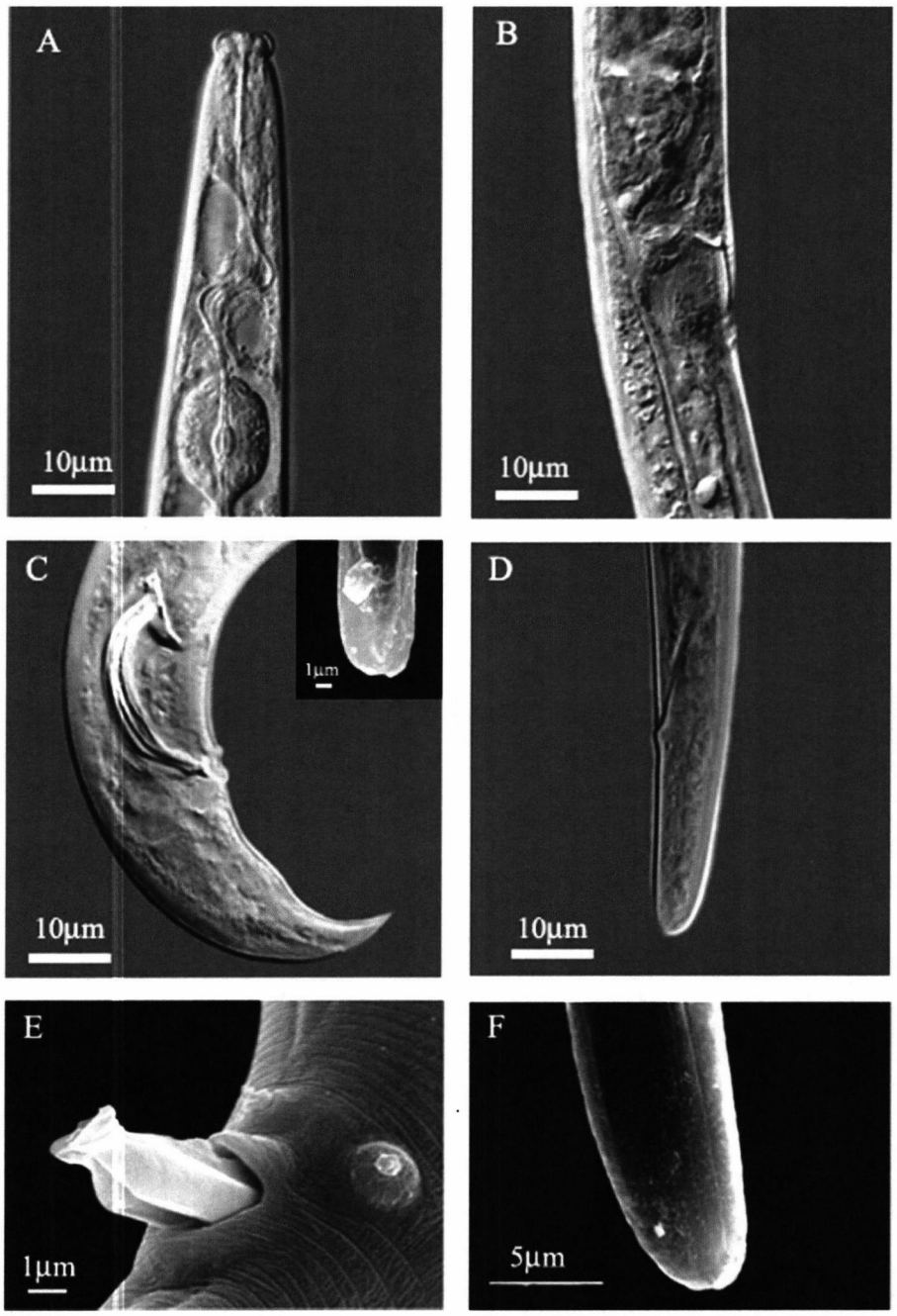

Fig. 9. Bursaphelenchus xylophilus. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; inset: Scanning electron micrograph (SEM) of bursa; D: LM of female tail; E: SEM of protracted spicule and cucullus; F: SEM of female tail tip.*

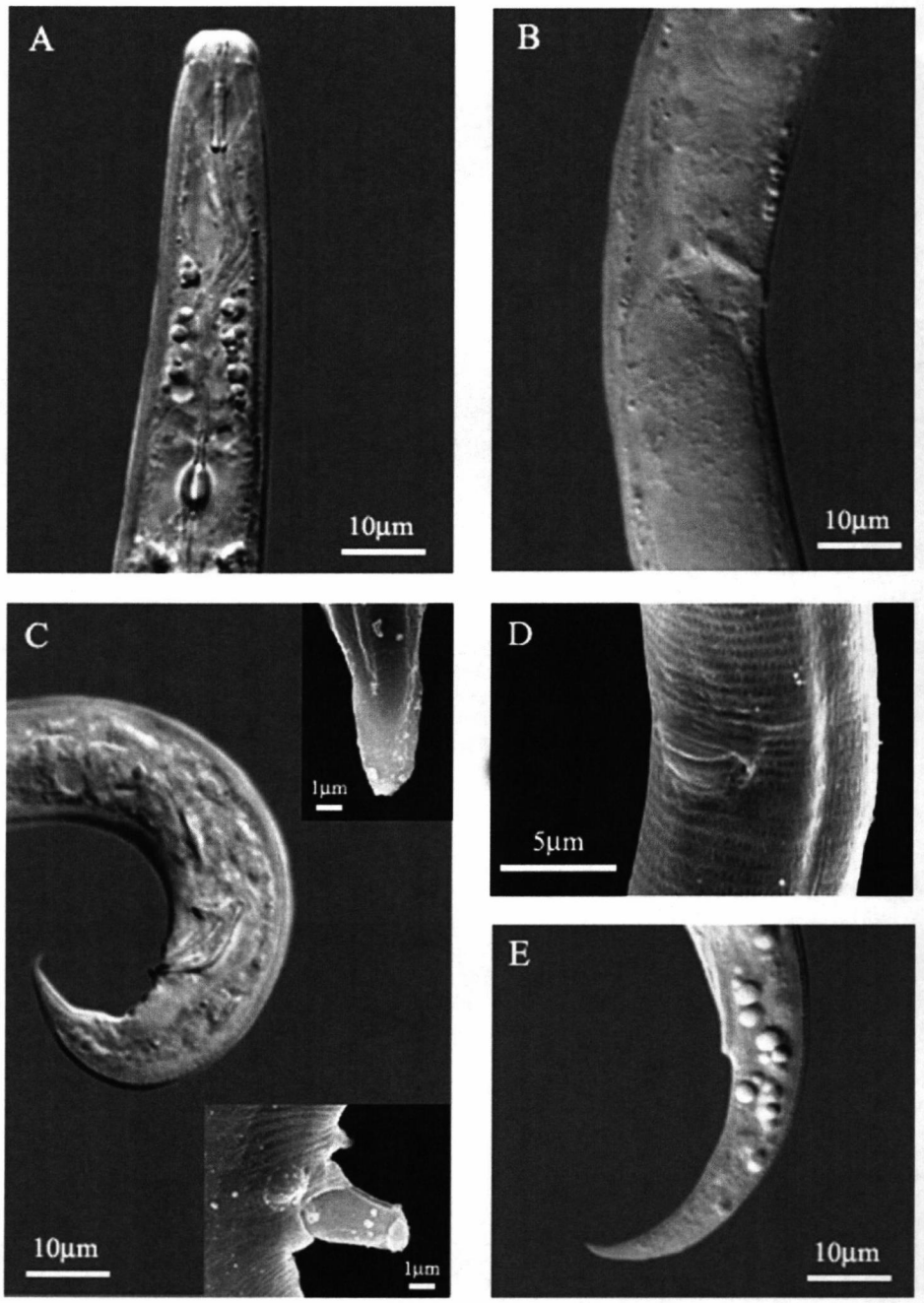

Fig. 10. Bursaphelenchus *sp. 1. A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; inset: Scanning electron micrograph (SEM) of bursa (top) and SEM of spicule protracted and cucullus (bottom); D: SEM of vulval region; E: LM of female tail.*

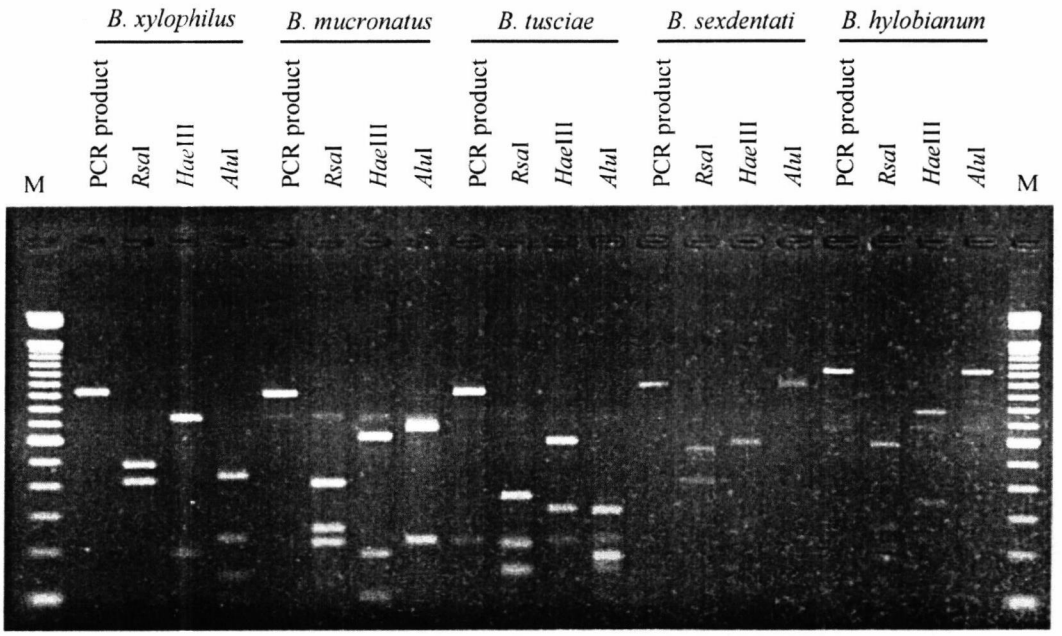

Fig. 11. *ITS-RFLP patterns of* Bursaphelenchus *isolates. Restriction fragments were obtained by digestion of the amplified ITS region of rDNA with the three named enzymes. M: 100 bb Marker (Invitrogene).*

Regardless of the underlying structural basis of polymorphism among populations, it is apparent that these genetic differences can discriminate within populations and are convenient diagnostic markers. Overall, ITS-RFLP has been established as a powerful and reproducible tool in the differentiation of many *Bursaphelenchus* species (Hoyer *et al.*, 1998; Braasch *et al.*, 1999) and has become an important feature in nematode diagnosis. ITS versatility, specificity, ease of experimental manipulation and availability of ITS databases should be increasingly applied in nematology.

This study, based on both morphological and molecular analysis, confirmed the identity of several *Bursaphelenchus* species reported from Portugal for the first time by Penas *et al.* (2002). Penas *et al.* (2002) had identified *B. hofmanni* from Portugal, although more recent studies have called into question the reliability of this initial identification, the species now being referred to as *Bursaphelenchus* sp. 1.

The ITS-RFLP patterns, as presented in this paper, were done whenever nematode numbers allowed for such analysis, or when morphological identification was doubtful. A more detailed characterisation of all Portuguese species, including morphology, morphometrics and DNA analysis is being conducted and will soon be published. *Bursaphelenchus hylobianum*, found for the first time in Europe (Penas *et al.*, 2002) and never previously observed in pine wood from natural stands, was successfully reared in maritime pine branch segments and may therefore be considered as a potential associate of *Pinus pinaster*. Other *Bursaphelenchus* species, in addition to *B. hylobianum*, were found associated with potential insect vectors (unpubl.).

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Sub - chapter III.2

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

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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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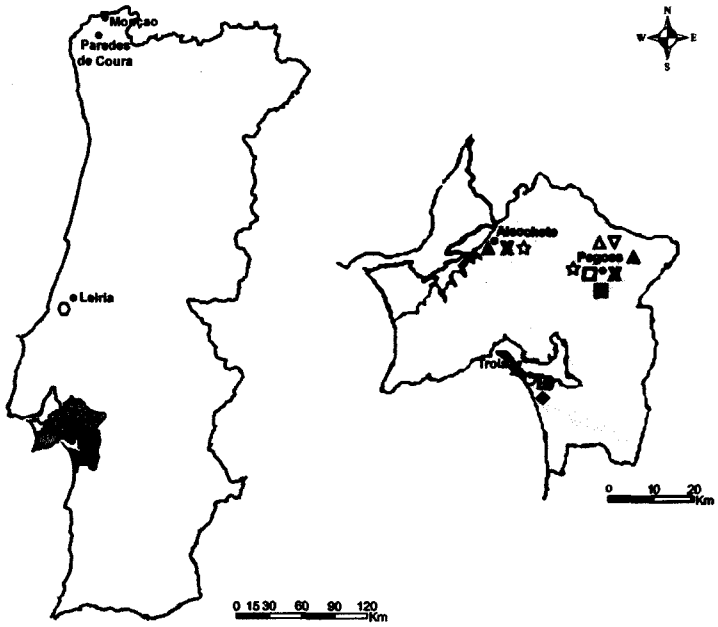

Figure 1 Sampling sites (•) and distribution of *Bursaphelenchus*–insect associations. ▩: region affected by *B. xylophilus*. Associations: *Bursaphelenchus* sp.–*Hylobius* sp. (○); *B. xylophilus*–*M. galloprovincialis* (x); *B. teratospicularis*–*O. erosus* (◯); *B. sexdentati*–*O. erosus* (□); *B. 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 teratospiculoris* *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).

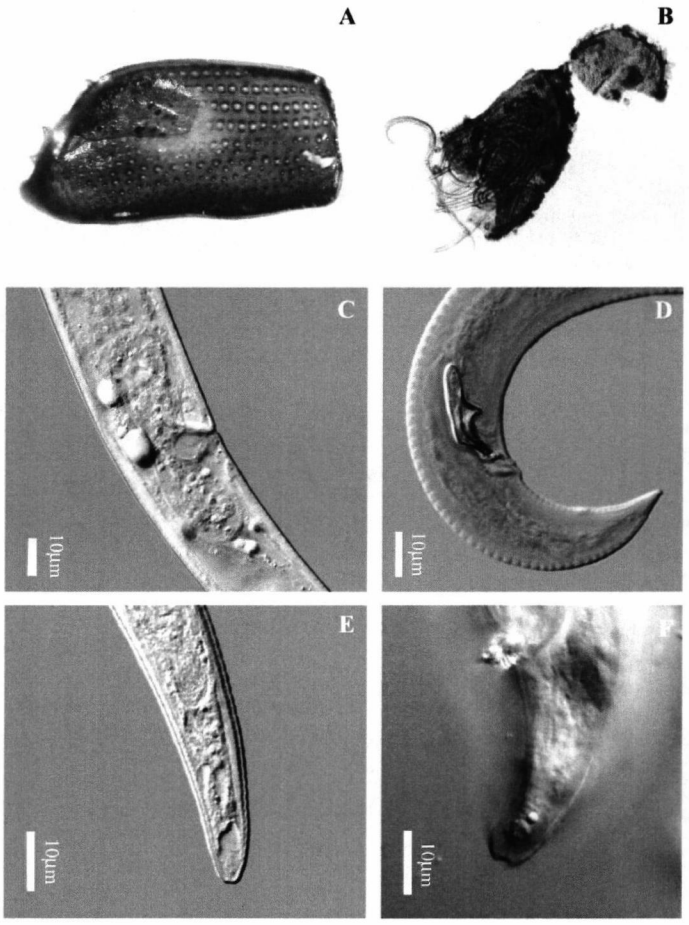

Figure 2 Adults of *Bursaphelenchus teratospicularis* associated with *Orthotomicus erosus*. (a) light micrograph (LM) of cocoon under the elytra, (b) cocoon with adults inside (LM), (c) vulval region (LM), (d) male tail (LM), (e) female tail (LM), (f) bursa on male tail tip (LM).

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.

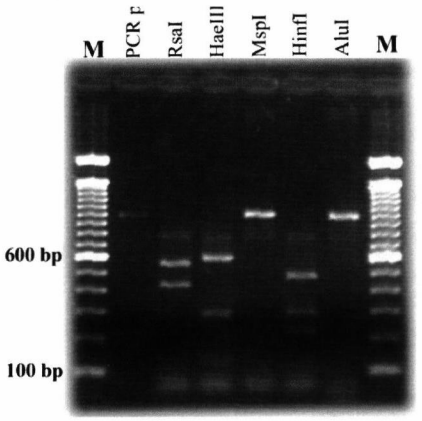

Figure 3 ITS-RFLP pattern of dauer juveniles of *Bursaphelenchus sexdentati* associated with *Orthotomicus erosus*.

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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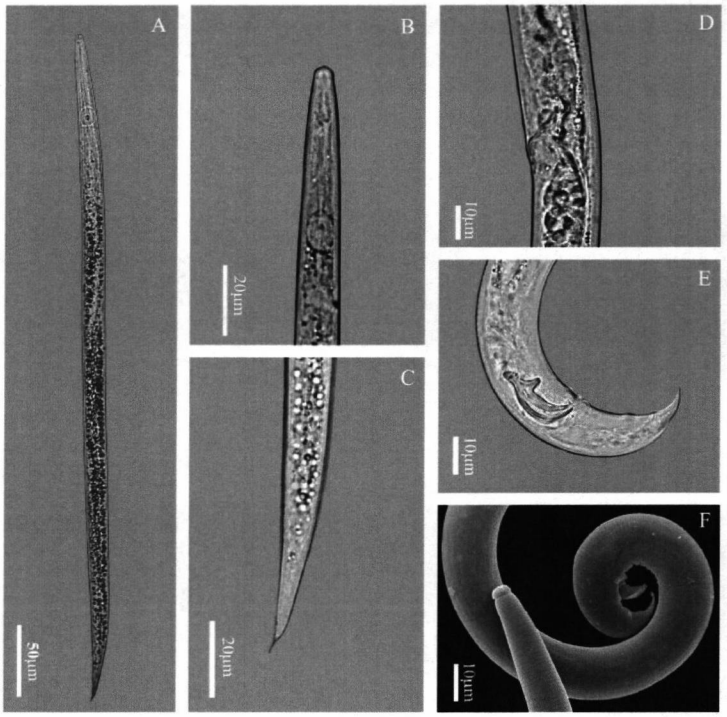

Figure 4 *Bursaphelenchus* sp. associated with *Hylobius* sp. Dauer juveniles isolated from the insect (a–c). (a) light micrograph (LM) of dauer juvenile, (b) anterior region (LM), (c) tail (LM). Adults obtained from pine branches inoculated with dauer juveniles (d–f), (d) vulval region (LM), (e) male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

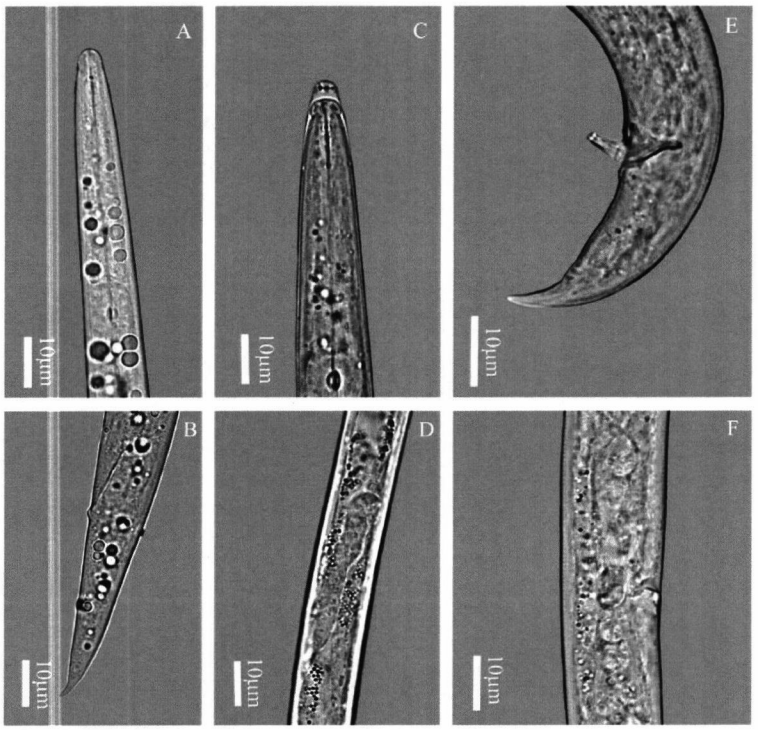

Figure 5 *Bursaphelenchus hellenicus* associated with *Hylurgus ligniperda*. (a) light micrograph (LM) of dauer juvenile anterior region, (b) tail of dauer juvenile (LM), (c) molt of dauer juvenile to fourth propagative juvenile (LM), (d) gonad of fourth juvenile stage (LM), (e) male tail (LM), (f) vulval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

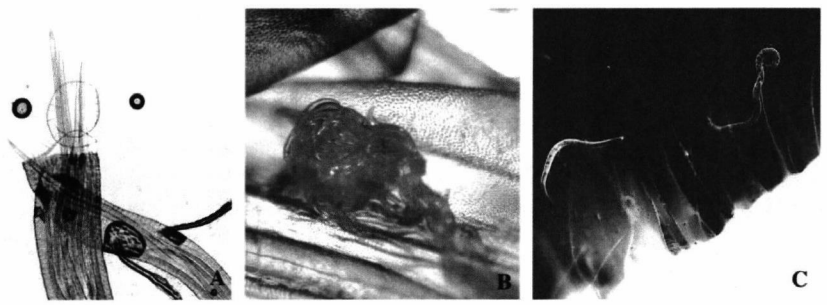

Figure 6 (a) *Bursaphelenchus xylophilus* inside the tracheae of *Monochamus galloprovincialis*, (b) *Bursaphelenchus* dauer juveniles on *Ips sexdentatus* wings, (c) *Bursaphelenchus* dauer juveniles on *Ips sexdentatus* hair.

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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.

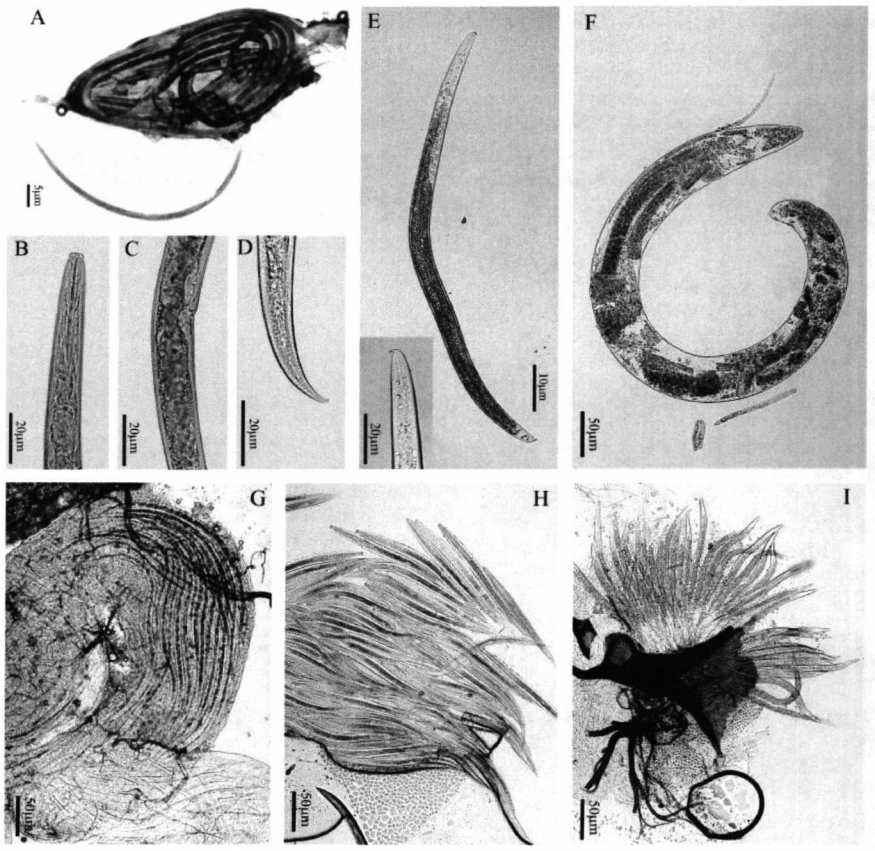

Figure 7 Other nematodes genera associated with the insects observed. *Ektaphelenchus* sp.2 females associated with *Hylurgus ligniperda* (a–d). (a) light micrograph (LM) of cocoon with females inside, (b) anterior region (LM), (c) vulval region (LM), (d) female tail (LM), (e) *Parasitaphelenchus* sp. (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.

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Sub - chapter III.3

Penas, A. C., Metge, K., Mota, M. & Valadas, V. (2006). *Bursaphelechus antoniae* sp. n. (Nematoda: Parasitaphelenchidae) associated with *Hylobius* sp. from *Pinus pinaster* in Portugal. *Nematology*, 8: 659-669.

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

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

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

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

Materials and methods

NEMATODE ISOLATION AND CULTURE

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

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

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

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

Morphobiometric observations

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

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

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

ITS-RFLP profiles

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

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

Sequencing

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

forward primer D2A 5′-ACA AGT ACC GTG AGG GAA AGT TG-3′ and reverse primer D3Br 5′-TCG GAA GGA ACC AGC TAC TA-3′. Sequencing PCR was performed with a Biometra T1 thermal cycler (Biometra, Göttingen, Germany). The PCR program consisted of an initial denaturation for 2 min, 30 s at 96°C, 35 cycles with 1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min extension at 72°C and a final extension for 6 min at 72°C. After completion of the PCR, small aliquots of the samples were separated electrophoretically using a 1.8% agarose gel and 0.5×TBE buffer.

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

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

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

Cross-breeding tests

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

Bursaphelenchus antoniae* sp. n.

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

Measurements

See Table 1.

Description

Male

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

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

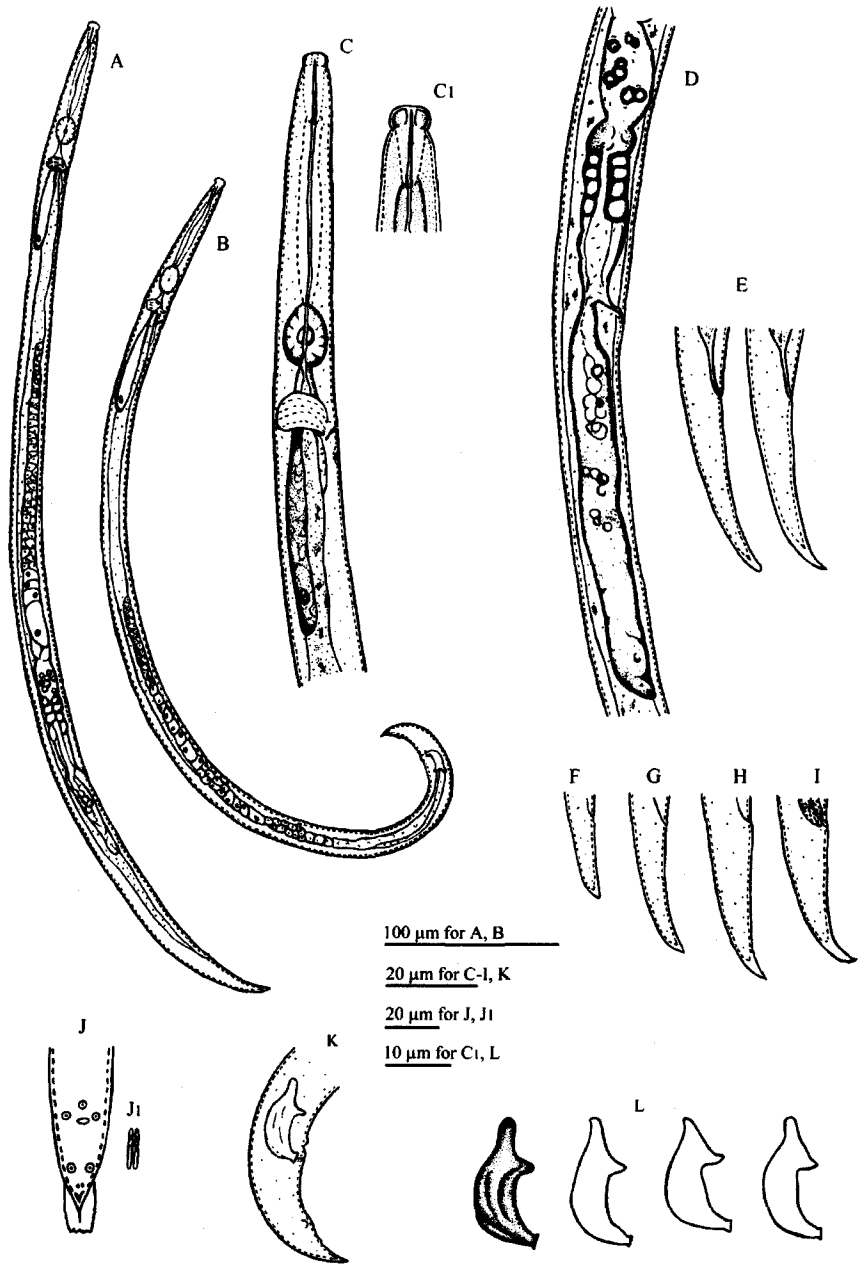

Fig. 1. Bursaphelenchus antoniae *sp. n. A: Entire female; B: Entire male; C: Female anterior region; C1: Head; D: Female vulval region; E: Female tails; F: Tail of J2; G: Tail of J3; H: Tail of J4 (female); I: Tail of J4 (male); J: Ventral view of male tail showing papillal disposition; J1: Spicules,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

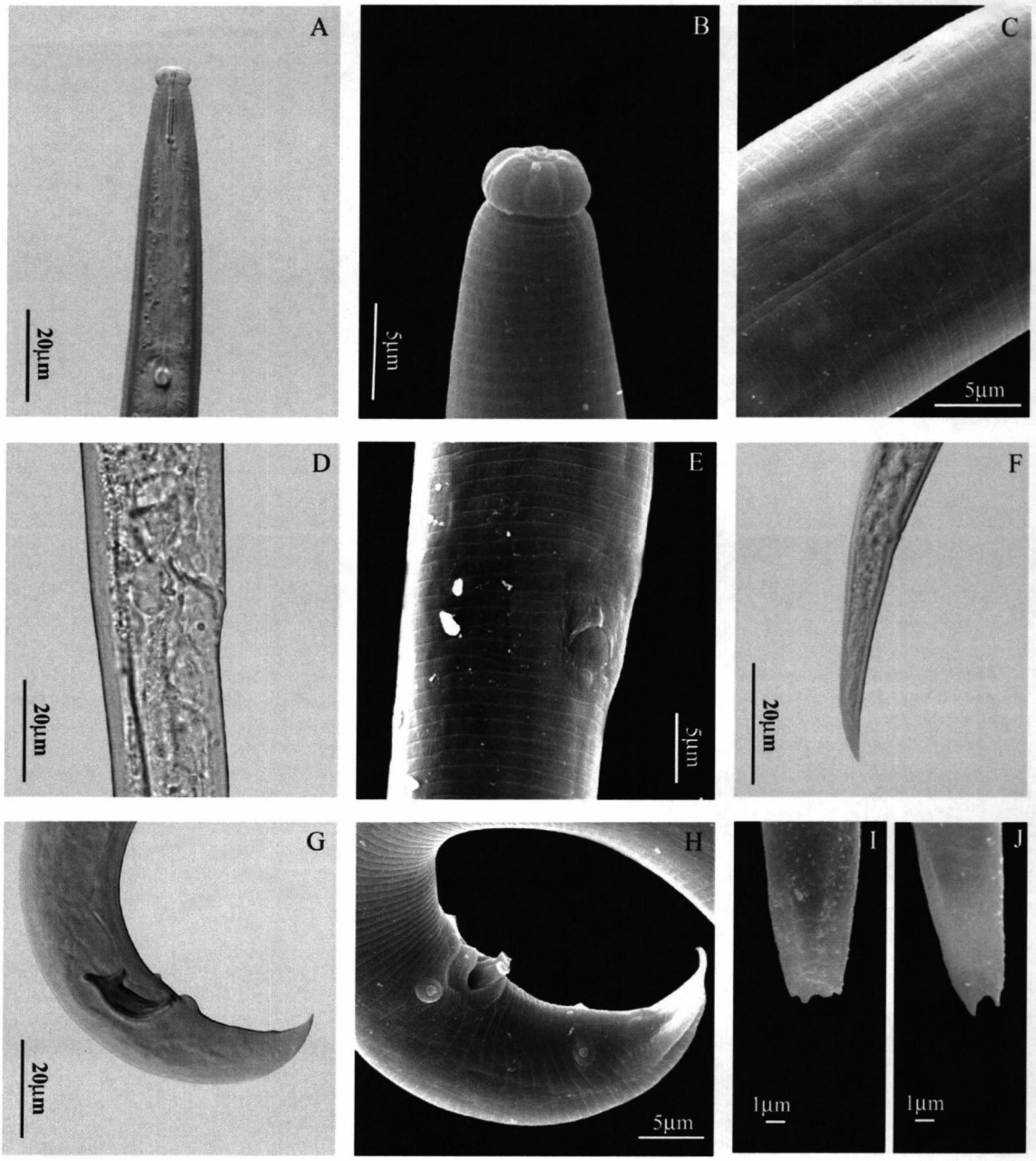

Fig. 2. Bursaphelenchus antoniae *sp. n. A: Light micrograph (LM) of anterior region; B: Scanning electron micrograph (SEM) of head; C: SEM of lateral field; D: LM of vulval region; E: SEM of vulval region; F: LM of female tail; G: LM of male tail; H: SEM of male tail; I, J: SEM of bursa.*

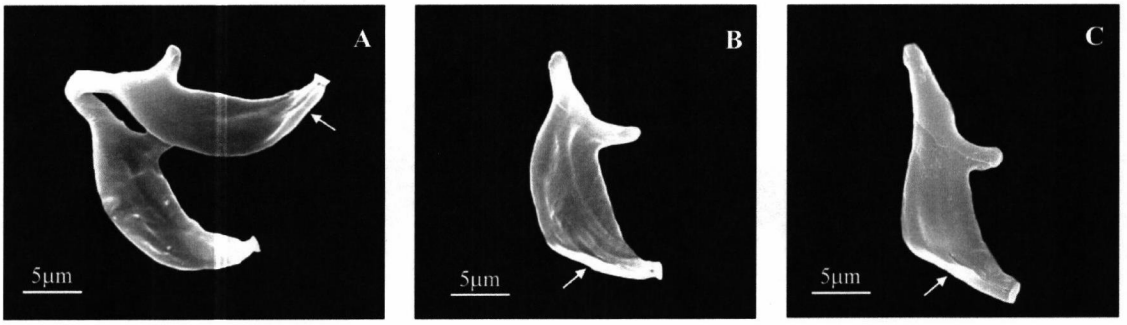

Fig. 3. *Excised spicules of* Bursaphelenchus antoniae *sp. n. (A, B) and* B. hylobianum *(C). Note the flattened wing-like structure in the distal third of the dorsal limb (arrows).*

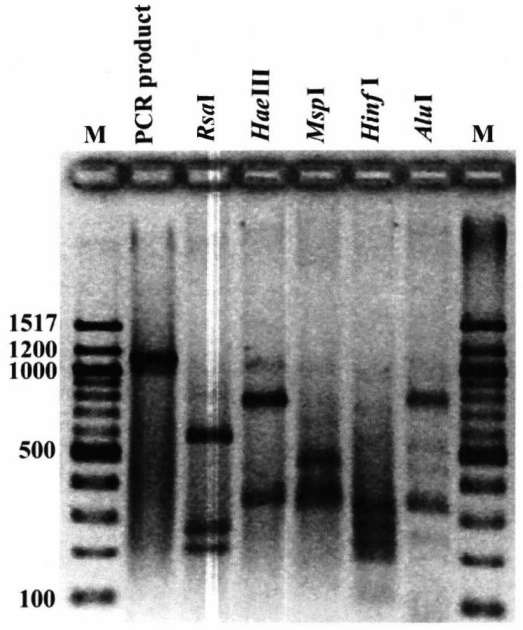

Fig. 4. *ITS-RFLP pattern of* Bursaphelenchus antoniae *sp. n.*

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

Female

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

Dauer juvenile

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

Juvenile stages

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

TYPE LOCALITY AND HABITAT

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

TYPE MATERIAL

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

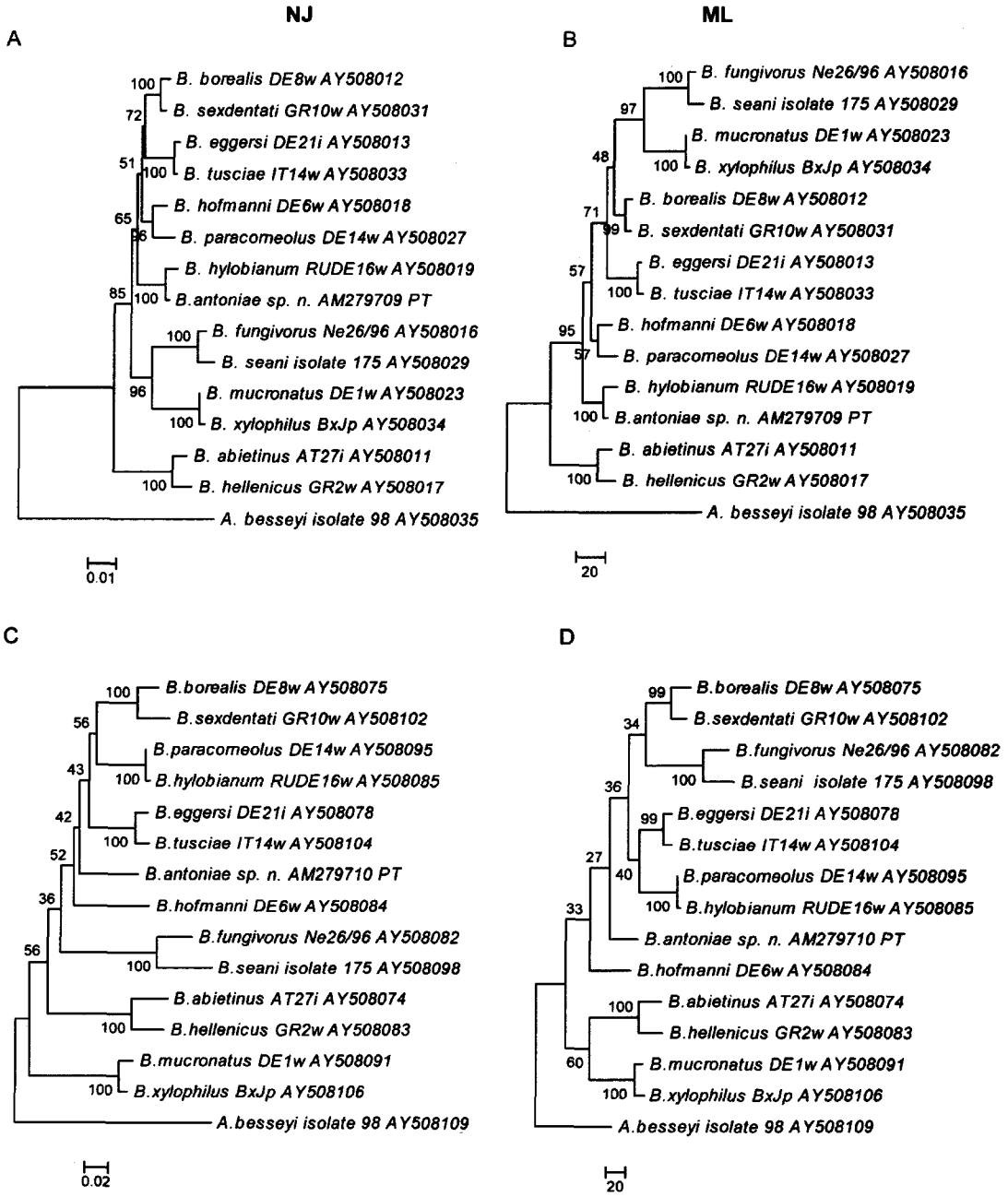

Fig. 5. *Phylogenetic relationships of* Bursaphelenchus antoniae *sp. n. and 13* Bursaphelenchus *species.* Aphelenchoides besseyi *is the outgroup. The global sequence alignments for tree constructions were calculated for 18S rDNA (A, B) and 28S D2/D3 domain (C, D) sequences by neighbour-joining (NJ)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

Taxonn

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

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

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

DIAGNOSIS AND RELATIONSHIPS

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

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

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

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

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

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

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

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

ITS-RFLP PROFILES

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

SEQUENCE ANALYSIS

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

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

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

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

* Total number of specimens of all propagative developmental stages.

CROSS-BREEDING TESTS

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

BIONOMICS

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

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Sub - chapter III.4

Penas, A. C., Bravo, M. A., Valadas, V. & Mota, M. (2007). Detailed morphobiometric studies of *Bursaphelenchus xylophilus* and characterization of other *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) associated *Pinus pinaster* in Portugal. *Journal of Nematode Morphology and Systematics*, 10: 137-163.

J. Nematode Morphol. Syst., 10 (2): 137- 163 (2007, publ. 2008)

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 J_2-J_3, J_3-J_4, J_{4F}-F, and J_{4M}-M; gonad length mean values of these four groups made possible to distinguish the non-moulting groups J_2, J_3, J_{4F}, J_{4M} 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 charaterized, 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 J_2-J_3, J_3-J_4, 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: J_2, J_3, J_{4F} 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 a 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 - Bxy_2 and Bxy_3, 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 (Bxy_1) while the other was inoculated and reared on a culture of non sporulating B. cinerea and measured later (Bxy_2 - measured after 15 days from a culture without subculturing and Bxy_3 – 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 Bxy_1 were compared with the data obtained of Bxy_2 and Bxy_3. 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 (J_1) 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	HinfI	AluI
B. hellenicus	1074	674 295 187	603 482 193	732 488	602 420 310	410 392 298 210
B. pinasteri	1080	600 475	1080	680 344	296 221 125	343 254 135
B. sexdentati	993	590 440	610 319 150	968	528 326 248	990
B. tusciae	953	362 223 163	620 325	577 313 127	460	319 244 206 185
B. xylophilus	958	508 439	723 208	582 391	283 265 149	453 242 156 100

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 (J_2), third (J_3) and fourth stage (J_4) 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 (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I, and *Alu*I) (Amersham BioSiences®) 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.

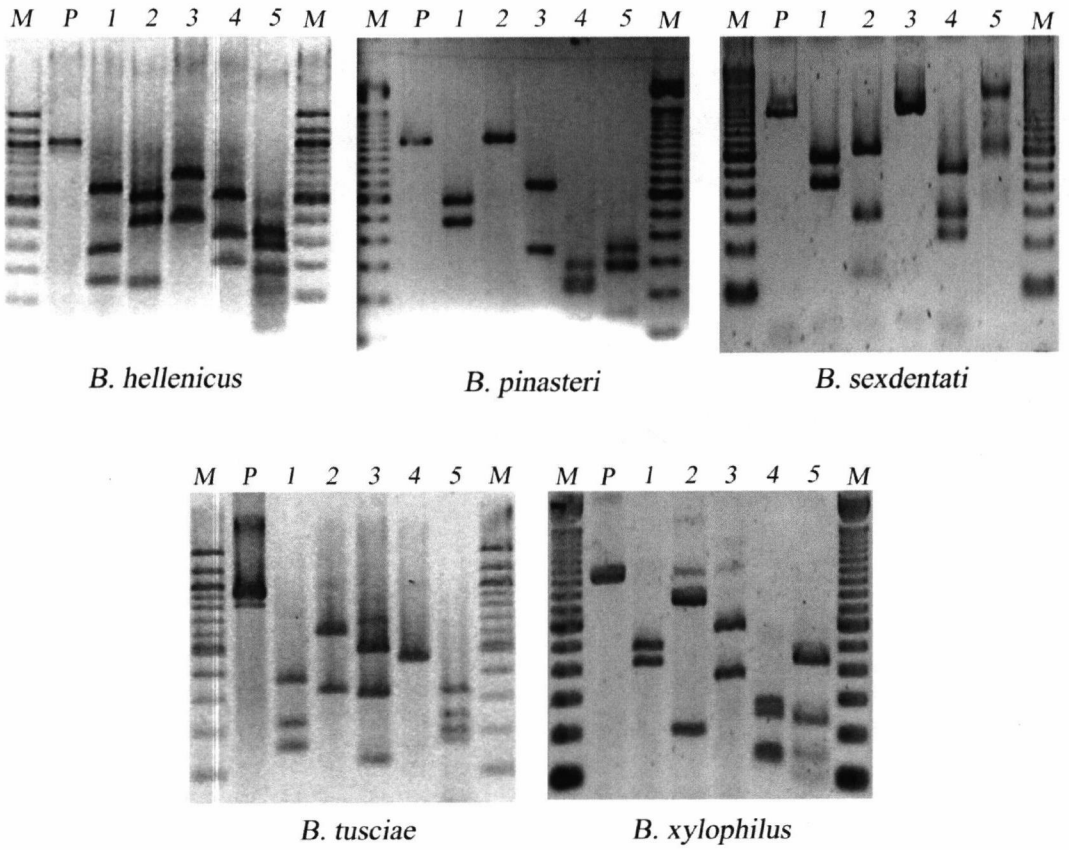

FIGURE 1. ITS-RFLP profiles of five *Bursaphelenchus* species. *Rsa*I (1), *Hae*III (2), *Msp*I (3), *Hinf*I (4) and *Alu*I (5) were the five restriction enzymes used for digestion of the amplified rDNA fragment of each species. M represents the molecular marker (100bp ladder, Invitrogen Life Technologies)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

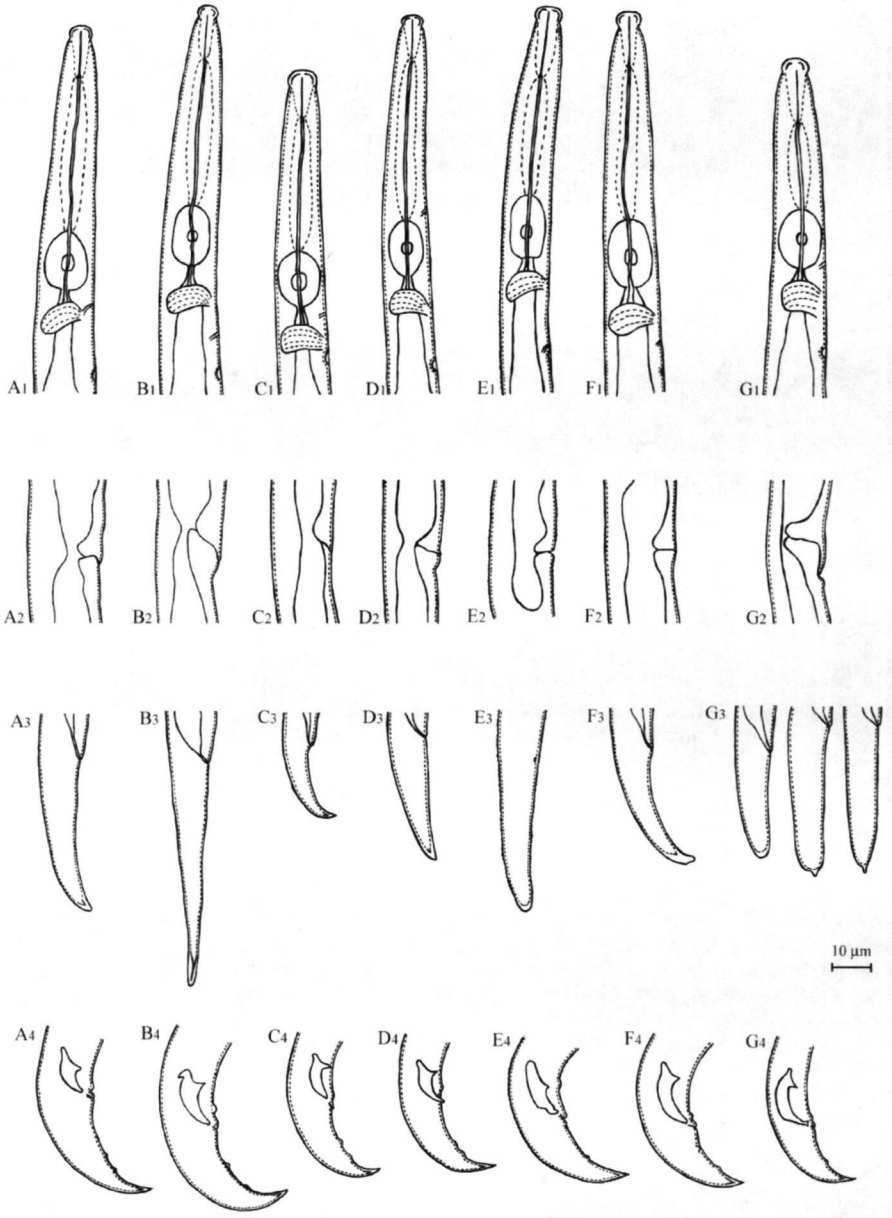

FIGURE 2. Camera lucida drawings of *Bursaphelenchus* species most important characters: anterior region (1), vulval region (2), female tail (3) and male tail (4). The species represented are: *B. hellenicus* (A), *B. leoni* (B), *B. pinasteri* (C), *B. sexdentati* (D), *B. teratospicularis* (E), *B.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

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

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

TABLE II. (Cont.)

Population	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♂♂
Character									
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 predo. 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)

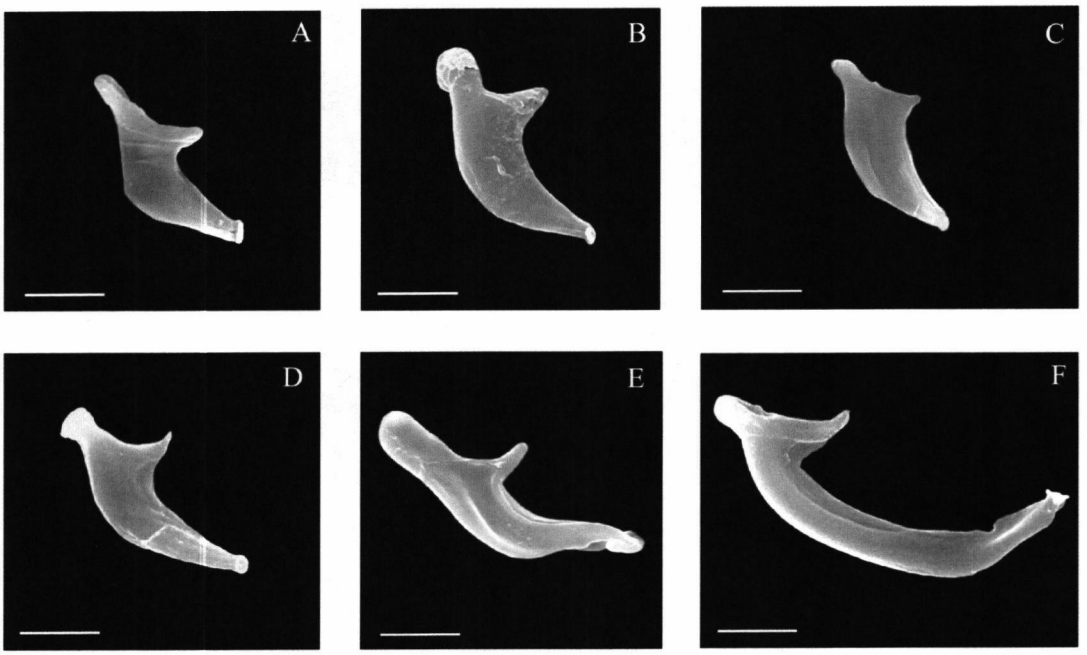

FIGURE 3. SEM pictures of excised spicules: A: *B. hellenicus*. B: *B. leoni*. C: *B. pinasteri*. D: *B. sexdentati*. E: *B. teratospicularis*. F: *B. xylophilus*. (Scale bar = 5 μm).

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

Material examined: Populations Bxy_1 and Bxy_4 were collected from wood material from Tróia (Setúbal), in the case of Bxy_1 20 females and 20 males were measured and in Bxy_2 10 females and 17 males. From populations collected from fungal cultures, Bxy_2 and Bxy_3 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

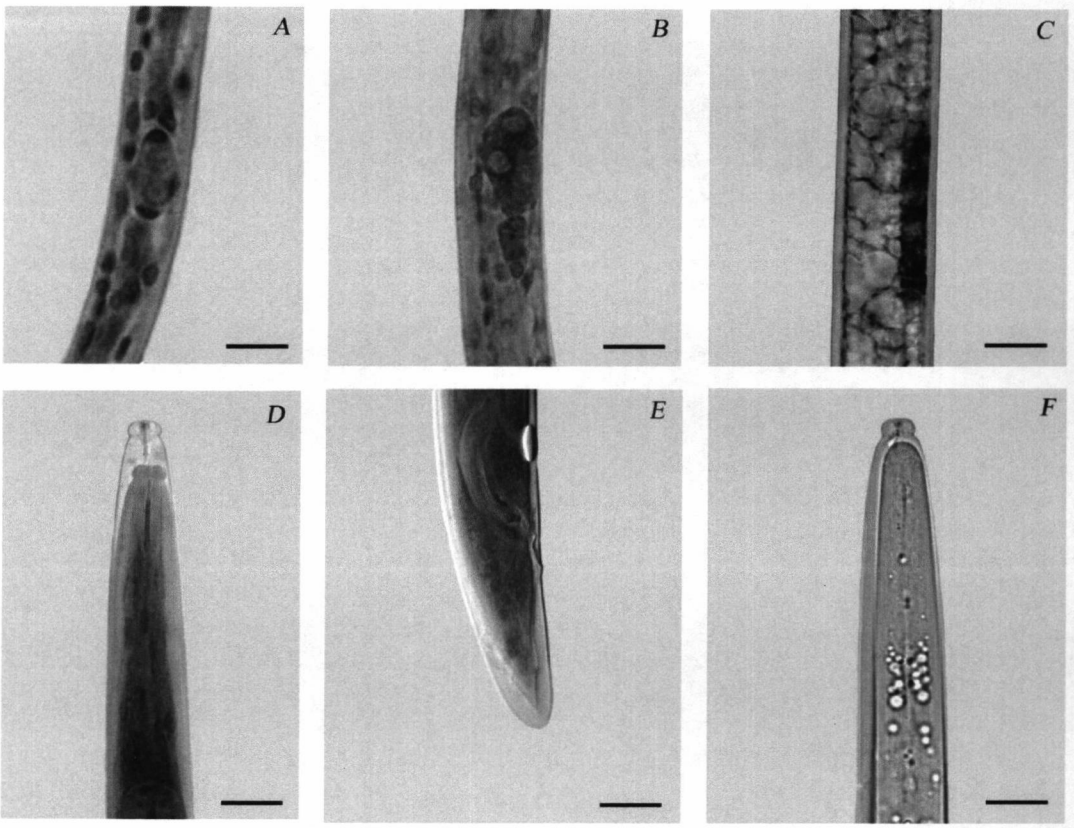

FIGURE 4. Light micrographs of *B. xylophilus* developmental stages. A: J_2 gonad. B: J_3 gonad. C: J_{III} gonad. D: Moulting from J_{4M} to male (anterior region). E: Moulting from J_{4M} to male (tail). F: Moulting from J_{III} to J_{IV} (head). (Scale bar = 10 μm).

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 ± SD (range).

Character	n	J_2	J_2-J_3	J_3	J_3-J_4	J_4		J_4^F	J_4^M	Adult		J_{III}	J_{IV}
		20	14	34	18	29♀♀	15♂♂	20♀♀	23♂♂	14♀♀	19♂♂	16	15
Body length		254.4±22.1	346.1±30.1	438.1±46.5	518.0±34.3	634±41.4	592.7±49.7	791.4±100.2	708.2±96.4	912.2±111.9	842.2±172.9	670.9±41.5	578.8±53.4
		(225-315)	(300-405)	(327-544)	(464-591)	(541-721)	(519-682)	(651-946)	(519-915)	(604-1091)	(605-1121)	(620-788)	(505-687)
a		28.5±3.2	26.3±2.5	32.6±2.9	32.4±1.8	39.6±2.1	36.9±4.3	36.5±3.0	36.2±3.3	41.9±5.3	41.5±5.1	37.0±3.0	41.0±2.9
		(24.3-34.6)	(23.6-32.4)	(27.2-36.8)	(29.0-34.9)	(35.1-42.7)	(34.6-49.7)	(31.4-41.5)	(28.8-45.8)	(33.6-51.9)	(31.5-49.7)	(32.7-43.9)	(36.1-44.8)
c		18.8±1.5	19.9±2.8	21.3±2.2	21.3±1.3	23.7±1.5	25.2±1.5	24.7±1.8	26.1±2.5	26.6±2.4	21.9±2.2	23.2±1.8	20.0±1.8
		(17.2-22.5)	(16.8-25.3)	(16.4-26.7)	(18.6-23.3)	(21.4-26.2)	(22.4-27.3)	(21.3-28.7)	(22.4-32.0)	(22.2-30.3)	(18.3-26)	(19.4-26.2)	(18.1-20.5)
c'		2.2±0.3	2.1±1.3	2.3±0.2	2.3±0.2	2.8±0.3	2.2±0.2	2.8±0.2	2.1±0.2	3.3 .3	2.5±0.1	2.4±0.3	3.3±0.3
		(1.7-2.8)	(1.7-2.5)	(1.9-3.0)	(2.0-2.8)	(2.3-3.3)	(1.7-2.4)	(2.3-3.2)	(1.7-2.5)	(2.7-4.1)	(2.4-2.9)	(2.0-2.9)	(2.7-3.88)
Stylet		8.8±1.2	10.7±0.6	11±0.7	11.8±0.4	12.3±0.6	12.4±0.9	12.9±0.8	12.6±0.8	13.6±0.9	13.3±1.0	11.4±0.9	-
		(6.5-10)	(10-11)	(10-13)	(11-12)	(11-13)	(11-14)	(11-14)	(11-13.5)	(12-15)	(11-15)	(10-13)	
Body diam.		9.1±1.0	13.6±0.9	13.5±0.9	16±1.2	16.0±1.0	15.3±1.6	21.6±1.3	19.6±1.6	21.9±2.4	20.2±2.7	18.2±1.2	14.1±0.8
		(7-11)	(12-15)	(12-15)	(14-19)	(14-18)	(12-17)	(19-23)	(17-23)	(17-25)	(15-26)	(15-20)	(13-16)
Median bulb		12.7±1.2	14.7±1.1	15.0±1.0	15.8±1.2	16.7±0.9	16.2±0.9	18.2±1.0	17.2±0.9	17.9±1.0	17.4±1.5	14.9±0.9	12.4±1.0
length		(10-15)	(13-17)	(13-18)	(14-18)	(15-18)	(15-18)	(17-20)	(16-20)	(17-20)	(15-22)	(13-16)	(11-15)
Median bulb		8.1±0.5	9.4±0.7	10.0±0.6	10.8±0.6	11.3±0.7	10.8±0.7	11.8±0.9	11.4±0.6	13.3±0.7	12.6±1.2	10.2±0.7	6.6±0.5
diam.		(7-9)	(8-10)	(9-11)	(10-12)	(10-13)	(10-12)	(10-13.5)	(10-12)	(12-14)	(11-15)	(9-11)	(6-7)
Gonad length		11.4±1.9	20.1±5.9	33.9±6.7	85.1±13.6	160.0±21.2	173.1±41.4	312.3±61.7	465.7±82.9	537.1±91.6	542.4±143.4	31.6±4.5	74.0±19.4
		(9-16)	(14-33)	(22-51)	(62-108)	(123-195)	(114-230)	(207-437)	(252-648)	(306-674)	(360-860)	(25-42)	(37-96)
Tail length		13.6±0.8	17.1±2.0	20.6±2.3	24.3±1.3	27.0±2.5	23.2±1.8	32.0±3.3	27.2±3.4	34.4±4.2	38.9±4.5	28.9±2.5	30.0±3.4
		(11-15)	(14-21)	(15-24)	(2 2-27)	(21-33)	(19-26)	(26-38)	(19-36)	(24-41)	(30-45)	(26-35)	(27-38)
Anal/cloacal		6.3±0.8	8.9±1.1	9.0±0.8	10.4±0.8	9.7±0.7	10.7±0.8	11.5±1.1	13.4±1.3	10.5±1.1	15.4±2.3	12.3±0.6	8.8±0.6
body diam.		(5-7)	(7-11)	(7-10)	(9-12)	(9-11)	(10-12)	(10-13)	(11-16)	(9-12)	(12-20)	(11-13)	(8-10)
L/gonad length		22.3±3.4	19.3±6.3	13.4±2.9	6.6±0.2	4.0±0.6	3.6±0.7	2.6±0.4	1.5±1.0	1.7±0.2	1.6±0.3	21.8±3.0	8.5±2.7
		(16.7-27.1)	(11.7-32.1)	(9.0-23.7)	(5.0-7.9)	(3.4-5.7)	(2.6-4.7)	(2.2-3.5)	(1.4-2.1)	(1.4-2.0)	(1.3-2.3)	(15.4-27.2)	(6.2-14.2)
Gonad length/L		4.6±0.9	6.5±1.4	7.8±1.4	16.4±2.0	25.3±3.2	29.4±5.8	39.3±4.9	65.5±5.2	58.8±7.0	64.1±10.0	4.7±0.8	12.7±2.9
* 100		(3.7-6.0)	(4.0-8.5)	(4.8-11.1)	(12.7-20.1)	(17.4-29.8)	(21.3-38.3)	(28.6-46.2)	(48.6-74.2)	(50.3-74.0)	(43.3-78.3)	(3.7-6.5)	(7.0-16.0)

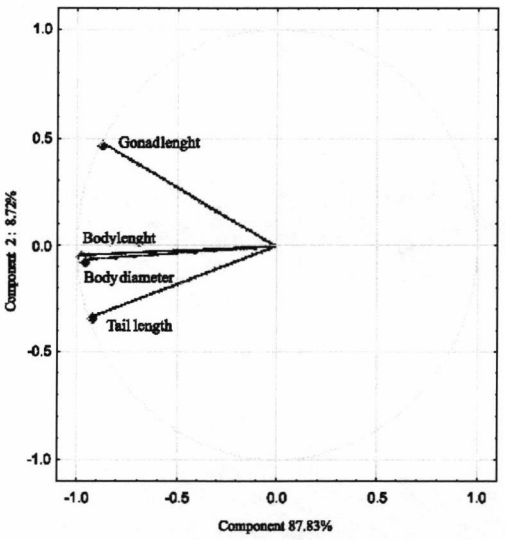

FIGURE 5. Bi-dimensional projection of four morphometric characters of moulting *B. xylophilus* based on a correlation matrix of the principal component analyses.

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 Bxy_1 were compared with data of the same population maintained on fungi 15 days (Bxy_2) and one year (Bxy_3) (Table II). The data show that Bxy_1 is smaller than Bxy_2 and Bxy_3. The higher values of the different characters of populations from fungi (Bxy_2 and Bxy_3) compared to the field population (Bxy_1) 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 (J_2-J_3), from third to fourth stage (J_3–J_4), 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 (J_2, J_3 and J_4), 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.

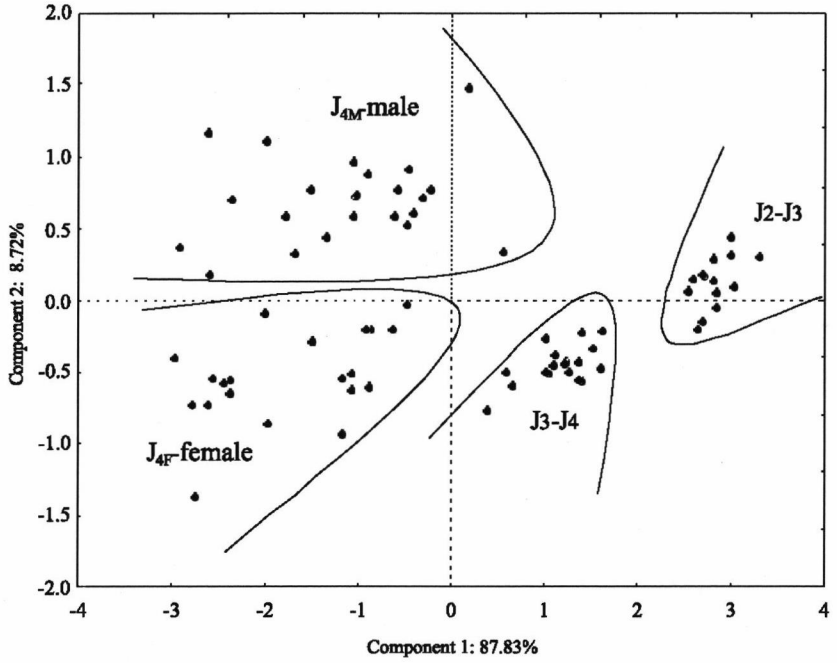

FIGURE 6. Bi-dimensional projection of 75 moulting *B. xylophilus* individuals and separation of the four groups based on a correlation matrix of the principal component analyses.

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).

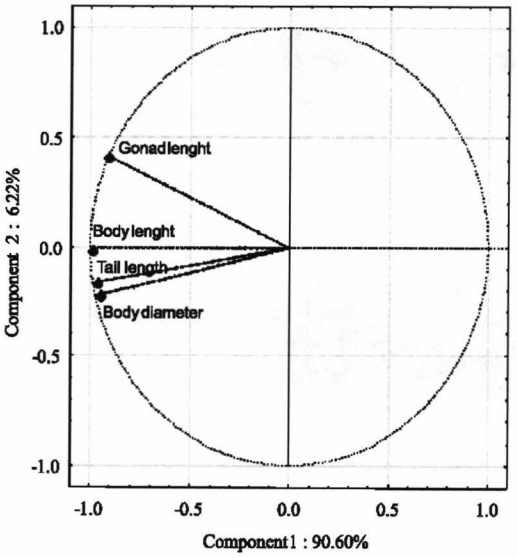

FIGURE 7. Bi-dimensional projection of four morphometric characters of *B. xylophilus* juvenile stages based on a correlation matrix of the principal component analyses.

***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 *Tomicus piniperdae*, *Ips sexdentatus* and *Hylurgus ligniperda* (Penas *et al.*, 2006a). *Bursaphelenchus hellenicus* can be maintained

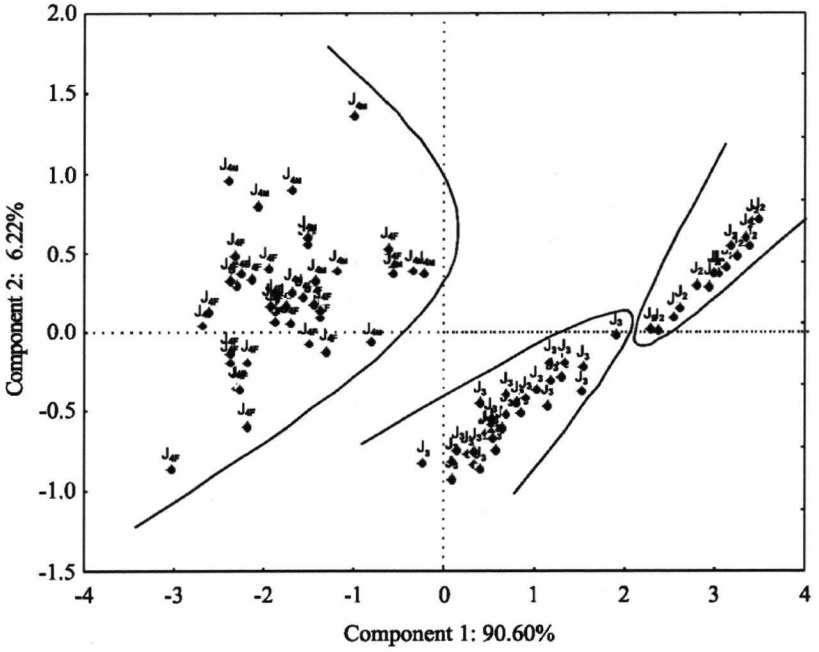

FIGURE 8. Bi-dimensional projection of 89 *B. xylophilus* individuals separated into three juvenile stages (J_2, J_3 and J_4) and separation of the groups based on a correlation matrix of the principal component analyses.

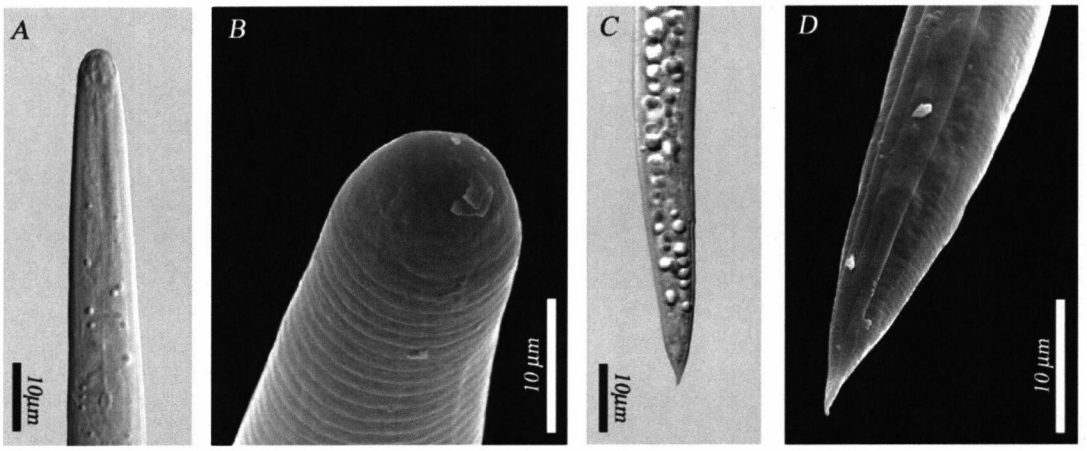

FIGURE 9. *Bursaphelenchus xylophilus* dauer juvenile (J_{IV}). A: Light micrograph (LM) of anterior region. B: Scanning electron micrograph (SEM) of anterior region. C: LM of posterior region. D: SEM of posterior region.

TABLA IV. Measurements of *Bursaphelenchus hellenicus* (in µm and in form: mean ± SD (range).

Population / Character	n	Bhe - Tróia 13 ♀♀	Bhe - Tróia 14 ♂♂
Body length		800.7±70.9 (607-908)	713.7±55.2 (637-802)
a		40.2±4.0 (30.4-46.1)	39.8±2.2 (36.6-43.9)
b		10.6±0.9 (8.0-11.6)	9.6±0.6 (8.9-10.6)
c		18.8±1.6 (14.8-21.1)	19.3±1.0 (17.4-21.2)
c'		3.7±0.3 (3.2-4.3)	2.6±0.2 (2.2-2.8)
V		73.8±3.3 (71.9-84.5)	-
Lip region diam.		6.9±0.3 (6.5-7.5)	6.8±0.4 (6.0-7.5)
Lip constriction diam.		5.8±0.3 (5.5-6.5)	5.7±0.3 (5-6)
Lip region height		3.8±0.3 (3.5-4.0)	3.9±0.2 (3.5-4.0)
Stylet		13.7±0.9 (12-15)	13.5±0.9 (12-15)
Pharynx length		75.7±1.8 (73-78)	73.9±2.4 (71-78)
Median bulb length		17.2±0.9 (15-19)	16.4±0.8 (15-17)
Median bulb diam.		12.7±0.7 (12-14)	11.5±0.5 (11-12)
Body diam. at middle of median bulb		12.7±0.8 (12-14)	15.4±0.9 (14-17)
Body diam. at base of median bulb		12.7±0.7 (12-14)	15.5±0.9 (14-17)
Distance anterior end to excretory pore		83.3±5.2 (78-96)	79.8±5.5 (70-88)
Distance anterior end to hemizonid		100.3±3.9 (95-104)	96.9±4.7 (90-106)
Distance ant. end to posterior end of pharyngeal glands		157.6±7.6 (146-173)	151.4±5.5 (141-161)
Body diam. at end of pharyngeal glands		18.3±1.0 (16-19)	17.3±1.0 (16-19)
Anterior genital branch		351.5±36.9 (297-425)	480.1±62.2 (377-568)
Posterior genital branch		109.2±11.9 (91-132)	-
Body diam. at vulva		19.5±1.4 (17-22)	-
Vulva to anus distance		176.9±9.9 (164-191)	-
Distance from anterior end to vulva		589.5±39.8 (513-673)	-
G1 (%)		44.3±7.2 (36.5-63.4)	-
G2 (%)		13.7±1.6 (10.8-16.5)	-
Anal/cloacal body diam.		11.6±0.9 (10-13)	14.4±0.9 (13.0-15.5)
Tail		42.7±1.8 (41-47)	37.1±2.7 (33-41)
T		-	67.1±4.7 (57.4-74.4)
Spicule (condylus to distal end)		-	15.5±0.7 (14.5-17.0)
Spicule (rostrum to distal end)		-	7.4±0.4 (7-8)
Spicule (curved median line)		-	12.0±0.5 (11-13)
Spicule (rostrum to condylus)		-	8.7±0.8 (7-10)
Distance single precloacal papilla to cloacal aperture		-	3.9±0.5 (3-5)
Distance cloacal aperture to first pair of postcloacal papillae		-	17.3±1.2 (15.5-19.5)
Distance cloacal aperture to second pair of postcloacal papillae		-	21.1±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, Ble_1, Ble_2 and Ble_3, extracted from wood material, collected from Grândola (Setúbal), Tróia (Setúbal) and Companhia das Lezírias (Santarém), respectively. From Ble_1 five females and six males were measured, from Ble_2 12 females and 13 males, and from Ble_3 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 ± SD (range).

Population		Ble$_1$-Grândola		Ble$_2$ - Tróia		Ble$_3$ - Companhia das Lezírias	
	n	5 ♀♀	6 ♂♂	12 ♀♀	13 ♂♂	17 ♀♀	18 ♂♂
Character							
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 - excr. 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	
Character	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 μm 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. Mail 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 (Ble_1) being considerably smaller then the others populations (Ble_2 and Ble_3). 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, Bpi_1 and Bpi_2, extracted from wood material, collected from Melides (Sines), and Murta, Pousadas (Santarém), respectively. From Bpi_1 14 females and 13 males were measured, and from Bpi_2 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 ± SD (range).

Population		Bpi_1 - Melides		Bpi_2 - Murta,Pousadas	
Character	n	14 ♀♀	13 ♂♂	9 ♀♀	4 ♂♂
Body length		597.2±53.9 (518-740)	526.2±31.8 (468-574)	693.1±30.0 (645-737)	559.5±51.3 (506-629)
a		39.5±2.7 (35.1-46.3)	39.0±1.8 (36.0-41.7)	43.5±2.2 (39.9-46.8)	44.7±3.3 (40.5-48.4)
b		9.9±0.8 (8.8-12.3)	8.9±0.4 (7.9-9.4)	10.9±0.6 (9.9-12.0)	9.4±0.5 (9-10)
c		23.1±2.3 (19.6-27.4)	19.1±1.8 (16.7-23.2)	23.2±1.9 (19.9-26.4)	19.5±1.2 (18-21)
c'		3.5±0.3 (3.0-4.1)	2.6±0.2 (2.3-3.0)	3.5±0.2 (3.1-3.8)	2.5±0.2 (2.4-2.7)
V		71.2±4.0 (57.8-74.3)	-	71.9±1.0 (70.5-73.4)	-
Lip region diam.		7.0±0.4 (6.5-8.0)	6.8±0.3 (6.5-7.0)	7.2±0.4 (7-8)	6.8±0.6 (6.0-7.5)
Lip constriction diam.		6.3±0.4 (5.5-7.0)	5.9±0.3 (5.5-6.5)	6.5±0.4 (6-7)	6.1±0.6 (5.5-7.0)
Lip region height		3.8±0.3 (3-4)	3.6±0.3 (3-4)	3.8±0.3 (3.5-4.0)	3.8±0.3 (3.5-4.0)
Stylet		11.0±0.6 (10-12)	11.0±1.0 (10-14)	12.5±1.1 (11.0-14.5)	11.3±0.5 (11-12)
Pharynx length		60.1±2.9 (54-64)	59.1±1.6 (56-62)	63.9±2.9 (57-67)	59.3±3.5 (55-63)
Median bulb length		15.3±0.9 (14-17)	14.5±0.5 (14-15)	16.7±1.1 (14.5-18.0)	14.8±1.0 (14-16)
Median bulb diam.		11.4±0.5 (11-12)	10.0±0.4 (9-11)	10.8±0.4 (10-11)	9.5±1.0 (9-11)
Body diam. at middle med. bulb		13.0±0.6 (12-14)	12.2±0.6 (11-13)	13.7±0.9 (12-15)	11.4±0.5 (11-12)
Body diam. at base median bulb		13.2±0.6 (12-14)	12.3±0.6 (11-13)	13.8±1.0 (12.0-15.5)	11.4±0.5 (11-12)
Distance ant. end - excretory pore		62.2±2.1 (58-65)	58.6±4.1 (50-64)	64.8±8.8 (57-77)	60.0±2.8 (58-62)
Distance ant. end - hemizonid		78.5±3.2 (72-83)	74.8±2.9 (70-78)	9.2±4.4 (74-84)	75.3±1.2 (74-76)
Distance ant. end - posterior end of pharyngeal glands		127.9±8.2 (108-139)	123.8±4.5 (115-131)	143.3±7.4 (127-152)	133.7±7.2 (129-142)
Body diam. at end phar. glands		14.6±0.6 (14-16)	13.3±0.7 (12-14)	14.8±1.2 (13-17)	12.3±0.6 (11.5-13.0)
Anterior genital branch		213.2±21.1 (185-257)	267.5±32.3 (219-326)	242.1±25.6 (199-282)	337.5±15.0 (316-350)
Posterior genital branch		72.2±5.1 (64-82)	-	74.9±8.2 (66-89)	-
Body diam. at vulva		14.9±0.5 (14-16)	-	14.9±1.1 (12-16)	-
Vulva to anus distance		137.9±6.9 (124-147)	-	164.2±7.9 (154-177)	-
Distance ant. end - vulva		423.9±27.0 (376-471)	-	498.4±24.8 (461-533)	-
G1 (%)		35.8±3.4 (30.3-42.3)	-	34.9±3.3 (29.4-40.5)	-
G2 (%)		12.2±1.1 (9.6-13.8)	-	10.8±1.3 (9.4-12.8)	-
Anal/cloacal body diam.		7.4±0.5 (6.5- 8.0)	10.7±0.7 (9.0-11.5)	8.7±0.7 (8-10)	11.5±0.7 (11.0-12.5)
Tail		26.0±2.5 (23-31)	27.6±2.0 (23-31)	30.0±3.0 (27-37)	28.8±2.2 (26-31)
T		-	42.6±13.7 (37.7-50.0)	-	60.5±3.3 (55.6-62.5)
Spicule (condylus to distal end)		-	13.3±0.6 (12-14)	-	12.6±0.8 (11.5-13.0)
Spicule (rostrum to distal end)		-	8.4±0.4 (7.5-9.0)	-	7.9±0.3 (7.5-8.0)
Spicule (curved median line)		-	11.8±0.5 (11.0-12.5)	-	11,5
Spicule (rostrum to condylus)		-	6.2±0.6 (5-7)	-	5.8±0.5 (5-6)
Distance from single precloacal papilla to cloacal aperture		-	3.5±0.7 (2.0-4.5)	-	3.6±0.3 (3.5-4.0)
Distance from cloacal aperture to first pair of postcloacal papillae		-	11.9±1.1 (10-14)	-	12.0±8.0 (11-13)
Distance cloacal aperture to second pair of postcl. papillae		-	15.9±1.0 (14-17)	-	15.8±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, Bse_1, Bse_2 and Bse_3, extracted from wood material, collected from Tróia (Setúbal), Companhia das Lezírias (Santarém) and Chamusca (Santarém), respectively. From Bse_1 9 females and 6 males were measured, from Bse_2 6 hembras and 10 machos, and from Bse_3 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 μm) 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 μm) 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), Lituania 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).

Population	Bse. - Tróia		Bse. - Companhia das Lezírias		Bse. - Chamusca	
Character / n	9 ♀♀	6 ♂♂	6 ♀♀	10 ♂♂	20 ♀♀	20 ♂♂
Body length	939.7±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 (21.7-26.0)	23.7±1.4 (21.7-26.0)	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±3.0 (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 - hemizonid	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 μm long and 10 μm 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, Btu_1 and Btu_2, extracted from wood material, collected from Santarém and Tróia (Setúbal), respectively. From Btu_1 two females and six males were measured, and from Btu_2 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 ± SD (range).

Population		Bte - Pegões	
	n	20 ♀♀	15 ♂♂
Character			
Body length		690.6±68.0 (597-931)	571.1±38.4 (497.0-648.0)
a		38.8±1.5 (34.4-40.9)	35.6±2.6 (29.2-39.4)
b		12.0±1.1 (10.3-15.3)	8.2±07 (6.8-9.2)
c		15.6±1.2 (14.0-18.8)	18.1±1.6 (13.8-20.7)
c'		3.7±0.3 (3.2-4.3)	2.3±0.2 (1.9-2.8)
V		78.8±0.7 (77.6-79.9)	-
Lip region diam.		7.5±0.5 (7-9)	7.4±0.5 (6.5-8.0)
Lip constriction diam.		6.8±0.3 (6.0-7.5)	6.5±0.4 (6-7)
Lip region height		3.6±0.4 (3-4)	3.8±0.4 (3-4)
Stylet		6.8±0.3 (6.0-7.5)	18.1±2.1 (15-22)
Pharynx length		76.2±4.7 (65-82)	69.7±4.4 (62-75)
Median bulb length		18.6±0.9 (17-21)	17.1±0.7 (16-18)
Median bulb diam.		10.2±0.7 (9-12)	9.7±0.7 (8-11)
Body diam. at middle of median bulb		14.1±0.8 (13.0-16.5)	13.4±1.0 (12-15)
Body diam. at base of median bulb		14.1±0.8 (13.0-16.5)	13.6±0.9 (12-15)
Distance from anterior end to excretory pore		107.4±10.9 (90-138)	92.5±5.3 (84-99)
Distance from anterior end to hemizonid		110.0±9.9 (94-137)	100.5±5.3 (92.0-108.0)
Distance ant. end - post. end of phar. glands		225.6±13.5 (202-250)	190.3±12.5 (159-210)
Body diam. at end of pharyngeal glands		17.4±1.6 (15-23)	15.7±0.9 (14-17)
Anterior genital branch		273.3±53.7 (201-406)	269.3±25.1 (224-302)
Posterior genital branch		19.8±2.3 (15-23)	-
Body diam. at vulva		17.8±1.5 (16.5-23.0)	-
Vulva to anus distance		105.8±10.7 (90-132)	-
Distance from anterior end to vulva		544.0±55.1 (477-741)	-
G1 (%)		39.4±5.7 (31.2-52.1)	-
G2 (%)		2.9±0.4 (2.4-3.5)	-
Anal/cloacal body diam.		17.8±1.5 (16.5-23.0)	13.7±0.8 (12-15)
Tail		45.3±5.5 (36-58)	37.1±2.7 (27-38)
T		-	47.3±5.0 (40.6-55.5)
Spicule (condylus to distal end)		-	17.7±0.8 (16.5-19.0)
Spicule (rostrum to distal end)		-	7.9±0.5 (7-9)
Spicule (curved median line)		-	14.3±1.2 (13.0-16.5)
Spicule (rostrum to condylus)		-	10.1±0.5 (9-11)
Distance from single precloacal papilla to cloacal aperture		-	9.1±1.6 (6.5-13.5)
Distance from cloacal aperture to postcloacal papillae pair		-	12.6±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 ± SD (range).

Population		Btu_1 - Santarém		Btu_2 - Tróia	
	n	2♀♀	6♂♂	5♀♀	6♂♂
Character					
Body length		633, 629	655±66.4 (594-773)	768±74.4 (676-871)	724±47.1 (674-814)
a		42.2, 40.6	43.9±3.0 (39.6-48.3)	37.3±1.1 (35.7-38.7)	39.1±2.4 (35.4-42.1)
b		11.3, 12.1	9.0±0.5 (8.5-9.7)	14.2±0.8 (13.0-14.8)	9.7±0.2 (9.4-10.1)
c		16.7, 20.3	24.3±1.6 (22.6-26.5)	18.7±0.8 (16.9-21.9)	24.4±3.1 (21.5-29.1)
c'		4.0, 3.4	2.2±0.1 (2.0-2.3)	4.0±0.5 (3.4-4.4)	2.3±0.3 (1.9-2.7)
V		72.8, 73.8	-	72.5±0.7 (72.0-75.5)	-
Lip region diam.		6.5, 6.0	6.2±0.3 (6.0-6.5)	6.8±0.4 (6-7)	6.5±0.4 (6-7)
Lip constriction diam.		5.5, 5.0	5.4±0.4 (5-6)	5.8±0.4 (5-6)	5.5±0.4 (5-6)
Lip region height		3.5, 3.0	3.4±0.2 (3.0-3.5)	3.6±0.2 (3.5-4.0)	3.7±0.3 (3.5-4.0)
Stylet		12.0, 11.5	12.8±0.8 (12-14)	15.2±0.8 (14-16)	13.8±0.8 (13-15)
Pharynx length		71, 68	72.5±4.1 (69-80)	74.8±3.9 (69-79)	74.8±5.1 (70-84)
Median bulb length		15, 16	15.5±1.0 (14-17)	18.2±1.3 (17-20)	18.7±2.4 (16-23)
Median bulb diam.		10, 10	10.2±0.7 (9-11)	11.7±0.4 (11.5-12.0)	11.9±1.3 (10.0-13.5)
Body diam. at middle of median bulb		13, 12	12.2±0.8 (11-13)	15.6±1.1 (14-17)	15.3±1.4 (13-17)
Body diam. at base of median bulb		13, 13	12.7±0.5 (12-13)	16.6±1.1 (15-18)	15.6±1.6 (13-18)
Distance anterior end to excretory pore		83, -	84.2±5.1 (79-94)	88.8±4.1 (85-94)	90.3±5.8 (84-99)
Distance from anterior end to hemizonid		93, 90	93.5±6.9 (88-107)	100.4±5.9 (95-110)	98.7±5.8 (95-110)
Distance from anterior end to posterior end of pharyngeal glands		126, 127	134.7±10.7 (121-149)	142.0±16.3 (117-155)	144.0±12.1 (130-161)
Body diam. at end of pharyngeal glands		14, 13	13.8±0.4 (13-14)	18.9±1.1 (17.5-20.0)	17.9±1.7 (14.5-19.0)
Anterior genital branch		257, 224	134.7±10.7 (121-149)	336.8±42.9 (282-401)	309.2±60.6 (235-420)
Posterior genital branch		83, 86	-	100.5±23.9 (76-122)	-
Body diam. at vulva		16, 15	-	20.2±1.7 (18.5-22.0)	-
Vulva to anus distance		132, 133	-	165±14.1 (144-183)	-
Distance from anterior end to vulva		461, 464	-	561.4±60.4 (490-636)	-
G1 (%)		40.6, 35.6	-	42.4±7.2 (34.9-53.5)	-
G2(%)		13.1, 13.7	-	13.0±3.4 (9.6-16.3)	-
Anal/cloacal body diam.		9.5, 9.0	12.2±0.8 (11-13)	10.7±0.4 (10-11)	13.1±1.1 (12-15)
Tail		38, 31	26.9±1.8 (25-30)	39.6±5.0 (35-48)	29.9±3.0 (24.5-33.0)
T		-	55.8±3.0 (51.2-59.9)	-	42.9±9.1 (32.6-59.0)
Spicule (condylus to distal end)		-	12.0±1.2 (10.0-13.5)	-	19.4±1.1 (18-21)
Spicule (rostrum to distal end)		-	18.7±1.5 (16-20)	-	12.8±0.5 (12.0-13.5)
Spicule (curved median line)		-	18.2±1.8 (16-20)	-	17.8±1.4 (16.0-19.5)
Spicule (rostrum to condylus)		-	7.4±0.6 (7.0-8.5)	-	7.6±0.9 (6.5-9.0)
Distance from single precloacal papilla to cloacal aperture		-	3.3±0.4 (3-4)	-	3.3±0.8(2.5-4.5)
Distance from cloacal aperture to first pair of postcloacal papillae		-	8.9±1.9 (6-11)	-	10.0±2.6 (6.5-13.5)
Distance from cloacal aperture to second pair of postcloacal papillae		-	15.3±1.5 (13.0-16.5)	-	16.2±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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CHAPTER IV. DISCUSSION

IV.1 Discussion

This is the first comprehensive study on the genus *Bursaphelenchus* from Portugal, and in it are described for the first time the species occurring in the country, their geographical location and abundance, association with insect vectors, and also characterization and discussion of the most important morphological characters and methodologies used in taxonomical studies on this genus.

Following the detection of the pathogenic pinewood nematode (PWN) *B. xylophilus* in Pegões, Portugal, in 1999 (Mota *et al.*, 1999), several surveys conducted for nematode identification were performed in the Setúbal peninsula and other selected high risk areas all over continental Portugal such as maritime ports, sawmills, lumber companies, etc. The outcome of such surveys allowed the identification of eight additional species of this genus in our country, namely: *B. antoniae* Penas, Metge, Mota & Valadas, 2006, *B. hellenicus* Skarmoutsos, Braasch & Michalopoulou, 1998, *B. leoni*, *B. mucronatus*, *B. pinasteri* Baujard, 1980, *B. sexdentati*, *B. teratospicularis* and *B. tusciae* Ambrogioni & Palmisano, 1998 (sub-chapter III.1, III.3 and III.4). All these species are recorded for the first time in Portugal, while *B. antoniae* was also described as a new species to science (sub-chapter III.3).

A common trait to all *Bursaphelenchus* species reported in this work is that they were found associated with maritime pine (*P. pinaster*), the surveyed host. Only *B. antoniae* was not collected directly from maritime pine wood, although it was recovered from *Hylobius* sp. Germar beetles which had emerged from *P. pinaster* stumps. Nevertheless, under laboratory conditions this species was maintained on small *P. pinaster* wood segments. Maritime pine is Portugal's most abundant forest species and occupies an area of approximately 971,000 ha (DGRF, 2006), being the main provider of wood, cellulose, agglomerates and furniture (Goes, 1991). As *P. pinaster* is more abundant in the north and center of the country, this might partially explain the higher diversity of *Bursaphelenchus* species found in these regions.

With this study, the total number of *Bursaphelenchus* species recorded from Portugal now totals ten, if we also consider *B. pinophilus* Brzeski & Baujard, 1997 which had already been previously reported by Braasch (2001). Worldwide, there are about 94 valid species of this nematode genus, of which about half occur in Europe

(Hunt, 2008). This means that in Portugal occur at least 10% of the world and 20% of the European species of this nematode genus, which can be considered to be a high number considering the relatively small size of the country (89 000 km^2), its peripherical geographic position within the European continent and the fact that only one conifer host, *P. pinaster*, was systematically analyzed in this study.

Following the detection of *B. xylophilus* in Portugal, the survey and description of new *Bursaphelenchus* species in Europe has also increased in recent years, increasing the knowledge on the distribution of many species. Nevertheless, due to their association with wood and wood products, *Bursaphelenchus* species are routinely intercepted in packaging and wood products in several European countries such as Finland (Tomminen, 1991), Germany (Braasch *et al.*, 2001) and Austria (Tomiczek *et al.*, 2003), a factor which must also be considered when studying the distribution of the species of this genus. The influence of human activities and transport can be seen by the record of *B. leoni* from South Africa (Braasch *et al.*, 1998) and a *Bursaphelenchus* sp. from Australia (Ridley *et al.*, 2001), as these nematodes now occur in areas from which pines are naturally absent (Mirov, 1967), which means that they have been introduced along with their host pines and for associated bark beetle vectors.

In Portugal, the most abundant nematode species considering all samples was *B. xylophilus*, which although initially confined to the denominated PWN "affected zone", south of Lisbon, was more recently reported from the center of Portugal near Lousã and Arganil (Coimbra district). The high frequency of occurrence of this species reflects the more intensive surveys conducted in its initial distribution area, where this nematode has been over the years responsible for an important mortality of adult maritime pine trees, with more than 700 000 symptomatic *P. pinaster* trees which had to be felled between 1999 and 2006 (Anonymous, 2006). It is important to mention that although *B. xylophilus* occurs on locations with mixed maritime pine and stone pine (*Pinus pinea* L.) forests, it has so far been only found associated with maritime pine in Portugal (Anonymous, 2006), and therefore the reasons why *P. pinea* is not attacked by this nematode in Portugal should be object of future studies. The pine sawyer *M. galloprovincialis* has been found to prefer maritime pine over *P. pinea* for feeding and laying eggs (Naves *et al.*, 2006), and according to some authors the stone

pine has a moderate resistance to the PWN (De Guiran & Boulbria, 1985; Evans *et al.*, 1996), which might help to explain the apparent absence of wilt disease in this species.

Along with *B. xylophilus*, *B. pinasteri* was the most frequently found species in the surveys. This species was recently synonymized with *Bursaphelenchus chitwoodi* Rühm, 1956 (J.B. Goodey, 1960) by Hunt (2008). *Bursaphelenchus pinasteri* was originally described associated with *P. pinaster* in France (Baujard, 1980), and in Portugal it appears to be very common in the same pine host. In the PWN "affected zone" of Setúbal peninsula this nematode may benefit from the abundance of dead and decaying maritime pine trees which appear each year due to *B. xylophilus* infections, along with the increasingly abundant populations of several bark and wood-boring insect species which have greatly benefited from the introduction of *B. xylophilus* and have now very high population levels in the Setúbal peninsula (Sousa *et al.*, 2005).

The other nematode species frequently encountered in wood samples can be considered to be "typical" Mediterranean species, such as *B. leoni*, *B. teratospicularis* and *B. tusciae*, which have been reported from countries like Spain (Escuer *et al.*, 2002; Escuer *et al.*, 2004), France (Baujard, 1980), Italy (Ambrogioni *et al.*, 1994; Ambrogioni & Caroppo, 1998) and Greece (Skarmoutsos & Skarmoutsos, 1999), suggesting that the Portuguese *Bursaphelenchus* fauna has a high component of "Mediterranean" *Bursaphelenchus* species, which is not surprising considering the influence of the Mediterranean-like climate characteristic of the majority of the country.

Conversely, *B. mucronatus* appears to be quite infrequent in Portugal as it was found only once and in a single location in the north of the country (sub-chapter III.1 and III.4). A similar situation can be found in Spain, where *B. mucronatus* is also infrequent and occurs mainly in Galicia and northern Spain (Escuer *et al.*, 2004). Nevertheless, worldwide *B. mucronatus* is a widespread species which can be found in conifer forests of Asia (Mamiya & Enda, 1979) and Europe (Ryss *et al.*, 2005), namely in France (Baujard *et al.*, 1979), Norway (McNamara & Stoen, 1988), Finland (Tomminen *et al.*, 1989), Sweden (Magnusson & Schroeder, 1989), Germany (Braasch, 1991) and Russia (Kulinich *et al.*, 1994). Our results suggest that *B. mucronatus*, preferring the boreal and colder climates and conifer hosts of more northern latitudes, may have the

southern limit of its vast distribution range in the northern strip of the Iberian Peninsula.

Overall, and with the exception of *B. xylophilus*, the casual agent of pine wilt disease on pines and susceptible conifers (e.g.: Linit, 1988; Kishi, 1995), the other nematode species found are considered strictly mycetophagous and colonize only weakened or dead pine trees (Hunt, 1993; Ryss *et al.*, 2005), although for some authors there have been suggestions of pathogenic effects to young pines, namely *B. mucronatus* (e.g.: Yang *et al.*, 1988; Braasch *et al.*, 1998). Nevertheless, such studies are usually not conclusive and have been performed mainly on pine seedlings, and thus the effective pathogenicity of these nematode species to healthy adult pine trees under field conditions remains to be effectively demonstrated both in Portugal and elsewhere.

The associations of *B. hellenicus* and *B. tusciae* with *P. pinaster* were here reported for the first time. These two species had been previously found in Europe on *Pinus brutia* Tenore, *Pinus sylvestris* L. and *Larix* sp. (Ryss *et al.*, 2005), which are species that do not occur or are extremely rare and localized in Portugal. Nevertheless, *B. tusciae* had also been reported from *P. pinea* and *B. hellenicus* from *Pinus halepensis* Mill. (Ryss *et al.*, 2005), and as these two pines are relatively common and widespread in southern Portugal, the possible association between the two nematode species and these two pines should be further investigated in our country.

Association of the different *Bursaphelenchus* species with their insect vectors was also studied in Portugal for the first time (sub-chapter III.2). The majority of the nematode species were found to have an ectophoretic association with insects of the family Scolytidae (Coleoptera), corroborating previous studies which have found that most species of the genus *Bursaphelenchus* are transmitted to dead or declining trees during oviposition of bark beetles of the family Scolytidae (e.g.: Braasch, 2001; Ryss *et al.*, 2005). Only two *Bursaphelenchus* species were found associated with non-scolytid beetles, namely *B. antoniae* and *B. xylophilus*, which were found in a Curculionidae and a Cerambycidae insect, respectively (sub-chapters III.2 and III.3). The recently described *B. antoniae* was found in *Hylobius* sp., while the closely related *B. hylobianum* (Korentchenko, 1980) Hunt, 1993 [with which *B. antoniae* was

misidentified in a previous report (sub-chapter III.1)], is also vectorized by a species of the same genus in Russia, namely *H. albosparsus* Boheman. A summary of the location of nematode sampling, associated vectors and abundance is presented in Table I.

Table I – Geographical distribution, association with insects and frequency of occurrence in wood of the *Bursaphelenchus* species found in continental Portugal.

Bursaphelenchus species	Locations (district)	Associated insects	Frequency of occurrence in wood material (%)	
			Inside Demarcated Zone (n= 3684)	Outside Demarcated Zone (n= 1129)
B. antoniae	Leiria	*Hylobius* sp.	a)	a)
B. hellenicus	Santarém, Setúbal, Sines and Viseu	*T. piniperda*, *I. sexdentatus* *H. ligniperda*	0.1	0.09
B. leoni	Aveiro, Castelo Branco, Coimbra, Lisboa, Porto, Santarém, Setúbal, Sines, Vila Real and Viseu	*Pityogenes* sp.	0.4	2.6
B. mucronatus	Coimbra	b)	0	0.09
B. pinasteri	Bragança, Évora, Lisboa, Santarém, Setúbal, Sines and Viseu	b)	3.6	0.3
B. pinophilus [c]	Unknown	b)	Unknown	Unknown
B. sexdentati	Coimbra, Porto, Santarém, Setúbal and Viseu	*O. erosus* *H. ligniperda*	0,08	0.4
B. teratospicularis	Castelo Branco, Santarém, Setúbal, Sines, Vila Real and Viseu	*O. erosus*	0.3	0.4
B. tusciae	Coimbra, Faro, Santarém, Setúbal, Sines and Viseu	*H. ligniperda*	0.3	0.6
B. xylophilus	"Affected Zone"	*M. galloprovincialis*	39	0

a) not found in wood material
b) not found associated with any insect
c) after Braasch *et al.*, 2001

Some of the nematode-insect associations described in sub-chapter III.2 had never been previously reported, such as the interaction between *B. hellenicus* and both *Ips sexdentatus* Boern. and *Hylurgus ligniperda* (Fabricius), *B. sexdentati* with both *H. ligniperda* and *Orthotomicus erosus* Wollaston and *B. tusciae* with *H. ligniperda*. In contrast, some nematode-insect associations which had been previously reported from other locations were not detected in Portugal despite the existence of suitable insect species, namely *B. sexdentati* with both *I. sexdentatus* (Rühm, 1960) and *Tomicus piniperda* (Linnaeus) (Braasch *et al.*, 1999), and *B. teratospicularis* with *Tomicus minor* (Hartig) (Kakuliya & Devdariani, 1965).

Further sampling on other locations may eventually detect such interactions in our country, although there is also the possibility that nematode species with widespread ranges may become associated with different insect vectors along diverse geographical locations, in accordance to the local assemblage and abundance of suitable vector species and/or existence of adequate conifer hosts. Such an example could be the association of *B. teratospicularis* with insects of the genus *Orthotomicus* Ferrari, as in Portugal this nematode was found associated with *O. erosus* while in Georgia the insect vector is a different species of the same genus, *O. proximus* (Eichhoff) (Kakuliya & Devdariani, 1965).

It is interesting to report that the scolytid species with the highest diversity of associated *Bursaphelenchus* spp. (three or four) was the bark beetle *H. ligniperda*, this being a rather non-aggressive insect which colonizes only very weakened or dead conifer trees, where it is found on the large stumps and roots of adult trees (Fabre & Carle, 1975; Sanchez & Alonso, 1986). In contrast, the much more "aggressive" bark beetle species *I. sexdentatus* and *T. piniperda*, which are known to attack and kill apparently healthy adult pine trees (e.g.: Ferreira & Ferreira, 1985; Sanchez & Alonso, 1986), were found to carry a common and single nematode species, *B. hellenicus*. Apparently, the lesser aggressive bark beetles are vectors of more *Bursaphelenchus* species than the more aggressive insects, in agreement with the generally accepted idea that most *Bursaphelenchus* are mycetophagous and require weakened or dead trees as appropriate hosts (Hunt, 1993; Ryss *et al.*, 2005). The relatively high number of conifer hosts (mainly of the genus *Pinus*) (see Ryss *et al.*, 2005), suggests that the

association of the mycetophagous *Bursaphelenchus* species with their hosts may be conditioned by both preferences of their local vector insects and the existence of suitable fungi communities in the hosts, which are known to vary according to the different bark and wood-boring insect communities that colonize the trees (Paine *et al.*, 1997). The complex interactions between such different species as insects, fungi, nematodes and tree hosts requires further study at a worldwide scale as it can contribute to understand the pathogenicity and aggressiveness of several species of bark and wood-boring insects and their associated fungi and nematodes towards their conifer hosts.

The abundance of the *Bursaphelenchus* species may depend on the population levels of their insect vectors, and therefore the apparent abundance of *B. sexdentati*, *B. teratospicularis* and *B. tusciae* may be a result of the abundance of their identified insect vectors *O. erosus* and *H. ligniperda*, which are among the most common and frequent insects colonizing dead and dying maritime pine trees in Portugal. In contrast, *B. antoniae* was found a single time in *Hylobius* sp. beetles collected from one location and afterwards was never detected on any of the wood samples, which may reflect the apparent rarity of the species of the genus *Hylobius* in Portugal (Bonifácio *et al.*, 1999; Naves *et al.*, 2000; Sousa *et al.*, 2000).

Nevertheless, some situations are not clear, such as the abundance of *B. pinasteri* (the nematode most frequently found on the sampled wood material other than *B. xylophilus*), although this nematode was never found associated with any of the surveyed insects (sub-chapter III.2). Furthermore, there are also no records for the vector(s) of this species worldwide (Vieira *et al.*, 2006), and thus it is possible that this nematode may be vectored by a less frequent scolityd beetle or even by an unsurveyed species from another distinct family of xylophagus Coleoptera, like the Bostrychidae Latreille or Anobiidae Fleming. Eventually, it can even be carried by an insect of a different insect order, as some other *Bursaphelenchus* species found associated with other insect orders such as the Hymenoptera and the Lepidoptera (e.g.: Giblin-Davis *et al.*, 1993; Rühm, 1956; Vieira *et al.*, 2006).

As previously mentioned, the pinewood nematode *B. xylophilus* was detected in a single species of the family Cerambycidae, the pine sawyer *M. galloprovincialis* (Sousa

et al., 2001; Sousa *et al.*, 2002). Insects of the genus *Monochamus* are considered to be the most important vectors of the PWN worldwide (e.g.: Kobayashi *et al.*, 1984; Linit, 1988; Kishi, 1995), and of the roughly 150 species distributed worldwide only five occur in Europe (Hellrigl, 1971). Of these, just two are native to the Iberian Peninsula, *M. galloprovincialis* and *M. sutor,* although only the first is present in Portugal (Vives, 2000). The close interaction between *B. xylophilus* and insects of the genus *Monochamus*, of which there are several examples in North America and Eastern Asia, was confirmed in Europe when the recently-introduced PWN was detected associated with the native *M. galloprovincialis*. Despite its recentness, this association has, nevertheless, been responsible for the death of several thousand maritime pine trees (Anonymous, 2006), thus causing profound economic, ecological and social impacts on the affected zone in Setúbal peninsula.

Subsequent studies have in fact proved that *M. galloprovincialis* does transmit the pinewood nematode through both maturation feeding (Naves *et al.*, 2007a) and oviposition activity of the adult beetles (Naves *et al.*, 2007b). Therefore, the pine sawyer *M. galloprovincialis* is an effective vector of *B. xylophilus*, along with six other species of the genus worldwide: *M. carolinensis* , *M. mutator* LeConte, *M. scutellatus* Say and *M. titillator* Fab. in North America, and *M. alternatus* and *M. saltuarius* (Gebler) in Northeast Asia (Linit, 1988; Kishi, 1995).

Besides insects of the genus *Monochamus*, other beetles of the family Coleoptera have been found to accasionally carry *B. xylophilus*, although with very low frequencies and nematode loads and therefore they are not classified as effective vectors of the PWN (Wingfield & Blanchette, 1983; Kobayashi, *et al.* 1984; Linit, 1988). In agreement with such reports, the PWN was never detected from any additional insect in Portugal, despite the more than 21 species from several insect families which were analyzed for its presence. In Portugal, *B. xylophilus* seems to establish a very close relationship with *M. galloprovincialis* since it was only found in this insect and no other nematode was isolated from it. An apparent common trait to most species of the *xylophilus* group such as *B. conicaudatus* Kanzaki & Futai, 2001, *B. luxuriosae* Kanzaki & Futai, 2003 and *B. doui* Braasch, Gu, Burgermeister & Zhang, 2005, is that nematodes are vectorized by cerambicyd beetles of the Laminae sub family and are carried on the vector´s tracheae

(Kanzaki & Futai, 2001, 2003; Kanzaki *et al.*, 2008), which was also found for *B. xylophilus* in Portugal.

Besides the genus *Bursaphelenchus*, species from other genera [*Ektaphelenchus*, *Parasitaphelenchus*, *Parasitorhabditis* (Fuchs, 1937) Chitwood, 1950 and *Contorthylenchus* Rühm, 1956] were also found associated with various scolytid and curculionid beetles, frequently in higher numbers than the *Bursaphelenchus* species (sub-chapter III.2). Although some aspects of the ecology of some of these nematode have already been studied [(e.g., *Parasitorhabditis* spp. being internal parasites of bark beetles (Massey, 1974)], the positive and negative interactions of such species with *Bursaphelenchus* nematodes aboard their insect vectors have not yet been studied, although it is possible that inter-specific competition may occur among the various ectophoretic nematodes for the limited space available on the folds and structures under the elytra and wings of the insect species selected for dispersion.

Precise identification of the various *Bursaphelenchus* species was made using morphological, biometrical and molecular characters. Morphometric measurements were made for all species except *B. mucronatus*, as the limited number of available individuals was used on molecular studies. All populations, with the exception of *B. antoniae*, were measured after being collected from pine wood from the field. Concerning the pine wood nematode, part of one collected population was also cultured on fungi, and size differences were observed as specimens from cultured populations displayed larger dimensions in all morphometric parameters (sub-chapter III.4). Such differences could be explained by the more adequate conditions of the cultured nematodes, with more favourable temperature and availability and quality of food. These results suggest that biometric measurements of nematode-cultured populations should be interpreted with some caution and, whenever possible, such biometric studies should be conducted on populations collected from the field. Nevertheless, some field-collected Portuguese populations exhibited large variation on the individual's size with both large and small nematodes coexisting.

In most cases, morphological characters alone allowed for a correct identification, although further confirmed on several occasions by molecular approaches (ITS-RFLP and rDNA sequencing analysis). Characters such as male spicule

shape, vulval region, female tail, head region, number of incisures in the lateral field and number and disposition of male caudal papillae provided a solid basis for specific identification of most species. Nevertheless, and especially for *B. xylophilus* and *B. sexdentati* these specific characters usually did not allow for precise identification, and need to be corroborated with the molecular techniques referred above.

The different species of the genus *Bursaphelenchus* are usually grouped according to their morphological and molecular characters (which are further detailed on the Introduction – chapter I). Following the classification proposed by three recent authors (Braasch, 2001; Ryss *et al.*, 2005 and Ye *et al.*, 2007), the species of *Bursaphelenchus* found in Portugal can be grouped as presented in Table II.

Table II – Inclusion of the different *Bursaphelenchus* species found in Portugal in the different groups proposed by Braasch (2001), Ryss *et al.* (2005) and Ye *et al.* (2007).

***Bursaphelenchus* spp.**	**Braasch, 2001**	**Ryss *et al.*, 2005**	**Ye *et al.*, 2007**
B. hellenicus	*hofmanni*	*piniperdae*	*abietinus*
B. leoni	*leoni*	*borealis*	Not aplicable
B. mucronatus	*xylophilus*	*xylophilus*	*xylophilus*
B. pinasteri	*hofmanni*	*piniperdae*	Not aplicable
B. pinophilus	*sexdentati*	*piniperdae*	Not aplicable
B. sexdentati	*sexdentati*	*piniperdae*	*sexdentati*
B. teratospicularis	Not defined	*eidmanni*	Not aplicable
B. tusciae	*eggersi*	*borealis*	*eggersi*
B. xylophilus	*xylophilus*	*xylophilus*	*xylophilus*

Considering the number of incisures in the lateral field suggested by Braasch (2001), the newly described *B. antoniae* should be included in the "*abietinus*" group (two incisures) along with *B. hylobianum*. On the other hand, following the dichotomous keys of Ryss *et al.* (2005), this new species should be included in the *piniperdae* – group, which also includes the closely related *B. hylobianum*.

The phylogenetic analysis of 14 *Bursaphelenchus* species, including *B. antoniae* is presented in sub-chapter III.3. The 18S rDNA sequencing tree shows that *B. antoniae* is grouped with *B. hylobianum,* although if we consider the 28S D2/D3 domain

sequencing this species is not included in a definite group. Comparing with the phylogenetic analysis of Ye *et al.* (2007) grouping the 14 *Bursaphelenchus* species from sub-chapter III.3, the clustering of species using the rDNA sequencing analysis suggest that *B. antoniae*, which was not studied by the previous authors, would probably be included in the "*hylobianum*" group.

In the case of *B. xylophilus*, most of the populations had males with the typical spicule shape, number and disposition of male caudal papillae and females with a long vulval flap and a rounded tail leading to a reliable identification, confirmed later by ITS-RFLP. Nevertheless, some of the *B. xylophilus* populations had females with distinct mucronated tails, making the morphological observations non conclusive. In these cases, an ITS-RFLP pattern of the population was absolutely required for the correct species identification. As discussed before, in only one occasion the molecular results identified *B. mucronatus*, a species very similar to *B. xylophilus*, with the mucronated female tail tip. In some occasions, only *B. xylophilus* juvenile stages were present in the samples, which made consistent diagnosis difficult and lead to further molecular analysis. The morphometric analysis of four characters (total body length, body diameter, tail length and gonad length) allowed the separation and confirmed the existence of three propagative juveniles stages outside the egg, J_2, J_3 and J_4 (sub-chapter III.4). These results are in total accordance with other studies developed for the same species in other countries (e.g.: Mamiya, 1975; Ishibashi *et al.*, 1978).

As already referred, *B. pinophilus* was not clearly identified from any of the samples analyzed, although this species has been previously reported for Portugal by Braasch (2001). *B. pinophilus* is closely related to *B. sexdentati* and to other species of the genus like *B. vallesianus* Braasch, Schönfeld, Polomski & Burgermeister, 2004, *B. borealis* Korenchenko, 1980, *B. naujaci* Baujard, 1980, *B. poligraphi* Fuchs, 1937 and *B. incurvus* Rühm, 1956, according to morphological and molecular studies (Braasch, 2001; Lange *et al.*, 2007). Although the characters analyzed suggested either *B. pinophilus* or *B. sexdentati*, the difficulty to separate this complex of species based on morphological characters lead to the classification of the populations studied in sub-chapter III.1 as "*Bursaphelenchus* spp.". In fact, the differentiation of both species, which was mainly based on spicule shape with presence or absence of cucullus and

female tail shape, is frequently confusing and even contradictory. In a large number of samples the precise identification of *B. sexdentati* or *B. pinophilus* was not achieved as such nematodes were analyzed by ITS-RFLP tests and were found to be *B. sexdentati*, it is reasonable to assume that the not identified isolates probably also correspond to *B. sexdentati* (sub-chapter III.1 and III.2).

This species was originally described by Rühm (1960) without a cucullus in the spicule tip and with a subcylindrical female tail with a bluntly rounded terminus. Such characteristics were later confirmed by Braasch (2001) and Ryss *et al.* (2005), and the presence of a cucullus in *B. pinophilus* allowed for the separation of both species. Nevertheless, Italian populations of *B. sexdentati* were reported as having a cucullus on the male spicule tip and a conoid female tail, (with rounded to pointed terminus) (Ambrogioni & Caroppo, 1998), similarly to the Portuguese populations studied in sub-chapter III.1 and III.4, which were confirmed to be *B. sexdentati* by ITS-RFLP analysis. Further studies on this complex of species is needed, as recent molecular studies (ITS-RFLP profiles and ITS1 and ITS2 sequencing) of different isolates of *B. sexdentati* from Europe (Portugal included) have distinguished a Central and a South European type of these species, which may in fact correspond to different species (Lange *et al.*, 2007).

For *B. teratospicularis* the characters studied suggest that this species may belong to a different genus. Immature females as well as some males were found in a cocoon-like structure under the elytra of the vector insects, *O. erosus*. Some characteristics of *B. teratospicularis* are clearly distinct from other *Bursaphelenchus* species such as the intestine ending in a blind sac and an anus that is very difficult to discern, coarsely annulated cuticle and instead of *dauer* juveniles, like the other *Bursaphelenchus* species, adult *B. teratospicularis* are transported inside cocoon-like structures by the insect vectors. The features just described match very well with the ones typical of the nematodes of the genus *Ektaphelenchus* (Thorne, 1935; Rühm, 1956; Massey, 1974; Hunt, 1993). The nematodes found in *O. erosus* were included in genus *Bursaphelenchus* because the males had a terminal bursa in the male tail tip, a characteristic typical of this genus and not reported in *Ektaphelenchus*. Nevertheless, rigorous and detailed studies on *B. teratospicularis* for a confirmation of its taxonomic identity should be conducted in the future.

The results presented on this thesis greatly increased the general knowledge on the *Bursaphelenchus* fauna associated with maritime pine in Portugal, studying for the first time it´s diversity, distribution, abundance and vector associations, along with diverse taxonomical studies. Nevertheless, there is still clearly an absence of knowledge concerning the species-diversity, abundance and host and vector associations of the various other nematode species which apparently cohabit with the *Bursaphelenchus* spp. in the Portuguese pines and share its vectors, which should be object of future studies as this biological knowledge can help to solve taxonomical problems such as the identity and the correct classification of the doubious *B. teratospicularis* associated with *O. erosus* previously referred.

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CHAPTER V. FINAL REMARKS

V.1 Final Remarks

The necessity to conduct the studies presented in this thesis arose after the detection of the pine wood nematode (PWN) *B. xylophilus* in Portugal in 1999, in what was the first confirmed presence of this pathogenic organism in a European country (Mota *et al.*, 1999). The economic, social and environmental consequence of the presence of the PWN in Portugal and in Europe required, over the years, an efficient and reliable identification of this organism on various surveys and studies, with the necessity to separate it from other morphologically similar species of the same genus which could cohabit the same host or vector. For that, it was also necessary to survey, identify and study the various species of the genus *Bursaphelenchus* associated with maritime pine *P. pinaster* in Portugal, a task which had not been previously done and became one of the most important objectives of this thesis.

There is now a record of ten species of this genus occurring in Portugal, of which nine where for the first time reported in this work, one new to science – *B. antoniae*. Only two species belong to the *xylophilus* group, and if one was recently introduced (*B. xylophilus*) the other appears to be quite infrequent and with a localized distribution (*B. mucronatus*), which may facilitate morphological identification of the nematodes found in wood samples collected from trees suspected of being infected with the pine wood nematode. Overall, these results contribute to the general characterization of the faunal biodiversity in Portugal and in Europe, and more specifically of the nematode fauna associated with maritime pine, its distribution, abundance and associated insect vectors.

Studies on the nematode-vector associations allowed the identification of several new interactions which had never been previously reported, although considering specifically the PWN the results agree with previous observations (Sousa *et al.*, 2001, 2002) and confirm the exclusive character of the interaction between *B. xylophilus* and its sole vector in Portugal *M. galloprovincialis*, which is also the only representative of its genus in the country.

The research conducted demonstrated that an observation and characterization of a few selected morphological characters can lead to a correct and

precise identification of some of the species, although one should always consider that a correct diagnosis can be problematic due to the slight and discrete morphological variations between closely related taxa, the complexity involved in the identification of the juvenile stages and the deterioration of the nematode samples (Vrain & McNamara, 1994).

Although the use of morphological and biometrical analysis frequently lead to consistent diagnosis, in some cases such as *B. xylophilus* and *B. sexdentati*, these methods were not sufficiently discriminative and did not allow for a rigorous identification, and thus a molecular approached was also required. The characterization of the ITS profile and the rDNA sequence was also necessary to confirm the identification of the new species, *B. antoniae*.

Overall, the different studies unequivocally demonstrated the complementary nature of using both morphological/biometrical and molecular methodologies, which jointly lead to more reliable and rigorous identifications. This should be taken into consideration following the very recent detection of the PWN on various locations outside its former "afected zone" in Portugal, which lead to the classification of the entire continental territory as an area affected by the PWN (MADRP, 2008) and will demand further surveys all over the country to determine the current and precise distribution of this pathogen.

As maritime pine is, until now, the sole conifer affected by *B. xylophilus* in Portugal, the studies conducted focused only on this pine species, which is the most widespread and important conifer in Portugal. Nevertheless, future studies on *Bursaphelenchus* diversity, hosts and vectors in Portugal should include other pine species such as *P. pinea* and *P. halepensis*, which have already been reported to host these and other *Bursaphelenchus* species in other Mediterranean countries (e.g.: Ambrogioni & Palmisano, 1998; Skarmoutsos & Skarmoutsos, 1999; Escuer *et al.*, 2002). The effective pathogenicity of the different *Bursaphelenchus* species should also be evaluated trough studies conducted under the climatic conditions occurring in Portugal and using local pine hosts. Additional species of the genus *Bursaphelenchus* may also be detected in future studies by surveying other insect species from the

families Scolitydae and Cerambycidae, or even from other non-surveyed families such as the Nitidulidae.

A detailed morphological and molecular study of *B. teratospicularis* should also be conducted in the future, in order to clarify the taxonomy of *B. teratospicularis* and discriminate it from similar *Ektaphelenchus* spp. also found on the elytra cocoons of their common vector insect, *O. erosus*.

The PWN has been present in Portugal for over a decade and is currently spreading into new areas, which means the economic, social and environmental impacts of its presence are likely to increase in the near future. Other nematodes of the *Bursaphelenchus* genus are constantly being intercepted and accidentally introduced all over Europe, as it also occurs with species from other animal and plant groups. A precocious and reliable taxonomical identification can mean the difference between a successful and a failed interception and introduction of one of those biological invaders. As shown by this study, the complementary use of both morphological and molecular techniques along with biological studies of the organisms can provide reliable identifications for even close-related species, in what can be defined as an 'integrative taxonomy' approach, aiming to delimit the units of life's diversity from multiple and complementary perspectives such as phylogeography, comparative morphology, population genetics, ecology, development, behaviour, etc. (Dayrat, 2005). Nevertheless, to perform such studies we need above all experienced taxonomists, a professional group which is becoming scarcer every year and which needs to be recognised as having an important role in preventing future biological invasions, which can eventually have impacts similar or even greater than the current undesirable presence of *B. xylophilus* in Portugal and in Europe.

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APPENDIX

Other manuscripts related to the subject of the thesis co-published by the author:

- Mota, M., Braasch, H., Bravo, M. A., Penas, A. C., Burgermeister, W., Metge, K. & Sousa, E. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology*, **1**: 727-734.

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Nematology, 1999, Vol. 1(7—8), 727-734

First report of *Bursaphelenchus xylophilus* in Portugal and in Europe

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Summary – A survey of aphelenchid nematodes (Nematoda: Aphelenchida) associated with maritime pine, *Pinus pinaster*, was conducted in Portugal in 1996 and 1999. A *Bursaphelenchus* species has been identified for the first time in the Iberian Peninsula. *B. xylophilus* is reported for the first time in Europe. It was found in very high numbers – up to 38 000 per 10 g of pine wood – inside a few declining trees infested with curculionid, cerambycid and scolytid beetles. Morphological observations, including shape of spicules, bursa, vulva, female tail end and stylet as well as morphometrics, were in accordance with the species description. Species-specific DNA fragment patterns were obtained using ITS-RFLP analysis, with five different restriction enzymes. The importance and implications of this finding are discussed.

Résumé – ***Première signalisation de* Bursaphelenchus xylophilus *au Portugal, at en Europe*** – Une enquête sur les nématodes Aphelenchides (Nematoda: Aphelenchida) associés au pin maritime (*Pinus pinaster*) a été réalisée au Portugal de 1996 à 1999. Une espèce de *Bursaphelenchus* a été identifiée pour la première fois dans la Péninsule Ibérique. *B. xylophilus* est signalé pour la première fois en Europe. Il a été trouvé en très grand nombre – jusqu'à 38 000 individus pour 10 g de bois de pin – dans des arbres dépérissants infestés par des Coléoptères Curculionides, Cérambycides et Scolytides. Les observations concernant la morphologie – en particulier la forme des spicules, la bourse, la vulve, l'extrémité de la queue de la femelle et le stylet – de même que les données morphométriques correspondent à la description de l'espèce. Des séquences de fragments d'ADN spécifique de l'espèce ont été obtenus par analyse ITS-RFLP à l'aide de cinq enzymes de restriction. L'importance et les implications de cette découverte sont discutées.

Keywords – ITS-RFLP, morphology, morphometrics, pinewood nematode (PWN), SEM.

The total area of forest trees in Portugal is about 3×10^6 ha, of which *Pinus* species occupy roughly 1.25×10^6 ha. Pine products include lumber, resin, pulp and pine seed, all very important economic products. *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle 1970, (Nematoda: Aphelenchoididae), is the causal agent of pine wilt disease (PWD) in certain *Pinus* species and is one of the few plant parasitic nematodes that kill a mature host within a relatively short period after penetration. The nematode, frequently referred to as "pine wood nematode" (PWN) is vectored by wood-boring beetles of the cerambycid genus *Monochamus*. *B. xylophilus* is native to North America and has been introduced by way of the timber trade to Japan, from where it has been spread into China, Taiwan and Korea.

B. xylophilus is a quarantine organism in the European Union (Directive 77/93 EEC). The pest risk analysis (PRA) for the territories of the European Union (as PRA area) on *B. xylophilus* and its vectors in the genus *Monochamus* (Evans *et al.*, 1996), elaborated by an expert group of the European Union, defines the key conclusions concerning the risk as follows: quarantine measures for *B. xylophilus* are justified because the nematode does not occur in the PRA area, the area is suitable for its establish-

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ment, susceptible host species occur, and the nematode is of potential economic importance to the PRA area. Pine wilt can be expected in the Mediterranean and continental dry region.

About 25 *Bursaphelenchus* species have been found in coniferous trees in Europe, namely Germany, Scandinavia, France, Poland, Italy, Greece, Austria, Bulgaria, Cyprus and Russia (Braasch *et al.*, 1998). Since 1996, an intensive survey for *Bursaphelenchus* species has been conducted in Austria, Germany, Italy and Greece under the frame of an EU-supported project, "Pest risk analysis of pinewood nematode related *Bursaphelenchus* species in view of South European pine wilting and wood imports from Asia". *B. mucronatus* (Mamiya & Enda, 1979) is the most abundant *Bursaphelenchus* species in Central and East Europe (Brzeski & Baujard, 1997; Brzeski & Brzeski, 1997; Braasch *et al.*, 1998), while *B. sexdentati* and *B. leoni* occurred most frequently in Southern Europe (Palmisano *et al.*, 1994; Philis & Braasch, 1996; Ambrogiani & Caroppo, 1998; Braasch *et al.*, 1998). However, *B. xylophilus* was not found in any of the above mentioned European countries. The taxonomy of *Bursaphelenchus* species found in pine wood is quite confusing (Mamiya & Kiyohara, 1972; Baujard, 1980) due to very similar morphological characters of some species. Recently, however, new techniques employing sex pheromones (Riga & Webster, 1992) as well as DNA analysis (Riga *et al.*, 1992; Braasch *et al.*, 1995; Irdani *et al.*, 1995; Hoyer *et al.*, 1998; Iwahori *et al.*, 1998) have been instruments in clarifying this group.

In Portugal, there are no results concerning a general survey of *B. xylophilus* or of other close and easily confused species, such as *B. mucronatus*. Macara (1994) has drawn attention to the danger of the presence of the nematode in Portugal and Europe. In response to an alert in 1994 by the government agency in charge of crop protection, "Direcção Geral de Protecção de Culturas"/ DGPC (Fernandes & Pereira, 1994), we initiated in 1996 a survey of these nematodes, as well as their cerambycid vectors, under a national research project. Preliminary results of the nematodes associated with pine trees have been reported (Mota, 1998). The purpose of this paper is to report the occurrence of the pine wood nematode *B. xylophilus* for the first time in Portugal and in Europe.

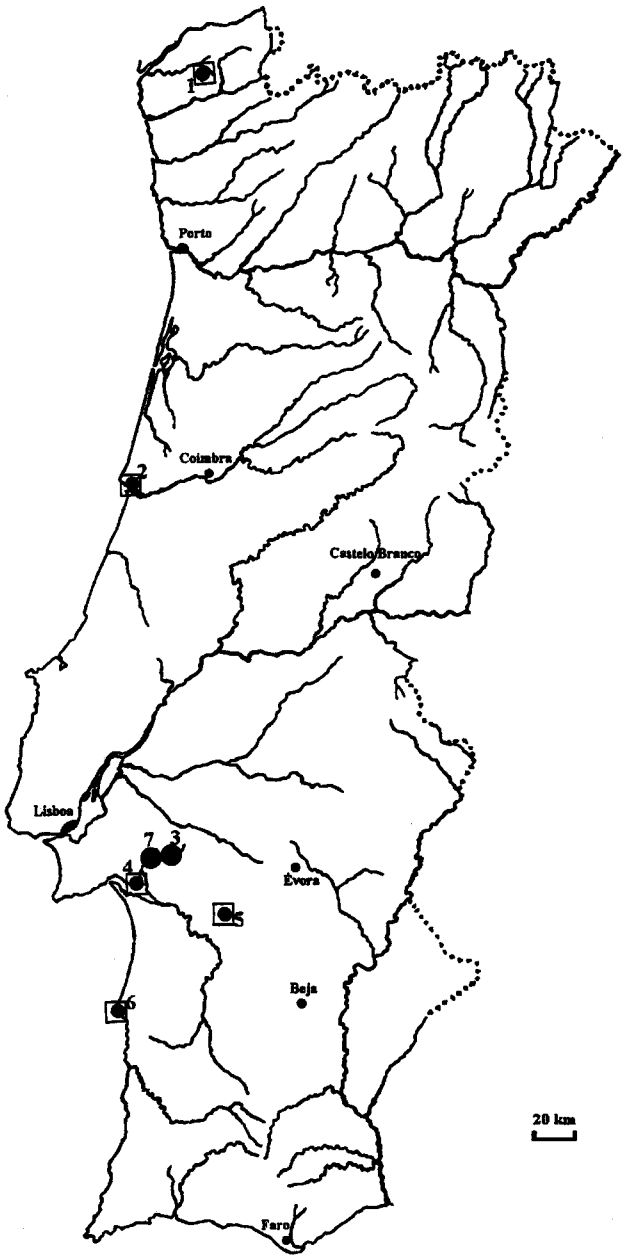

Fig. 1. *Survey of* Bursaphelenchus xylophilus *in Portugal, 1999. 1: Paredes de Coura; 2: Quiaios, Figueira da Foz; 3: Marateca/Pegoes; 4: Gambia; 5: Monte de Canelas; 6: Sines; 7: Vale de Landeira. 3 and 7 indicate sites with positive identifications of* B. xylophilus.

Materials and methods

Pine trees were surveyed in several regions in Portugal (Fig. 1, sampling sites 1-7). Wood material was collected from different parts of the tree and processed in water using different extraction methods. Nematodes were collected mostly from the Baermann funnel and processed within 48 h. Photographic observations of heat-relaxed

specimens were performed with the help of a Leitz Dialux 20 or a Zeiss Axioskop microscope and utilizing an Olympus DP-10 digital camera or a Sony CCDmIRIS video camera. Measurements were done on FA 4:1 (formalin, propionic acid, distilled water) fixed and glycerin mounted material using standard methods, and with the help of a drawing tube ("camera lucida"). Specimens were prepared for SEM as previously described (Mota & Eisenback, 1993). A JEOL 35 or a Zeiss DSM 940 SEM with Oxford G 1500 cryo preservation were used.

Isolation of nematode DNA

Nematodes (5 to 20 adult animals) were homogenized in Eppendorf tubes using micropestles (Eppendorf™). DNA isolation was carried out using the Dynal DNA Direct System I Kit (Dynal™), following the protocol provided with the kit. In short, the homogenate was first incubated with paramagnetic particles in lysis buffer for 5 to 10 min at room temperature. The paramagnetic particle/DNA complex was then separated using a magnet and washed three times using washing buffer. The complex was mixed with resuspension buffer and DNA released by incubating at 65°C for 5 min. DNA concentration was determined fluorimetrically using a DyNA Quant 200 fluorometer (Pharmacia™) and the fluorescent dye Hoe 33258.

ITS-RFLP procedure

Nematode rDNA containing the internal transcribed spacer regions ITS1 and ITS2 was amplified using forward primer 5′-CGTAACAAGGTAGCTGTAG-3′ (Ferris *et al.*, 1993) and reverse primer 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain, 1993). The PCR mixture (50 μl) contained 0.6 μM of each primer, 2 units Taq DNA polymerase (Stratagene™), 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM dNTP's (Boehringer Mannheim™) and 2 ng DNA template. Amplification was carried out using a Perkin Elmer™ 9600 thermocycler employing an initial denaturation at 94°C for 2.5 min, 40 reaction cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 5 min. After completion of the PCR, 5 μl aliquots of the reaction mixture were resolved by electrophoresis in a 1.8% agarose gel and DNA fragments were visualized by staining in 1 μg/ml ethidium bromide. Suitable aliquots of the amplified DNA were digested with 3 units of the restriction endonucleases *Alu* I, *Hae* III, *Hinf* I, *Msp* I and *Rsa* I (Gibco™) following the manufacturer's instructions. Restriction fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

The *Bursaphelenchus* isolates used by ITS-RFLP technique for comparison with the Portuguese strain were *B. xylophilus* US 15 from Illinois, USA and *B. mucronatus* DE-4(w) from Templin, Germany.

Results

B. xylophilus was found in 2 sites, "Herdade Moinho Novo" (HMN; site 3 in Fig. 1) and "Herdade Vale de Landeira" (HVL; site 7 in Fig. 1), about 3 km from each other, in the Marateca/ Pegões region in Portugal (Fig. 1), in a few *Pinus pinaster* trees which had died recently. Samples from the other sites were without *B. xylophilus*. Observations of *Bursaphelenchus* specimens were made on males and females of populations from both sites. The main morphological characters for the HMN population are shown in light microscopy, LM (Fig. 2) and scanning electron microscopy, SEM (Fig. 3) and include the typical head shape, with offset lips, characteristic of *Bursaphelenchus* species. The stylet of both males and females is short, weak and with very small, inconspicuous knobs. The female vulva clearly shows the distinct and long overlapping vulval flap, with the vulva at an approximately 90° angle with the surface. The female tail terminus is round, occasionally displaying a minute inconspicuous terminus, resembling a mucron. The post-uterine sac is clearly seen and is very long. The male spicule is curved and has a characteristic cucullus in the shape of a flattened disc. Male genital papillae are observed in characteristic number and order.

Morphometrics of this population are shown in Table 1. Overall, and apart from body length (L), greater than the lectotypes measured by Nickle *et al.* (1981) and Mamiya and Kiyohara (1979), they are very much in the same range of *B. xylophilus* measurements.

In addition to morphological determinations, ITS-RFLP analysis was employed to determine species affiliation of the samples. Amplified rDNA of the nematodes was digested with five restriction enzymes which had been shown to produce species-specific fragment patterns (Hoyer *et al.*, 1998). As shown in Fig. 4, identical fragment patterns were obtained with DNA from an authentic *B. xylophilus* isolate from the USA, and DNA from the nematodes collected in Portugal. In contrast, *B. mucronatus* yielded totally different restriction fragment patterns.

General morphological observations as well as ITS-RFLP profiles of the HVL population match the *B. xy-*

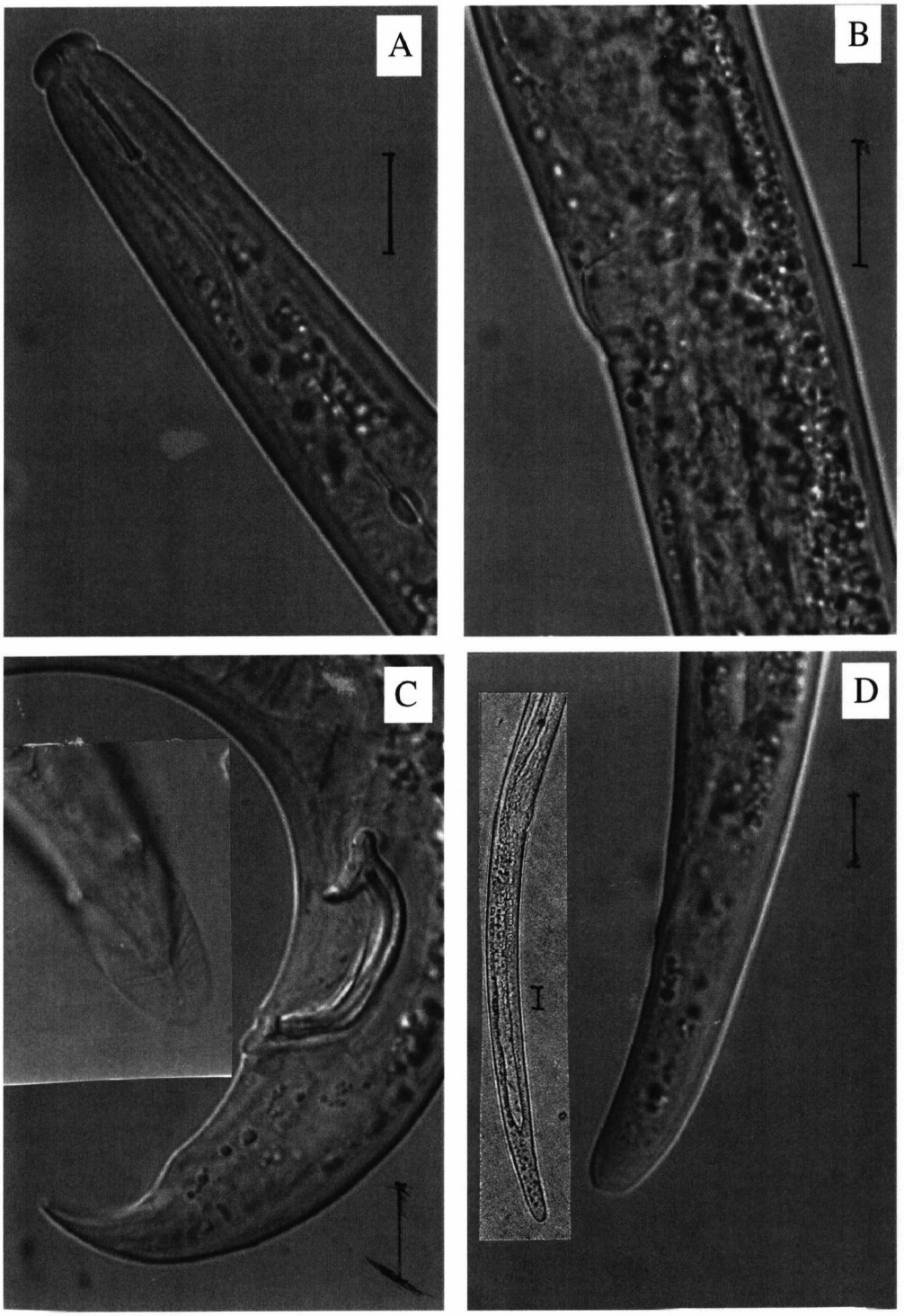

Fig. 2. *Light microscope (LM) observations of males and females of the Portuguese HMN population of* Bursaphelenchus xylophilus. *A: female head region with distinct labial region and stylet with very small knobs; B: female vulva and long vulval flap; C: male tail showing curved spicule with condylus and enlarged flattened cucullus (inset: ventral view showing bursa); D: round female tail terminus (inset: female tail with long post-uterine sac). (Scale bars = 10 μm).*

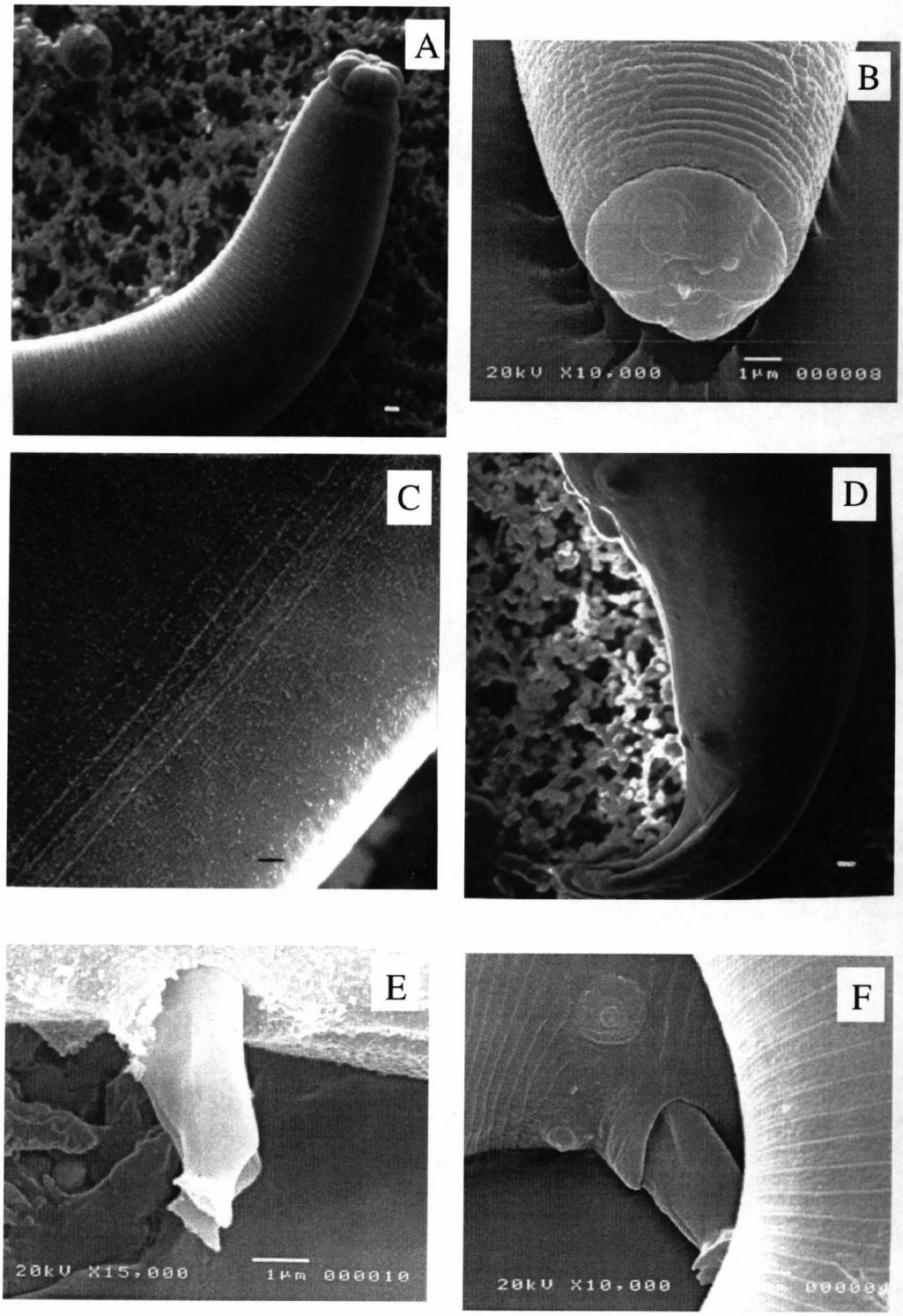

Fig. 3. *Scanning electron microscope (SEM) observations of males and females of the Portuguese HMN population of* Bursaphelenchus xylophilus*: A: female head region showing clear offset lip region; B: view of female lip region; C: female cuticle showing 4 lateral lines; D: mail tail; E, F: details of mail tail showing spicule tip (cucullus) and papillae. (Scale bars = 1 µm).*

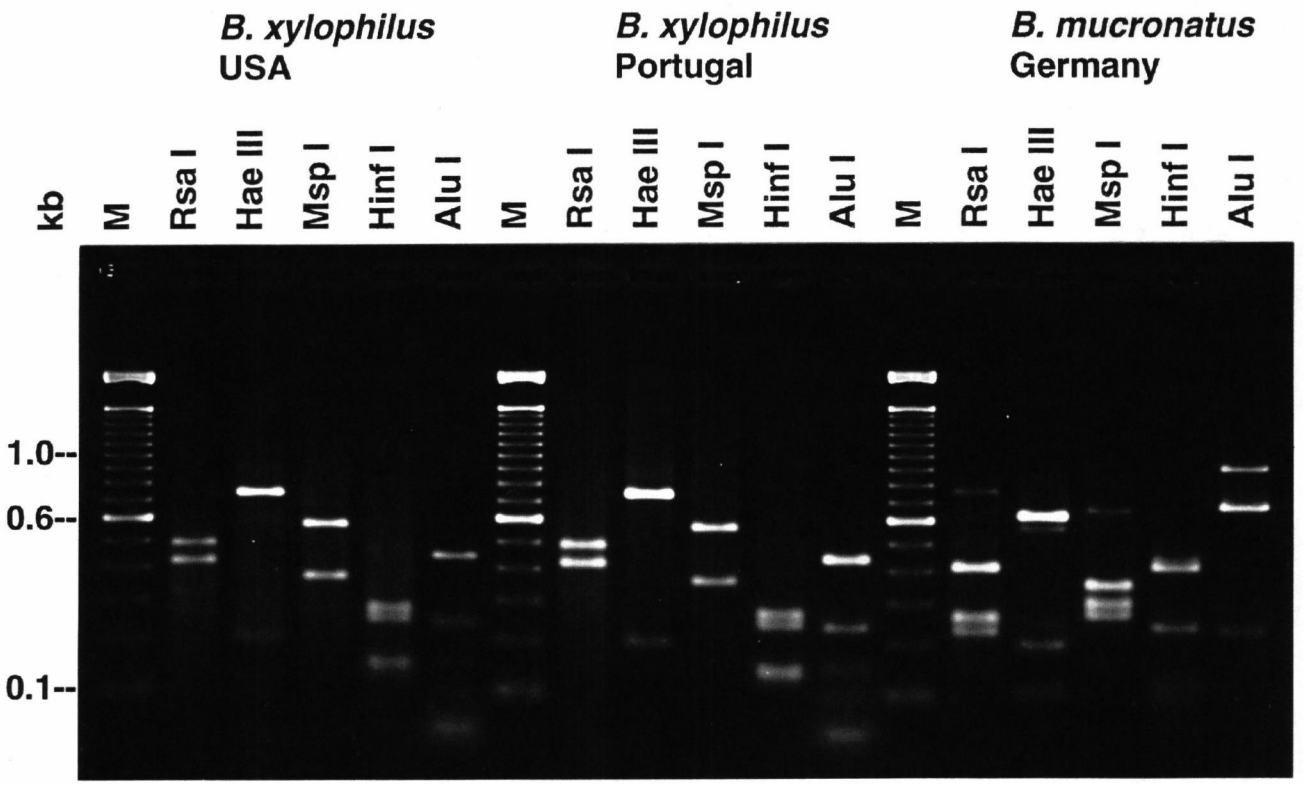

Fig. 4. *ITS-RFLP patterns of* Bursaphelenchus xylophilus *isolates from USA (US 15) and Portugal compared with one* B. mucronatus *isolate (DE-4w) from Germany. Restriction fragments were obtained by digestion of the amplified ITS regions of rDNA with the five named restriction enzymes.* B. mucronatus *showed some weak signals of partially digested fragments in each lane.*

Table 1. *Morphometrics (mean and range) of* Bursaphelenchus xylophilus *(HMN population, from Marateca/Pegões region in Portugal). All measurements in μm.*

	Females		Males	
n	12		12	
L	1050	(890-1290)	1030	(800-1300)
Stylet	12.3	(11-15)	12.6	(11-16)
a	50.0	(41.2-58.3)	49.4	(43.7-56.1)
b	13.83	(12.7-16.4)	13.28	(11.1-14.9)
c	26.6	(21.8-31.8)	28.0	(23.7-31.6)
c′	2.75	(1.7-3.7)	2.35	(2.0-2.6)
V(fem.)/T(male) %	73.3	(70.3-75.7)	55.2	(46.4-64.8)
Spicules (measured along chord)			24	(22-25)

lophilus characteristics, and will be detailed in a coming paper. Wood and bark breeding insects collected from one *B. xylophilus* infested pine tree in the Marateca/Pegões region included curculionid (*Pissodes* sp.), cerambycid (*Arhopalus* sp. and suspected *Monochamus* sp. larvae) and scolytid (*Hylastes opacus*) species.

Discussion

The nematodes found in dead and declining pines (*P. pinaster*) in two sites in Portugal were clearly identified as *Bursaphelenchus xylophilus* by morphological criteria and ITS-RFLP patterns. *B. xylophilus* can be distinguished from other *Bursaphelenchus* species by the presence of the following three characters: the typically shaped spicules with a cucullus (disc-like expansion) at their distal extremity; the anterior vulval lip developed as a distinct flap; the rounded tail terminus of females. The female tail of the similar species, *B. mucronatus*, and of some American *B. xylophilus* populations has a mu-

cronate terminus, but *B. mucronatus* never has a round tail. The males of the two species can hardly be differentiated morphologically. The populations from Portugal accurately fit the light microscopic morphological features of *B. xylophilus* (Fig. 2). Additionally, electron microscopical examination (Fig. 3) revealed structures known for this species (Nickle *et al.*, 1981; Yin *et al.*, 1988; Hunt, 1993): lateral field with 4 lines; an adanal pair of caudal papillae just before the anus; two postanal pairs just before caudal alae origin and a single median papilla just preanal.

In recent years, additional techniques have been introduced to differentiate *Bursaphelenchus* species, including DNA analysis (Beckenbach *et al.*, 1992; Riga *et al.*, 1992; Braasch *et al.*, 1995; Iwahori *et al.*, 1998), sex pheromones and attraction (Riga & Webster, 1992) as well as inter- and intraspecific cross-fertilization (Riga *et al.*, 1992; Braasch, 1994). The morphological identification of *B. xylophilus* was unequivocally confirmed by ITS-RFLP analysis. In previous investigations, species-specific ITS-RFLP patterns were established for *B. xylophilus, B. mucronatus, B. fraudulentus, B. leoni* and *B. sexdentati* (Hoyer *et al.*, 1998) and, more recently, for *B. fungivorus, B. hofmanni, B. borealis, B. poligraphi* and *B. eggersi* (Braasch *et al.*, publication submitted). In these investigations, restriction fragment patterns were produced using the same five enzymes as described in Materials and methods, and all of these species could be differentiated from each other by two or more of the five restriction fragment patterns obtained. As shown in Fig. 4, *B. xylophilus* and *B. mucronatus* differ in each of the five restriction fragment patterns obtained, and the American and Portuguese *B. xylophilus* provenances are identical with respect to all patterns obtained using the five enzymes.

This discovery of *B. xylophilus* will certainly prompt concern in Europe about the extent of the presence of this pest. Portuguese and European Union authorities have been alerted and immediate measures have been taken. Among these are included isolation of the infested area, burning of the infested trees and launching of a national survey to evaluate the presence of *B. xylophilus* in Portugal. Investigations on the extent of the nematode establishment, its vector in Portugal, the damage caused under the given climatic conditions and the possible pathway of introduction are urgently needed.

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Short communication

Bursaphelenchus xylophilus (Nematoda; Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal

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Bursaphelenchus xylophilus (Steiner & Bührer, 1934) Nickle, 1970 is the causal agent of pine wilt disease (PWD) in *Pinus*. It is a quarantine organism in the European Union (Directive 77/93, EEC) and an impediment to international trade of forest products. A pest risk analysis for *B. xylophilus* and its vectors in the genus *Monochamus* for the territories of the European Union concluded that, due to the frequent occurrence of susceptible pine hosts in Europe and the presence of suitable insect vectors, PWD could have important economic and ecological consequences if it was introduced in Europe (Evans *et al.*, 1996).

During a survey of aphelenchid nematodes associated with maritime pine, *Pinus pinaster*, carried out in Portugal in 1999, *B. xylophilus* was reported for the first time in Europe near Setúbal (40 km southeast of Lisbon) (Mota *et al.*, 1999). A wider and more detailed survey was conducted in the rest of the country from November 1999 to January 2000 within a national Program for Nematode Eradication. *B. xylophilus* was not found in any of the 2753 pine trees sampled outside the affected zone, confirming that the infestation is restricted to the Setúbal peninsula (Anon., 1999).

Worldwide, several insects have been described in association with the pinewood nematode, namely species of Cerambycidae, Buprestidae and Curculionidae, but longhorn beetles in the genus *Monochamus* are considered to be the most important vectors (Linit, 1988; Kishi, 1995). Two *Monochamus* species, *M. sutor* (Linnaeus) and *M. galloprovincialis* (Olivier) were reported to occur in Portugal, respectively by Oliveira (1882) and Zuzarte (1985).

Materials and methods

To investigate the role of *Monochamus* spp. in the PWD cycle in Portugal, adult *P. pinaster* trees infested with *B. xylophilus* were felled in the beginning of 2000 and the attacked logs and branches kept at 26°C. Emerging adult *M. galloprovincialis* were dissected and crushed in Syracuse dishes and kept in water for 24 h at 20-24°C. Two methods were used to obtain adult nematode populations. Three to four-year old branches were collected from uninfested maritime pine trees and cut into 12-15 cm long segments with a diameter of 4-5 cm. The segments of the extremities of each branch were kept as controls and only the intermediate segments were used for the inoculations. Each branch segment was inoculated by dipping 0.5 ml of a concentrated nematode suspension into two small holes drilled radially to the centre of the branch at 4-5 cm from the ends. Both extremities of each segment were covered with paraffin and the inoculation holes were sealed. A total of 20 segments (one for the dauer juveniles from each insect) were inoculated. Each segment was placed in a sealed polythylene bag in a plastic box and incubated at 26°C for 4 weeks. After this period, each branch segment was cut into 3 mm thick disks which were submerged in water for 48 h using a modified Baermann technique for the extraction of the nematodes. Simultaneously, dauer juveniles collected from another six *Monochamus* adults were placed on plates of potato dextrose agar (PDA) with the fungus *Botrytis cinerea* and incubated at 26°C for rearing to the adult stage.

The adult nematodes were identified in temporary slides using the light microscope.

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Results

A total of 69 *M. galloprovincialis galloprovincialis* adults, 30 females and 39 males, were examined. On average, 54% (18 females and 19 males) were infested with nematodes. The insects carried between six and 72 000 nematodes, averaging 2300 and 2000 in individual infested females and males, respectively. Most commonly, there were between some hundreds and a few thousand nematodes per insect.

The three propagative nematode stages, as well as females and males, were extracted from all the inoculated branch segments; in some of them the number of nematodes had increased considerably. Although some of the dauerlarvae displayed a tail terminus with an axial and distinct mucron, only females having rounded tails ("r" form) were found (Fig. 1). All morphological characters of both sexes were typical of the species as described by Mota *et al.* (1999). No other nematode species were found in the segments inoculated with dauer juveniles collected from *M. galloprovincialis* or from the control segments.

Discussion

As in the USA and Japan, a *Monochamus* species appears to be the most important vector of the PWN. This is the first report of *M. galloprovincialis* associated with *B. xylophilus*, and the confirmation that a European *Monochamus* species appears to be a suitable vector of *B. xylophilus*.

This insect species had already been found associated with the closely related *Bursaphelenchus mucronatus* in Europe, namely in Sweden (Magnusson & Schroeder, 1989), Finland (Tomminen *et al.*, 1989; Tomminen, 1990), Italy (Palmisano *et al.*, 1992) and Germany (Braasch *et al.*, 1999).Other insect species could also be involved in the disease cycle in Portugal and are being monitored for the presence of the nematode. A more detailed study concerning the insect/nematode association in the disease transmission is being developed and will be presented in future publications.

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Fig. 1. *Dauer juveniles of* Bursaphelenchus xylophilus *within tracheae of* Monochamus galloprovincialis.

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Precision and Selection of Extraction Methods of Aphelenchid Nematodes from Maritime Pine Wood, *Pinus pinaster* L.

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Abstract: Four extraction methods for *Bursaphelenchus xylophilus* and other aphelenchid nematodes were compared on the number of nematodes per gram recovered, and on the precision of the mean number of nematodes per gram of pine wood. The number of nematodes per gram recovered by each method, in addition to its inherent shortcomings when the actual number of nematodes is unknown, failed to provide clear rankings among the extraction methods. The precision of the mean number of nematodes per gram did provide clear guidelines for selection. Selection of the method may be based on prior knowledge about the range of nematodes to be expected or the independence of precision from the mean number of nematodes.

Key words: Bursaphelenchus xylophilus, extraction methods, maritime pine, pinewood nematode, precision.

The order Aphelenchida includes several economically important species, mainly within the genera *Aphelenchoides* and *Bursaphelenchus*. A number of *Bursaphelenchus* species are frequently associated with insects and trees, and *B. xylophilus* is an important pest and pathogen of conifers of the genus *Pinus*. It may be responsible for "pine wilt disease" and has been reported to cause major damage in native pines in Japan, China, and Korea as well as some exotic species in the United States and Canada (Evans et al., 1996). Recently (Mota et al., 1999), *B. xylophilus* was found for the first time in Europe in maritime pine, *Pinus pinaster*, from southern Portugal.

The selection of extraction methods and quantification are important in ecological studies, and the Baermann funnel is often used. Selection of the most appropriate extraction method usually involves the comparison of different methods using a number of samples or, when feasible, subsamples followed by comparison of statistical analyses to establish which method extracts more nematodes. For a number of reasons, *a priori* knowledge of how many nematodes are present in a sample is the exception (Griesbach et al., 1999; Hoshino and Togashi, 1999; McSorley and Parrado, 1987), and different counts may result from differences in the efficiency of the methods or from differences in the number of nematodes actually present in samples.

Attempts to account for this uncertainty involve the mixing of soil prior to extraction, but mixing does not necessarily improve the spatial homogeneity of nematodes in a sample (McSorley and Parrado, 1987). Reference samples with low numbers of nematodes (Griesbach et al., 1999) might reduce but not eliminate the uncertain. Addition of nematode inoculum has been employed (McSorley and Frederick, 1991; Stetina et al., 1997) in the hope that final numbers of nematodes will accurately reflect the amount of inoculum.

However, an alternative approach based on precision rather than efficiency is available and does not require knowing the actual number of nematodes present in a sample *a priori*. Precision is defined as the closeness of repeated measurements of the same quantity (Sokal and Rohlf, 1995) and, for a given sample of size n, can be expressed (McSorley, 1987) by

$$D = (t^2 s^2 / n)^{1/2} \quad (1)$$

where D is the half-length of the 1-α confidence interval of the mean, t is the value of Student's t distribution with n-1 degrees of freedom and a type-I error probability α, and s^2 is the variance of the sample. Expressing D as a proportion of the mean ($\bar{Y}$), D′ is

$$D' = (t^2 s^2 \bar{Y}^{-2}/n)^{1/2} \quad (2)$$

which implies that the greater the value of D′, the lesser the relative precision of the mean.

When a series of samples are available, the relation between their variances and means can be described in a number of ways, including the negative binomial distribution or Taylor's power law. Taylor's power law covers a wider range of distributions, whereas the negative binomial distribution, which has severe ecological limitations (Taylor et al., 1979), is not independent of sample size, with its parameter k reaching ± infinity at randomness (Elliot, 1979).

Taylor's power law can be expressed by

$$s^2 = a \bar{Y}^b \quad (3)$$

where a is a sampling factor and b can be interpreted as an index of aggregation ranging (when a = 1) from a near-regular (b → 0) through random (b = 1), to a highly aggregated (b → ∞) distribution of organisms (Taylor, 1961).

Fitting Taylor's power law to a set of samples by regression techniques is better accomplished in linear form, usually taking the decimal logarithms of both terms of eq. (3),

$$\log s^2 = \log a + b \log \bar{Y} \quad (4)$$

Even if the aggregation index b frequently appears to

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be independent from the mean (Elliot, 1979), as implied by eqs. (3) and (4), independence may break down at low densities (Taylor, 1961) and a straight line may not be the best description of the relationship between the logarithm of the variances and of the means. Therefore, a curvilinear model could be more appropriate, with the relationship between the mean and the variance expressed by

$$\log s^2 = \log a + b \log \bar{Y} + c (\log \bar{Y})^2 + d (\log \bar{Y})^3 + \ldots + w (\log \bar{Y})^x + \ldots \quad (5)$$

where b, c, d, . . ., w, . . . can be equal or different from zero. Once fitted, the model described in eq. (5), can be used to replace s^2 in eq. (2) by its value, providing the general equation

$$D' = \{t^2 a \bar{Y}^{[-2 + b + c \log \bar{Y} + d (\log \bar{Y})^2 + \ldots + w (\log \bar{Y})^{x-1} + \ldots]} / n\}^{1/2} \quad (6)$$

Therefore, the change of the relative precision of any extraction method can be investigated for the whole range of the expected mean number of nematodes $\bar{Y}$ for a given type-I error probability and sample size.

In addition to the selection of extraction methods, eq. (6) can be used to determine the number of samples needed to attain a desired precision with a stated type-I error probability. For this, an iterative solution of eq. (6) should be made to obtain the desired value of D', with the appropriate value of t for each sample size *n* or type-I error, α.

The objectives of this study are to investigate both approaches (number of nematodes extracted and precision of nematode extraction), without adding nematodes or assuming reference samples, for their adequacy and usefulness to compare and select extraction methods of *B. xylophilus* and other aphelenchid nematodes from maritime pine wood.

Materials and Methods

Branches or trunks of maritime pine were collected from Quiaios (site 1, branches only) and Pegões (sites 2 and 3, branches only; site 4, only a portion of the trunk), Portugal. In total, four trees were sampled. The number of branches varied, depending on tree size and weight of wood extracted. Distribution of selected branches was casual, with no particular pattern. The material collected at each site was cut in 1 to 2-cm pieces, thoroughly mixed, randomly divided in 20 portions with the same weight (200 g in site 1, 100 g in site 2, 15 g in site 3, and 8 g in site 4), and randomly assigned to four extraction methods (five replicates per method per mixed site sample). A single operator performed all procedures, at 18–22 °C.

Method 1, trays (TR): A three-layered plastic net, nylon tissue, and paper tissue were fitted to a plastic tray and covered with wood samples. Water was added until the wood was completely soaked. After 48 hours, nematodes were collected on a 38-µm-pore-size sieve and counted.

Method 2, Baermann funnels with a plastic net and paper tissue (BP): Wood samples were soaked and immersed in water over paper tissue and a plastic net. After 24 hours, the first sample of nematodes was taken directly from the funnels (16-cm-diam.), the water was replaced, and a second sample taken after another 24-hour period.

Method 3, Baermann funnels with nylon tissue (BN): Method 3 was similar to method 2, except that nylon tissue with a 90-µm-pore-size was used instead of paper tissue and a plastic net.

Method 4, flasks (FL): Wood samples were immersed in water in 1-liter-capacity plastic flasks for 48 hours and sieved through 710 and 38-µm-pore-size sieves. Nematodes collected in the smaller-pore sieve had to be separated from the wood material using the procedure described for method 2, but only after 24 hours.

All replicates of all methods were microscopically examined for the presence of nematodes and to obtain a preliminary evaluation of abundance. Whenever preliminary counts exceeded 100 nematodes per replicate, the suspensions were diluted with water and nematodes were counted in 1-ml aliquots. Abundance was always expressed on a per-gram basis.

Data analysis of number of nematodes: Extraction methods were compared independently at each site by one-tailed Student's *t* tests after data transformation by Box-Cox transforms (Box and Cox, 1964) or, when transformation failed to homogenize variances, by one-tailed Mann-Whitney U tests. An experiment-wise type-I error of 0.05 was adopted for the six, one-tailed comparisons of each site, using the Dunn-Šidák method (Sokal and Rohlf, 1995). A least-squares regression-based approach was also followed, involving the fit of linear models to Box-Cox transformed data. Forward stepwise selection with replication was used. The candidate model included qualitative variables only, namely sites and extraction methods (coded as 1, 0) as well as all interactions among them. An experiment-wise type-I error of 0.05 was adopted for the coefficients.

Data analysis of precision: Samples were jackknifed by calculating *n* means and variances for each sample after removing one different value each time (Efron, 1982) and logarithms taken to fit a regression model by the least-squares method. The candidate model included up to the third degree of log $\bar{Y}$, plus four qualitative variables in which the extraction methods were coded as 1, 0, and all interactions among the independent variables. Model selection was done by forward stepwise selection with an experiment-wise error rate of 0.05 for the coefficients. After replacing the qualitative variables by their values, the resulting equations were used to compare the relative precision of extraction methods, replacing s^2 in eq. (2), as described for eq. (6).

Results

Selection of extraction methods by number of nematodes: Two aphelenchid species were identified: *Laimaphelenchus pensobrinus,* Massey, was present in all four samples and *Bursaphelenchus xylophilus,* Steiner & Buhrer (Nickle), in samples 2, 3, and 4. Mean values and standard errors of nematodes per gram in each site and extraction method are shown in Table 1, together with the results of mean comparisons for site by Student's *t* or Mann-Whitney U tests. Significant differences were found only at sites 2 and 3. In site 2, FL and BP did not differ in nematode extraction and neither did TR and BN, and TR and BN extracted significantly more nematodes than the former. In site 3 the lack of "transitivity" prevented a consistent interpretation of data (Chew, 1976) in the sense that, for example, FL is not lower than TR, TR is not lower than BP, but FL is significantly lower then BP.

Therefore, a different approach that does not allow this lack of "transitivity" to occur was followed, and a least-squares regression model was fit to the data. The resulting equation included site variables only and was written as

$$Y = (0.924 - 0.083\ S_1 + 0.738\ S_3 + 0.557\ S_4)^{1/\lambda} \quad (7)$$

where Y is back-transformed data of nematodes per gram, S_i are the sites, and $\lambda = 0.075$ is the estimated value of the Box-Cox transformation. The significance levels of the model and coefficients were near 0, the significance level of lack of fit was 0.06, and the adjusted coefficient of determination was $R^2_{adj} = 0.984$. Because the qualitative variables where coded as 1 when present and as 0 otherwise, eq. (7) reduces to $Y = 0.841^{1/\lambda}$ for site 1, $Y = 0.924^{1/\lambda}$ for site 2, $Y = 1.662^{1/\lambda}$ for site 3, and $Y = 1.481^{1/\lambda}$ for site 4.

Selection of extraction methods by precision: The selected model can be written as

$$s^{2'} = a + b\bar{Y}' + c\bar{Y}'^2 + d\ BN + e\ FL + f\bar{Y}'\ TR + g\bar{Y}'\ BP + h\bar{Y}'\ FL + i\bar{Y}'^3\ BP + j\bar{Y}'^3\ FL \quad (8)$$

where $s^{2'}$ and $\bar{Y}'$ are, respectively, logarithmically transformed variances and means, and TR, BP, BN, and FL are qualitative variables representing the methods. The significance level of the model was near 0 and of the coefficients was always less than 0.005, and the adjusted coefficient of determination was $R^2_{adj} = 0.993$. Because all qualitative variables were present in the selected model, the relationship between the variance and the mean was significantly different in all methods. When the qualitative variables were replaced by their value (1 when used, 0 otherwise), four different equations for variance were obtained. For trays (TR)

$$s^2 = 0.056\ \bar{Y}^{(0.948 + 0.379 \log \bar{Y})} \quad (9)$$

for Baermann funnels with a plastic net and paper tissue (BP)

$$s^2 = 0.056\ \bar{Y}^{[1.893 + 0.379 \log \bar{Y} - 0.095 (\log \bar{Y})^2]} \quad (10)$$

for Baermann funnels with nylon tissue (BN)

$$s^2 = 0.030\ \bar{Y}^{(1.172 + 0.379 \log \bar{Y})} \quad (11)$$

and for flasks (FL)

$$s^2 = 0.129\ \bar{Y}^{[2.168 + 0.379 \log \bar{Y} - 0.183 (\log \bar{Y})^2]} \quad (12)$$

The next step was to substitute the variances obtained in (9) through (12) into eq. (2) and express (for $n = 5$ and $\alpha = 0.05$) D′ in terms of the mean number of nematodes per gram as described in eq. (6), with the result shown in Figure 1.

Discussion

The selection of extraction methods by abundance of recovered nematodes requires that statistically significant differences are present. This approach, when applied here to the number of nematodes per gram recovered by the four methods, shows an apparent relationship between sites and extraction methods, with no differences among methods in sites 1 and 4, differences in sites 2 and 3, and with the results for site 3 preventing clear conclusions because of the lack of "transitivity."

Therefore, this analysis could not support conclusive selection of extraction method. An alternative analysis was done, still using the number of nematodes per gram recovered by the four methods, and least-squares regression was used to investigate differences among methods, sites, and interactions between methods and sites. According to the selected model, shown in eq. (7), neither significant differences among methods nor interactions between methods and sites were found.

TABLE 1. Mean and standard error of nematodes (number per gram) collected in four sites by trays (TR), Baermann funnels with a plastic net and paper tissue (BP), Baermann funnels with nylon tissue (BN), and flasks (FL).

Site 1		Site 2		Site 3		Site 4	
FL	0.077 ± 0.022 a	FL	0.236 ± 0.051 a	FL	695.333 ± 53.909 a	BP	153.500 ± 33.755 a
BP	0.129 ± 0.027 a	BP	0.274 ± 0.032 a	TR	778.000 ± 107.831 ab	BN	192.150 ± 20.458 a
TR	0.136 ± 0.063 a	TR	0.504 ± 0.090 b	BN	917.027 ± 214.973 ab	FL	211.375 ± 48.180 a
BN	0.178 ± 0.050 a	BN	0.554 ± 0.042 b	BP	1202.213 ± 231.204 b	TR	216.025 ± 12.852 a

For each site, means followed by the same letter do not differ significantly at an experiment-wise type-I error rate of 0.05. In all samples, $n = 5$, except TR, site 1, and FL, site 4, where $n = 4$.

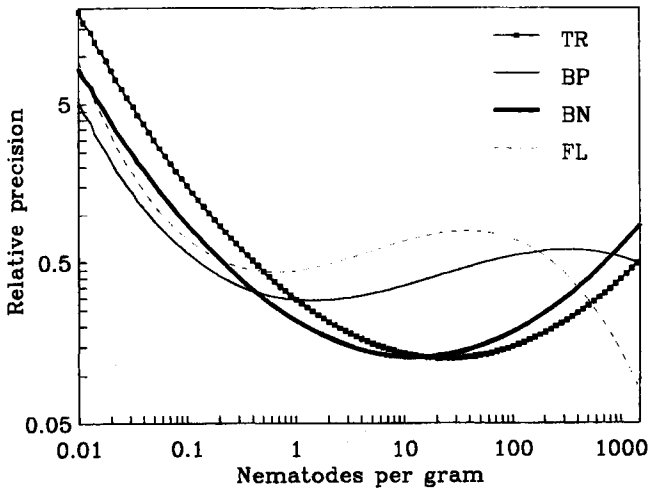

FIG. 1. Change of relative precision D′ with the mean number of nematodes per gram (for $n = 5$ and $\alpha = 0.05$) in trays (TR), Baermann funnels with a plastic net and paper tissue (BP), Baermann funnels with nylon tissue (BN), and flasks (FL).

Again, no support was available for the selection of any extraction method.

According to eq. (8), all extraction methods significantly differed in the relationship between variance and mean. Regardless of the method, variance was not independent of the mean number of nematodes per gram, and a general trend for greater precision with increasing numbers of nematodes was recognized (Fig. 1).

Nevertheless, two extraction methods groups can be identified on the basis of relative precision. In one, comprised of TR and BN, precision increases with the mean until a maximum is reached at about 22 and 12 nematodes per gram, respectively, and decreases thereafter. The other was comprised of BP and FL.

Differences of methods in terms of precision suggest that selection of the extraction method may require some prior knowledge of the approximate number of nematodes per gram to be found. If the number of nematodes per gram is less than 0.4, extraction with Baermann funnels with a plastic net and paper tissue (BP) is recommended; if between 0.4 and 14, Baermann funnels with nylon tissue (BN) should be used; trays (TR) are recommended for a range of 14 to 500 nematodes per gram; and flasks (FL) should be used for higher numbers of nematodes (500 to 1,500 per gram).

However, this *a priori* knowledge is frequently unavailable or it may be more feasible to select only one extraction method. Because the more precise method changes with the number of nematodes, a criterion other than selecting the method that maximizes precision should probably be adopted. In this case, the criterion is, undoubtedly, to select the method with a relative precision more independent from the mean number of nematodes per gram, which means that Baermann funnels with a plastic net and paper tissue (BP) should be used. After a sharp increase, precision of this method remained essentially constant when the mean number of nematodes increased. By either criteria, selecting an extraction method is possible.

We do not believe that different masses influenced the results, although this would need to be investigated in further research with more samples; the different sample weights relate to the different tree sizes and amounts of tissue required for extraction, although the results were always in reference to a weight unit.

Selection of extraction methods by precision implies that the knowledge of the exact number of nematodes present in samples is no longer a goal in itself. It also implies that the selection of a method having the least error is no longer necessarily possible. This approach provides researchers with a flexible tool to select an extraction method, though obviously not error-free, with an error distribution that has known bounds. In addition, emphasis in precision independency from the mean number of nematodes may be an important part of the experimental design, especially when predictions based on nematode numbers are desired.

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Preliminary survey for insects associated with *Bursaphelenchus xylophilus* in Portugal

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The detection of *Bursaphelenchus xylophilus* in *Pinus pinaster* trees in Portugal in 1999 led the official services to implement exceptional and urgent control measures to prevent the spread of the pest. Because the pest is vector-transmitted, special attention was paid to the insects associated with infested trees. A survey comprising 21 species of insects, from six families, with a total of 1367 specimens, was made in order to evaluate their possible role as vectors of the nematode in Portugal. Five insect species were found to carry other *Bursaphelenchus* spp., but only *Monochamus galloprovincialis* was associated with *B. xylophilus*.

Introduction

In 1999-03, *Bursaphelenchus xylophilus* was found for the first time in Europe, 40 km south-east of Lisbon (PT), associated with dead trees of maritime pine (*Pinus pinaster*) (Mota *et al.*, 1999). A national eradication programme comprising exceptional and urgent control measures was adopted to prevent spread of the pest, and a survey conducted in the rest of the country, by the end of 1999, showed that the infestation in Portugal was confined to the Setúbal Peninsula and surrounding areas – the 'affected zone' (GANP, 1999) or 'quarantine area' (IPPC, 2001).

Transmission of the pest requires an insect vector with a larval development phase occurring in dying or dead trees infested with *B. xylophilus*. Cerambycid beetles of the genus *Monochamus* are considered to be the most important vectors of the nematode in North America and Japan. Nevertheless, several other insects have also been described worldwide to carry *B. xylophilus*: 21 species of *Cerambycidae*, two species of *Curculionidae* and one genus of *Buprestidae* (Linit, 1988). In the quarantine area, several of these species, or similar species of the same genera, can be found colonizing *B. xylophilus*-infested trees. However, *Monochamus galloprovincialis* is the only *Monochamus* species present in this area (Sousa *et al.*, 2000) and appears to be a suitable vector of *B. xylophilus* (Sousa *et al.*, 2001). To clarify the insect component of pine wilt disease in Portugal, a survey was conducted to evaluate the possibility that other woodborers may carry the nematode.

Materials and methods

Fifteen adult trees of *P. pinaster* with an average diameter (BHD) of 31.0 ± 6.14 cm infested with *B. xylophilus* were felled in the winter of 1999/2000 in the Pegões region (Fig. 1), north-east of Setúbal city. The trees presented different wilting conditions, ranging from initial symptoms to almost completely dry. Branches and trunk were cut off in sections and the whole trunk debarked. All adult insects present in the trunk and branches were collected and identified. Wood material with the presence of immature stages was kept in controlled conditions at 26 ± 2 °C to allow the emergence of adult insects. In the winter of 2000/2001, another six *B. xylophilus*-infested trees (average BHD 34.0 ± 10.5 cm) were felled at Tróia (Fig. 1), and the wood sections were kept under natural conditions until adult insects emerged.

Emerging insects were dissected and crushed in Syracuse dishes and kept individually in water for 24 h at 22 ± 2 °C. When nematodes were present, the dauer larvae obtained from each insect were inoculated into separate 13.5 ± 1 cm sections of 3- to 4-year-old branches of *P. pinaster* sealed at both ends with paraffin. All sections were placed in sealed plastic bags and stored at 26 ± 2 °C for 4 weeks. After this period, nematodes were extracted using a modified Baermann technique, in which small 3-mm discs cut from the branch sections remain

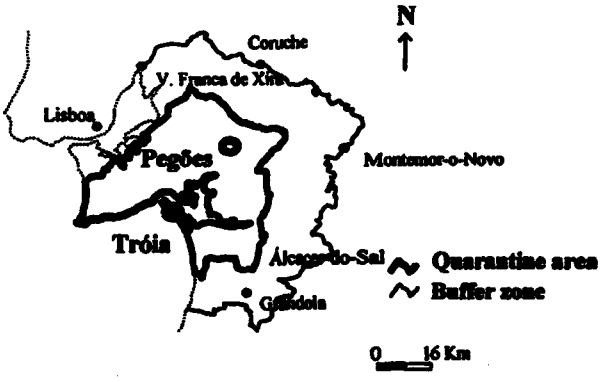

Fig. 1 Location of the two study plots (Pegões and Tróia) in relation to the quarantine area and buffer zone, in the Setúbal area south-east of Lisbon.

Table 1 Insects that emerged from trees of *Pinus pinaster* infested with *Bursaphelenchus xylophilus* and were screened for the nematode

Family/species	No. of observed insects per year		Insects with *Bursaphelenchus* dauer larvae (%)		*Bursaphelenchus* species associated with insects	
	2000	2001	2000	2001	2000	2001
Coleoptera						
Cerambycidae						
Monochamus galloprovincialis	82	151	53	13	*B. xylophilus*	*B. xylophilus*
Arhopalus ferus	26	–	–	–	–	–
Arhopalus syriacus	23	39	–	–	–	–
Pogonocherus perroudi	19	36	–	–	–	–
Acanthocinus griseus	17	52	–	–	–	–
Spondilys buprestoides	–	2	–	–	–	–
Rhagium inquisitor	1	–	–	–	–	–
Ergates faber	1	–	–	–	–	–
Scolytidae						
Orthotomicus erosus	314	38	30	–	Three other species	–
Tomicus piniperda	87	61	21	–	Two other species	–
Ips sexdentatus	65	3	78	–	One other species	–
Hylurgus ligniperda	55	–	24	–	Two other species	–
Pityogenes sp.	–	122	–	–	–	–
Hylastes linearis	9	2	–	–	–	–
Hylastes attenuatus	1	–	–	–	–	–
Buprestidae						
Chrysobothris solieri	14	35	–	–	–	–
Calcophora mariana	4	1	–	–	–	–
Phaenops cyanea	2	1	–	–	–	–
Curculionidae						
Pissodes castaneus	30	54	–	–	–	–
Eremotes porcatus	18	–	–	–	–	–
Elateridae						
Unidentified sp.	10	–	–	–	–	–
Hymenoptera						
Siricidae						
Sirex noctilio	1	–	–	–	–	–

submerged in water for 48 h. Adult nematodes were identified in temporary slide mounts by light microscopy.

Results

Twenty-one species of bark and wood-boring beetles were screened for the presence of nematodes, out of a total of 1367 insects (770 insects in 2000 and 597 in 2001). Eight species of *Cerambycidae* (440 specimens), seven of *Scolytidae* (757 specimens), three *Buprestidae* (57 specimens), two *Curculionidae* (102 specimens) and one *Elateridae* (10 specimens) were checked for nematodes (Table 1). A male of *Sirex noctilio* (Hymenoptera; *Siricidae*) also emerged from the wood material and was analysed.

Nematodes of the genus *Bursaphelenchus* other than *B. xylophilus* were found in four species of *Scolytidae*. Considering insects from Pegões, *Orthotomicus erosus* was found carrying three different *Bursaphelenchus* spp., *Hylurgus ligniperda* two other species and both *Tomicus piniperda* and *Ips sexdentatus* one *Bursaphelenchus* species each. Scolytids from Tróia were not found to carry *Bursaphelenchus* nematodes. All these insects, as well as *Hylastes linearis* and the weevil *Pissodes castaneus*, carried other nematodes belonging to different genera of the *Aphelenchoididae* or other taxonomic groups.

Only *M. galloprovincialis galloprovincialis* was found carrying *B. xylophilus*. No other nematode species was found associated with this beetle, and none of the other cerambycid species screened carried any nematodes (Table 1). The percentage of *M. galloprovincialis* carrying *B. xylophilus* reached 53% (41/82) in 2000 and only 13% (19/151) in 2001. No differences were detected between males and females in *B. xylophilus* infestation. In the Pegões population, 48% of the males and 53% of females were infested, whereas only 13% of the males and 12% of the females from the Tróia population carried *B. xylophilus*.

Differences in the mean number of nematodes per insect were found between populations (Pegões: 3751 ± 1787; Tróia: 10 700 ± 2792), with large variability in both populations: from six to 72 000 nematodes in 2000 and from 20 to 42 400

Table 2 Percentage of infected *Monochamus galloprovincialis* with the indicated numbers of *Bursaphelenchus xylophilus* in their bodies, in the study areas at Pegões (2000) and Tróia (2001)

	Number per insect (thousands)			
Study area	< 1	1–5	5–10	> 10
Pegões	58	27	10	5
Tróia	32	16	5	47

nematodes in 2001. In the Tróia population, almost half the infested insects were found to be carrying more than 10 000 nematodes each, whereas more than half the insects from Pegões had less than 1000 nematodes each (Table 2).

Discussion

Although five *Bursaphelenchus* species were found associated with bark beetles, and new studies concerning these interactions are being initiated, no scolytid beetles were found to carry *B. xylophilus* in Portugal. This conclusion is consistent with reports from Japan and North America, where bark beetles are also not associated with this nematode (Mamiya & Enda, 1972; Wingfield & Blanchette, 1983; Linit, 1988; Bowers *et al.*, 1992).

Seven cerambycids and all the buprestids and curculionids surveyed were found not to carry *B. xylophilus*. Many such species are very common in pines in Portugal and may reach large numbers in decaying trees, e.g. *Pissodes castaneus* and *Arhopalus syriacus*. It may be noted that, in North America and Japan, some of these species (or similar ones of the same genera) have been reported to carry low numbers of *B. xylophilus*, namely *Arhopalus* spp., *Acanthocinus griseus*, *Chrysobothris* spp. and *Pissodes* spp. (Mamiya & Enda, 1972; Linit *et al.*, 1983; Wingfield & Blanchette, 1983).

Our results showed that *M. galloprovincialis* is the only vector of *B. xylophilus* in Portugal. This cerambycid has already been found carrying the closely related *B. mucronatus* in several European countries (Magnusson & Schroeder, 1989; Tomminen *et al.*, 1989; Palmisano *et al.*, 1992; Ambrogioni *et al.*, 1994; Braasch *et al.*, 1999) but, in Portugal, *B. mucronatus* does not occur in the quarantine area.

The numbers of nematodes per beetle found in this study, both average (3751–10 700) and maximum (42 400–72 000), were of the same order of magnitude but rather less than in other studies on *Monochamus alternatus* in Japan (Kobayashi *et al.*, 1984) and *Monochamus carolinensis* in the USA (Linit *et al.*, 1983; Wingfield & Blanchette, 1983). However, the percentage of *M. galloprovincialis* carrying *B. xylophilus* in Portugal (13–53%) was markedly lower than in the other studies. From 18 studies in Japan in different sites and/or years, the mean percentage of *M. alternatus* carrying the nematode was 71% (Kobayashi *et al.*, 1984). Percentage infection of *M. carolinensis* was 79–98% (Linit *et al.*, 1983; Wingfield & Blanchette, 1983). The reason for this difference could be that the other authors targeted trees known to be suffering from pine wilt disease (and therefore certainly infected with nematodes), whereas in the present study, the trees were selected because they were wilting or dead, without establishing the cause.

Differences were found in the percentage of infested individuals and the mean number of nematodes per insect between the two surveyed populations. These results are consistent with those reported from the USA and Japan concerning other *Monochamus* species (Linit, 1988). This variability may be associated with either intraspecific population factors or environmental conditions, as the two populations were surveyed in different years and locations.

The number of nematodes carried by individuals of *M. galloprovincialis* is less than that carried by the most efficient vectors of *B. xylophilus* in other parts of the world (*M. alternatus* and *M. carolinensis*). However, there is no doubt that the association created in Portugal between the nematode and a new vector (*M. galloprovincialis*) is adequate to ensure effective dissemination of the nematode throughout Portugal and further in Europe, unless contained. Further research will help to clarify the epidemiological cycle of the disease in Portugal and to define better control methods for the eradication programme.

Prospection préliminaire pour les insectes associés à *Bursaphelenchus xylophilus* au Portugal

La détection de *Bursaphelenchus xylophilus* sur des arbres de *Pinus pinaster* au Portugal en 1999 a conduit les services officiels à mettre en oeuvre des mesures de lutte exceptionnelles et urgentes pour empêcher la dissémination du ravageur. Cet organisme est transmis par des vecteurs, et une attention particulière a donc été portée aux insectes associés aux arbres infestés. Une prospection portant sur 21 espèces d'insectes de six familles, avec 1367 spécimens au total, a été conduite pour évaluer leur rôle éventuel de vecteurs du nématode au Portugal. Cinq espèces portaient des *Bursaphelenchus* spp., mais seul *Monochamus galloprovincialis* était associé à *B. xylophilus*.

Предварительное обследование на выявление насекомых, связанных с *Bursaphelenchus xylophilus*, в Португалии

Обнаружение *Bursaphelenchus xylophilus* на деревьях *Pinus pinaster* в Португалии в 1999 году заставило официальные службы применить чрезвычайные и срочные меры по предотвращению распространения нематоды. Поскольку она расселяется с помощью переносчиков, особое внимание было обращено на насекомых, связанных с зараженными деревьями. Для оценки их возможной роли в качестве переносчиков нематоды в Португалии было проведено обследование 1367 особей, принадлежащих 21 виду насекомых из 6 семейств. Пять видов насекомых было обнаружено в качестве носителей других видов *Bursaphelenchus* spp., и только *Monochamus galloprovincialis* был связан с *B. xylophilus*.

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