ASSESSMENT OF THE GENETIC DIVERSITY
OF
THE PINEWOOD NEMATODE,
BURSAPHELENCHUS XYLOPHILUS, IN PORTUGAL

Avaliação da diversidade genética do nemátode da madeira do pinheiro, *Bursaphelenchus xylophilus*, em Portugal

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ABSTRACT

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, has a wide distribution in North America, and is present throughout most of the territories of Canada and the United States. During the last century, this species has been transported by man to several non-native regions of the world, associated with trade and the global flow of forest products. Up to date, this invasive species has been reported from Asia (PR China, Japan, Korea and Taiwan) and more recently in Europe (Portugal). Due to the impact on native pine forests of these regions, this nematode species, the causal agent of pine wilt disease, is of great economic importance worldwide. In Portugal, the distribution of the PWN has been constrained to a relatevily small área (500 000 ha) in the south of Lisbon (Setúbal Peninsula); however, it has become the most serious threat to pine forests in the country. Until recently, no consensus had emerged on the possible pathway of the PWN introduction in Portugal. Several hypotheses have been put forward to explain this introduction, such as an origin from endemic areas where the nematode naturally occurs (North America), or non-endemic areas where the nematode behaves as an exotic pest (East Asia). Random amplified polymorphic DNA (RAPD-PCR) and satellite DNA (satDNA) techniques were used in order to assess the level of genetic variability and genetic relationships, among several isolates of the PWN, representative of the entire affected area in Portugal. In the case of RAPD-PCR, 24 Portuguese isolates, plus two additional isolates of *B. xylophilus*, representing North America and East Asia were included. *B. mucronatus* was used as an out-group. Twenty-eight random primers generated a total of 640 DNA fragments. With satDNA, 206 *Msp*I sequence repeats were obtained from 21 Portuguese isolates of *B. xylophilus*. Both molecular methods revealed a high genetic similarity among the Portuguese isolates, and the low level of genetic diversity strongly suggests that they were dispersed recently from a single introduction, and from East Asia. The lack of apparent relationship between the genetic variability and the geographic distribution of the PWN within the affected area, suggests that the recent introduction of this pest (and pathogen) in Portugal has been uniformly distributed since its establishment, probably following the natural distribution and expansion of the insect vector.

AVALIAÇÃO DA DIVERSIDADE GENÉTICA DO NEMÁTODE DA MADEIRA DO PINHEIRO, *BURSAPHELENCHUS XYLOPHILUS*, EM PORTUGAL

RESUMO

O nemátode da madeira do pinheiro (NMP), *Bursaphelenchus xylophilus*, tem uma extensa distribuição na América do Norte, e encontra-se actualmente distribuído ao longo da maioria dos territórios de Canadá e dos Estados Unidos. Durante o último século, esta espécie foi transportada pelo Homem para outras regiões do mundo (não-nativas), associadas com o comércio e o fluxo global de produtos de origem florestal. Actualmente, esta espécie invasiva está reportada para algumas regiões do SE asiático (China, Japão, Coreia e Taiwan) e mais recentemente para a Europa (Portugal). Devido ao impacto que este organismo agente da doença da murchidão dos pinheiros causa nas florestas nativas destas regiões esta espécie assume uma elevada importância económica a nível mundial. Em Portugal, a distribuição do NMP encontra-se confinada a uma área restrita e limitada (500 000 ha), a sul de Lisboa (península de Setúbal); contudo, constitui uma das maiores ameaças às florestas de pinheiro do país e da UE. Até recentemente, nenhum consenso existia quanto à origem do NMP em Portugal. Diversas hipóteses têm sido colocadas para explicar esta introdução, nomeadamente a partir de zonas onde o nemátode ocorre naturalmente (América do Norte), ou de outras áreas (não-nativas) onde o nemátode se comporta como um espécie invasiva (Leste da Ásia). A fim de avaliar a variabilidade genética do NMP proveniente da área afectada em Portugal, foram utilizadas várias técnicas moleculares, designadamente o *random amplified polymorphic DNA* (RAPD-PCR) e o *satellite DNA* (satDNA). No caso do RAPD-PCR, foram utilizados 24 isolados do NMP provenientes de Portugal, 1 proveniente da América do Norte e 1 da Ásia, tendo sido utilizado como *out-group* um isolado de *B. mucronatus*. A partir dos 28 RAPD *primers* utilizados obtiveram-se 640 fragmentos. No caso do satDNA, foram utilizados 21 isolados do NMP provenientes de Portugal, obtendo-se no total 206 sequências da família *MspI*. Ambos os métodos revelaram uma elevada similaridade genética entre os vários isolados do NMP da área afectada em Portugal. O nível reduzido de diversidade genética obtido entre os isolados portugueses do NMP, permite concluir que se trata de uma única introdução deste organismo em Portugal, e proveniente de uma região asiática. A inexistência de uma de correlação entre a variabilidade genética e a distribuição geográfica do NMP dentro da área afectada em Portugal, indica que o NMP se encontra distribuído de forma uniforme ao longo de toda a área afectada, provavelmente relacionado com a distribuição e a expansão natural do insecto vector.

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CONTENTS

CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

For millions of years the distribution of the world's biota has been constrained by natural barriers. However, with increasing globalization and the breaking down of geographical boundaries, new biological invasions by non-indigenous species have become a global environmental issue, often causing severe outbreaks with economic and ecological disruption in various ecosystems (Liebhold *et al.*, 1995; Sakai *et al.*, 2001).

In forest ecosystems the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970, is considered one of the most important pests and pathogens in the world. The general fear of establishment of the PWN, the causal agent of the pine wilt disease (PWD), into countries where conifer forests assume great importance, stems from the devastating damage caused by this nematode to pine forests (Mamiya, 2004; Shin and Han, 2006). The introduction of the PWN into non-native areas (outside of North America) is primarily associated with trade and the global flow of forest products (Bergdahl, 1999; Webster, 2004). Unmanufactured wood, especially in raw log form, has been identified as one of the most high-risk pathways of movement of forest insects and pathogens into new environments, between continents (Evans *et al.*, 1996; Tkacz, 2002). Many of the *Bursaphelenchus* species, including the PWN, have been routinely intercepted in packaging and wood products in several countries, e.g. Austria (Tomiczek *et al.*, 2003), China (Gu *et al.*, 2006), Finland (Tomminen, 1991) and Germany (Braasch *et al.*, 2001). Furthermore, the recent detections of the PWN in packaging wood imported from countries considered free of this pest, due to the repeated use and circulation of this type of wood material, e.g. Brazil, Belgium, Italy and Spain (Gu *et al.*, 2006), undoubtedly stresses the importance of trade globalization for the potential entry/establishment of this pathogen into endemic forests worldwide.

The damage by this invasive species is clearly demonstrated by the devastation caused in non-native regions where the disease became established, e.g. Japan and China (Yang, 2004; Shimazu, 2006). The introduction of this nematode into non-native areas has resulted in huge annual losses due to the effects on increased mortality and growth loss of the pine forest (26 million m^3 of timber lost since 1945 in Japan), and by the increased costs in management procedures and disease control (Mamiya, 2004; Shimazu, 2006). In addition, the introduction of this pest has resulted in vast and irreversible changes to the native forest ecosystems including tree species conversions, wildlife habitat destruction, soil and water conservation and loss of biodiversity (Kiyohara and Bolla, 1990; Suzuki, 2002).

The PWN is already established for more then 100 years in Japan (Yano, 1913), and in the past two decades the new reports of pine wilt disease came mainly from East Asia (Cheng *et al.*, 1983; Yi *et al.*, 1989). However, in 1999 the PWN was reported for the first time in Portugal and in Europe (Mota *et al.*, 1999). Following this finding, there has been considerable activity in both delineating the extent of the infested area and preventing the spread to the remainder of the country and the European Union (EU) (directive 2001/218/EC). The potential threat of the PWN to coniferous forests is real and the most effective way of reducing this threat is to be more restrictive to the importation of wood products, and to carry a rigorous inspection system for wood material (Evans *et al.*, 1996; Gu *et al.*, 2006). Therefore, specific measures have been applied in Portugal in order to control and eradicate the PWN and its insect vector, and in each EU member country, national surveys were performed to determine whether the nematode is present in other territories beside Portugal (directive 2001/218/EC).

The current situation in Portugal assumes great importance not only because of the economic implications, but also through the destruction of the pine forest in the area where the PWN became established (Setúbal Península). On the other hand, pine forests occupy a huge area of the continental territory (1.25×10^6 ha) representing one of the greatest natural resources of the country, namely in the form of timber (*Pinus pinaster* Ait.), wood products and pine nuts (*P. pinea* L.). Consequently, strict requirements have been imposed on all wood movements from the affected area to other regions in Portugal, as well as to other EU member states. These measures have had serious implications for the timber industry within the affected area, creating a significant impact on the national economy and markets of wood industries (Mota & Vieira, 2004).

The occurrence of pine wilt disease in Portugal is presently limited to a relatively small area (ca. 500 000 ha). Nevertheless, the potential danger of spread of this disease assumes a high phytosanitary risk because of the wide distribution of both the insect vector (*Monochamus galloprovincialis* Oliv.) and the known susceptible host (*P. pinaster*) in Portugal (Rodrigues, 2006).

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) (Iwahori *et al.*, 2004; Mota *et al.*, 2004). These hypotheses were recently tested, suggesting a possible double introduction of the PWN in Portugal (Metge and Burgermeister, 2006), both from East Asian countries. Although this study incorporates a large number of different isolates from different regions of the world, concerning Portugal it is restricted to the use of three isolates only, and representative of a small area of the full affected area. Therefore, the study of the genetic diversity of the PWN within the affected area in Portugal may provide additional clues for the pathway analysis of this pest in our country, and relevant information for ongoing studies on the bioecology of the nematode and the disease process.

LITERATURE REVIEW

PWN BIOLOGY, DISTRIBUTION AND DISEASE DISSEMINATION

PWN is considered a native species from North America, where it is distributed throughout Canada and USA (Robbins, 1982; Bowers *et al.*, 1992; Sutherland and Peterson, 1999), and also with a single report from Mexico (Dwinell, 1993). In these regions, the PWN has been associated with several conifer species: blue spruce and white spruce (*Picea* spp.), atlas cedar and deodara cedar (*Cedrus* spp.), eastern larch and european larch (*Larix* spp.), balsam fir (*Abies* spp.) and douglas fir (*Pseudotsuga* spp.), however, it is mainly found in pine species (*Pinus* spp.) (Robins, 1982; Bowers *et al.*, 1992).

B. xylophilus has both phytophagous (transmission by feeding) and mycophagous (transmission by oviposition) phases of development (Figure I). The nematode is carried by *Monochamus* beetles that feed on twigs in the crowns of healthy trees. Later the female beetles lay their eggs in damaged or dying trees as well as in freshly cut stems with bark. Fourth stage juveniles ("dauer" larvae) of *B. xylophilus* are carried under the elytra (wing cases) and in the tracheae (breathing tubes) of the beetles and migrate into the tree through the wounds caused by feeding or oviposition beetles.

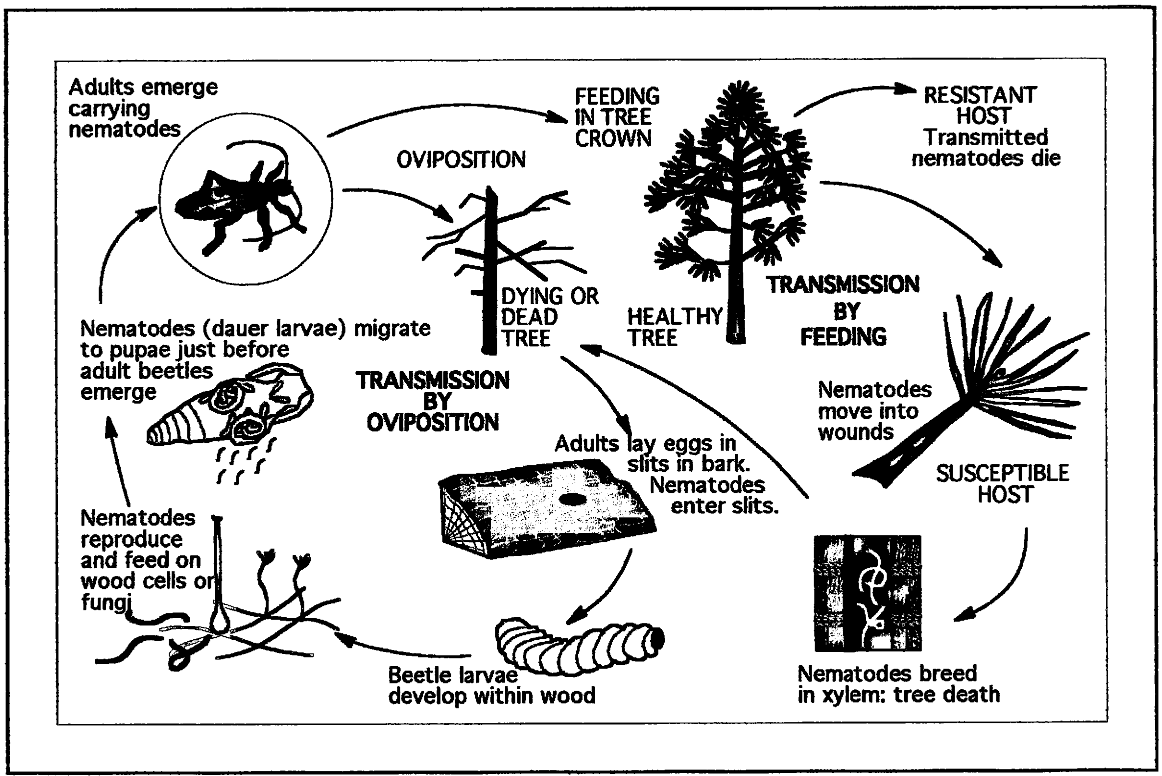

FIGURE I: Schematic representation of the inter-relationships between the pinewood nematode, *Bursaphelenchus xylophilus*, and its vectors in the genus *Monochamus* (adapted from Fielding and Evans, 1996).

Transmission during maturation feeding is the initiation of the phytophagous phase of the nematode, which has the greatest importance for the potential development of pine wilt disease. In a suitable tree species and under favorable climatic conditions, the nematodes multiply quickly in susceptible trees, feed on plant tissues and move from the cambium into the xylem. Their generation time is 6 days at 20°C and 3 days at 30°C. The nematodes contribute to plant death by blocking water conductance through the xylem. The damaged trees become available for oviposition by *Monochamus* spp. females when nematodes enter the tree through the oviposition slits in the bark. In dead trees, the nematodes feed on fungi, in particular on blue stain fungi. *Monochamus* larvae develop initially in the cambium and then burrow into the wood, where the nematodes congregate in the vicinity of the pupal chambers formed by the mature beetle larvae. When the new beetle emerges, the nematodes migrate into the tracheae and to the area beneath the elytra of the beetles. The presence of suitable fungi in the trees encourages nematode reproduction and survival and, consequently, increases the number of nematodes carried by the emerging beetles (Mamiya, 1984; Linit, 1988; Kishi, 1995; Fielding and Evans, 1996).

The introduction and spread of this species into new areas has also been aided by the high phenotypic plasticity of the nematode, including excellent adaptation for resistance in the host tree (i.e. long periods of starvation) and dispersion (ectophoretic insect association) (Mamiya, 1984). In the native host species of North America, the nematode does not cause disease, since both plant and nematode have co-evolved for a very long time and thus the trees have become resistant/tolerant to its presence (Kiyohara and Bolla, 1990), except in some exotic *Pinus* spp. plantations (Evans *et al.*, 1996). On the other hand, this scenario changes drastically when this organism reaches non-native habitats.

It is assumed that the presence of the PWN in Japan is the result of an accidental introduction by means of contaminated wood products from the USA (California) to the southern Japanese island of Kyushu, in the beginning of the 20th century (Yano, 1913). However, only in 1971 was the PWN associated with the high mortality of pine trees and identified as the causal agent of PWD, mainly of Japanese black pine (*P. thunbergii* Parl.) and Japanese red pine (*P. densiflora* Sieb. and Zucc.) (Kiyohara and Tokushige, 1971). In spite of the numerous efforts to control the nematode and the insect vector (*M. alternatus* Hope), the disease spread throughout the entire country, with the exception of the most Northern prefectures of Aomori and Hokkaido, occupying nowadays 28% of the total pine forest area (Mamiya, 2004; Shimazu, 2006).

During the eighties, the PWN was reported in other East Asia countries as well. In 1983 it was found for the first time in mainland China, associated with dead and dying Japanese black pine, in Nanjing (Jiangsu Province) (Cheng *et al.*, 1983). The situation in China assumes great importance either by the continuous spreading of the disease (up to date more then 20 million pine trees destroyed) among different regions of the country (Jiangsu Province, Anhui Province, Guangdong Province, Zhejiang Province, Shandong Province and Hubei Province) mainly due to human factors, and secondly by the potential threat to other areas where all the conditions that determine the establishment of the disease are present, and which are still free of the PWN (Yang, 2004).

In Taiwan the first report of the PWN occurred in 1985, identified from a luchu pine (*P. luchuensis* Mayr.) stand displaying 50% mortality, in the Taipei prefecture (Tjean and Jan, 1985a). It has also been reported from Japanese black pine in Taoyeun prefecture (Tjean and Jan, 1985b).

In 1989, the PWN was detected in South Korea, in Pusan (the largest harbor city located in the extreme southern part of the country), associated with the Japanese black pine and Japanese red pine (Yi *et al.*, 1989). Although the area of distribution of the disease was controlled until 1997, and limited in relatively small areas in the southern part of the country (La *et al.*, 1999), in the last years a continuous spread of the disease has been observed, and more recently it has been reported simultaneously from new different areas (Mokpo, Sinan, Yeongam, Daegu, Gumi, Andong, Gyeongbuk, Gangneung and Donghae), constituting today the major forest pest in the country (Shin and Han, 2006).

In 1999, the PWN was reported for the first time in Portugal, and in Europe, associated with maritime pine (*P. pinaster*) (Mota *et al.*, 1999), and with a single species as the insect vector (*M. galloprovincialis*) (Sousa *et al.*, 2001). After the initial detection, a national survey was carried out along the pine forests, and a quarantine area was established where the nematode occurred, in the Peninsula of Setúbal (ca. 30 km SE of Lisbon). The PWN affected area covers 510,000 ha, surrounded by a buffer zone of 500,000 ha more, for safety reasons. Although the initial affected area persists as almost identical from 1999, in the last survey/eradication campaign the number of declining trees in the demarcated area increased significantly within the affected zone, followed by an expansion of the delimited area, particularly to the south of the country (Sines, corresponding to the south point). As a result of this trend, new prevention measures were established by the EU, i.e., the implementation of a 3 km phytosanitary strip surrounding the entire quarantine area, where all the pine trees are to be cut and removed until the end of 2007 (Rodrigues, 2006).

PINEWOOD NEMATODE TAXONOMY: MORPHOLOGICAL APPROACHES

The genus *Bursaphelenchus* was established by Fuchs (1937) and includes nematodes that are associated with insects and dead or dying trees, mainly conifers, and which have an ectophoretic stage. Most species are fungal feeders and are either transmitted to dead or dying trees during oviposition by insect vectors, or to healthy trees during maturation feeding of their insect vectors (Hunt, 1993). The genus is mainly distributed in the northern hemisphere, however a few number of species have been reported outside of this geographical range (South Africa), associated with plantations of pine species (for a detailed information see Ryss *et al.*, 2005, in the Appendix of this thesis).

The current concern on the introduction of the PWN into new areas has increased the interest and the knowledge of this genus and the number of species recorded worldwide. Up to date, the genus comprise 85 described species, 10 of which where described in the last two years, mainly from East Asia (Vieira *et al.*, 2006). In Portugal, until the report of the PWN in 1999, no knowledge of this genus was available. At the moment, 10 species have been reported for the country, associated with maritime pine trees (Penas *et al.*, 2004), including the description of a new species to science, *B. antoniae* Penas, Metge, Mota and Valadas, 2006 (Penas *et al.*, 2006).

The economic importance posed by the PWN clearly reinforced the need for an accurate diagnosis of the species, where morphological studies remain the standard method for routine identification. Different criteria may be used to divide the large number of nominal species of the genus *Bursaphelenchus*, into smaller and more convenient species groupings. Tarjan and Baéza-Aragon (1982) were the first to attempt the assembly of morphological identification keys for this genus, providing a detailed classification of the spicule characters and other useful morphological diagnostic data. Braasch (2001), and for the species associated with conifer trees in Europe (28 at that time), proposed the establishment of the species groups based on the number of lateral lines (nine different groups), followed by the distribution of the male papillae, spicule shape, presence and size of the female vulval flap and the shape of female tail.

Yet, an integrated morphological identification system to all the species of the genus has been lacking. Furthermore, the fact that more then 70% of these species occur in pine trees makes the identification even more uncertain. Therefore, Ryss *et al.* ellaborated a synopsis of the genus in order to provide an identification system to all the nominal species, where the spicule structure is the main diagnostic character to separate the species into groups (cf. Appendix of this thesis). The six species groups (*aberrans*-group, *borealis*-group, *eidmanni*-group, *hunti*-group, *piniperdae*-group and *xylophilus*-group) are merely recognized as identification units in order to facilitate species identification. However, some of these groups could be considered as natural, i.e. phylogenetically related (e.g. the *xylophilus*-group) (Ryss *et al.*, 2005).

Despite the clear separation of the members of the *xylophilus*-group (*B. baujardi* Walia, Negi, Bajaj and Kalia, 2003; *B. conicaudatus* Kanzaki, Tsuda and Futai, 2000; *B. doui* Braasch, Gu, Burgermeister and Zhang, 2004; *B. fraudulentus* Rhüm, 1956; *B. kolymensis* Korentchenko, 1980; *B. luxuriosae* Kanzaki and Futai, 2003; *B. mucronatus* Mamiya and Enda, 1979; *B. singaporensis* Gu, Zhang, Braasch and Burgermeister 2005; *B. xylophilus*) from other groups based solely on the male spicule shape, the variability and overlapping in range of several other taxonomic characters within some species of this group is such that their accurate identification is difficult.

One of the major characters used for distinguishing the PWN from all other members is the shape of the female tail, i.e. rounded, and lacking a distinct mucron. However, specimens of *B. xylophilus* from North America show a wide variation in female tail shape, showing variations from rounded to a mucronated form, similar to the female tail of *B. mucronatus* (Wingfield *et al.*, 1983). In addition to the morphological similarities between *B. xylophilus* and *B. mucronatus,* these two species are capable of genetic exchange, either directly or via intermediate forms (Guiran and Bruguier, 1989), which clearly compromise the identification at the species level using morphological data only. Furthermore, the presence of males or juvenile stages alone deemed to be an unreliable method in the identification at the species level within the *xylophilus*-group, as well as for the differentiation of geographic isolates.

PINEWOOD NEMATODE TAXONOMY: MOLECULAR APPROACHES

Due to the limitations and constrains of morphological observations between *Bursaphelenchus* species, alternative molecular tools have become a valuable instrument for species and sub-specific separation. Initially these molecular tools were mainly developed for the differentiation of some species of the *xylophilus*-group, such as *B. xylophilus* and *B. mucronatus*, in order to achieve a better understanding of the relationships, and the clear identification of the *B. xylophilus* isolates.

The first methods used for the *Bursaphelenchus* species identification and isolates separation were based on protein profiles (Hotchkin and Giblin, 1984) and enzyme electrophoresis (Guiran *et al.*, 1985). However, the value of these methods was limited by differential gene expression during the life cycle of the nematode or by the response to external environmental influences (Harmey and Harmey, 1993). Immunological approaches have also been used for species-specific identification, using polyclonal antibodies that could differentiate specific antigens of certain *B. xylophilus* isolates (Lawler and Harmey, 1993), as well as monoclonal phage antibodies (Fonseca *et al.*, 2006).

With the expansion of DNA-based methodologies, new alternatives, independent of the development stage and phenotypic variation due to external influences (Harmey and Harmey, 1993), have been able to detect genetic variation that can be exploited or adapted for taxonomic and diagnostic purposes. Bolla and co-workers (1988) differentiated *B. xylophilus* pathotypes using restriction enzyme analyses and hybridization with total genomic DNA. Others have used cloned DNA hybridization probes from *C. elegans* (Abad *et al.*, 1991), or *Bursaphelenchus*, based on ribosomal probes (Webster *et al.*, 1990), DNA probes (Abad *et al.*, 1991; Tàres *et al.*, 1992) and satellite DNA (Tàres *et al.*, 1994), for a more reliable characterization of the species, and for the differentiation of specific and intraspecific groups.

The development of the polymerase chain reaction (PCR) (Mullis *et al.*, 1986) promoted the improvement of some of the previous methods, and the establishment of new methods where only small amounts of DNA are required. The amplification of specific genomic regions is a highly effective methodology to detect inter- and intra-specific variations among *taxa*.

Species-specific DNA fragments have been amplified using primers derived from a cloned repetitive DNA sequence (Harmey and Harmey, 1993). ITS-RFLP has been used mainly for *Bursaphelenchus* species identification (Burgermeister *et al.*, 2005), while other methods have been carried out for the specific-species detection of *B. xylophilus*, namely PCR-based diagnostics with species-specific primers (Kang *et al.*, 2004; Matsunaga and Togashi, 2004; Li *et al.*, 2004; Leal *et al.*, 2005), real-time PCR assay (Cao *et al.*, 2005), and PCR amplification using satellite DNA-based primers (Castagnone *et al.*, 2005).

Concerning the assessment of the relationships among isolates with different geographical origins the following molecular methods have been applied: sequencing of heat shock protein genes, hsp70 (Beckenbach et al, 1992), sequence of rDNA ITS regions (Iwahori *et al.*, 1998; Beckenbach *et al.*, 1999; Kanzaki and Futai, 2002; Megte *et al.*, 2006), sequence of D2 and D3 of the 28S gene (Metge *et al.*, 2006). The random amplified polymorphic DNA technique (RAPD) has also been used for the study of intra-specific variation of PWN isolates from China (Zheng *et al.*, 1998; Zhang *et al.*, 1999), Japan (Kusano *et al.*, 1999), and a mixture of different geographical isolates (Braasch *et al.*, 1995; Irdani *et al.*, 1995; Wang *et al.*, 2001; Zhang *et al.*, 2002). Recently, a more integrated study has been conducted using several isolates each from the native regions (Canada and USA) and non-indigenous areas (China, Japan, Korea and Portugal) (Metge and Burgermeister, 2006).

BASIS AND OBJECTIVES OF THIS THESIS

Populations of an introduced invasive organism are expected to be genetically more diverse if they are derived from multiple introductions from different origins, as compared to the situation following a single introduction. An evaluation of molecular genetic variation of invasive populations may also allow identification of the source population or populations. These phylogeographic patterns might be viewed as DNA fingerprinting at the level of populations or localities (Sakai *et al.*, 2001). The limited studies on the number of isolates used from the affected area in Portugal do not clearly elucidate the number of possible introductions of this pest in our country, as well as the genetic diversity of the PWN established within the affected area.

The natural dispersion of the PWN is always dependent on its vector beetle (in the Portuguese case, *M. galloprovincialis*), which can carry hundreds to thousands of nematodes (Linit, 1988). Vector flight is influenced by many factors, e.g. prevailing winds and landscape structure including forest coverage, availability of found resources (pine trees) (Linit, 1988; Takasu *et al.*, 2000). However, the spreading of the nematode could also happen via human activities, in some cases caused by the transport of infested wood or products to new areas inside the country. In fact, this may be the single most important factor involved in the spread of the PWN. Therefore, careful pathway analyses based on genetic markers could be useful to trace the possible way(s) of the disease spread, and to prevent further unintentional transport of the pest to nematode-free areas.

The incidence of pine wilt disease is closely related to environmental conditions. Different biotic and abiotic stress factors influence tree infection, however, variation in host specificity and pathogenicity have been reported for different PWN isolates, from both native and non-native regions (Kiyhoara and Bolla, 1990). The recognition of genetic differences among different isolates could be useful for the selection of different isolates (groups) to be implemented on pathogenetic tests.

Hence, based on the above features the present thesis was developed with the following objectives:

- To characterize the genetic diversity of the PWN in the affected area in Portugal, and to establish the relationship among the different isolates.
- To investigate whether pine wilt disease in Portugal originated from a single introduction or repeated introductions of the PWN.
- To investigate possible traces of spreading of the PWN, among the affected area in Portugal.

CHAPTER II

LACK OF GENETIC VARIATION OF *BURSAPHELENCHUS XYLOPHILUS* IN PORTUGAL REVEALED BY RAPD-PCR ANALYSES

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ABSTRACT

Random Amplified Polymorphic DNA (RAPD-PCR) technique was used to assess the level of genetic variability and genetic relationships among 24 Portuguese isolates of pinewood nematode, *Bursaphelenchus xylophilus*. The isolates represent the main infested areas of Portugal. Two additional isolates of *B. xylophilus* representing North America and East Asia were included, and *B. mucronatus* was used as out-group. Twenty-eight random primers generated a total of 640 DNA fragments. The Nei and Li similarity index revealed a high genetic similarity among the Portuguese isolates (above 90%). Hierarchical cluster analysis was performed to illustrate the relatedness among the isolates. No indication for separate groups among the Portuguese isolates was obtained, and the low level of genetic diversity strongly suggests that they were dispersed recently from a single introduction. The lack of apparent relationship between the genetic and the geographic matrices of the Portuguese isolates limits the use of this technique for following recent pathways of distribution. Genetic distance of the Portuguese isolates towards an isolate from China was much lower as compared to an isolate from the USA. This confirmed previous results suggesting an East Asian origin of the Portuguese *B. xylophilus*.

Key words: pinewood nematode, DNA fingerprinting, RAPD, similarity.

INTRODUCTION

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934) Nickle, 1970, the pinewood nematode (PWN), is the causal agent of pine wilt disease (PWD) and one of the most important pests and pathogens of conifer forests worldwide (Evans et al., 1996). Although considered a native species to North America (Rutherford et al., 1990), in the past century it has been introduced and spread into non-native areas, first Japan (Yano, 1913; Kiyohara and Tokushige, 1971), then China (Cheng et al., 1983), Taiwan (Tzean and Jan, 1985) and Korea (Yi et al., 1989), and more recently into the European continent in Portugal (Mota et al., 1999). The impact of this invasion into non-native areas is to damage endemic natural resources, mainly in pine forests, not only by the huge economic loss of wood, but also due to the social importance of pine forests in some countries such as Japan (Mamiya, 2004; Yang, 2004; Rodrigues, 2006).

The introduction and spread of this species into new areas depend on appropriate environmental conditions (mean summer temperature above 20°C), the presence of a suitable/susceptible host tree (mainly *Pinus* spp.) and the presence of a proper insect vector (usually a *Monochamus* sp.) (Mamiya, 1984; Linit, 1988; Kishi, 1995; Evans et al., 1996). Although the expression and expansion of pine wilt disease depend on a range of biological and physical factors, PWN displays a wide range of pathogenicity (Kiyohara and Bolla, 1990; Sutherland et al., 1991) and is associated with various host species across a wide geographical distribution (for detailed information see Ryss et al., 2005).

In Portugal, PWN is associated with maritime pine (*Pinus pinaster* Ait.) (Mota et al., 1999; Penas et al., 2004) and with a longhorn cerambycid beetle (*Monochamus galloprovincialis* Oliv.) as the insect vector (Sousa et al., 2001). The geographic distribution of PWN is confined to an area 20 km south of Lisbon (Peninsula de Setúbal), occupying 510,000 ha of the continental area. A buffer zone of approximately 500,000 ha free of PWN was established for safety reasons (Rodrigues, 2006). Due to the phytosanitary measures implemented by the Portuguese government following the European Union directives (77/93 updated as 2000/29/EC), the nematode has been confined to this part of the country since its detection in 1999 (Penas et al., 2004; Rodrigues, 2006); however, each year thousands of trees displaying symptoms of PWD have been cut and removed (Rodrigues, 2006).

Several molecular biological techniques have been used for the study of genetic variability among different geographical isolates of *B. xylophilus*. Initially, the genetic differentiation of some populations was achieved by the use of restriction analyses and hybridization with total genomic DNA (Bolla et al., 1988), or by applying DNA probes (Webster et al., 1990; Abad et al., 1991; Tàres et al., 1993). Other studies using the heat shock protein *Hsp70* gene (Beckenbach et al., 1992), PCR-RFLP and rDNA sequencing (Iwahori et al., 1998; Beckenbach et al., 1999) demonstrated some genetic differences among different isolates.

The RAPD-PCR technique has also been used for the study of intra-specific variation of PWN isolates from China (Zheng et al., 1998; Zhang et al., 1999), Japan (Kusano et al., 1999) and a mixture of different geographical isolates (Braasch et al., 1995; Irdani et al., 1995a, 1995b; Wang et al., 2001; Zhang et al., 2002). Recently, a more integrated study has been conducted using several isolates each from the native regions (Canada and USA) and non-indigenous areas (China, Japan, Korea and Portugal) (Metge and Burgermeister, 2006).

The introduction of a species into a new area can be used as a natural cause study, where the species must be able to cope with a range of new environmental pressures (Sakai et al., 2001). The genetic diversity among the Portuguese isolates of *B. xylophilus* is not known since available information is restricted to only three isolates from adjacent blocks of the affected area (Metge and Burgermeister, 2006). Two groups have observed significant degrees of differentiation among different isolates from countries where PWN has become established (Zheng et al., 1998; Metge and Burgermeister, 2006).

In this study, we have applied the RAPD-PCR technique to determine genetic distances among isolates of PWN from 24 locations within the affected area in Portugal. Cluster analysis of genetic relationships was used to examine whether the Portuguese *B. xylophilus* originated from a single introduction or multiple introductions, and an attempt was made to trace the spreading of *B. xylophilus* from its point of introduction throughout the affected area in Portugal.

MATERIALS AND METHODS

Nematode isolates: In 2005, during the annual survey for PWN carried out by PROLUNP (http://www.dgrf.min-agricultura.pt/prolunp), a total of 250 pine wood samples were collected from *P. pinaster* (maritime pine) trees displaying symptoms of PWD from the 28 blocks that compose the affected area in Portugal. The division of the affected area into blocks follows the experimental design established by PROLUNP for the practical purpose of survey and eradication of PWN (Fig. 1). Wood samples, 40 to 80 g each, were collected from pine trees at 1.5 m from the base of the trunk using a 1.2-cm-diam. low-speed drill and stored in small plastic bags. Nematodes were extracted using Baermann funnel technique and processed within 48 hr.

Culturing geographic isolates: From each positive sample (presence of *B. xylophilus*), the nematodes were collected and cultured on *Botrytis cinerea* Pars., grown on potato dextrose agar (PDA) and incubated at 25°C for 2 wk. After successful rearing, 24 isolates were selected, representing 24 different blocks (the four remaining blocks were excluded partly due to unsuccessful rearing of some cultures and the limited number of sample slots in the electrophoresis apparatus). From each isolate, 100 to 200 nematodes (without separation according to sex or developmental stage) were collected and washed several times in distilled water, transferred to a 1.5-ml Eppendorf tube with distilled water and stored at -80°C until use. All isolates have been confirmed as *B. xylophilus* by ITS-RFLP (data not shown). The additional isolates used were: one *B. xylophilus* from Nanjing, China (BBA code: Ne12/02) isolated from *P. thunbergii* Parl. and kept in fungus culture since 2002, one *B. xylophilus* from Missouri, USA (BBA code: N5/00) from an unknown source and kept in fungus culture since 2000 and one *B. mucronatus* from Brandenburg, Germany (BBA code: DE-4w) isolated from *P. sylvestris* L. and kept in fungus culture since 1996, as an outgroup.

DNA extraction: DNA extraction was performed using the QIAmp DNA Micro Kit (Qiagen, Germany). The nematodes were placed in 1.5-ml microcentrifuge tubes and pelleted by centrifugation at 9,000*g* for 2 min, and the supernatant discarded. To the pellet, 30 µl of ATL buffer was added, and the nematodes were homogenized using Eppendorf micropestles (Eppendorf, Hamburg, Germany). The homogenate was mixed with an additional 150 µl of the

ATL buffer and further processed according to the manufacturer's instructions. DNA concentrations were measured fluorimetrically using the fluorescent dye Hoechst 33258 and a DyNa Quant 200 fluorimeter (Pharmacia Biotech, Germany).

RAPD-PCR procedure: For this study, 30 oligonucleotide decamer primers (MWG, Germany) were used (Table 1). These primers were selected because they gave suitable results for the comparison of *B. xylophilus* isolates in previous studies (Braasch et al., 1995; Metge and Burgermeister, 2006; Gonçalo Silva, unpub. data). All RAPD reactions were performed as described by Schmitz et al. (1998), with slight modifications. Each PCR reaction (25 µl) contained Stoffel buffer (10 mM Tris, pH 8.3, 10 mM KCl), 4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of primer, 5 units of AmpliTaq DNA Polymerase Stoffel fragment (Applied Biosystems, Germany) and 4 ng of DNA template. Amplification was performed in a Perkin Elmer 9600 thermocycler (Applied Biosystems). The PCR was started by an initial denaturation step at 94°C for 2.5 min, followed by 40 cycles of 20 sec at 92°C, 15 sec at 38°C, 1 min at 72°C and a final extension at 72°C for 7 min. The rate of heating from 38°C to 72°C was regulated to 0.3°C/sec. After amplification, 10 µl aliquots of the reaction mixture were loaded onto a 2% agarose gel in TAE running buffer and electrophoresed for approximately 4 hr at 80 volts. The gel was stained in a 1 µg/ml ethidium bromide-water solution for 30 min and photographed with a UV system (Gel Jet Imager 2005, Intas, Germany). For each primer, PCR reactions were set up in individual 0.2-ml tubes. Twenty-seven *B. xylophilus* isolates were included: 25 from Portugal, including one isolate used as replicate; one from the USA; one from China; and one *B. mucronatus* isolate from Germany. In total, 840 reactions were performed, corresponding to 30 primers x 28 individual samples.

Data collection and analyses: The distinct RAPD products of each primer were run electrophoretically twice to ensure that no bands were artifacts. The RAPD fingerprint patterns obtained were converted into binary data matrices by scoring the presence of a band as 1 and its absence as 0. Bands that were not reproducible were excluded from the analyses. Faint and visually indistinguishable bands were ignored as genetic markers. The binary matrix was subjected to the MSVP ver. 3.12d software, using the Nei and Li coefficient (Nei and Li, 1979) to generate a matrix of genetic distances. The cluster analyses of genetic distances were performed with the unweighted pair-group method using arithmetic averages (UPGMA) in the module SAHN (sequential, agglomerative, hierarchical and nested clustering method) of NTSYS-PC ver. 2.1 (Rholf, 2000). The dendrograms were constructed with the TREE option of NTSYS-PC. The cophenetic correlation coefficient was calculated to provide statistical support for the dendrograms obtained, and Mantel's test (Mantel, 1967) was performed to check the goodness-of-fit of the cluster analysis to the matrix on which it was based. To evaluate the robustness of dendrograms, bootstrap values (1,000 replications) were calculated using the software TREECON ver. 1.3b (Van de Peer, 1997). The relationships between the Nei and Li genetic distance matrix and the geographic distance matrix were assessed using Mantel's test. In this case, the geographic distance between two isolates (only for the Portuguese isolates) was defined as the linear distance between the sites.

RESULTS

With the exception of primers Z9 and Z17, which amplified a large number of products causing difficulties for reliable band scoring, all 28 remaining primers were used for evaluation of amplification products and construction of the binary matrix. A total of 471 RAPD markers were scored for the isolates of *B. xylophilus*. These included 24 Portuguese isolates and a duplicate sample of isolate PT09 (termed PT09') for control of reproducibility, and one isolate each from Asia (Nanjing, China) and North America (Missouri, USA). A total of 222 RAPD markers were scored for the isolate of the out-group species, *B. mucronatus* (Brandenburg, Germany) (Table 1). The RAPD profiles were different with each of the primers. Depending on the primer, variable total numbers of amplified bands were obtained, as shown in Table 1. Figure 2 presents the RAPD profiles obtained from two of the 28 different primers used in order to illustrate the banding patterns observed. Within the Portuguese isolates, the banding patterns revealed a large number of monomorphic genetic markers in comparison to the polymorphic genetic markers; however, intraspecific polymorphism was revealed in a small proportion in some isolates (Table 2).

The genetic similarity matrix based on the Nei and Li coefficient is presented in Table 3. The lowest similarity (approximately 50%) was reached between the American isolate and all the other *B. xylophilus* isolates. A high genetic similarity was observed between the Portuguese isolates and the isolate from China, ranging from 84% to 94%. Within the Portuguese isolates, the genetic distances reached very low values for all combinations of isolates. More than 90% of the pair-wise combinations had more than 95% genetic similarity, and the remaining pair-wise combinations were still above 90% similarity (Table 3). The pair-wise combinations between isolate PT09 and its duplicate sample (PT09') expectedly showed an extremely high genetic similarity (99%), thus illustrating the reproducibility of RAPD profiles obtained with each primer. As expected, *B. mucronatus*, used as an outgroup, showed very low similarity (around 15%) towards the *B. xylophilus* isolates.

Cluster analysis of the genetic distances was conducted using the UPGMA algorithm, based upon Nei and Li's similarity matrix. This generated a dendrogram indicating the relationships among the *B. xylophilus* isolates used in this study (Fig. 3). The cophenetic correlation coefficient between the dendrogram and the original distance matrix of the RAPD profiles was significant, with a high correlation value r = 0.99 (1 = best possible fit). The dendrogram obtained clearly illustrated the outgroup position of the *B. mucronatus* isolate and the large intraspecific distances between the isolate from the USA and the other isolates from China and Portugal, all of which is supported by a high bootstrap value. The position of the Chinese isolate was found to be close to the group of the Portuguese isolates, with strong support by a high bootstrap interaction node value. Within the Portuguese isolates, a remarkable degree of similarity was obtained for all 24 isolates representing the entire affected area in Portugal. Although some primers revealed a different number of polymorphic bands for some isolates, all isolates were positioned together in the same, unique cluster (Table 2; Fig. 3).

UPGMA dendrograms were also constructed (based on Pearson product-moment correlation coefficient, using the software package Gel Compare ver. 4.1) for each single primer using the profile intensity generated for the 28 isolates, and similar results were obtained, i.e., *B. mucronatus* was separated as an outgroup, the USA isolate was always clearly separated from the other *B. xylophilus* isolates and the Portuguese isolates were very close to each other and close to the Chinese isolate (data not shown).

The relationship between Nei and Li's genetic similarity matrix and the geographic distance matrix was estimated using Mantel's test. The r value obtained (0.212) revealed a low correlation between the genetic distances of the Portuguese isolates and their distribution among the affected area in Portugal.

DISCUSSION

Several studies have demonstrated intraspecific variability of *B. xylophilus* isolates from different geographical areas using RAPD-PCR (Braasch et al., 1995; Irdani et al., 1995a, 1995b; Zheng et al., 1998; Kusano et al., 1999; Zhang et al., 1999; Wang et al., 2001; Zhang et al., 2002; Wu et al., 2005; Metge and Burgermeister, 2006; Zhang et al., 2006). These previous investigations revealed a significant degree of genetic divergence among different isolates of *B. xylophilus*. Metge and Burgermeister (2006) examined a number of isolates (15 from North America, 12 from Asia and three from Portugal) using RAPD-PCR and ISSR-PCR. They obtained two major clusters: one including the isolates from North America (Canada and the USA) displaying a high level of genetic diversity, and a second cluster including all isolates from non-native areas (China, Korea, Japan and Portugal), with less genetic diversity. However, other estimates of similarity across isolates collected in different areas in China ranged from 46% to 95%, showing significant differences among some non-native isolates (Zheng et al., 1998; Zhang et al., 1999). This is the first study where a significant number of *B. xylophilus* isolates from the affected area in Portugal was analyzed. The number of polymorphisms detected among 471 RAPD markers obtained using 28 primers was very low, reflecting a high genetic homogeneity among the 24 isolates examined. Low values of genetic distance were obtained in all pairwise comparisons; and the resulting UPGMA dendrogram suggested a low level of genetic divergence among the Portuguese isolates. Genetic distance of the Portuguese isolates from an isolate from China was much lower compared to an isolate from the USA. This confirmed previous results suggesting an East Asian origin of the Portuguese *B. xylophilus* (Metge and Burgermeister, 2006).

An objective of our investigation was to determine whether the Portuguese *B. xylophilus* originated from a single introduction or repeated introductions of the pest. Populations of an introduced invasive organism are expected to be genetically more diverse if they are derived from multiple introductions from different origins, as compared to the situation following a single introduction. Metge and Burgermeister (2006) suggested the possibility of two *B. xylophilus* introductions to Portugal from East Asia. This was based on their finding that one of their three Portuguese isolates clustered apart from the others among the isolates from East Asian countries. The three isolates were obtained from adjacent blocks of the affected area. However, the isolate that clustered separately had been maintained in culture since 1999, whereas the other two isolates were obtained in 2003 and kept in culture for only two years. Culturing of *B. xylophilus* isolates for up to 10 years on *Botrytis cinerea* malt agar may lead to small changes in RAPD profiles which are presumably caused by genetic shift (Metge et al., 2004). Culture-dependent genetic shift may therefore present an alternative explanation for the separate position of one of the three isolates studied by Metge and Burgermeister (2006). To avoid a possible genetic shift during culturing, all Portuguese isolates used in our study were collected from pine trees and reared in culture for only two weeks before DNA extraction and RAPD-PCR. No indication of separate groups of isolates was obtained in the dendrogram, and the low level of genetic diversity strongly suggests that they were dispersed recently from a single introduction.

Another intention of our study was to see whether the pathways of spreading of *B. xylophilus* from its point of introduction throughout the affected area in Portugal could be traced using RAPD-based markers. The presence of an international seaport (Setúbal) in the center of the affected area suggests a high probability for entry of PWN through this harbor. The local dispersion of PWN is always dependent on its vector beetle (*Monochamus galloprovincialis*), which can carry hundreds to thousands of nematodes (Linit, 1988). Vector flight is influenced by many factors, e.g., prevailing winds and landscape structure, including forest coverage. In analogy to findings in Japan by Takasu et al. (2000), a nearly concentric expansion of PWN from its initial site may be tentatively assumed. This hypothesis is supported by the position of two isolates collected near the seaport (PT24 and PT17) at the root of the dendrogram. Likewise, three isolates from neighboring areas (PT23, PT11 and PT05) were placed at the bottom of the dendrogram. However, genetic differentiation was inconsistent with the geographic distances of the remaining isolates. Mantel's test showed a low correlation value (0.212) between the matrices of genetic and geographic distances. Apparently, the high degree of similarity in RAPD profiles of the Portuguese *B. xylophilus* isolates limits the use of this technique for following recent pathways of distribution. Another problem lies in the correct assessment of the pathways and mode of transportation. In our preliminary attempt of correlation, the geographical distance matrix was based on linear distances between sites of sampling. In reality, long-distance spreading may not happen only by vector flight, but in some cases be caused by the transport of infested wood or wood products to new areas inside the country. In this way, genetically identical PWN populations could be found at distant sites, and careful pathway analyses based on genetic markers could be very useful to clarify the situation and prevent further unintentional transport of the pest.

Recently, Castagnone-Sereno et al. (2006) identified 18.5% variable sequence positions in cloned repeats of the Msp I satellite DNA (146 bp) of *B. xylophilus* isolates. His phylogenetic study based on satellite DNA variation revealed considerable diversity among Portuguese *B. xylophilus* isolates which appeared to correlate reasonably with geographic distances. Thus, satellite DNA seems to have a higher rate of genetic variation with time, compared to RAPD markers, and it is perhaps better suited to follow short-term changes in *B. xylophilus* populations following PWN introduction to Portugal. New comparative studies using satellite DNA are in progress (Castagnone-Sereno and Vieira, unpublished work) in order to provide more information about the genetic structure of the Portuguese isolates and to elucidate their pathways of spreading in the affected area in Portugal.

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Table 1: Primer sequences and total number of randomly amplified DNA-PCR bands produced by each primer, applied to 27 *B. xylophilus* isolates and one *B. mucronatus* isolate.

Primer*	Sequence	*B. xylophilus* markers [n]	*B. mucronatus* markers [n]	Sum of all markers (*B. xylophilus* + *B. mucronatus*) [n]
Z01	TCT GTG CCA C	12	7	16
Z02	CCT ACG GGG A	22	3	24
Z03	CAG CAC CGC A	16	9	23
Z04	AGG CTG TGC T	7	4	9
Z05	TCC CAT GCT G	20	10	28
Z06	GTG CCG TTC A	19	9	25
Z07	CCA GGA GGA C	12	6	18
Z08	GGG TGG GTA A	21	10	31
Z10	CCG ACA AAC C	23	13	29
Z11	CTC AGT CGC A	18	7	25
Z12	TCA ACG GGA C	10	10	19
Z13	GAC TAA GCC C	11	8	17
Z14	TCG GAG GTT C	13	8	19
Z15	CAG GGC TTT C	10	7	16
Z16	TCC CCA TCA C	12	4	15
Z18	AGG GTC TGT G	18	6	23
Z19	GTG CGA GCA A	27	6	32
Z20	ACT TTG GAG G	13	5	15
B07	GGT GAC GCA G	19	5	24
Re6	CGG AAT TCG C	14	8	20
Re8	CGA TCG ATG C	18	6	23
Re9	GGA AGC TTC G	17	7	23
Re10	CCC TGC AGG C	18	10	23
Y01	GTG GCA TCT C	11	8	16
Y04	GGC TGC AAT G	19	11	27
Y06	AAG GCT CAC C	26	12	37
Y08	AGG CAG AGC A	23	12	33
Y16	GGG CCA ATG T	22	11	30
Total		471	222	640

*Primers Z09 (CAC CCC AGT C) and Z17 (CCT TCC CAC T) were excluded because they produced complex patterns of amplification products precluding reliable band scoring.

Table 2: Number of RAPD-PCR markers among the Portuguese *B. xylophilus* isolates.

Primer	Total of bands (*a*)	Polymorphic bands (*b*)	Polymorphism % (*b/a* x 100)
Z01	7	3	42.9
Z02	16	6	37.5
Z03	13	5	38.5
Z04	6	2	33.3
Z05	15	10	66.7
Z06	15	1	6.7
Z07	10	2	20.0
Z08	16	4	25.0
Z10	17	3	17.6
Z11	15	6	40.0
Z12	6	1	16.7
Z13	9	3	33.3
Z14	8	2	25.0
Z15	8	3	37.5
Z16	8	3	37.5
Z18	13	2	15.4
Z19	22	6	27.3
Z20	9	1	11.1
B07	10	4	40.0
Re6	10	1	10.0
Re8	16	6	37.5
Re9	11	5	45.5
Re10	13	7	53.8
Y01	10	1	10.0
Y04	14	4	28.6
Y06	18	9	50.0
Y08	19	2	10.5
Y16	16	4	25.0
Total	350	106	

Table 3: Similarity matrix (Nei and Li coefficient) among 27 isolates of *B. xylophilus* and one isolate of *B. mucronatus* based on 640 RAPD markers.

	PT01	PT02	PT03	PT04	PT05	PT06	PT07	PT08	PT09	PT10	PT11	PT12	PT13	PT14	PT15	PT16	PT17	PT18	PT9'	PT19	PT20	PT21	PT22	PT23	PT24	CH01	US01	BmDE
PT01	1.00																											
PT02	0.996	1.00																										
PT03	0.985	0.985	1.00																									
PT04	0.983	0.983	0.979	1.00																								
PT05	0.956	0.956	0.960	0.966	1.00																							
PT06	0.965	0.965	0.973	0.975	0.956	1.00																						
PT07	0.969	0.969	0.977	0.975	0.964	0.969	1.00																					
PT08	0.973	0.969	0.969	0.979	0.964	0.965	0.973	1.00																				
PT09	0.967	0.967	0.974	0.972	0.953	0.982	0.970	0.967	1.00																			
PT10	0.979	0.979	0.978	0.992	0.973	0.978	0.982	0.986	0.976	1.00																		
PT11	0.968	0.968	0.957	0.955	0.974	0.938	0.942	0.946	0.935	0.951	1.00																	
PT12	0.990	0.990	0.990	0.984	0.958	0.975	0.971	0.967	0.972	0.980	0.962	1.00																
PT13	0.981	0.984	0.984	0.978	0.960	0.976	0.973	0.969	0.974	0.982	0.957	0.990	1.00															
PT14	0.969	0.969	0.976	0.978	0.963	0.984	0.976	0.972	0.986	0.986	0.941	0.978	0.984	1.00														
PT15	0.950	0.950	0.954	0.956	0.941	0.969	0.954	0.950	0.959	0.963	0.924	0.956	0.961	0.965	1.00													
PT16	0.958	0.958	0.969	0.963	0.944	0.969	0.957	0.953	0.978	0.967	0.930	0.967	0.969	0.976	0.954	1.00												
PT17	0.923	0.926	0.933	0.935	0.917	0.929	0.944	0.926	0.926	0.938	0.905	0.931	0.933	0.932	0.911	0.918	1.00											
PT18	0.959	0.959	0.967	0.969	0.950	0.970	0.963	0.959	0.980	0.972	0.935	0.969	0.970	0.978	0.963	0.966	0.923	1.00										
PT9'	0.965	0.965	0.972	0.970	0.951	0.980	0.969	0.965	0.998	0.974	0.933	0.970	0.972	0.988	0.957	0.976	0.925	0.978	1.00									
PT19	0.969	0.969	0.976	0.978	0.959	0.984	0.972	0.968	0.994	0.982	0.940	0.978	0.980	0.992	0.965	0.980	0.932	0.986	0.992	1.00								
PT20	0.979	0.975	0.979	0.965	0.943	0.967	0.959	0.959	0.976	0.965	0.947	0.981	0.975	0.978	0.952	0.967	0.916	0.969	0.978	0.978	1.00							
PT21	0.977	0.977	0.980	0.971	0.948	0.972	0.961	0.957	0.982	0.970	0.949	0.986	0.980	0.980	0.957	0.972	0.921	0.978	0.980	0.988	0.990	1.00						
PT22	0.977	0.977	0.984	0.974	0.952	0.976	0.965	0.961	0.982	0.974	0.949	0.986	0.984	0.988	0.961	0.976	0.925	0.974	0.984	0.988	0.990	0.992	1.00					
PT23	0.940	0.940	0.951	0.941	0.927	0.946	0.935	0.932	0.948	0.944	0.917	0.949	0.950	0.950	0.936	0.958	0.898	0.940	0.946	0.953	0.945	0.954	0.954	1.00				
PT24	0.923	0.927	0.937	0.928	0.918	0.933	0.949	0.926	0.950	0.935	0.901	0.928	0.937	0.944	0.919	0.937	0.962	0.935	0.948	0.948	0.932	0.937	0.937	0.912	1.00			
CH01	0.875	0.872	0.878	0.880	0.863	0.885	0.878	0.890	0.894	0.887	0.847	0.876	0.881	0.895	0.879	0.882	0.854	0.879	0.896	0.891	0.888	0.881	0.888	0.858	0.868	1.00		
US01	0.496	0.500	0.500	0.493	0.493	0.496	0.492	0.490	0.497	0.493	0.503	0.501	0.504	0.502	0.500	0.494	0.486	0.499	0.500	0.500	0.509	0.506	0.510	0.494	0.485	0.486	1.00	
BmDE	0.149	0.145	0.146	0.142	0.140	0.143	0.142	0.145	0.140	0.143	0.145	0.146	0.143	0.140	0.145	0.138	0.143	0.144	0.140	0.141	0.150	0.148	0.143	0.139	0.143	0.159	0.135	1.00

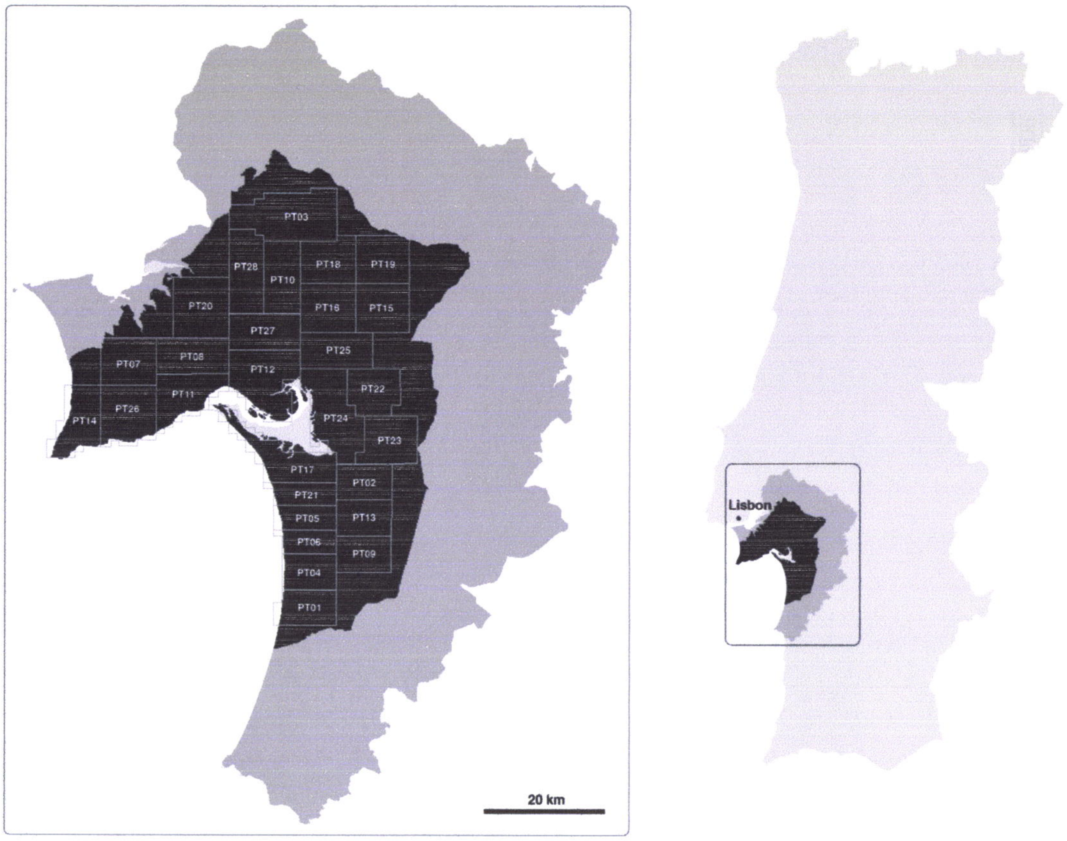

Figure 1: Right: Portugal continental and location of the quarantine area. Left: Location of *Bursaphelenchus xylophilus* isolates obtained from different blocks within the affected area. Black: the area affected by the PWN; dark grey: the buffer area, established for safety reasons (free of PWN).

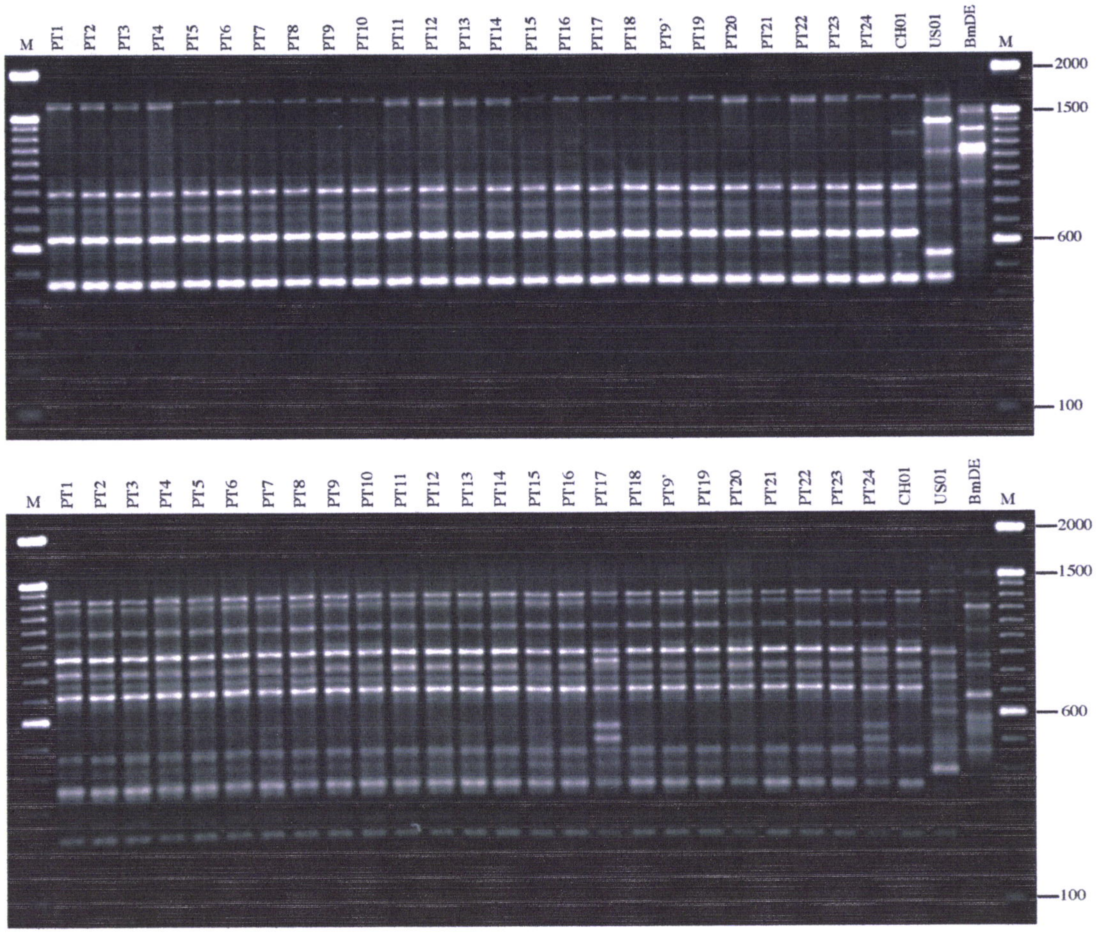

Figure 2: RAPD profiles generated by primer Z12 (above) and Y16 (below). M: marker (100 bp ladder, Invitrogen); PT1-PT24: *B. xylophilus* isolates from Portugal; CH01: *B. xylophilus* isolate from China (BBA code: Ne12/02); US01: *B. xylophilus* isolate from USA (BBA code: N5/00); BmDE: *B. mucronatus* from Germany (BBA code: DE-4w).

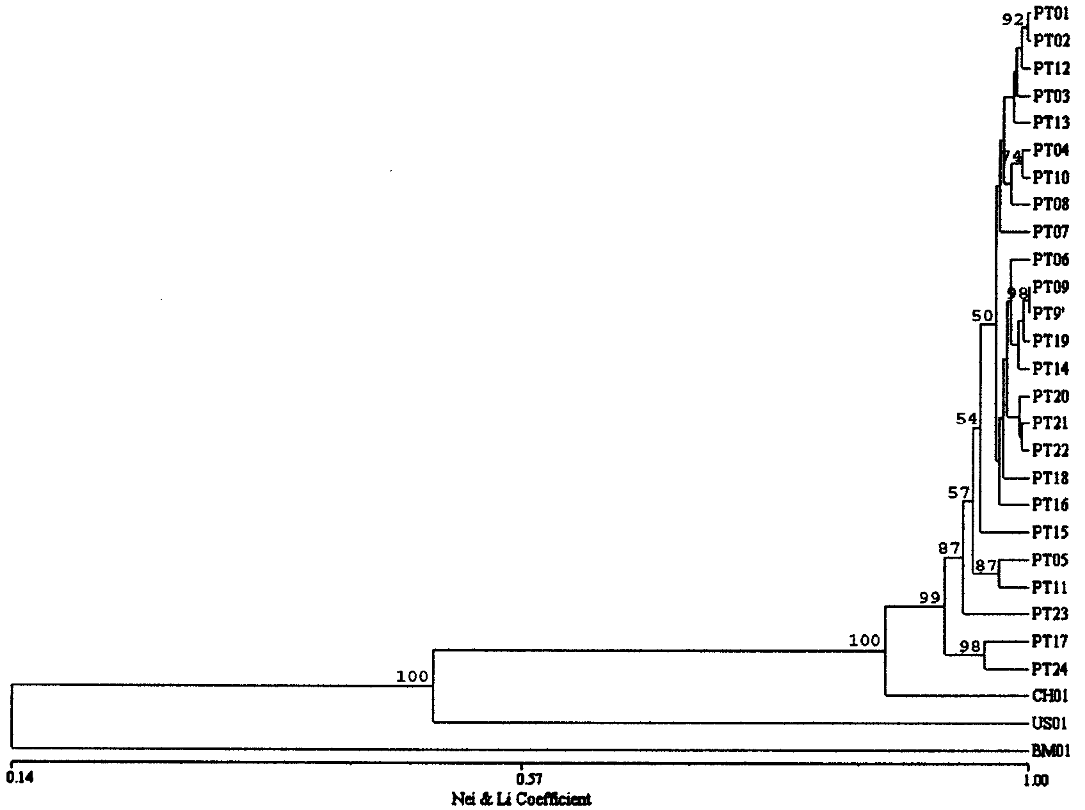

Figure 3: UPGMA tree inferred from 640 RAPD markers for 27 *B. xylophilus* isolates and one *B. mucronatus* isolate as the out-group.

CHAPTER III

MSPI SATELLITE DNA VARIABILITY AMONG PORTUGUESE ISOLATES OF THE PINEWOOD NEMATODE, *BURSAPHELENCHUS XYLOPHILUS*

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ABSTRACT

The *Msp*I satellite DNA was analyzed from *Bursaphelenchus xylophilus* isolates distributed within the area affected by pine wilt disease in Portugal. A total of 206 *Msp*I repeats from twenty-one isolates were studied. The nucleotide alignment of these clones shows that most of them share a homogeneous sequence length of 146 bp, with rare exceptions. The average nucleotide variability among the 206 repeats was 7.07%. The nucleotide alignment of all the repeats revealed no specific nucleotide substitutions, which could discriminate each isolate or groups of geographically close isolates. A variability analysis, intra and inter-isolates, showed similar and low genetic divergence respectively, which is congruent with previous RAPD-PCR data that indicated very little isolate differentiation throughout most of the *B. xylophilus* distribution in Portugal. Analysis of distribution of the variability along the sequence monomers suggests that satellite DNA repeats are subject to some degree of constraint, revealing highly conserved domains, whereas others show high nucleotide variability.

Key words: pinewood nematode, *Bursaphelenchus xylophilus*, satellite DNA, Portugal.

INTRODUCTION

Many eukaryotic genomes possess large amounts of non coding DNA, arranged in highly repetitive tandem units, commonly referred to as satellite DNA. These sequences are generally A-T rich and show variability affecting monomer size, nucleotide sequence, copy number, and appear to be primarily clustered within the heterochromatin at centromeres and other heterochromatic regions of chromosomes (Charlesworth *et al.*, 1994). The biological function of these DNA sequences remains undefinable, although it has been proposed that they are involved in intragenomic mechanisms, such as centromeric condensation, sister chromatid pairing, karyotypic evolution and chromosome arrangement (Plohl *et al.*, 2004; Shapiro and Sternberg, 2005). Consequently, owing to their usual confinement within a given species, there is a wide variation of satellite DNAs among animals, displaying genetic variability and providing a suitable target region for phylogenetic markers and fingerprinting opportunities. Due to its lower functional constraints, the evolutionary rate of satellite DNA has been proposed to be rapid, and supported by a number of satellite DNA families that have proved to be species-specific. Furthermore, several studies have demonstrated the usefulness of satellite DNA as genetic markers for monitoring genetic diversity at populations level (Dover, 1986; Hall *et al.*, 2003; Plohl *et al.*, 2004).

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, has a wide distribution in North America, being found throughout most of the territories of Canada and the United States. During the last century, this species has been transported by man to several non-native regions of the world, associated with trade and the global flow of forest products (Evans *et al.*, 1996; Webster, 2004). Up to date it has been reported from Asia (PR China, Japan, Korea and Taiwan) and more recently from Europe (Portugal) (Mota *et al.*, 1999). Due to the impact on the native pine forest of these regions, this nematode species and causal agent of pine wilt disease (PWD) assumes great economic importance worldwide. In Portugal the distribution of the PWN has been constrained to a small region south of Lisbon, however, it has become the most serious threat to pine forests in the country (Rodrigues, 2006).

Although several relevant data on satellite DNA have been documented for various plant parasitic nematodes, e.g. *Meloidogyne* species (Castagnone-Sereno *et al.*, 2000; Mestrovick *et al.*, 2005), few studies have been conducted with the PWN. Tàres *et al.* (1993) described the *Msp*I satellite DNA of *B. xylophilus*, which constitutes up to 30% of its genome. It consist of repetition units slightly A+T rich and 160 bp long. The sequence information available is restricted to thirteen monomers obtained from the same Japanese *B. xylophilus* isolate (Tarès *et al.*, 1993). However, hybridization patterns of *B. xylophilus* isolates, from different geographical locations (North America and Japan) with the *Msp*I satellite DNA family, revealed the existence of several polymorphisms, resulting in different profiles for each isolate (Tarès *et al.*, 1994).

In order to obtain more information on the genetic variability of the Portuguese *B. xylophilus*, we cloned and analyzed 206 *Msp*I satellite DNA sequences from twenty-one isolates, covering most of the geographical distribution of this species in the affected area in Portugal. This report provides the first large-scale characterization of the overall variability of this satellite DNA family within this species.

MATERIALS AND METHODS

Nematode isolates

The geographical location of all the *B. xylophilus* isolates used in this study is shown in Fig. 1. The isolates were previously (in 2005) isolated from wood samples of maritime pine (*Pinus pinaster* Ait.) and kept in *Botrytis cinerea* Pars. growing in malt agar (MA). All isolates were re-cultured on *B. cinerea* growing on MA, and stored in an incubator at 25°C during 2 weeks. From each isolate several hundred nematodes (without separation according to sex or developmental stage) were collected and washed several times in distilled water, transferred to a 1.5 ml Eppendorf tube with distilled water, and stored at 14°C until needed.

Isolation of MspI satellite DNA

Amplification of satellite DNA was performed based on a single worm PCR procedure (Castagnone et al., 2005), avoiding the extraction of the genomic DNA. For each isolate, three nematodes were transferred to a dry thin walled PCR tube, covered with 5 µl lysis buffer (50mM KCl, 10 MM Tris pH 8.2, 2.5 mM $MgCl_2$, 60 mg ml^{-1} proteinase K, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin), and overlaid with mineral oil. Tubes were kept at -80 °C for 45 min, and immediately transferred to 60 °C for 60 min and then 95 °C for 15 min in a thermal cycler. The satellite DNA repeats were amplified with specific primers, J10-1 (5′-GGTGTCTAGTATAATATCAGAG-3′) and J10-2Rc (5′-GTGAATTAGTGACGACGGAGTG-3′) (Castagnone et al., 2005), designed according to the sequence derived from the *MSp*I satellite DNA family previously characterized for *B. xylophilus* (Tarès et al., 1993). PCR was carried out in 25 µl reaction mixtures containing 5 µl buffer (and 3 nematodes), 2.5 mM dNTP, 100 ng of each of the primers and 0.5 Unit of *Taq* DNA polymerase (Q-Biogene) using a TRIO-Thermoblock thermal cycler (Biometra). After denaturation at 94 °C for 5 min, cycling was performed for 25 cycles of 30 s each at 94 °C, 1 min at 64 °C and 1 min at 72 °C, with a postcycling extension at 72 °C for 5 min. The resulting fragments were separated on 1.3% agarose gel and stained with ethidium bromide. Bands corresponding to a monomer were recovered from the agarose gel, using a gel extraction column (MinElute Gel Extraction Kit, Qiagen Inc.). Satellite DNA fragments recovered from the gel were ligated into the plasmid vector pGEM-T using protocols provided by the manufacturer (pGEM-T Vector System, Promega). The ligation was used to transform competent *Escherichia coli* DH5∂ cells, which were spread on L-agar plates with ampicillin, and grown overnight at 37 °C. The positive recombinant clones where identified as white colonies, and 12 random recombinant were amplified by PCR using SP6 and T7 universal primers (94 °C for 2 min, followed by 35 cycles of: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; followed by a 7 min final extension at 72 °C) and insert size was estimated on agarose gels. Clones with inserts of approximately 150-bp were used to inoculate 3 ml LB, growth overnight at 37 °C, and mini-prepped to extract plasmid DNA (QIAprep Spin Miniprep Kit, QIAGEN, Inc.). The positive clones were sequenced in one direction by Genome Express (Meylan, France).

Sequence analysis

Pairwise sequence alignment and multiple alignments were carried out with the program CLUSTAL X 1.81 (Thompson et al., 1997). Sequence homology searches were performed in GenBank using Blastn 2.2.2 with default parameters (Altschul *et al.*, 1997). Genetic distances were calculated according to Kimura's (1980) two-parameter method. All positions, including gaps, were equally weighed. For phylogenetic analyses preliminary tests were carried out with two methods (UPGMA and NJ) using the MEGA v.3 program (Kumar *et al.*, 2004). Sequence variation across satellite repeats was investigated as described in Hall *et al.* (2003): the occurrence of the most frequent base in each nucleotide position was calculated and plotted against nucleotide position. The average percent occurrence and standard deviations were also calculated.

RESULTS AND DISCUSSION

As already expected from the previously published data on the *Msp*I satellite DNA family of *B. xylophilus*, the amplified PCR products using specific primers previously designed close to the both ends of the monomers sequence (Castagnone *et al.*, 2005), produced a ladder pattern of monomers with approximately 150 bp, followed by bands of corresponding dimers (300 bp), trimers (450 bp), and so on (Fig. 2). The band of approximately 150 bp of each isolate was independently eluted from gel and cloned. A total of 206 clones were sequenced from twenty-one isolates, from seventeen different blocks of the affected area in Portugal (Table 1). The nucleotide alignment of these clones show that most of them (197) shared a homogeneous sequence length of 146 bp. The very few exceptions occurred in some monomer units, with a length of 145 bp (PT1a_10, PT6_8, PT17_9), while in others were 148 bp long (PT6_4, PT13_3, PT13_6, PT13_7, PT13_8, PT13_9). All the repeats are A + T rich with a total average content of 57,1%, similar to the previously defined consensus sequence (59,6%) of a *B. xylophilus* Japanese isolate, for the same number of nucleotides (Tàres *et al.*, 1993). Although satellite DNAs have been characterized in other nematodes species, such as phytoparasitic species of the genus *Meloidogyne* (Piotte *et al.*, 1994; Castagnone-Sereno *et al.*, 2000; Mestrovic *et al.*, 2006) or entomopathogenic nematodes of the genus *Heterorhabditis* (Abadon *et al.*, 1998), BLAST search does not provide significant similarity with other sequences in GenBank database, neither with particular subdomains shared with other recognized satDNAs.

The nucleotide alignment of the 206 *Msp*I satellite DNA sequences revealed no specific nucleotide substitutions, which would clearly discriminate a specific isolate or groups of isolates (not shown). Even in some cases, more then two clones shared an identical nucleotide sequence (e.g. PT1a_11, PT6_2, PT16_12; and PT1_2, PT7_5, PT14_12) derived from distinctive isolates. The only exception was found for the sequence repeats of the PT13 isolate, suggesting a possible partial homogenization only shared within this isolate. The average sequence variability of each single sequenced satellite monomer to the calculated consensus sequence was 7.07%, a higher average comparing with the 3.9% average result previously obtained, but using only 13 repeats (Tarès *et al.*, 1993).

Homogenization of a repetitive sequence occurs via different intragenomic mechanisms that lead to lower nucleotide variability among interbreeding genomes, when compared with genomes that do not exchange (Elder and Turner, 1995; Dover, 2002). A variability analysis was carried out on the within and between genetic distances of the twenty-one isolates to assess the degree and trends of diversity in *B. xylophilus*. The genetic distances, based on the Kimura two-parameter model, ranged from 0.065±0.014 to 0.176±0.025 within the satellite DNA isolates sequences (Table 1), and 0.081 to 0.163 between isolates (Table 2), showed similar and low genetic divergence among intra- and inter-isolate variation. This is in agreement with previous studies that revealed a high genetic similarity among the Portuguese *B. xylophilus* isolates. Under this premise, and behind such lack of inter-isolates differentiation, these results clearly reinforce the idea of a single introduction of this invasive species into the Portuguese territory (Vieira *et al.*, 2007). Furthermore, phylogenetic inference (UPGMA and NJ) based on the 206 satellite DNA sequences shows a broad polytomy, where the sequence repeats intermingles regardless of the isolate, or geographical location (not shown). Previous preliminary results, based on the *Msp*I satellite DNA family, suggest a fairly positive correlation between the genetic diversity and the geographic distribution of some *B. xylophilus* in Portugal (Castagnone-Sereno, 2006). However, the overall analyses of the 206 satellite DNA sequences herein displayed, suggest that the intra-isolate variation is a reflex of the variability of this satellite DNA family itself, distributed uniformly in all the affected area, without a specific correlation and spreading of the disease within the different geographical blocks of the affected area. Altogether, these data suggest that the recent introduction of *B. xylophilus* in Portugal is uniformly distributed since its establishment, and probably following the natural distribution and expansion of the insect vector (Vieira *et al.*, 2007).

Although no function has been attributed for the majority of the satellites families, particular roles related to the heterochromatin condensation (Ugarkovic *et al.*, 1996; Pons *et al.*, 1997), maintenance of the functional centromeres (Willard, 1990) has been proposed for some satellite DNA. In order to verify the sequence variation of the *Msp*I family, disregarding their origin (isolate repeat sequences and geographical distribution), but for a functional point of view, the same approach conducted for the centromeric repeats of *Arabidopsis* populations and for the ∂-satellite DNA in humans was herein followed (Hall *et al.*, 2003), i.e., the percentage of occurrence of the most frequent base for each nucleotide position was taken as a measure of variability and plotted against nucleotide position (Fig. 3). In an overall analysis, this satellite family revealed 31.5% of all nucleotides with a frequency of 100%, whether 63% of the remaining nucleotides reside within 1 S.D. from the total average 92.96 ± 11.27. In addition, 5.5% represent a highly polymorphic nucleotide position, with frequencies below 2 S.D. from the total average. Following these results, there are specific regions exhibiting certain highly conserved domains, whereas others show a considerable variation and significantly different than the mean (Fig. 3), as previously mentioned for the 13 sequenced monomers of a Japanese *B. xylophilus* isolate (Tarès *et al.*, 1993). As discussed for other satellite DNA families of other organisms (e.g. *Arabidopsis*, humans), the preservation of both conserved and variables domains across satellite sequences, along with the strict conservation of the sequence length, strongly suggest that the evolution of the satellite repeats is constrained, i.e., the highly conserved domains indicate that some repeat regions may be under selective pressure to maintain a particular DNA sequence, whereas other regions evolve without constraint (Hall *et al.*, 2003).

LITERATURE CITED

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Table 1: ***Bursaphelenchus xylophilus*** **isolate list, number of complete monomers obtained, and mean distance (Kimura two-parameter) within each isolate ± standard error (S.E.).**

Block	Isolate code	*N*	Isolate mean distance (Kimura two-parameter)	S.E.
PT1	PT1	9	0.122	0.019
	PT1a	11	0.102	0.016
	PT1b	12	0.082	0.013
PT2	PT2	5	0.150	0.025
PT3	PT3	7	0.169	0.026
PT4	PT4	11	0.065	0.014
	PT4a	12	0.141	0.021
PT6	PT6	9	0.113	0.016
PT7	PT7	11	0.094	0.014
PT8	PT8	12	0.176	0.025
PT10	PT10	6	0.154	0.026
PT13	PT13	10	0.089	0.015
PT14	PT14	10	0.072	0.014
PT15	PT15	8	0.150	0.024
PT16	PT16	10	0.086	0.015
PT17	PT17	12	0.150	0.024
PT19	PT19	7	0.174	0.026
PT20	PT20	11	0.146	0.023
PT21	PT21	11	0.158	0.026
	PT21a	11	0.112	0.018
PT24	PT24	11	0.090	0.014
Total		206		

Table 2: Pairwise comparisons of Kimura two-parameter genetic distance (below diagonal) between the Portuguese *B. xylophilus* isolates, with standard error (above diagonal).

	PT1	PT1a	PT1b	PT2	PT3	PT4	PT4a	PT6	PT7	PT8	PT10	PT13	PT14	PT15	PT16	PT17	PT19	PT20	PT21	PT21a	PT24
PT1		0.02	0.02	0.02	0.021	0.017	0.020	0.018	0.018	0.021	0.020	0.018	0.016	0.021	0.016	0.018	0.020	0.020	0.023	0.018	0.019
PT1a	0.12		0.01	0.02	0.020	0.013	0.017	0.014	0.014	0.022	0.019	0.014	0.013	0.020	0.013	0.018	0.020	0.020	0.021	0.015	0.013
PT1b	0.11	0.09		0.02	0.019	0.012	0.016	0.013	0.013	0.021	0.018	0.013	0.012	0.019	0.013	0.016	0.019	0.019	0.020	0.014	0.013
PT2	0.14	0.12	0.11		0.022	0.017	0.019	0.018	0.018	0.022	0.021	0.018	0.018	0.020	0.018	0.020	0.021	0.021	0.021	0.019	0.018
PT3	0.15	0.14	0.13	0.16		0.020	0.021	0.020	0.021	0.023	0.022	0.020	0.019	0.023	0.019	0.022	0.023	0.023	0.023	0.020	0.020
PT4	0.11	0.08	0.08	0.11	0.14		0.016	0.013	0.012	0.022	0.019	0.013	0.013	0.020	0.013	0.017	0.019	0.020	0.021	0.014	0.012
PT4a	0.14	0.12	0.12	0.13	0.15	0.112		0.017	0.017	0.022	0.020	0.017	0.017	0.020	0.016	0.020	0.021	0.021	0.021	0.018	0.016
PT6	0.13	0.10	0.09	0.13	0.139	0.091	0.127		0.015	0.022	0.020	0.013	0.013	0.020	0.013	0.018	0.020	0.020	0.021	0.015	0.014
PT7	0.12	0.09	0.09	0.12	0.143	0.076	0.120	0.105		0.023	0.019	0.014	0.014	0.020	0.013	0.018	0.021	0.021	0.021	0.016	0.013
PT8	0.15	0.16	0.15	0.16	0.167	0.158	0.161	0.160	0.165		0.022	0.022	0.021	0.022	0.021	0.022	0.022	0.022	0.023	0.021	0.022
PT10	0.14	0.13	0.12	0.13	0.159	0.122	0.135	0.137	0.129	0.157		0.019	0.019	0.021	0.019	0.020	0.021	0.021	0.022	0.019	0.019
PT13	0.12	0.10	0.09	0.12	0.144	0.084	0.12	0.102	0.100	0.163	0.134		0.014	0.019	0.014	0.018	0.019	0.020	0.020	0.015	0.013
PT14	0.10	0.09	0.08	0.12	0.131	0.071	0.117	0.092	0.089	0.150	0.126	0.087		0.020	0.013	0.016	0.019	0.019	0.021	0.014	0.013
PT15	0.14	0.13	0.13	0.13	0.162	0.130	0.139	0.139	0.137	0.159	0.136	0.137	0.133		0.020	0.021	0.021	0.021	0.022	0.020	0.020
PT16	0.11	0.09	0.08	0.12	0.137	0.075	0.119	0.096	0.091	0.158	0.129	0.091	0.077	0.135		0.017	0.019	0.020	0.021	0.01	0.013
PT17	0.13	0.13	0.12	0.14	0.156	0.120	0.142	0.133	0.132	0.160	0.143	0.133	0.115	0.148	0.124		0.021	0.020	0.022	0.02	0.02
PT19	0.14	0.14	0.13	0.15	0.160	0.136	0.149	0.144	0.144	0.163	0.148	0.143	0.135	0.150	0.139	0.152		0.021	0.022	0.02	0.020
PT20	0.14	0.14	0.13	0.14	0.159	0.131	0.142	0.139	0.140	0.154	0.136	0.138	0.128	0.137	0.133	0.143	0.148		0.022	0.02	0.02
PT21	0.16	0.14	0.14	0.14	0.159	0.137	0.144	0.145	0.141	0.165	0.145	0.147	0.143	0.145	0.145	0.157	0.157	0.149		0.02	0.02
PT21a	0.12	0.11	0.10	0.12	0.140	0.095	0.124	0.109	0.106	0.151	0.127	0.106	0.095	0.131	0.101	0.127	0.137	0.130	0.139		0.02
PT24	0.12	0.09	0.09	0.12	0.138	0.079	0.115	0.099	0.092	0.155	0.124	0.092	0.085	0.127	0.088	0.125	0.137	0.130	0.135	0.10	

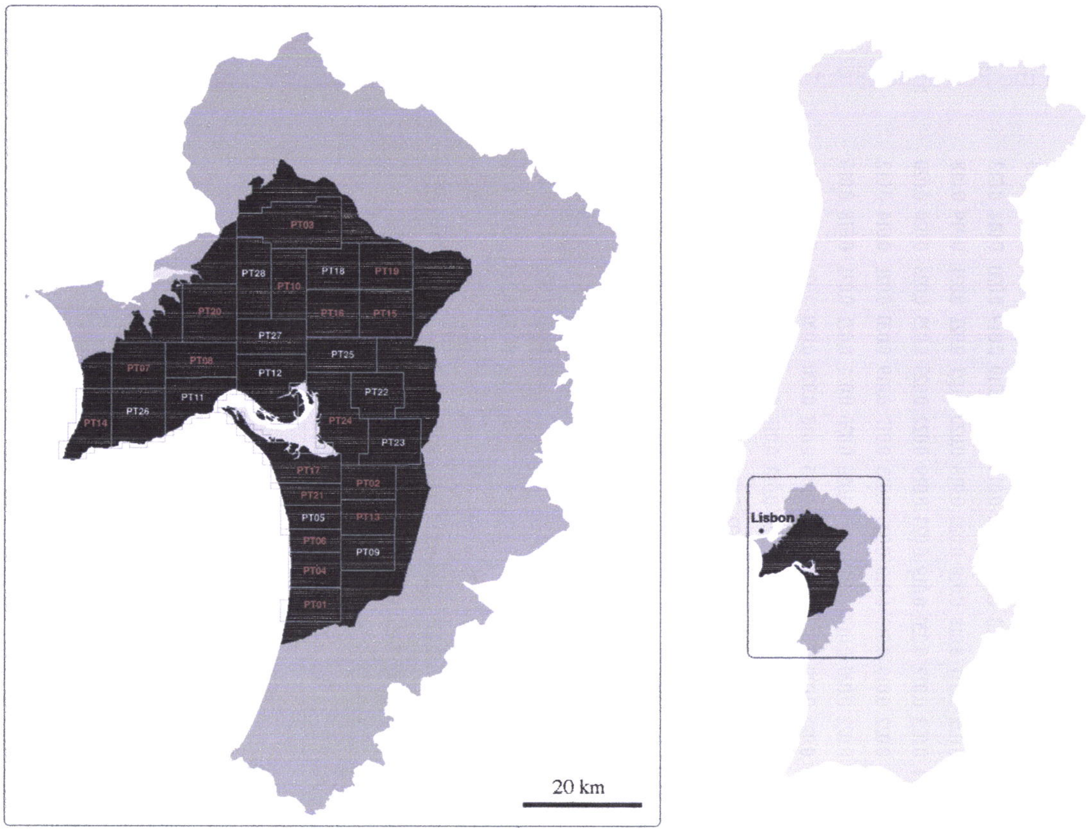

Figure 1: Right: Portugal continental and location of the quarantine area. Left: Location of *Bursaphelenchus xylophilus* isolates obtained from different blocks within the affected area. Black: the area affected by the PWN; dark grey: the buffer area, established for safety reasons (free of PWN).

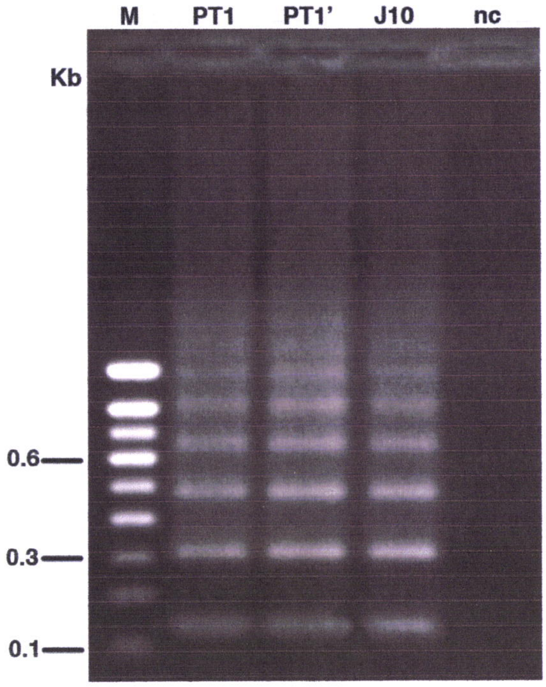

Figure 2: PCR amplification using the single nematode protocol, using satellite DNA primers set specific for *MspI*, of *Bursaphelenchus xylophilus*. M: DNA size marker (100 bp ladder); PT1 and PT1': *B. xylophilus* (isolate PT1 from Portugal); J10: *B. xylophilus* (isolate J10 from Japan); nc: negative control.

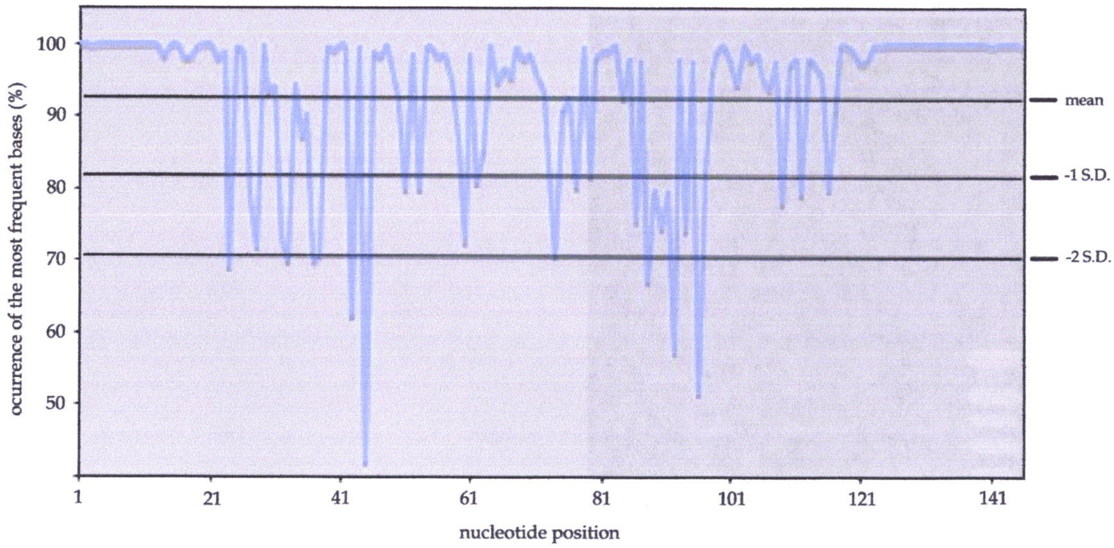

Figure 3: Nucleotide variation across the *Msp*I satellite repeats. Percentage of occurrence of the most frequent base for each nucleotide position plotted against nucleotide positions. Horizontal lines represent the average (mean line) with -1 and -2 standard deviation, respectively.

CHAPTER IV

GENERAL CONCLUSIONS

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The way of introduction of the PWN to non-endemic areas has been primarily attributed to several hypotheses related with human activities, especially by the movement of infected wood products, between long (among continents and countries) and short (within a country) levels of distance. However, the short distance level of the disease spreading is attributed to the biological development of the insect vector as well. The genetic diversity of an exotic species in a new established area is always dependent on the diversity of the initial colonizers. An understanding of the role played in the Portuguese situation has been hindered by the lack of detailed studies from the isolates distributed in this region. In this thesis the performed studies were aimed at understanding the degree of genetic diversity among several isolates of the affected area in Portugal and the possible pathways of the disease spread within the affected area.

GENETIC DIVERSITY OF PWN IN PORTUGAL

The native forms of an organism are the major source of genetic variation, regularly displaying a higher level of genetic diversity when compared with those populations found in non-native areas and due to its artificial establishment. The effect of human activities on spreading the PWN into new areas is well documented, and variation on the PWN, at different levels, can explain a substantial part of the within-isolate variation observed from different geographical areas. Genetic variation among the PWN isolates is certainly not new. According to previous studies, the isolates collected from the USA and Canada exhibit a high level of diversity, the greatest level of diversity being reached among isolates collected in some areas of Canada (Iwahori *et al.*, 1998). On the other hand, isolates found in the non-endemic areas express a low level of genetic diversity. Indeed, even in some of the non-native areas the genetic variation reaches some heterogeneity among some of the PWN isolates. Nevertheless, the degree of this variation could be limited by several hypotheses, i.e. the origin of the isolate (endemic area *vs.* non-endemic area), or by the number of introduced isolates. Furthermore, the number of individuals present in the infected wood products that reach the new site of infection could also limit the genetic variation of the initial introduction.

In Portugal, the extension of this genetic variation has not been clear. Recently, the origin of the PWN in Portugal was stated as being from an Asia region, and by a possible double introduction. If the introduction of this pathogen occured at lEast twice (even from non-native regions), different levels of genetic variability among the affected area in Portugal are to be expected, since a relative degree of variability in the Portuguese isolates was shown (Metge and Burgermeister, 2006). Still, this result might be due to a genetic shift of one of the isolates kept in fungal culture for a long period of time (Chapter II). The fact that the Portuguese *B. xylophilus* isolates used in these studies show a high genetic similarity, using RAPD-PCR and satellite DNA (Chapter II and Chapter III) clearly exclude the idea of a possible double introduction in Portugal. Furthermore, and based on the comparisons made in Paper I, the Portuguese isolates display a close genetic similarity with the East Asia isolate, confirming the results previously obtained by other authors (Metge and Burgermeister, 2006).

DISPERSAL OF THE PWN IN THE AFFECTED AREA

According to the data generated from other countries, the detection of the PWN is consistently coincident with port areas, associated with the trade of goods between countries. Initially the main concern came from those countries where the PWN was already naturally or artificially established. However, the report of several detections of PWN in wood products originating from PWN-free countries increased the unpredictable introduction of this pathogen into new areas. It has been shown (Chapter II and Chapter III) that the lack of genetic diversity among the PWN isolates in Portugal reflect a single introduction. Furthermore, the proximity of the international sea harbor in the Setúbal Península could determine the initial point of introduction, as considered in Paper I.

The evolution of a forest disease within a country is guided by a widely studied framework involving two main processes: 1) transport of contaminated wood by human activities and 2) biological development of the insect vector. In Portugal, the PWN distribution is limited to a relatively small area and no other detection has been reported outside this area. Initially, this question was addressed by the correlation between the results obtained by the RAPD-PCR and the linear distances of each isolate (Chapter II). However, this correlation was too weak to establish any geographic correlation and isolate distribution, and consequently to predict any pattern for the evolution of the disease. Secondly, the results obtained by satellite DNA analysis also reveal a high polytomic distribution of the sequence clones, i.e., the genetic variability found in each isolate is a reflection of the variation of the *Msp*I satellite DNA family in *B. xylophilus*, showing an intermingled relationship among sequence clones homogeneously distributed within all the affected area (Chapter III). In addition, the insect vector species occurs throughout the affected area. Such overlapping distribution of the insect vector may provide the main source of spreading of the pine wilt disease in Portugal (Chapter II).

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APPENDIX

A SYNOPSIS OF THE GENUS *BURSAPHELENCHUS* FUCHS, 1937 (APHELENCHIDA: PARASITAPHELENCHIDAE) WITH KEYS TO SPECIES

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A synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species

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Summary – The 75 valid species of the genus *Bursaphelenchus* are listed together with their synonyms. Diagnostic characters and their states are discussed and illustrated. Tabular and traditional text keys are provided for the genus. Two new subspecies are proposed to distinguish populations of *B. piniperdae* and *B. poligraphi*, as described by Rühm (1956), from the original descriptions of these species published by Fuchs (1937). Known records of *Bursaphelenchus* species with their associated natural vectors, plants and plant families are given. Dendrograms of species relationships (UPGMA, standard distance: mean character difference) based on combined taxonomic characters and also on spicule characters only, are provided. Discussion as to whether the species groups are natural or artificial (and therefore purely diagnostic) is based on their relationships in the dendrogram and the vector and associated plant ranges of the species. Of the six species groups distinguished, two appear to represent natural assemblages, these being the *xylophilus*-group (with ten species) and the *hunti*-group (seven species), of which two, *B. cocophilus* and *B. dongguanensis*, form the *cocophilus*-cluster which is separated on the dendrogram from the main clusters. The remaining four species groups appear to be artificial and purely diagnostic in function, namely the *aberrans*-group (four species); the *eidmanni*-group (six species); the *borealis*-group (five species), and the *piniperdae*-group (43 species). Two new subspecies, both in the *piniperdae*-group, *viz. B. piniperdae ruehmpiniperdae* n. subsp. and *B. poligraphi ruehmpoligraphi* n. subsp., are proposed and diagnosed from *B. piniperdae piniperdae* and *B. poligraphi poligraphi* the respective type subspecies. *Bursaphelenchus dongguanensis* is regarded as being a valid member of the genus and its transfer to *Parasitaphelenchus* is rejected.

Keywords – associated plants, dendrogram, key, morphology, new subspecies, taxonomy, vectors.

The genus *Bursaphelenchus* Fuchs, 1937 was established by Fuchs (1937) and includes nematodes that are associated with insects and dead or dying, mainly coniferous, trees and which have an ectophoretic stage. The type species is *B. piniperdae* Fuchs, 1937. Most species are fungal feeders and are either transmitted to dead or dying trees during oviposition by insect vectors, or to healthy trees during maturation feeding of their insect vectors. The majority of vectors are beetles, mostly from the Scolytidae, Cerambycidae, Curculionidae and Buprestidae (see Appendix). Until recently, only one species of the genus, *Bursaphelenchus cocophilus* (Cobb, 1919) Baujard, 1989, was recorded outside of the northern hemisphere. However, with the record of *B. leoni* Baujard, 1980 in South Africa (Braasch *et al.*, 1998), and more recently a *Bursaphelenchus* sp. from dying pine (*Pinus halepensis* Miller) in Australia (Ridley *et al.*, 2001), the known range of the genus has significantly increased. Of the total number of known species, approximately 70% are associated with conifers, mainly *Pinus* spp. (Vieira *et al.*, 2003; Braasch, 2004a).

In western Europe the species composition, distribution and associated plants of *Bursaphelenchus* have been studied especially thoroughly in Austria, Germany, Greece, Italy (Braasch *et al.*, 2000; Braasch, 2001, 2004a), Finland (Tomminen *et al.*, 1989), Cyprus (Braasch & Philis, 2002), Portugal (Penas *et al.*, 2004) and Spain (Abelleira *et al.*, 2003). In Eastern Europe, the longest species lists have been published for Georgia (Kurashvili *et al.*, 1980) and Russia (Korentchenko, 1980; Braasch, 2001).

In Asia, first in Japan (Mamiya & Kiyohara, 1972) and later in China (Cheng, 1983), Taiwan (Tzean & Jan, 1985)

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and Korea (Yi *et al.*, 1989), special attention was paid to this group after the detection of the pathogenicity of the pine wood nematode, *B. xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, in pine trees in Japan (Kiyohara & Tokushige, 1971). More recently, new species and reports have increased our knowledge of *Bursaphelenchus* species diversity within this broad area (Dan & Yu, 2003; Kanzaki & Futai, 2003; Tomiczek *et al.*, 2003; Braasch, 2004b; Palmisano *et al.*, 2004).

Bursaphelenchus xylophilus is considered to be indigenous to North America (Robbins, 1982; Rutherford *et al.*, 1990). On the American continent several other *Bursaphelenchus* species have been recorded, a number being described as new to science (Steiner, 1932; Massey, 1974; Thong & Webster, 1983; Giblin-Davis *et al.*, 1993). In the Caribbean and Latin American regions several species have also been found (Loof, 1964; Perez & Plumas, 1999), although the major focus has been on the red ring nematode, *B. cocophilus* (Cobb, 1919; Dean, 1979; Araújo *et al.*, 1998; Harrison & Jones, 2003).

According to Braasch (2001), the American continent has a species list that differs almost completely from those of Europe and Asia, the following species being common to all three regions: *B. xylophilus* (apparently introduced from America where it is the native species), *B. fraudulentus* Rühm, 1956 and *B. mucronatus* Mamiya & Enda, 1979. The *Bursaphelenchus* species of Europe and Asia may be divided into three assemblages; two groups being represented by species found in only one continent and the third with species widely distributed in both continents. Detailed data on species distribution, associated plants and vectors are given in Table 2 and Appendix.

Recent studies have suggested that some *Bursaphelenchus* species may, under particular circumstances, be pathogenic to young pines (Mamiya, 1999; Braasch *et al.*, 2000; Michalopoulos-Skarmoutsos *et al.*, 2004). However, within the genus, only *B. cocophilus* and *B. xylophilus* are officially recognised as agricultural and forestry pests of world importance.

Bursaphelenchus cocophilus, otherwise known as the red ring nematode, uses the palm weevil, *Rhynchophorus palmarum* L., as host and vector. The nematode is responsible for the devastating red ring disease of coconut palm (*Cocos nucifera* L.), oil palm (*Elaeis guineensis* Jacquin), and other palms (Dean, 1979; Griffith & Koshy, 1990). In Venezuela, over a period of more than 10 years, 35% of oil palms died from red ring disease and, in Tobago, more than 80% losses were reported in coconut plantations (Esser & Meredith, 1987; Brammer & Crow, 2001). This species, which is restricted to the American continent, is recorded from a huge area having a tropical climate, including Central and South America and many of the Caribbean islands. It is morphologically distinct from other species of *Bursaphelenchus* and was previously placed in its own genus – *Rhadinaphelenchus* J.B. Goodey, 1960. Taking into consideration the large area where coconut palms are grown, this species is regarded as one of the most important nematode pests in the tropics (Griffith & Koshy, 1990; Brammer & Crow, 2001).

Bursaphelenchus xylophilus, also known as the pine wood nematode (PWN) and the causal agent of pine wilt disease, is associated with cerambycid beetles, particularly *Monochamus* spp. It is a pest of many commercially important forestry trees, including pine, spruce, fir, larch and other conifers, thus playing an important role in world and national economies. In 2000, approximately 580 000 ha of pine forest in Japan were estimated to be infested by this species, an area corresponding to 28% of the total area of pine forest (Mamiya, 2004). The damage caused, and rapid spread in Japan and in other Asian countries (Mamiya, 1984, 2004; Yang, 2004), as well the recent detection of PWN in Portugal (Mota *et al.*, 1999) has increased concern that the disease may be disseminated to regions where it is currently absent. For this reason, a number of political measures have been taken, including an EU directive (77/93 updated as 2000/29/EC) aimed at preventing the introduction and spread of this pathogen in Europe by implementing special phytosanitary measures for solid wood packaging materials exported from countries where the nematode has been recorded.

Because of the commercial implications, accurate diagnosis of *B. xylophilus* is critical. Identification requires a high level of expertise as it is morphologically difficult to distinguish from other, similar species of *Bursaphelenchus* (Bolla & Wood, 2004; Braasch, 2004a). In this scenario, special attention is given to those species belonging to the pine wood nematode species complex (PWNSC), a complex of morphologically similar species, such as *B. xylophilus* and *B. mucronatus*, which may be capable of genetic exchange, either directly or *via* intermediate forms (Rutherford *et al.*, 1990). In addition, several other species of *Bursaphelenchus* are morphologically similar to *B. xylophilus* and share a combination of characters, including the distinctive angular shape of spicules, presence of four lateral lines and the large vulval flap in females (Braasch, 2001). Taxonomically these species may be considered as the *xylophilus*-group, a group that includes the following nematodes: *B. xylophilus*; *B. abruptus* Giblin-Davis, Mundo-Ocampo, Baldwin, Norden & Batra, 1993; *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000; *B. fraudulentus*; *B. kolymensis* Korentchenko, 1980; and *B. mucronatus* (see Braasch, 2001; Kanzaki & Futai, 2003).

With increasing globalisation and the breaking down of geographical boundaries, new biological invasions by non-indigenous species have become a global environmental problem. According to the Convention on Biological Diversity (CBD), accurate identification to diagnose dangerous invasive species at an early stage is the most important initial phase of programmes for monitoring and control of the environment. Precise data on the distribution of accurately identified world pests, including the PWNSC and *B. cocophilus*, is therefore necessary to counteract such potent threats.

Morphology remains the standard method for routine identification of nematode species. In the case of *Bursaphelenchus*, several characteristics have been used, including male spicule shape, presence or absence of a vulval flap and its size, female tail shape, *etc.* Light microscopical observations have been supplemented by the use of scanning electron microscopy (SEM) (Brzeski & Baujard, 1997; Braasch, 1998, 2000; Penas *et al.*, 2004). Other techniques, such as sex pheromone analysis, have also been used for species separation (Riga & Webster, 1992), although precise identification and diagnosis of the species belonging to the pine wood nematode species complex remains a difficult task.

Due to the limitations and constraints of morphological observations, molecular methods have recently become a valuable tool for separating *Bursaphelenchus* species (Tarès *et al.*, 1993; Hoyer *et al.*, 1998; Mota *et al.*, 1999; Liao *et al.*, 2001; Kanzaki & Futai, 2002b; Abad, 2004; Iwahori *et al.*, 2004). Of major interest is: *i*) the molecular characterisation of the nematode rDNA, and in particular the ITS regions (ITS-1 and ITS-2), which appear to be highly conserved within a species (Hoyer *et al.*, 1998; Liao *et al.*, 2001); *ii*) satellite DNA as a species-specific probe (Tarès *et al.*, 1993; Abad, 2004); and *iii*) homologous DNA probes (Tarès *et al.*, 1992). Intra-specific variability using RAPD-PCR techniques (Braasch *et al.*, 1995; Zhang *et al.*, 2002) and DNA base sequences (18S, 5.8S, ITS1 and ITS2 of rDNA, and mithochondrial cytochrome oxidase subunit I (COI) gene) (Beckenbach *et al.*, 1999; Kanzaki & Futai, 2002b; Iwahori *et al.*, 2004) has proved very useful for evaluating genetic distances and for assisting the development of phylogenies and pathway analysis of world populations of the pine wood nematode.

The objectives of this paper are: *i*) to compile a list of valid species and their synonyms; *ii*) to create a catalogue of the best morphological characters previously used by taxonomists of the genus; *iii*) to use these data to construct text and tabular keys to the genus (the tabular key may be later used to develop a computer-aided identification system of the genus); *iv*) to perform a critical comparison of the original descriptions of the species; *v*) to review the published records of each species, in order to analyse possible links of nematode species with specific taxa of associated insect vectors and host plants; and *vi*) to construct a dendrogram of the phenetic similarities of the species based on the tabular key to the genus and then to attempt to verify the clusters so formed by linking with published records of their vector taxa and associated plants.

Material and methods

In this paper, data from the original descriptions of the species were used in addition to other taxonomical studies on the genus plus recent morphological investigations of various species. Material from the collections of the University of Évora (Évora), Institute of Parasitology RAS (Moscow) and the Zoological Institute RAS (St Petersburg), as well as the collection of Drs Ana Catarina Penas and Maria Antónia Bravo, National Agricultural Station (Oeiras, Portugal) were also used.

As male morphology is most relevant for species identification, two columns have been added to the tabular key to give an idea of how many specimens were assessed for the characters used (see Table 1). These columns are: N_lit = the number of males studied from literature sources (drawings, photographs, specific measurements and descriptions of every character listed in the table); and N_coll = the number of specimens studied from various collections.

The following species were studied from mounted material in various slide collections (Table 1): *B. borealis* Korentchenko, 1980, *B. eroshenkii* Kolossova, 1998, *B. glochis* Brzeski & Baujard, 1997, *B. hylobianum* (Korentchenko, 1980) Hunt, 1993, *B. kolymensis*, *B. mucronatus*, *B. pinophilus* Brzeski & Baujard, 1997, *B. tusciae* Ambrogioni & Palmisano, 1998 and *B. xylophilus*. *Aphelenchoides ritzemabosi* (Schwartz, 1911) Steiner & Buhrer, 1932 was used as an outgroup.

The taxonomic analysis and keys are mainly based on a detailed study of literature data supplemented by available collection material. In the catalogue of the diagnostic characters used in the tabular key, references to the main publications are cited when a character was proposed as being of species diagnostic value or was used in keys, differential diagnoses, or in the taxonomic descriptions. A uniform nomenclature of the character states for each character was necessary as different authors have either used various terms for the same character state, or one name to cover different character states (see section on the characters for the tabular key). Line drawings of the diagnostic characters and their states (Figs 2-23) are provided to illustrate accurately each of the character states used in the keys and thereby avoid any ambiguity stemming from subjective interpretation of the descriptive terms employed. The drawings were prepared from original material, slides in our collections, or adapted from published taxonomic descriptions.

A summarised range of the character variability in published descriptions of the species was accepted herein as the range of the character for this species (*e.g.*, a suite of alternative forms for qualitative characters and the minimum and maximum values for quantitative characters). If information on a particular character was absent in the published descriptions and could not be inferred from the illustrations, the species was regarded as indeterminate for this character and was marked by a '?' symbol in the tabular key.

A minimum level of difference between similar species of at least three characters was established for any species to be considered as valid. This criterion was used to appraise the taxonomic status of all currently described *Bursaphelenchus* species. All published species descriptions and illustrations were considered to be reliable unless proof to the contrary existed.

The number of valid species in this overview is greater than in previous reviews of the genus, an increase due partly to the criteria used and partly because of additional valid species revealed by a detailed study of the previously insufficiently known species proposed in the Chinese, Georgian, German and Russian literature.

Detailed study of character variability in a larger set of species may necessitate revision of the taxonomic status of the nominal taxa proposed herein. However, the purpose of this analysis is to attempt to evaluate the diagnostic data for all *Bursaphelenchus* species and to define groups of similar species in order to aid further taxonomic research using morphological and molecular methods.

In the species list that follows, references to the pertinent literature, including page numbers, taxonomic information, notes, *etc.*, are cited in square brackets and in a smaller point. This should facilitate referral to the original source.

Genus *Bursaphelenchus* Fuchs, 1937 [p. 366]
= *Aphelenchoides* (*Bursaphelenchus*) Fuchs, 1937 (Rühm, 1956)

[p. 218, type-species *Bursaphelenchus piniperdae* Fuchs, 1937]
= *Devibursaphelenchus* Kakulia, 1967
[pp. 441-442, type-species *Devibursaphelenchus typographi* Kakulia, 1967 = *Bursaphelenchus typographi*]
= *Huntaphelenchoides* Nickle, 1970
[p. 379, Figs 16, 46, 66, 87, type-species *Bursaphelenchus fungivorus* Franklin & Hooper, 1962]
= *Omemeea* Massey, 1971a
[p. 289, type-species *Omemeea maxbassiensis* Massey, 1971 = *Bursaphelenchus maxbassiensis*]
= *Teragramia* Massey, 1974
[p. 213, type-species *Teragramia willi* Massey, 1974 = *Bursaphelenchus willi*]
= *Ipsaphelenchus* Lieutier & Laumond, 1978
[p. 192, type-species *Ipsaphelenchus silvestris* Lieutier & Laumond, 1978 = *Bursaphelenchus silvestris*]
= *Rhadinaphelenchus* J.B. Goodey, 1960b
[pp. 99, 102, type-species *Aphelenchus cocophilus* Cobb, 1919 = *Bursaphelenchus cocophilus*]

DIAGNOSIS

Based on Nickle (1970), Yin *et al.* (1988), Hunt (1993) and Braasch (2001).

Adult

Parasitaphelenchidae. Mature female vermiform. Male tail strongly curved ventrally, tip with terminal bursa-like flap of cuticle, tail tip evenly tapering, not spicate. Body length 0.3-1.7 mm. Cuticle annuli fine, 1 μm wide or less. Oral disc absent, lips cup-like, lateral lips narrower than others. Stylet less than 30 μm long, slender with narrow lumen, basal knobs weak. Anus and rectum functional.

Male

Spicules separate, hook-like, sometimes linear, but never strongly curved. Spicule rostrum usually prominent and separated from condylus (Figs 1, 2A, D-F), but sometimes fused with condylus to form compact capitulum

(Fig. 2B). Two or more pairs of caudal papillae present, one adanal and one to four pairs postanal. Gubernaculum absent.

Female

Tail subconoid, evenly tapering; tip usually smooth, sometimes with simple mucro, but never spicate or with four tubercles; anterior vulval flap present or absent. Postuterine sac present, usually 3-6 vulval body diam. long; V = 64-92; c′ = 7 or less.

Dispersal juvenile (insect associate)

Ectophoretic, with single exception of *B. hylobianum*, the juveniles of which were found in the haemocoel of the curculionid host (Coleoptera: Curculionidae).

RELATIONSHIPS

The main diagnostic feature of the Parasitaphelenchidae is the presence of a bursa-like flap of cuticle surrounding the terminal region of the male tail. The family currently contains two valid genera: *Bursaphelenchus* Fuchs 1937; and *Parasitaphelenchus* Fuchs, 1930. *Bursaphelenchus* may be distinguished from *Parasitaphelenchus* in that the insect-associated juvenile (dispersal juvenile, J3/J4) is usually ectophoretic *vs* the endoparasitic fourth-stage juvenile being located in the insect haemocoel in *Parasitaphelenchus*; the spicules are separate in *Bursaphelenchus vs* usually partially fused in *Parasitaphelenchus*; and the male tail of *Bursaphelenchus* is strongly recurved *vs* more or less straight in *Parasitaphelenchus*.

Bursaphelenchus differs from the morphologically closest Aphelenchoididae genera (*Aphelenchoides* Fischer, 1894; *Laimaphelenchus* Fuchs, 1937; *Megadorus* J.B. Goodey, 1960; *Ruehmapahelenchus* J.B. Goodey, 1963; *Schistonchus* Cobb, 1927 (Fuchs, 1937); *Sheraphelenchus* Nickle, 1970; *Tylaphelenchus* Rühm, 1956; *Anomyctus* Allen, 1940) in the presence of a small bursa-like flap of cuticle on the tip of the male tail *vs* males lacking a bursa-like flap. *Bursaphelenchus* differs from the genera of the family Ektaphelenchidae (*Ektaphelenchus* Fuchs, 1937; *Cryptaphelenchus* Fuchs, 1937; *Cryptaphelenchoides* J.B. Goodey, 1960; *Ektaphelenchoides* Baujard, 1984) in having a functional anus and rectum in the female and in having a narrow stylet lumen *vs* females lacking a functional anus and rectum and stylet usually with a wide lumen.

TYPE SPECIES

Bursaphelenchus piniperdae piniperdae[1] Fuchs, 1937 (by original designation) [pp. 366-370, Figs 66-69] *nec Aphelenchoides* (*Bursaphelenchus*) *piniperdae apud* Rühm, 1956 [pp. 218, 229-230, Fig. 61]
= *Aphelenchoides piniperdae* (Fuchs, 1937) T. Goodey, 1951 [p. 166]

OTHER SPECIES

B. aberrans Fang, Zhuo & Zhao, 2002b [pp. 791-794, Fig. 1, Table 1]
B. abietinus Braasch & Schmutzenhofer, 2000 [pp. 2-5, Figs 1-3, Table 1]
B. abruptus Giblin-Davis, Mundo-Ocampo, Baldwin, Norden & Batra, 1993 [pp. 161-172, Figs 1-6]
B. baujardi Walia, Negi, Bajaj & Kalia, 2003 [pp. 3-5, Fig. 1]
B. bestiolus Massey, 1974 [p. 182, Fig. 121]
B. borealis Korentchenko, 1980 [pp. 1768-1772, Figs 1, 2]
B. chitwoodi Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *chitwoodi* Rühm, 1956 [pp. 219, 231, Fig. 62]
B. cocophilus (Cobb, 1919) Baujard, 1989 [p. 324]
= *Aphelenchus cocophilus* Cobb, 1919 [pp. 203-210]
= *Aphelenchus* (*Chitinoaphelenchus*) *cocophilus* (Cobb, 1919) Micoletzky, 1922 [pp. 586-587]
= *Aphelenchoides cocophilus* (Cobb, 1919) T. Goodey, 1933 [pp. 217-219. Figs 91, 92]
= *Chitinoaphelenchus cocophilus* (Cobb, 1919) Chitwood *in* Corbett, 1959 [pp. 83-86]
= *Rhadinaphelenchus cocophilus* (Cobb, 1919) J.B. Goodey, 1960b [pp. 98-101, Fig. 1]
B. conicaudatus Kanzaki, Tsuda & Futai, 2000 [pp. 165-168, Fig. 1, Table 1]
B. corneolus Massey, 1966 [p. 428, Fig. 10]
B. crenati Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *crenati* Rühm, 1956 [pp. 219, 227-228, Fig. 59]
B. cryphali (Fuchs, 1930) J.B. Goodey, 1960a [p. 116]
= *Parasitaphelenchus cryphali* Fuchs, 1930 [pp. 635-636, Figs 172, 173]
= *Aphelenchoides cryphali* (Fuchs, 1930) Fuchs, 1937 [p. 331]
= *Shistonchus cryphali* (Fuchs, 1930) Skrjabin, Shikhobalova, Sobolev, Paramonov & Sudarikov, 1954 [p. 310]
= *Aphelenchoides* (*Bursaphelenchus*) *cryphali* (Fuchs, 1930) Rühm, 1956 [pp. 220, 234-235, Fig. 65]

species inquirenda apud Tarjan & Baeza-Aragon, 1982 [p. 127]
B. digitulus Loof, 1964 [pp. 203, 235-237, Fig. 14]
B. dongguanensis Fang, Zhao & Zhuo, 2002a [pp. 109-111; Fig. 1][2]
= *Parasitaphelenchus dongguanensis* (Fang, Zhao & Zhuo, 2002) Kaisa, 2005 [pp. 3-5, Figs 1-9, Table 1]
B. eggersi Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *eggersi* Rühm, 1956 [pp. 219, 231-233, Fig. 63]
B. eidmanni Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *eidmanni* Rühm, 1956 [pp. 220, 238-239, Fig. 69]
B. elytrus Massey, 1971b [pp. 167-168, Fig. 5 (a-e)]
B. eremus Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *eremus* Rühm, 1956 [pp. 219, 225-226, Fig. 57]
B. eroshenkii Kolossova, 1998 [pp. 161-164, Figs 1, 2]
B. erosus Kurashvili, Kakulia & Devdariani, 1980 [pp. 88-89, Fig. 18]
B. eucarpus Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *eucarpus* Rühm, 1956 [pp. 219, 226-227, Fig. 58]
B. fraudulentus Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *fraudulentus* Rühm, 1956 [pp. 220, 240-241, Fig. 71]
B. fuchsi Kruglik & Eroshenko, 2004 [pp. 96-98, Fig. 1]
B. fungivorus Franklin & Hooper, 1962 [pp. 136-139, Figs 1, 2]
= *Huntaphelenchoides fungivorus* (Franklin & Hooper, 1962) Nickle, 1970 [p. 389]
B. georgicus Devdariani, Kakulia & Khavatashili, 1980 [pp. 457-458, Fig. 1]
nomen nudum apud Hunt, 1993 [p. 134]
B. glochis Brzeski & Baujard, 1997 [pp. 313-317, Figs 45-63, Tables 7, 8]
B. gonzalezi Loof, 1964 [pp. 204-205, 237-239, Fig. 15]
= *Huntaphelenchoides gonzalezi* (Loof, 1964) Nickle, 1970 [p. 389]
B. hellenicus Skarmoutsos, Braasch & Michalopoulou, 1998 [pp. 625-628, Figs 1, 2]
B. hofmanni Braasch, 1998 [pp. 616-620, Figs 1, 2]
B. hunanensis Yin, Fang & Tarjan, 1988 [pp. 3, 4, Figs 1-11, Tables 1, 2]
B. hunti (Steiner, 1935) Giblin & Kaya, 1983 [pp. 48-49][3]
= *Aphelenchoides hunti* Steiner, 1935 [p. 106, Fig. 27]
= *Huntaphelenchoides hunti* (Steiner, 1935) Nickle, 1970 [pp. 379, 381, 389-390, Figs 16, 46, 66, 87]
B. hylobianum (Korentchenko, 1980) Hunt, 1993 [p. 132][4]
= *Parasitaphelenchus hylobianum* Korentchenko, 1980 [pp. 1776-1779, Figs 5, 6, Tables 5, 6]
B. idius Rühm, 1956. (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *idius* Rühm, 1956 [pp. 220, 236-237, Fig. 67]
B. incurvus Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *incurvus* Rühm, 1956 [pp. 220, 228-229, Fig. 60]
B. kevini Giblin, Swan & Kaya, 1984 [pp. 178-182, Figs 1-5, Table 1]
B. kolymensis Korentchenko, 1980 [pp. 1772-1776, Figs 3, 4, Tables 3, 4] (Magnusson & Kulinich, 1996) [pp. 156-159, Figs 1, 2 (redescription of type material with emended diagnosis)]
B. leoni Baujard, 1980 [pp. 170-172, Fig. 2]
B. lini Braasch, 2004b [pp. 3-7, Figs 1, 2, Table 1]
B. luxuriosae Kanzaki & Futai, 2003 [pp. 565-569, Figs 1, 2, Tables 1-3]
B. maxbassiensis (Massey, 1971) Baujard, 1989 [p. 323]
= *Omemeea maxbassiensis* Massey, 1971a [pp. 289-291, Fig. 1]
B. minutus Walia, Negi, Bajaj & Kalia, 2003 [pp. 1-3, Fig. 1]
B. mucronatus Mamiya & Enda, 1979 [pp. 354-356, Fig. 1]
B. naujaci Baujard, 1980 [pp. 168-170, Fig. 1]
= *B. bakeri apud* Tarjan & Baeza-Aragon, 1982 [pp. 127, 130] *nec* Rühm, 1964 (= junior synonym of *B. sexdentati* Rühm, 1960)
B. newmexicanus Massey, 1974 [pp. 186,188, Fig. 124]
B. nuesslini Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *nuesslini* Rühm, 1956 [pp. 219, 237-238, Fig. 68]
B. paracorneolus Braasch, 2000 [pp. 177-181, Figs 1-3, Table 1]
B. pinasteri Baujard, 1980 [pp. 172-175, Fig. 3]
= *B. chitwoodi apud* Tarjan & Baeza-Aragon, 1982 [p. 131] (Hunt, 1993, p. 132) *nec B. chitwoodi* Rühm, 1956
Bursaphelenchus piniperdae ruehmpiniperdae n. subsp.[1]
= *Aphelenchoides* (*Bursaphelenchus*) *piniperdae* (Fuchs, 1937) Rühm, 1956 [pp. 218, 229-230, Fig. 61] *nec Bursaphelenchus piniperdae* Fuchs, 1937
B. pinophilus Brzeski & Baujard, 1997 [p. 310, Figs 20-44, Tables 5, 6]
B. pityogeni Massey, 1974 [pp. 186, 190, Fig. 125]
B. poligraphi poligraphi[5] Fuchs, 1937 [pp. 370-372, Figs 70-73] (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides poligraphi* (Fuchs, 1937) T. Goodey, 1951 [p. 166]

B. poligraphi ruehmpoligraphi n. subsp.[5]
= *Aphelenchoides* (*Bursaphelenchus*) *poligraphi apud* Rühm, 1956 [pp. 219, 233-234, Fig. 64] nec *B. poligraphi* Fuchs, 1937
B. rainulfi Braasch & Burgermeister, 2002 [pp. 973-976, Figs 1, 2, Tables 1, 2]
B. ratzeburgii Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *ratzeburgii* Rühm, 1956 [pp. 218, 224-225, Fig. 56]
B. sachsi Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *sachsi* Rühm, 1956 [pp. 220, 235-236, Fig. 66]
B. scolyti Massey, 1974 [pp. 190-191, Fig. 126]
B. seani Giblin & Kaya, 1983 [pp. 40-41, Figs 1-4]
B. sexdentati Rühm, 1960 (Hunt, 1993) [p. 133]
= *Aphelenchoides* (*Bursaphelenchus*) *sexdentati* Rühm, 1960 [pp. 205-207, Fig. 2]
= *B. bakeri* Rühm, 1964 [p. 220]; Tarjan & Baeza-Aragon, 1982 [pp. 127, 130, 137]
B. silvestris (Lieutier & Laumond, 1978) Baujard, 1980 [p. 175]
= *Ipsaphelenchus silvestris* Lieutier & Laumond, 1978 [pp. 192-194, Fig. 3]
B. sinensis Palmisano, Ambrogioni, Tomiszek & Brandstetter, 2004 [pp. 57-62, Figs 1-3, Table 1]
B. steineri Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Aphelenchoides*) *steineri* Rühm, 1956 [pp. 212-214, Fig. 52]
B. sutoricus Devdariani, 1974 [pp. 710-711, Fig. 2 (erroneously named *Bursaphelenchus welchi* on p. 711)]
= *B. xerokarterus apud* Tarjan & Baeza-Aragon, 1982 [p. 131] *nec B. xerokarterus* Rühm, 1956
B. sychnus Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *sychnus* Rühm, 1956 [pp. 220, 239-240, Fig. 70]
B. talonus (Thorne, 1935) J.B. Goodey, 1960a [p. 117]
= *Aphelenchoides talonus* Thorne, 1935 [pp. 132, 137-138, Fig. 5 (e-g)]
= *Aphelenchoides* (*Bursaphelenchus*) *talonus* (Thorne, 1935) Rühm, 1956 [p. 241]
B. teratospicularis Kakulia & Devdariani, 1965 [pp. 187-191, Fig. 1]
B. thailandae Braasch & Braasch-Bidasak, 2002 [pp. 854-859, Figs 2, 3, Tables 1, 2]
B. tritrunculus Massey, 1974 [pp. 190, 193, 194, Fig. 128]
B. tusciae Ambrogioni & Palmisano, 1998 [pp. 242-248, Figs 1-7, Table 1]
B. typographi (Kakulia, 1967) Ebsary, 1991 [p. 91]
= *Devibursaphelenchus typographi* Kakulia, 1967 [pp. 439-442, Figs 1, 2]
B. vallesianus Braasch, Shönfeld, Polomski & Burgermeister, 2004 [pp. 72-78, Figs 1-4, Tables 1-3]
B. varicauda Thong & Webster, 1983 [pp. 312-313, Figs 1, 2]
B. wekuae Kurashvili, Kakulia & Devdariani, 1980 [pp. 86-87, Fig. 17]
B. wilfordi Massey, 1964 [pp. 151-153, Fig. 8 (c-f)]
B. willi (Massey, 1974) Baujard, 1989 [p. 323]
= *Teragramia willi* Massey, 1974 [pp. 213, 215-216, Fig. 144]
B. xerokarterus Rühm, 1956 (J.B. Goodey, 1960a) [p. 116]
= *Aphelenchoides* (*Bursaphelenchus*) *xerokarterus* Rühm, 1956 [pp. 219, 222-224, Fig. 55]
B. xylophilus (Steiner & Buhrer, 1934) Nickle, 1970 [p. 390] [Nickle *et al.*, 1981, pp. 391-392, Figs 1-18 (redescription, designation of lectotype; successful mating experiments between *B. lignicolus* and *B. xylophilus*)]
= *Aphelenchoides xylophilus* Steiner & Buhrer, 1934 [pp. 950-951 Fig. 1]
= *Paraphelenchoides xylophilus* (Steiner & Buhrer, 1934) Haque, 1967 [pp. 1251-1253]
= *Bursaphelenchus lignicolus* Mamiya & Kiyohara, 1972 [p. 121, Fig. 1]

SPECIES INQUIRENDAE VEL INCERTAE SEDIS

Bursaphelenchus conurus (Steiner, 1932) J.B. Goodey, 1960a [p. 117, but see also Rühm, 1956, p. 241]
= *Aphelenchoides conurus* Steiner, 1932 [pp. 442-443, Fig. 4]
species incertae sedis apud Tarjan & Baeza-Aragon, 1982 [p. 127]
species inquirenda apud Hunt, 1993 [p. 133]
Bursaphelenchus ruehmi Baker, 1962 [p. 200][6]
= *Aphelenchoides* (*Bursaphelenchus*) *conjunctus apud* Rühm, 1956 [pp. 220, 241] *nec Aphelenchoides conjunctus* Fuchs, 1930
= *Bursaphelenchus conjunctus* (Fuchs, 1930) Andrássy, 1958 [p. 185]
= *Bursaphelenchus conjunctus apud* J.B. Goodey, 1960a [p. 116] *nec Aphelenchoides conjunctus* Fuchs, 1930
= *Bursaphelenchus ruehmi* J.B. Goodey, 1963 [p. 146] (= junior objective homonym)
species indeterminata apud Tarjan & Baeza-Aragon, 1982 [p. 131]
species inquirenda apud Hunt, 1993 [p. 133]

DEPARTURES TO OTHER GENERA

Laimaphelenchus lignophilus (Körner, 1954) Goodey, 1960a [p. 116]
= *Aphelenchoides lignophilus* Körner, 1954 [pp. 344-345, Fig. 59]
= *Bursaphelenchus lignophilus* (Körner, 1954) Meyl, 1961 [p. 83]
Aphelenchoides conjunctus (Fuchs, 1930) Filipjev, 1934 [p. 215][6] *nec Aphelenchoides* (*Bursaphelenchus*) *conjunctus apud* Rühm, 1956 and *B. conjunctus apud* J.B. Goodey, 1960a (= *Bursaphelenchus ruehmi* Baker, 1962)
= *Parasitaphelenchus conjunctus* Fuchs, 1930 [pp. 629-630, Figs 162-165]
= *Aphelenchoides* (*Schistonchus*) *conjunctus* (Fuchs, 1930) Filipjev, 1934 [p. 215]
= *Shistonchus conjunctus* (Fuchs, 1930) Skrjabin, Shikhobalova, Sobolev, Paramonov & Sudarikov, 1954 [p. 310]
species incertae sedis apud Tarjan & Baeza-Aragon, 1982 [pp. 125-126, no bursa]

NOMINA NUDA

Bursaphelenchus populneus Kakulia, Devdariani & Maglakelidze, 1980 [p. 109]
nomen nudum apud Hunt, 1993 [p. 134]
Bursaphelenchus tbilisensis Kakulia, Devdariani & Maglakelidze, 1980 [pp. 109-110]
nomen nudum apud Hunt, 1993 [p. 134]

ANNOTATIONS TO THE SPECIES LIST

[1] *Bursaphelenchus piniperdae.* Description and illustrations of this, the type species, in Rühm (1956) appear to represent a different taxon to that described in the original paper by Fuchs (1937) (see Table 1). Taxonomists have not recorded this species since 1980 (last record: Caucasus, Kurashvili *et al.*, 1980). For more precise determination the species is included in Table 1, in the text key to *Bursaphelenchus* and in the trees of phenetic similarities (Figs 24, 25) as separate subspecies, namely *B. piniperdae piniperdae* Fuchs, 1937 and *B. piniperdae ruehmpiniperdae* n. subsp. (= *B. piniperdae apud* Rühm, 1956 *nec B. piniperdae piniperdae* Fuchs, 1937). *B. piniperdae ruehmpiniperdae* n. subsp. differs from *B. piniperdae piniperdae* in having the stylet 18-19 μm long *vs* 11-12 μm in *B. p. piniperdae*; spicule length, measured along arc, of 14-19 *vs* 12-14 μm in *B. p. piniperdae*; ratio spicule length/capitulum width of 2.5 *vs* 1.5 in *B. p. piniperdae*; ratio depth of capitulum depression/capitulum width = 0.4 *vs* 0.2 in *B. p. piniperdae*; spicule tip finely rounded *vs* bluntly rounded in *B. p. piniperdae*; and tail of dispersal juvenile pointed *vs* narrowly rounded in *B. p. piniperdae*.

It is important that the type species proposed by Fuchs (1937) is redescribed to modern standards so that taxonomic relationships can be unequivocally established.

[2] *Bursaphelenchus dongguanensis.* Kaisa (2005) transferred *B. dongguanensis* to the genus *Parasitaphelenchus*, thereby proposing the combination *P. dongguanensis* (Fang, Zhao & Zhuo, 2002) Kaisa, 2005. The new combination was based on an analysis of the published description of the species as no collection specimens were available for study. Kaisa studied collection material and published descriptions of nine out of 14 valid *Parasitaphelenchus* species and argued the case for transferring the species to *Parasitaphelenchus* on the basis of the a, c and V indexes of *B. dongguanensis* and the fact that the male tail was not strongly recurved. The presence of endoparasitic juveniles in *B. dongguanensis* was not established as the species was described only from the dead wood of wilted *Pinus massoniana*. The male tail recurvature in *B. dongguanensis* is very weak, although a similar tail curvature was illustrated for the type species *Bursaphelenchus piniperdae* by Fuchs (1937) and Rühm (1956), and also occurs in several other *Bursaphelenchus* species. The actual form of the male body was not illustrated when the species was proposed by Fang *et al.* (2002), the body of both male and female being depicted in an artificial U-shaped form (as in some of the older nematological publications), rather than as the heat relaxed habitus. In addition, *B. dongguanensis* was fixed in TAF, a process which in our experience makes nematodes too soft to draw conclusions about the real body shape. The spicules of *B. dongguanensis* are not fused. As all other quantitative characters overlap between *Parasitaphelenchus* and *Bursaphelenchus*, these cannot be considered as arguments to support the transference of *B. dongguanensis* to the genus *Parasitaphelenchus*. Additional support for this decision may be derived by comparing *B. dongguanensis* with the type species of both genera, namely *Bursaphelenchus piniperdae* Fuchs, 1937 and *Parasitaphelenchus uncinatus* (Fuchs, 1929) Fuchs, 1930. Males of *P. uncinatus* have only one pair of postcloacal papillae located near the bursal flap, whereas *B. dongguanensis* males have two such pairs. Males of *B. piniperdae* have one pair of large postcloacal papillae and three pairs of small glandpapillae (illustrated in Fuchs, 1937 and Rühm, 1956). *Bursaphe-*

lenchus xylophilus, a widely distributed species often considered a 'typical' species for the genus, has two pairs of male postcloacal papillae located near the bursal flap, the same situation as in *B. dongguanensis*. We therefore do not accept the combination *Parasitaphelenchus dongguanensis* (Fang, Zhao & Zhuo, 2002) Kaisa, 2005 as valid and the species is returned to the genus *Bursaphelenchus*.

[3] The original description of *Bursaphelenchus hunti* (= *Aphelenchoides hunti*) by Steiner (1935) and the illustration in this paper (Fig. 27) were based only on nematodes from bulbs of *Lilium tigrinum* (Liliaceae) intercepted from Japan, not from fruits of tomatillo, *Physalis ixocarpa* (Solanaceae) intercepted from Mexico (see Nickle, 1970, p. 390).

[4] *Bursaphelenchus hylobianum* juveniles reportedly inhabit the insect haemocoel and this species is apparently the only endoparasite within the genus. Korentchenko (1980) described this species as belonging to the genus *Parasitaphelenchus*, but Hunt (1993, p. 134) argued that the male tail morphology, spicule structure and disposition of the nine caudal papillae are characters of *Bursaphelenchus*, and transferred the species accordingly.

[5] *Bursaphelenchus poligraphi*. The description and illustrations of this species by Rühm (1956) are slightly different from those in the original paper by Fuchs (1937) (Table 1). This species has not been recently redescribed, although DNA profiles attributed to this species have been published (Braasch *et al.*, 1999, 2004). To facilitate more exact identification, this species is included in Table 1, in the text of key to *Bursaphelenchus* and in the trees of phenetic similarities (Figs 24, 25) as the subspecies: *B. poligraphi poligraphi* Fuchs, 1937 and *B. poligraphi ruehmpoligraphi* n. subsp. (= *B. poligraphi apud* Rühm, 1956 *nec B. poligraphi poligraphi* Fuchs, 1937). *B. poligraphi ruehmpoligraphi* n. subsp. differs from *B. poligraphi poligraphi* in having the spicule rostrum thorn-like *vs* conical in *B. p. poligraphi*; bursal flap conical *vs* oval to rounded in *B. p. poligraphi*; male tail terminus pointed *vs* rounded in *B. p. poligraphi*; spicule slender with the ratio of male spicule length (measured along the arc) to its width (measured posterior to rostrum in lateral view) being 5 or more, *vs* spicule stout and corresponding ratio <4 in *B. p. poligraphi*; ratio of spicule length to capitulum width = 2.5 or more *vs* 2.0 or less in *B. p. poligraphi*; spicule length along arc > 15-18 μm *vs* 11-13 μm in *B. p. poligraphi*; and stylet 12-14 μm long *vs* 10 μm in *B. p. poligraphi*.

[6] *Aphelenchoides conjunctus*. As described by Fuchs (1930), this species has all the features of aphelenchoidid nematodes (pharynx form, male spicule shape, female tail, male tail mucronate and lacking a bursa, two pairs of male postanal papillae, stylet = 8 μm, spicule length along arc = 14-18 μm). It may be considered as *species inquirenda* within *Aphelenchoides*, but not *Bursaphelenchus*, because of the absence of a terminal bursa and the spicule shape.

Baker (1962, p. 200) showed that that the species attributed to *B. conjunctus* by Rühm was different from the original description of Fuchs (1930). Rühm's species has a bursal flap in the male and therefore belongs to the genus *Bursaphelenchus*. Rühm's material was renamed by Baker (1962) as *B. ruehmi*. Baker also pointed out that *B. conjunctus apud* Rühm (= *B. ruehmi*) had also been mentioned by J.B. Goodey (1960). The same species was referred to as *B. conjunctus* by Andrássy (1958, p. 185). In this review, *B. conjunctus* Fuchs, 1930 is considered to be a *species inquirenda* within the genus *Aphelenchoides* whereas *B. conjunctus apud* Rühm, 1956 (= *B. ruehmi*) *nec B. conjunctus* Fuchs, 1930 is considered herein as *species inquirenda* within *Bursaphelenchus*.

SOME REMARKS ON THE GENUS

i) The generic differences between *Bursaphelenchus* and *Parasitaphelenchus* were discussed in detail by Hunt (1993) and emended by Kaisa (2005).

ii) In this account, following the argument in Thong and Webster (1991) and Mamiya (1984), the term 'dispersal juvenile' is used instead of 'dauerlarva'. The insect associated dispersal juvenile is a juvenile stage specialised for a phoretic transmission by an insect vector to a new habitat. In *Parasitaphelenchus*, the parasitic (fourth-stage) juvenile is found as an endoparasite in the insect haemocoel, whereas in *Bursaphelenchus* the dispersal juvenile (J3/J4) is ectophoretic, although exceptionally, as in *B. hylobianum*, it appears to be endoparasitic.

iii) Vulva position: V = 82 and more in *Parasitaphelenchus*: (Hunt mentioned 85% or more, but Kaisa stressed that *P. acroposthion*, according to Steiner (1932), has 82% as the minimum value); whereas in *Bursaphelenchus*, V = 80 or less. However, at least four species of *Bursaphelenchus* (*B. typographi*, *B. digitulus*, *B. erosus* and *B. dongguanensis*) have V = 85 and more.

iv) Male spicules: Spicules are partially fused in *Parasitaphelenchus*, although Kaisa (2005) reported that the spicules were not fused on slide material of *P. gallagheri* and *P. procercus*, or in Figures 38 and 40 of the original description of *P. papillatus* Fuchs, 1937. In *Bursaphelenchus* the spicules are usually separate, but were reported to be partially fused in some species (Hunt, 1993).

v) Male tail curvature: The male tail is not strongly recurved in *Parasitaphelenchus*, but is so shaped in *Bursaphelenchus*. In the type-species *Bursaphelenchus piniperdae*, as well as in *B. poligraphi*, *B. digitulus* and several other species, the male tail is not strongly recurved.

vi) Kaisa (2005) also considered the following characters as distinguishing the genera: a-index $\geqslant 29$ in *Parasitaphelenchus*, but <29 in *Bursaphelenchus* (however, more than 70 *Bursaphelenchus* spp. have an a-index > 29 and 31 *Bursaphelenchus* species have a > 40); c-index $\geqslant 40$ in *Parasitaphelenchus*, but <40 in *Bursaphelenchus* (but *B. eidmanni*, *B. poligraphi*, *B. dongguanensis*, *B. erosus* and *B. typographi* have a female c-index > 40).

vii) Of the listed characters, the most important one is biological, endoparasitic juveniles being the diagnostic feature of *Parasitaphelenchus*. Significant overlaps between the two genera may be found in the other listed characters, the most reliable of these being the recurved tail of *Bursaphelenchus vs* more or less straight in *Parasitaphelenchus*, and the usually separate spicules in *Bursaphelenchus vs* usually partially fused in *Parasitaphelenchus*.

viii) According to Mayr (1969) the genus taxon is a monophyletic group of species separated from other genera by a distinct gap (in morphological and other characters) and occupying a distinctly separate niche. *Parasitaphelenchus* is distinctly different from *Bursaphelenchus* in the endoparasitic habit of the fourth-stage juvenile *vs* the ectophoretic dispersal juvenile (J3/J4) of *Bursaphelenchus*. Thus, *Parasitaphelenchus* is more specialised to insect parasitism and may have evolved from the genus *Bursaphelenchus*, the insect vector in the *Bursaphelenchus* cycle becoming the host of the parasitic juveniles of *Parasitaphelenchus*. As a result of this specialisation, a sclerotised mouth hook developed in the infective third-stage juveniles of *Parasitaphelenchus* to facilitate invasion of the bark beetle grubs (Hunt, 1993). This structure, as well as the endoparasitic habit of the juveniles, may be considered as synapomorphies of *Parasitaphelenchus*.

ix) Among the generic synonyms of *Bursaphelenchus*, the genus *Rhadinaphelenchus* J.B. Goodey, 1960, which was synonymised with *Bursaphelenchus* by Baujard (1989), is of most interest. The only species of the genus, *Rhadinaphelenchus cocophilus* (Cobb, 1919) J.B. Goodey, 1960 is now considered to belong to *Bursaphelenchus* (Baujard, 1989; Giblin-Davis *et al.*, 1989, 2003; Giblin-Davis, 1993; Fang *et al.*, 2002a; see also discussion in Hunt, 1993). The most similar species to *B. cocophilus* is *B. dongguanensis* which has a similar spicule structure and an a-index >80. *Bursaphelenchus cocophilus* may be placed in the *hunti*-species group on the basis of spicule structure (lamina wide, dorsal and ventral limb well separate, see Figure 2A). Vectors of the group do not include members of the Scolytidae, but are restricted to beetles of the family Curculionidae and various Hymenoptera (Halictidae and Anthophoridae).

BIONOMICS

The phoretic juveniles are associated with insects. Vectors are mainly Coleoptera, particularly the Scolytidae, but also the Buprestidae, Cerambycidae and Curculionidae. Some species are associated with the insect orders Hymenoptera (Halictidae) or Lepidoptera (Sesiidae).

Associated plants are mainly trees, particularly Pinaceae, but also include trees from other families, including Araliaceae, Areaceae, Betulaceae, Cupressaceae, Fagaceae, Juglandaceae, Moraceae, Oleaceae, Rosaceae, Rubiaceae, Salicaceae, and Ulmaceae, as well as herbaceous plants belonging to Alliaceae and Solanaceae.

Species groups

Different criteria may be used to divide the large number of nominal species of the genus *Bursaphelenchus* into smaller, more convenient, 'species groups'. Tarjan and Baeza-Aragon (1982) proposed terminology for the spicule structure (Fig. 1) in *Bursaphelenchus* and gave a detailed classification of spicule characters and their states. Giblin and Kaya (1983) used this terminology to construct a species grouping which was based mainly on the shape of the spicules, complicated copulatory structures described and illustrated for all species of the genus. The classification of Braasch (2001, 2004a), on the other hand, is based on the number of incisures in the lateral field, number and arrangement of the male caudal papillae, presence of a vulval flap in the female, and shape of the female tail. Unfortunately, these characters are available for only some of the nominal species, thereby limiting the utility of this scheme.

In this paper only the spicule structure is used to separate the species into groups. These species groups are intended purely as identification units in order to facilitate species identification. However, some of these groups may be natural (*i.e.*, phylogenetically based). The different parts of the spicule are illustrated in Figure 1. The most important spicule characters are the shape of the rostrum (a derivation of the ventral limb of the ancestral aphelenchoid spicule) and the shape of the condylus (derived from the dorsal spicule limb).

In the following dichotomous key, which is based on spicule structure, the six species groups are keyed out first and are then followed by keys for each species group. For each species group a brief diagnosis and list of species introduce the corresponding key, the species donating the group name being listed first (*i.e.*, *B. hunti* is listed first in the species list of the *hunti*-group).

When constructing the text keys, two approaches for the identification process were employed. The first approach was to separate one species from the current set of species by a 'unique character'. The second approach was to split the current set of species into several non-overlapping subsets of species using an appropriate 'group character', the condition being that each species of the current set has only one of several alternative states of such a character. Unique characters are very rare in a large genus such as *Bursaphelenchus*, an example being the head region structure in *B. maxbassiensis* where the first head annulus is distinctly larger in diameter than the other annuli and strongly offset. Among the group characters, the type of spicule structure is the best, sorting the genus into six, non-overlapping, species groups. However, within the *piniperdae*-group, the most speciose of all the groups, it is difficult to select diagnostic characters because of the large variability and overlapping of characters amongst the many nominal species. In an attempt to overcome this difficulty, species of the *piniperdae*-group, therefore, appear more than once in the text key.

Key to the species groups

1. Dorsal and ventral limbs of male spicule not joined at spicule tip; spicule tip broad and blunt (Fig. 2A) .... *hunti*-group
– Dorsal and ventral limbs of male spicule joined at spicule tip; spicule tip narrow and conoid 2

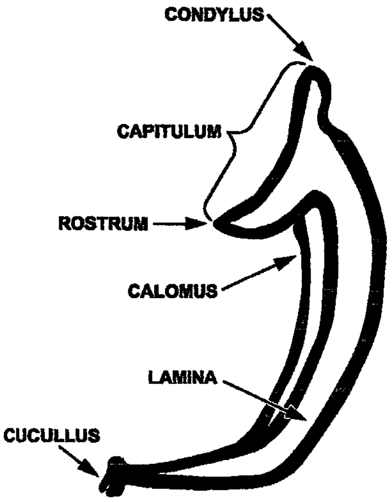

Fig. 1. *Male spicule (lateral view) showing constituent parts.*

2. Capitulum compact, rostrum and condylus fused (Fig. 2B) .... *aberrans*-group
– Capitulum elongate, rostrum and condylus well developed and separate 3
3. Spicule linear, with small rostrum located halfway along its length (Fig. 2C) .... *eidmanni*-group
– Spicule hook-like, with prominent rostrum located more anteriorly 4
4. Condylus recurved posteriorly (Fig. 2D) .... *borealis*-group
– Condylus straight or indistinct (Fig. 2E, F) 5
5. Capitulum flattened anteriorly, condylus small, dorsal contour of lamina distinctly angular in last third; cucullus usually present (Fig. 2E)................. .... *xylophilus*-group
– Capitulum concave anteriorly; condylus elongate, dorsal contour of lamina smoothly curved or angular at midpoint, cucullus usually absent, but small cucullus sometimes present (Fig. 2F) .... *piniperdae*-group

Keys to the species of *Bursaphelenchus* Fuchs, 1937

These keys are based mainly on descriptions in the literature and on collection material, as listed in Table 1 (columns N_lit and N_col).

THE *HUNTI*-GROUP

Dorsal and ventral limbs of spicule not joined at tip, which is broad and blunt.

Species: *B. hunti, B. cocophilus, B. dongguanensis, B. fungivorus, B. gonzalezi, B. kevini* and *B. seani.*

1. Index a > 80 2
– Index a < 65 3
2. Index c′ = 5.6 or more, weak fifth lateral line present centrally, male bursa oval to rounded in ventral view (Fig. 21B), spicule rostrum conical to rounded (Fig. 6B, C) .... *B. cocophilus*
– Index c′ = 2.2 or less, four lateral lines, male bursa truncate in ventral view (Fig. 21C), spicule rostrum thorn-like (Fig. 6A) .... *B. dongguanensis*
3. Female tail terminus mucronate (Fig. 3A) .... *B. kevini*
– Female tail terminus pointed (Fig. 3B) 4
– Female tail terminus rounded (Fig. 3C) 5
4. Junction between spicule rostrum and lamina of spicule angular (Fig. 20A), ratio female genital postuterine branch length to vulval body diam. = 2 or less .... *B. seani*
– Junction between rostrum and lamina of spicule smoothly curved (Fig. 20B), ratio female genital postuterine branch length to vulval body diam. = 2.9 or more .... *B. gonzalezi*
5. Lateral field with three incisures (Fig. 13B) .... *B. hunti*
– Lateral field with four incisures (Fig. 13C) .... *B. fungivorus*

THE *ABERRANS*-GROUP

Male spicule capitulum compact, rostrum and condylus fused.

Species: *B. aberrans, B. idius, B. elytrus, B. sinensis.*

1. Female tail tip strongly recurved (Fig. 10A) .... *B. aberrans*
– Female tail tip not strongly recurved (tail tip straight or slightly curved ventrally (Fig. 10B) 2
2. Female index c′ = 2.7 or less, six incisures in lateral field (Fig. 13E) .... *B. idius*
– Female index c′ = 3.2 or more, four or fewer incisures in lateral field (Fig. 13A-C) 3
3. Vulval flap absent (Fig. 7B), male bursa conical in ventral view (Fig. 21A), spicule length measured along arc = 24 μm or more, found in America .... *B. elytrus*
– Vulval flap present (Fig. 7A), male bursa rounded in ventral view (Fig. 21B), spicule length measured along arc = 22 μm or less, found in Asia and Europe .... *B. sinensis*

THE *EIDMANNI*-GROUP

Spicule straight, linear, small conical rostrum located midway along spicule.

Species: *B. eidmanni, B. digitulus, B. erosus, B. steineri, B. teratospicularis, B. typographi.*

1. Female tail tip with distinct mucro (Fig. 3A) 2
– Female tail tip without mucro, digitate to rounded (Fig. 3C, D) 4
2. Female postuterine branch length/vulva-anus distance < 0.3 (Fig. 23A, B) .... *B. erosus*
– Female postuterine branch length/vulva-anus distance > 0.3 (Fig. 23C, D) 3
3. Female index V = 74 or less, male bursa minute and conical in ventral view (Fig. 21A); ratio of spicule length along arc to its width (excluding rostrum) = 10 or more (Fig. 16D) .... *B. steineri*
– Female index V = 84 or more, male bursa rounded in ventral view (Fig. 21B), sometimes with slightly m-shaped posterior line; ratio of spicule length along arc to its width (excluding rostrum) = 6 or less (Fig. 16A, B) .... *B. digitulus*
4. Female postuterine branch = 4 or more vulval body diam. long (Fig. 5C, D) .... *B. eidmanni*
– Female postuterine branch = 1.5 or less vulval body diam. long (Fig. 5A) 5
5. Index V = 80 or less, spicule length along arc = 15 μm or more .... *B. teratospicularis*
– Index V = 85 or more, spicule length along arc = 12 μm or less .... *B. typographi*

THE *BOREALIS*-GROUP

Spicule condylus recurved posteriorly.

Species: *B. borealis, B. cryphali, B. leoni, B. silvestris, B. tusciae.*

1. Vulval flap absent (Fig. 7B) .... *B. cryphali*
– Vulval flap present (Fig. 7A) 2

2. Male bursa oval or rounded in ventral view (Fig. 21B) .... *B. silvestris*
– Male bursa truncate in ventral view (Fig. 21C)..... 3
3. Female index c′ = 5 or more .... *B. leoni*
– Female index c′ = 4.5 or less 4
4. Female postuterine branch. = 4.7 or more vulval body diam. long (Fig. 5C, D), male spicule condylus tip rounded (Fig. 9B) .... *B. tusciae*
– Female postuterine branch = 3.5 or less vulval body diam. long (Fig. 5A, B), male spicule condylus tip pointed (Fig. 9C) .... *B. borealis*

THE *XYLOPHILUS*-GROUP

Spicule narrow, capitulum flattened, condylus small, lamina angular in posterior third, cucullus present (except in *B. crenati*).

Species: *B. xylophilus*, *B. abruptus*, *B. baujardi*, *B. conicaudatus*, *B. crenati*, *B. eroshenkii*, *B. fraudulentus*, *B. kolymensis*, *B. luxuriosae*, *B. mucronatus*.

1. Spicule cucullus absent (Fig. 4C) .... *B. crenati*
– Spicule cucullus present (Fig. 4A) 2
2. Vulval flap absent (Fig. 7B), five lateral incisures (Fig. 13D) .... *B. eroshenkii*
– Vulval flap present (Fig. 7A), lateral field with other number of incisures (Fig. 13A-C) 3
3. Spicule condylus reduced to indistinct, not offset from capitulum-calomus angle (Fig. 9D).......... .... *B. conicaudatus*
– Spicule condylus well developed, rounded (Fig. 9B) 4
4. Female tail tip strongly recurved (Fig. 10A)....... .... *B. luxuriosae*
– Female tail tip straight or slightly curved ventrally (Fig. 10B) 5
5. Female tail tip truncate or finely rounded (V-shaped) (Fig. 3C, E) .... *B. abruptus*
– Female tail tip mucronate, pointed or broadly rounded (U-shaped) (Fig. 3A, B, D) 6
6. Excretory pore located at median bulb level or more anterior (Fig. 8C, D) 7
– Excretory pore located posterior to median bulb (Fig. 8A, B) 9
7. Spicule rostrum rounded to digitate (Fig. 6C), spicule length along arc = 21 μm or less ..... *B. kolymensis*
– Spicule rostrum sharply conical to pointed (Fig. 6B), spicule length along arc = 22 μm or more 8
8. Angle between line along capitulum (condylus-rostrum) and line extending spicule tip = 30° or less (lines appear to be parallel) (Fig. 11B, C) .... *B. baujardi*
– Angle between line along capitulum (condylus-rostrum) and line extending spicule tip = 45° or more (Fig. 11A) .... *B. fraudulentus*
9. Male bursa truncate in ventral view (Fig. 21C), depth of capitulum depression/capitulum width > 0.1 (Fig. 19B); dorsal contour of spicule lamina smoothly curved (Fig. 15A) .... *B. mucronatus*
– Male bursa oval to rounded in ventral view (Fig. 21B), ratio of depth of capitulum depression/capitulum width > 0.1 (Fig. 19A); dorsal contour of spicule lamina distinctly angular in posterior third (Fig. 15C) 10
10. Female tail tip usually broadly rounded (Fig. 3D); spicule rostrum-calomus junction angular (Fig. 20A), male tail terminus (lateral view) pointed (Fig. 22B) .... *B. xylophilus*
– Female tail tip mucronate to pointed (Fig. 3A, B); spicule rostrum-calomus junction smoothly curved (Fig. 20B), male tail terminus shape (lateral view) narrowly rounded (Fig. 22C) ...... *B. fraudulentus*

THE *PINIPERDAE*-GROUP

Spicule stout, capitulum concave, rostrum and condylus well developed, condylus elongated, lamina smoothly curved or angular at midpoint, cucullus absent or present.

Species: *B. piniperdae* (consisting of two subspecies: *B. piniperdae piniperdae* Fuchs, 1937 and *B. piniperdae ruehmpiniperdae* n. subsp.), *B. abietinus*, *B. bestiolus*, *B. chitwoodi*, *B. corneolus*, *B. eggersi*, *B. eremus*, *B. eucarpus*, *B. fuchsi*, *B. georgicus*, *B. glochis*, *B. hellenicus*, *B. hofmanni*, *B. hunanensis*, *B. hylobianum*, *B. incurvus*, *B. lini*, *B. maxbassiensis*, *B. minutus*, *B. naujaci*, *B. newmexicanus*, *B. nuesslini*, *B. paracorneolus*, *B. pinasteri*, *B. pinophilus*, *B. pityogeni*, *B. poligraphi* (consisting of two subspecies: *B. poligraphi poligraph* Fuchs, 1937 and *B. poligraphi ruehmpoligraphi* n. subsp.), *B. rainulfi*, *B. ratzeburgii*, *B. sachsi*, *B. scolyti*, *B. sexdentati*, *B. sutoricus*, *B. sychnus*, *B. talonus*, *B. thailandae*, *B. tritrunculus*, *B. vallesianus*, *B. varicauda*, *B. wekuae*, *B. wilfordi*, *B. willi*, *B. xerokarterus*.

1. Anterior head annulus distinctly larger in diam. than others and offset (Fig. 17C) ..... *B. maxbassiensis*
– Head annuli of equal diam. or annulation indistinct under light microscope (Fig. 17A, B) 2
2. Female tail tip with mucro (Fig. 3A) 3
– Female tail tip pointed (Fig. 3B) 14
– Female tail tip finely rounded (V-shaped) (Fig. 3C) 31
– Female tail tip broadly rounded (U-shaped) (Fig. 3D) 50
3. Male spicule tip with cucullus (Fig. 4A) 4
– Male spicule tip without cucullus, sharp to angular (Fig. 4B) 5
– Male spicule tip without cucullus, finely rounded to digitate (Fig. 4C) 6
– Male spicule tip without cucullus, bluntly rounded to widely rounded (Fig. 4D) 11
4. Excretory pore located at median bulb level (Fig. 8C) .... *B. pinophilus*
– Excretory pore located at nerve ring or posterior (Fig. 8A) .... *B. fuchsi*
5. Female vulval flap present (Fig. 7A) .... *B. varicauda*
– Female vulval flap absent (Fig. 7B) ..... *B. wekuae*
6. Male index c < 14 .... *B. sutoricus*
– Male index c = 15 or more 7
7. Female tail tip strongly recurved (Fig. 10A) .... *B. xerokarterus*
– Female tail tip straight or slightly curved ventrally (Fig. 10B) 8
8. Female postuterine branch < 1 vulval body diam. long (Fig. 5A) .... *B. chitwoodi*
– Female postuterine branch > 2.6 vulval body diam. long (Fig. 5B, C) 9
9. Excretory pore located between nerve ring and median bulb (Fig. 8B) .... *B. pinasteri*
– Excretory pore located at nerve ring level or posterior (Fig. 8A) 10
10. Male spicule condylus truncate (Fig. 9A), female vulval flap absent (Fig. 7B) .... *B. eucarpus*
– Male spicule condylus rounded (Fig. 9B), small, but distinct, female vulval flap present (Fig. 7A) .... *B. varicauda*
11. Female postuterine branch = 5 or more vulval body diam. long (Fig. 5C, D) .... *B. naujaci*
– Female postuterine branch = 4 or less vulval body diam. long (Fig. 5A, B) 12
12. Male spicule length along arc = 26 μm or more, female index c = 14 or less .... *B. tritrunculus*
– Male spicule length along arc = 17 μm or less, female index c = 20 or more 13
13. Female tail tip strongly recurved (Fig. 10A), male bursa truncate in ventral view (Fig. 21C) .... *B. ratzeburgii*
– Female tail tip straight or slightly curved ventrally (Fig. 10B), male bursa conical in ventral view (Fig. 21A) .... *B. thailandae*
14. Spicule tip with cucullus (Fig. 4A) 15
– Spicule tip without cucullus, sharp to finely rounded or digitate (Fig. 4B, C) 18
– Spicule tip without cucullus, bluntly rounded to widely rounded (Fig. 4D) .... *B. thailandae*
– Spicule tip without cucullus, broadly truncate (Fig. 4E) .... *B. hylobianum*
15. Female postuterine branch = 1 or less vulval body diam. long (Fig. 5A) .... *B. minutus*
– Female postuterine branch = 4 or more vulval body diam. long (Fig. 5C) 16
– Female postuterine branch = 2-3 vulval body diam. long (Fig. 5B) 17
16. Female tail tip strongly recurved (Fig. 10A), male bursa conical in ventral view (Fig. 21A), stylet length = 12 μm or less .... *B. corneolus*
– Female tail tip straight or slightly curved ventrally (Fig. 10B), male bursa truncate in ventral view (Fig. 21C), stylet length = 16 μm or more ..... *B. fuchsi*
17. Two lateral incisures (Fig. 13A), one pair of male postanal papillae (Fig. 12A) .... *B. abietinus*
– Three lateral incisures (Fig. 13B), two pairs of male postanal papillae (Fig. 12B) ..... *B. paracorneolus*
18. Ratio of male spicule length along arc to its width measured posterior to rostrum < 3 (Fig. 16A) .... *B. wilfordi*
– Ratio of male spicule length along arc to its width measured posterior to rostrum = 3.5 or more (Fig. 16B-D) 19
19. Female tail tip strongly recurved (Fig. 10A) 20

– Female tail tip straight or slightly curved ventrally (Fig. 10B) ... 22

20. Male spicule length along arc = 18 μm or more, two pairs of male postanal papillae (Fig. 12B), ratio of spicule length (along arc) to capitulum width (distance between ends of rostrum and condylus) = 2.5 or more (Fig. 18C) ... *B. glochis*

– Male spicule length along arc = 15 μm or less, one pair of male postanal papillae (Fig. 12A), ratio of spicule length (along arc) to capitulum width (distance between ends of rostrum and condylus) = 2.1 or less (Fig. 18B) ... 21

21. Male spicule rostrum sharply pointed, short (Fig. 6B), spicular lamina dorsal line smoothly curved (Fig. 15A) female index $c > 20$, excretory pore located posterior to median bulb (Fig. 8A, B) ... *B. xerokarterus*

– Male spicule rostrum narrowly rounded to digitate, long (Fig. 6C), spicular lamina dorsal line angular (Fig. 15B), female index $c < 20$, excretory pore located at median bulb level (Fig. 8C) ... *B. rainulfi*

22. Spicule condylus pointed (Fig. 9C) ... *B. eremus*

– Spicule condylus blunt; rounded or truncate (Fig. 9A, B) ... 23

23. Spicular lamina dorsal line angular (Fig. 15B) ... *B. sachsi*

– Spicular lamina dorsal line smoothly curved (Fig. 15A) ... 24

24. Spicule condylus truncate (Fig. 9A) ... 25

– Spicule condylus rounded (Fig. 9B) ... 27

25. Male bursa truncate in ventral view (Fig. 21C), female postuterine branch < 3.5 vulval body diam. long (Fig. 5B) and 0.3 or less of vulva-anus distance (Fig. 23B) ... *B. eucarpus*

– Male bursa oval or conical in ventral view (Fig. 21A, B), female postuterine branch > 5 vulval body diam. long (Fig. 5C, D) and 0.5 or more of vulva-anus distance (Fig. 23C, D) ... 26

26. Male spicule rostrum conical (Fig. 6B), spicule stout, ratio male spicule length along arc to its width measured posterior to rostrum (lateral view) < 4 (Fig. 16B); four pairs of male postanal papillae (Fig. 12D.1, D.2) ... *B. poligraphi poligraphi*

– Male spicule rostrum thorn-like (Fig. 6A), spicule slender, ratio male spicule length along arc to its width measured posterior to rostrum (lateral view) = 5 or more (Fig. 16C); two pairs of male postanal papillae (Fig. 12B) ... *B. poligraphi ruehmpoligraphi* n. subsp.

27. Female index $c' = 5.8$ or more ... *B. wekuae*

– Female index $c' = 4.9$ or less ... 28

28. Stylet length = 19 μm or more, male spicule rostrum rounded (Fig. 6C), female postuterine branch length < 1 vulval body diam. long (Fig. 5A) ... *B. hunanensis*

– Stylet length less than 15 μm, male spicule rostrum conical or pointed (Fig. 6B), female postuterine branch length > 2 vulval body diam. long (Fig. 5B, C) ... 29

29. Male bursa truncate in ventral view (Fig. 21C), female index $c = 13$ or less ... *B. sychnus*

– Male bursa oval, rounded or conical in ventral view (Fig. 21A, B), female index $c = 19$ or more ... 30

30. Female vulval flap absent (Fig. 7B), female postuterine branch length 2 or less vulval body diam. long (Fig. 5B), female index $V = 82$ or more ... *B. georgicus*

– Female vulval flap present (Fig. 7A), female postuterine branch length 3.5 or more vulval body diam. long (Fig. 5C), female index $V = 77$ or less ... *B. pinasteri*

31. Male spicule tip (lateral view) with cucullus (Fig. 4A) ... 32

– Male spicule tip without cucullus, bluntly rounded to widely rounded or broadly truncate (Fig. 4D, E) ... 38

– Male spicule tip without cucullus, sharp, finely rounded or digitate (Fig. 4B, C) ... 41

32. Excretory pore located at median bulb level (Fig. 8C) ... *B. vallesianus*

– Excretory pore located at nerve ring or posterior (Fig. 8A) ... 33

33. Female postuterine branch = 4 or more vulval body diam. long (Fig. 5C) and extending for 0.7 of vulva-anus distance or more (Fig. 23D) ... 34

– Female postuterine branch = 3 or less vulval body diam. long (Fig. 5B) and extending for 0.6 of vulva-anus distance or less (Fig. 23B, C) ... 35

34. Female tail tip strongly recurved (Fig. 10A), male bursa conical in ventral view (Fig. 21A), stylet length = 12 μm or less ... *B. corneolus*

– Female tail tip straight or slightly curved ventrally (Fig. 10B), male bursa truncate in ventral view (Fig. 21C), stylet = 16 μm or more .... *B. fuchsi*

35\. Two lateral incisures (Fig. 13A) ...... *B. abietinus*

– Three lateral incisures (Fig. 13B) 36

36\. One pair of male postanal papillae (Fig. 12A); angle between line along capitulum (condylus-rostrum) and line extending the spicule end = 15° or more with intersection point dorsal (Fig. 11D) .... *B. hofmanni*

– Two pairs male postanal papillae (Fig. 12B), angle between line along capitulum (condylus-rostrum) and line extending spicule end = 20° or more with intersection point ventral (Fig. 11B) 37

37\. Male bursa conical, oval or rounded in ventral view (Fig. 21A, B), female tail tip straight or slightly curved ventrally (Fig. 10B) .... *B. hellenicus*

– Male bursa truncate in ventral view (Fig. 21C), female tail tip strongly recurved (Fig. 10A) .... *B. paracorneolus*

38\. Male spicule rostrum pointed (Fig. 6B) 39

– Male spicule rostrum thorn-like or rounded (Fig. 6A, C) 40

39\. Female postuterine branch = 1.5 or less body diam. long (Fig. 5A), female vulval flap absent (Fig. 7B), spicule condylus with rounded tip (Fig. 9B), male bursa truncate or rounded in ventral view (Fig. 21B, C) .... *B. lini*

– Female postuterine branch = 6 or more body diam. long (Fig. 5D), female vulval flap present (Fig. 7A), spicule condylus with pointed tip (Fig. 9C), male bursa conical in ventral view (Fig. 21A) .... *B. bestiolus*

40\. Male bursa truncate in ventral view (Fig. 21C), spicule rostrum thorn-like (Fig. 6A), ratio depth of capitulum depression/capitulum width > 0.2 (Fig. 19C) .... *B. pityogeni*

– Male bursa conical in ventral view (Fig. 21A), spicule rostrum digitate (Fig. 6C), ratio depth of capitulum depression/capitulum width = 0.1 or less (Fig. 19A) .... *B. talonus*

41\. Female tail tip strongly recurved (Fig. 10A) 42

– Female tail tip straight or slightly curved ventrally (Fig. 10B) 46

42\. Angle between line along capitulum (condylus-rostrum) and line extending spicule tip varying from 19° with ventral intersection point, to 9° with intersection point dorsal (lines look parallel, Fig. 11C) 43

– Angle between line along capitulum (condylus-rostrum) and line extending spicule tip = 20-44° with intersection point ventral (Fig. 11B) 44

43\. Excretory pore located at nerve ring or posterior (Fig. 8A), male bursa truncate in ventral view (Fig. 21C), two pairs of male postanal papillae (Fig. 12B) .... *B. scolyti*

– Excretory pore located at median bulb level (Fig. 8C), male bursa oval to rounded in ventral view (Fig. 21B), one pair of male postanal papillae (Fig. 12A) .... *B. rainulfi*

44\. Male spicule rostrum digitate (Fig. 6C), male bursa rounded in ventral view (Fig. 21B), female index V = 70 or less .... *B. eggersi*

– Male spicule rostrum sharply conical to pointed (Fig. 6B), male bursa conical in ventral view (Fig. 21A), female index V = 71 or more 45

45\. Male spicule condylus short, spicule length along arc = 18 μm or more, ratio spicule length (along arc) to capitulum width (distance between ends of rostrum and condylus) = 2.5 or more (Fig. 18C), female index c′ = 4.2 or more .... *B. glochis*

– Male spicule condylus long, spicule length along arc = 16 μm or less, ratio spicule length (along arc) to capitulum width (distance between ends of rostrum and condylus) = 2.2 or less (Fig. 18B), female index c′ = 3.6 or less .... *B. nuesslini*

46\. Ratio of female postuterine branch length to vulva-anus distance < 0.2 (Fig. 23A) ..... *B. hunanensis*

– Ratio of female postuterine branch length to vulva-anus distance > 0.5 (Fig. 23C, D) 47

47\. Male spicule condylus truncate (Fig. 9A) 48

– Male spicule condylus rounded (Fig. 9B) 49

48\. Male spicule rostrum conical (Fig. 6B), spicule stout, ratio male spicule length along arc to its width measured posterior to rostrum (lateral view) < 4 (Fig. 16B) four pairs of male postanal papillae (Fig. 12D.1, D.2) .... *B. poligraphi poligraphi*

– Male spicule rostrum thorn-like (Fig. 6A), spicule slender, ratio male spicule length along arc to its width measured posterior to rostrum (lateral view) = 5 or more (Fig. 16C), two pairs of male postanal papillae (Fig. 12B) .... *B. poligraphi ruehmpoligraphi* n. subsp.

49. Male spicule rostrum small and conical (Fig. 6B), excretory pore located at median bulb or between nerve ring and median bulb (Fig. 8B, C); one pair of male postanal papillae (Fig. 12A), male bursa truncate in ventral view (Fig. 21C) .... *B. newmexicanus*

– Male spicule rostrum large and digitate (Fig. 6C), excretory pore located at nerve ring or posterior (Fig. 8A); two pairs of male postanal papillae (Fig. 12B), male bursa rounded in ventral view (Fig. 21B) .... *B. varicauda*

50. Male spicule tip with cucullus (Fig. 4A) .... *B. hellenicus*

– Male spicule tip without cucullus, bluntly rounded to broadly truncate (Fig. 4D, E) 51

– Male spicule tip without cucullus, sharp to finely rounded or digitate (Fig. 4B, C) 53

51. Female postuterine branch = 3 or less vulval body diam. long (Fig. 5B); female index c = 14 or less, male spicule extremely wide, ratio: spicule length along arc to its width measured posterior to rostrum (lateral view) = 3 or less (Fig. 16A), one pair of male postanal papillae (Fig. 12A) .... *B. willi*

– Female postuterine branch = 5 or more vulval body diam. long (Fig. 5C, D); female index c = 19 or more, male spicule more slender, ratio: spicule length along arc to its width measured posterior to rostrum (lateral view) = 4 or more (Fig. 16B), two or more pairs of male postanal papillae (Fig. 12B, C.1, C.2, D.1, D.2) 52

52. Male spicule condylus truncate (Fig. 9A), small female vulval flap present (Fig. 7A), male bursa truncate in ventral view (Fig. 21C) ..... *B. naujaci*

– Male spicule condylus rounded (Fig. 9B), female vulval flap absent (Fig. 7B), male bursa oval to rounded in ventral view (Fig. 21B) .... *B. piniperdae piniperdae*

53. Male bursa truncate in ventral view (Fig. 21C), one pair of male postanal papillae (Fig. 12A), male spicule condylus truncate (Fig. 9A) .... *B. incurvus*

– Male bursa oval to rounded in ventral view (Fig. 21B), two or more pairs of male postanal papillae (Fig. 12B, C.1, C.2, D.1, D.2), male spicule condylus rounded (Fig. 9B) 54

54. Male spicule rostrum rounded (Fig. 6C), spicule length along arc = 17 μm or less, female vulval flap present (Fig. 7A) .... *B. varicauda*

– Male spicule rostrum sharply pointed (Fig. 6A, B), spicule length along arc = 17 μm or more, female vulval flap absent (Fig. 7B) 55

55. Four pairs of male postanal papillae (one pair papillae and three pairs of gland papillae) (Fig. 12D.1, D.2), spicule length along arc = 19 μm or less, ratio spicule length (along arc) to capitulum width (distance between ends of rostrum and condylus) = 2.5 or more (Fig. 18C) .... *B. piniperdae ruehmpiniperdae* n. subsp.

– Two pairs of male postanal papillae (Fig. 12B), spicule length along arc = 19 μm or more, ratio spicule length (along arc) to capitulum width (distance between ends of rostrum and condylus) = 2.2 or less (Fig. 18B) .... *B. sexdentati*

Tabular key to *Bursaphelenchus* species

The characters in this tabular, polytomous, or multientry key (see Table 1) were selected from keys, differential diagnoses and original descriptions of *Bursaphelenchus* species. Character states are standardised and illustrated because different authors have either used different expressions for the same character state or the same expression for different states. To split the measured characters and ratios into their optimum states, a particular search for the 'borders' between the various character states was undertaken in order to minimise overlap of character-states between species. The order of characters in the tabular key to (Table 1) is a compromise between their significance in identification and the availability of data on the character for the majority of nominal species within the genus. For instance, the position of the excretory pore and the number of lateral lines are very important diagnostic characters, but are known only for 60 and 37, respectively, of the 75 species in the genus. Characters C1-C15 are ordered according to their efficacy in splitting the largest group of the previous step to the smallest subgroups of species, thus decreasing the number of identification steps. Characters C16-32 are ordered as in the species description: measurements, ratios and qualitative characters first for both sexes (stylet and cephalic annuli), then for male spicule, male and female, correspondingly. *Aphelenchoides ritzemabosi* is included in Table 1 as the outgroup for the analysis of similarity of species (below). Data for the outgroup

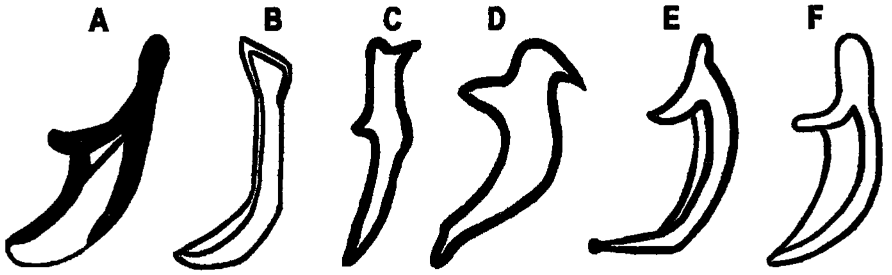

Fig. 2. *Character 1: Spicule structure. A: Dorsal and ventral limbs not joined at spicule tip, which is broad and blunt* (hunti-*group*)*; B: Capitulum compact, rostrum and condylus fused* (aberrans-*group*)*; C: Spicule linear, small conical rostrum in middle of ventral limb* (eidmanni-*group*)*; D: Condylus recurved posteriorly* (borealis-*group*)*; E: Narrow, capitulum flattened, condylus small, lamina angular in last third, cucullus present* (xylophilus-*group*)*; F: Stout, capitulum concave, condylus elongated, lamina smoothly curved or angular at midpoint, cucullus usually absent although small cucullus sometimes present* (piniperdae-*group*).

species were taken from the slide collection of the Zoological Institute (St Petersburg) as well as from Siddiqi (1974). To make the cluster analysis of the outgroup and ingroups representative, the state 4 (male bursa absent) in C25 was included. Two additional columns are: N_lit = number of studied male specimens (figures and descriptions) in the literature sources; and N_col = number of studied male specimens in collection materials.

DIAGNOSTIC CHARACTERS

C1: Spicule structure (Fig. 2)

1: dorsal and ventral limbs not joining at spicule tip, which is broad and blunt (*hunti*-group) (Fig. 2A);
2: capitulum compact, rostrum and condylus fused (*aberrans*-group) (Fig. 2B);
3: spicule linear, small conical rostrum located at *ca* half of spicule length (*eidmanni*-group) (Fig. 2C);
4: condylus recurved posteriorly (*borealis*-group) (Fig. 2D);
5: narrow, capitulum flattened, condylus small, lamina angular in last third, cucullus generally present (*xylophilus*-group) (Fig. 2E);
6: stout, capitulum concave, condylus elongate, lamina smoothly curved or angular at midpoint, cucullus usually absent, but small cucullus sometimes present (*piniperdae*-group) (Fig. 2F).

Note: species groups were employed by Giblin and Kaya (1983) and Braasch (2001). Here, species groups are based on spicule structure and are considered to be purely diagnostic.

C2: Female tail tip (Fig. 3)

1: mucronate (Fig. 3A);
2: pointed (Fig. 3B);
3: finely rounded (V-shaped) (Fig. 3C);
4: broadly rounded (U-shaped) (Fig. 3D);
5: truncate (Fig. 3E).

Note: this character was used by Rühm (1956), Tarjan and Baeza-Aragon (1982), Thong and Webster (1983), Yin *et al.* (1988) and Braasch (2001).

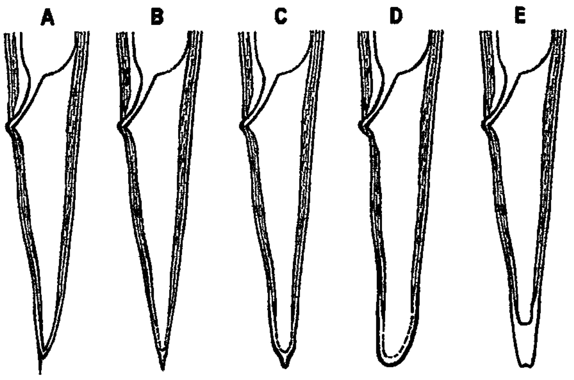

Fig. 3. *Character 2: Female tail tip. A: Mucronate; B: Pointed; C: Finely rounded (V-shaped); D: Broadly rounded (U-shaped); E: Truncate.*

C3: Male spicule tip (lateral view) (Fig. 4)

1: with cucullus (Fig. 4A);
2: without cucullus, sharp to angular (Fig. 4B);
3: without cucullus, finely rounded to digitate (Fig. 4C);
4: without cucullus, bluntly rounded to widely rounded (Fig. 4D);
5: without cucullus, broadly truncate (Fig. 4E).

Note: this character was used by Tarjan and Baeza-Aragon (1982), Yin *et al.* (1988) and Braasch and Schmutzenhofer (2000).

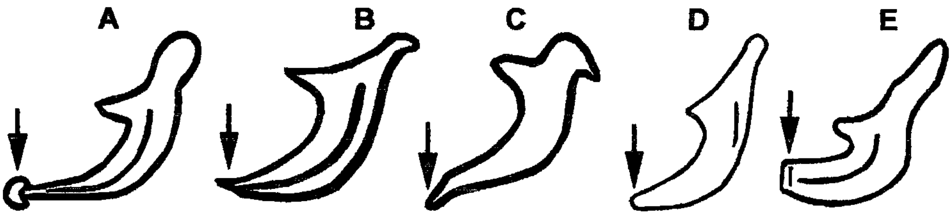

Fig. 4. *Character 3: Male spicule tip (lateral view). A: With cucullus; B: Sharp to angular, cucullus absent; C: Finely rounded to digitate, cucullus absent; D: Bluntly rounded to widely rounded, cucullus absent; E: Broadly truncate, cucullus absent.*

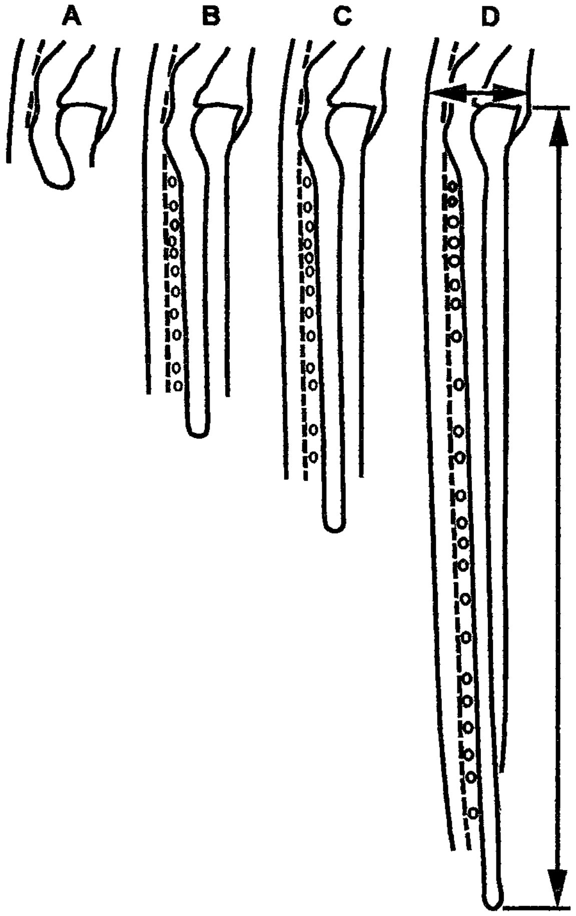

Fig. 5. *Character 4: Ratio of female genital postuterine branch length to vulval body diameter. A: 1.5 or less; B: 1.6-3.5; C: 3.6-6.3; D: 6.4 or more. (Note: Method of measuring is shown in D.)*

C4: Ratio of female postuterine branch length to vulval body diameter (Fig. 5)

1: 1.5 or less (Fig. 5A);
2: 1.6-3.5 (Fig. 5B);
3: 3.6-6.3 (Fig. 5C);
4: 6.4 or more (Fig. 5D).

Note: this character was used by Thong and Webster (1983).

C5: Male spicule rostrum (Fig. 6)

1: thorn-like (Fig. 6A);
2: sharply conical to pointed or acute (Fig. 6B);
3: digitate (Fig. 6C);
4: bluntly conical to almost flattened (Fig. 6D).

Note: this character was used by Rühm (1956) and Yin *et al.* (1988).

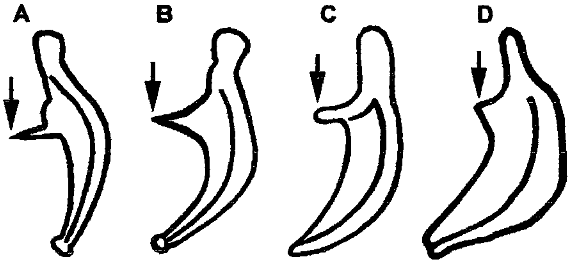

Fig. 6. *Character 5: Male spicule rostrum. A: Thorn-like; B: Sharply conical to pointed or acute; C: Digitate; D: Bluntly conical.*

C6: Female vulval flap (Fig. 7)

1: present (Fig. 7A);
2: absent (Fig. 7B).

Note: this character was used by Lieutier and Laumond (1979), Tarjan and Baeza-Aragon (1982), Giblin and Kaya (1983), Yin *et al.* (1988) and Braasch (2001).

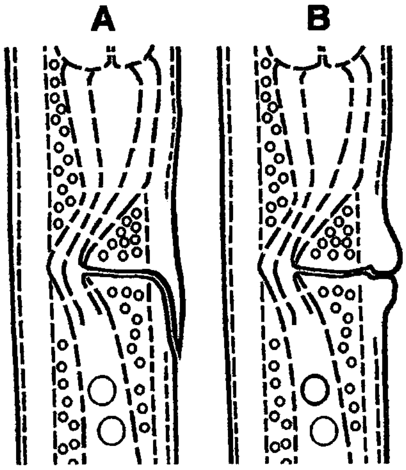

Fig. 7. *Character 6: Female vulval flap. A: Present; B: Absent.*

C7: Excretory pore position (Fig. 8)

1: at nerve ring or posterior (Fig. 8A);
2: between nerve ring and median bulb (Fig. 8B);
3: at median bulb (Fig. 8C);
4: anterior to median bulb (Fig. 8D).

Note: this character was used by Fuchs (1937), Massey (1971), Thong and Webster (1983) and Walia *et al.* (2003).

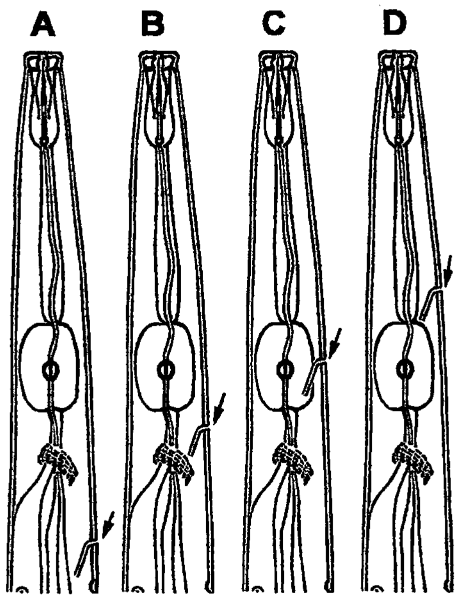

Fig. 8. *Character 7: Excretory pore position (arrows). A: At nerve ring or posterior; B: Between nerve ring and median bulb; C: At median bulb; D: Anterior to median bulb.*

C8: Male spicule condylus shape (Fig. 9)

1: truncate (Fig. 9A);
2: rounded (Fig. 9B);
3: pointed (Fig. 9C);
4: reduced to indistinct, not offset from capitulum-calomus angle (Fig. 9D).

Note: this character was used by Tarjan and Baeza-Aragon (1982), Yin *et al.* (1988) and Braasch (2001).

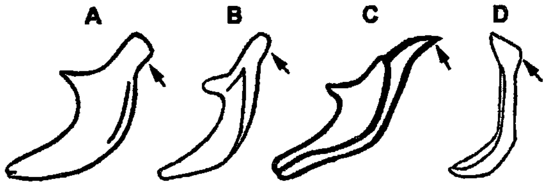

Fig. 9. *Character 8: Male spicule condylus shape. A: Truncate; B: Rounded; C: Pointed; D: Reduced or indistinct, not offset from capitulum-calomus angle.*

C9: Female tail tip curvature (Fig. 10)

1: Female tail tip strongly recurved (Fig. 10A);
2: Female tail tip straight or slightly curved ventrally (Fig. 10B).

Note: this character was used by Braasch and Schmutzenhofer (2000) and Braasch (2001).

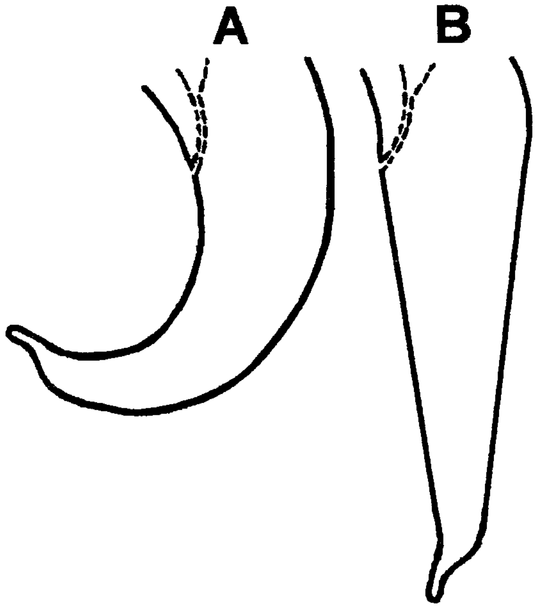

Fig. 10. *Character 9: Female tail tip ventral curvature. A: Tail tip strongly recurved; B: Tail tip straight or slightly curved ventrally.*

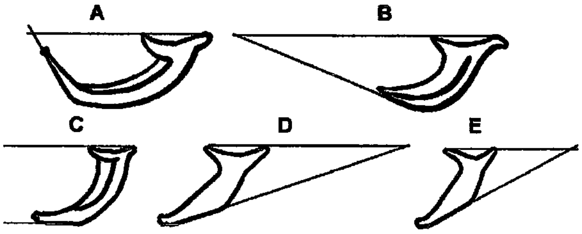

Fig. 11. *Character 10: Angle between lines: along capitulum (condylus-rostrum) and extending the spicule end, in degrees. A: 45° and more, point of intersection ventral; B: 20-44°, point of intersection ventral; C: From 19° with point of intersection ventral, to 9° with point of intersection dorsal; D: 10-29°, point of intersection dorsal; E: More than 30°, point of intersection dorsal.*

C10: Angle between line along capitulum (condylus-rostrum) and line extending the spicule tip, in degrees (Fig. 11)

1: 45° and more, intersection point ventral (Fig. 11A);
2: 20-44°, intersection point ventral (Fig. 11B);
3: from 19° with intersection point ventral, to 9° with intersection point dorsal (Fig. 11C);
4: 10-29°, intersection point dorsal (Fig. 11D);
5: more than 30°, intersection point dorsal (Fig. 11E).

Note: this character was used, as a qualitative one, by Giblin-Davis *et al.* (1993), Kolossova (1998) and Kanzaki and Futai (2003). Here the character is quantified.

C11: Number of pairs of male postanal papillae (including glandpapillae) (Fig. 12)

1: one (Fig. 12A);
2: two (Fig. 12B);
3: three (Fig. 12C.1, C.2);
4: four (Fig. 12D.1, D.2).

Note: this character was used by Fuchs (1937), Rühm (1956), Franklin and Hooper (1962), Tarjan and Baeza-Aragon (1982), Brzeski and Baujard (1997), Braasch and Schmutzenhofer (2000), Braasch (2001) and Kanzaki and Futai (2002a, 2003).

C12: Number of lateral incisures (Fig. 13)

1: two (*i.e.*, one band in lateral field) (Fig. 13A);
2: three (*i.e.*, two bands in lateral field) (Fig. 13B);
3: four (*i.e.*, three bands in lateral field) (Fig. 13C);
4: five (*i.e.*, four bands in lateral field) (Fig. 13D);
5: six (*i.e.*, five bands in lateral field) (Fig. 13E).

Note: this character was used by Baujard (1980), Yin *et al.* (1988), Braasch *et al.* (1998), Braasch and Schmutzenhofer (2000), Braasch (2001) and Braasch and Braasch-Bidasak (2002). All the species descriptions with 'lateral field lines absent' are considered here as having an unknown number of lines and are marked by '?' in the tabular key.

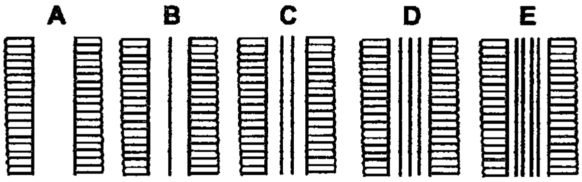

Fig. 13. *Character 12: Number of lateral incisures. A: Two incisures (i.e., lateral field in one band); B: Three incisures (i.e., two bands in lateral field); C: Four incisures (i.e., three bands in lateral field); D: Five incisures (i.e., four bands in lateral field); E: Six incisures (i.e., five bands in lateral field).*

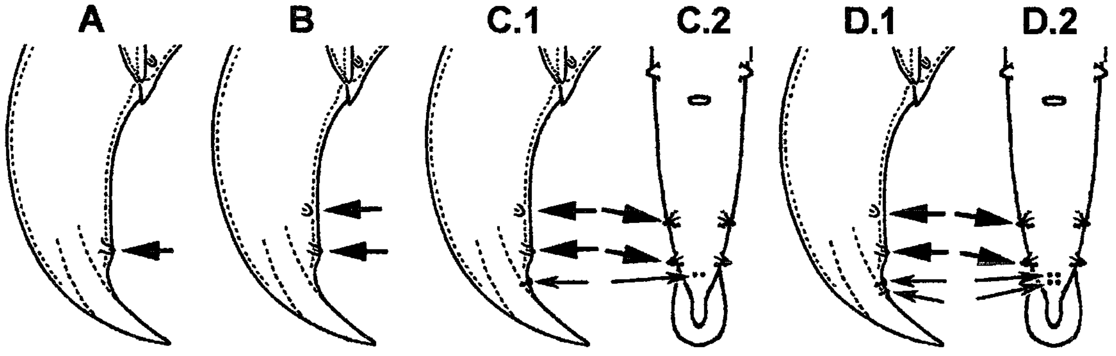

Fig. 12. *Character 11: Number of pairs of male postanal papillae (including glandpapillae). Lateral view: A: One; B: Two; C.1: Three; D.1: Four. Ventral view: C.2: Three; D.2: Four. Large papillae marked by large arrows, small papillae (glandpapillae) by small arrows.*

C13: Male spicule lamina midpoint (Fig. 14)

1: exceptionally broad to mitten-shaped (Fig. 14A);
2: not exceptionally broad (Fig. 14B).

Note: this character was used by Yin *et al.* (1988).

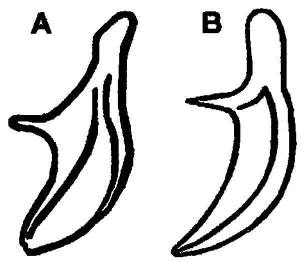

Fig. 14. *Character 13. Midpoint of male spicule lamina. A: Exceptionally broad to mitten-shaped; B: Not exceptionally broad.*

C14: Male spicule lamina dorsal line (Fig. 15)

1: smoothly and symmetrically curved (Fig. 15A);
2: angular at midpoint (Fig. 15B);
3: angular in last third or a quarter part (Fig. 15C).

Note: this character was used by Franklin and Hooper (1962) and Yin *et al.* (1988).

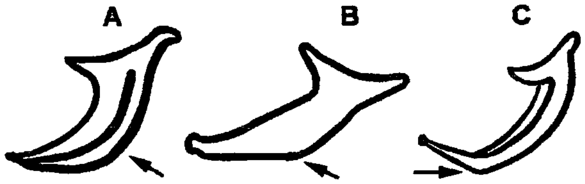

Fig. 15. *Character 14: Spicule lamina dorsal contour. A: Smoothly and symmetrically curved; B: Angular at midpoint; C: Angular in last third or quarter.*

C15: Ratio of male spicule length along arc to its width measured posterior to rostrum (lateral view) (Fig. 16)

1: <3.4 (Fig. 16A);
2: 3.4-5.8 (Fig. 16B);
3: 5.9-9.0 (Fig. 16C);
4: >9.0 (Fig. 16D).

Note: this character was used as a qualitative one by Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988). Here the character is quantified.

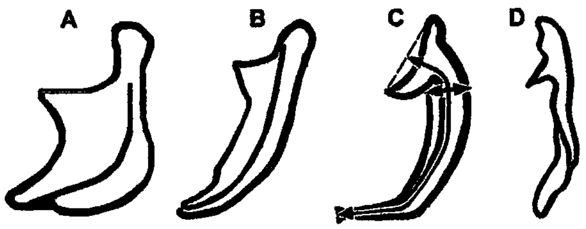

Fig. 16. *Character 15: Ratio of male spicule length measured along arc to its width measured posterior to rostrum (lateral view). A: Less than 3.4; B: 3.4-5.8; C: 5.9-9.0; D: More than 9.0. (Note: Method of measuring is shown in C.)*

C16: Stylet length:

1: <11 μm;
2: 11-19 μm;
3: >19 μm.

Note: this character was used by Fuchs (1937), Rühm (1956), Massey (1971), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C17: Cephalic annuli (Fig. 17)

1: indistinct under light microscope (Fig. 17A);
2: distinct under light microscope, of equal diameter (Fig. 17B);
3: distinct under light microscope, anterior annulus distinctly larger in diameter than others and offset (Fig. 17c).

Note: this character was used by Massey (1971a).

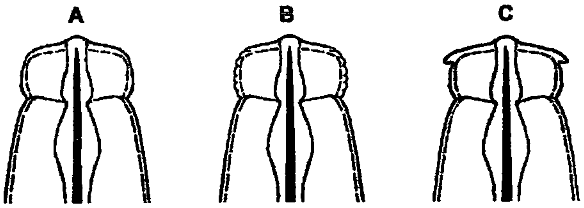

Fig. 17. *Character 17: Cephalic annuli. A: Indistinct or absent under light microscope (LM); B: Distinct under LM, of equal width; C: Distinct under LM, anterior annulus distinctly larger in diameter than others and offset.*

C18: Male spicule length measured along arc (method of measuring is shown in Fig. 16C)

1: <13 μm;
2: 13-23 μm;
3: >23 μm.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C19: Ratio of spicule length (along arc)/capitulum width (distance between ends of rostrum and condylus) (Fig. 18)

1: <1.5 (Fig. 18A);
2: 1.5-2.2 (Fig. 18B);
3: 2.3-3.0 (Fig. 18C);
4: 3.1-4.0 (Fig. 18D);
5: >4.0 (Fig. 18E).

Note: this character was used as a qualitative one by Yin *et al.* (1988). Here the character is quantified.

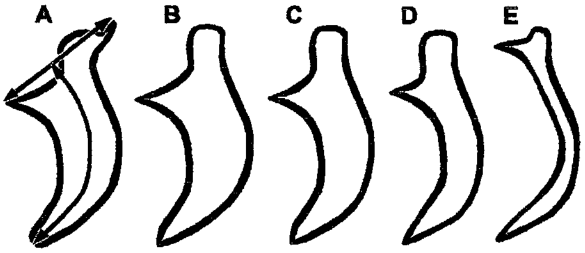

Fig. 18. *Character 19: Ratio of spicule length (along arc) / capitulum width (distance between ends of rostrum and condylus). A: Less 1.5; B: 1.5-2.2; C: 2.3-3.0; D: 3.1-4.0; E: More than 4.0. (Note: Method of measuring is shown in A.)*

C20: Ratio of depth of capitulum depression/capitulum width (Fig. 19)

1: 0.1 or less (Fig. 19A);
2: 0.11-0.20 (Fig. 19B);
3: >0.2 (Fig. 19C).

Note: this character was used as a qualitative one by Yin *et al.* (1988). Here the character is quantified.

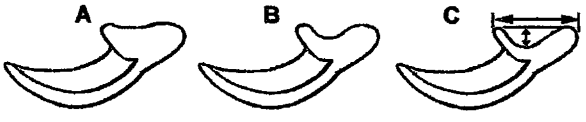

Fig. 19. *Character 20: Ratio of depth of capitulum depression / capitulum width. A: 0.1 or less; B: 0.11-0.20; C: More than 0.2. (Note: Method of measuring is shown in C.)*

C21: Junction of spicule rostrum and calomus (Fig. 20)

1: angular (Fig. 20A);
2: smoothly curved (Fig. 20B).

Note: this character is used here for the first time.

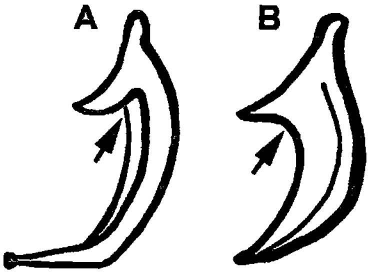

Fig. 20. *Character 21: Junction of rostrum and calomus in male spicule. A: Angular; B: Smoothly curved.*

C22: Male body length

1: <360 μm;
2: 370-710 μm;
3: 720 μm or more.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C23: Male index a

1: 27 or less;
2: 28-79;
3: >80.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C24: Male index c

1: 14 or less;
2: 15-50;
3: >50.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C25: Male bursal flap shape (ventral view) (Fig. 21)

1: conical to finely pointed (Fig. 21A);
2: oval to rounded (Fig. 21B);
3: truncate, posterior edge straight or curved inwards (Fig. 21C);
4: absent.

Note: this character was used by Rühm (1956), Giblin and Kaya (1983) and Braasch and Schmutzenhofer (2000). A fourth state (bursal flap absent) is added for the outgroup used in the analysis of the general phenetic similarity (*Aphelenchoides ritzemabosi*). Males of all species of *Bursaphelenchus* have a bursal flap, this being the main diagnostic feature for the genus and also for the family Parasitaphelenchidae.

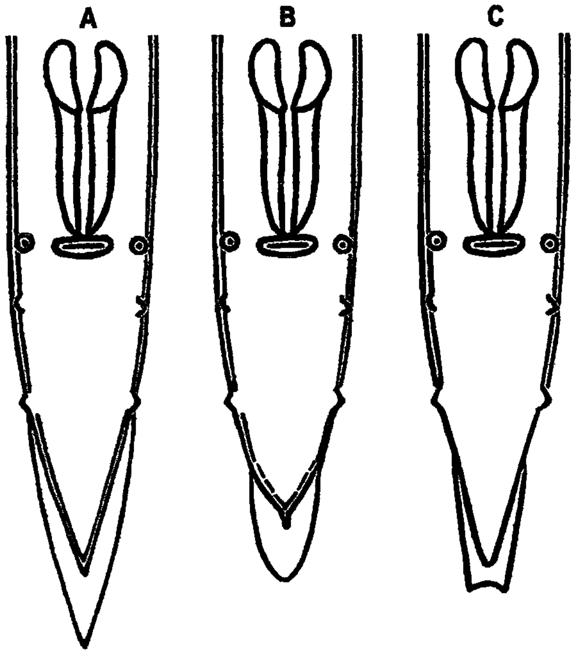

Fig. 21. *Character 25: Male bursa shape (ventral view). A: Conical to finely pointed; B: Oval to rounded; C: Truncate, posterior edge straight or curved inwards.*

C26: Male tail terminus shape (lateral view) (Fig. 22)

1: mucronate (Fig. 22A);
2: pointed (Fig. 22B);
3: narrowly rounded (Fig. 22C);
4: rounded (Fig. 22D).

Note: this character was used by Braasch (1998) and Braasch and Schmutzenhofer (2000).

C27: Female body length

1: <390 μm;
2: 400-1400 μm;
3: >1400 μm.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C28: Female index a

1: 27 or less;
2: 28-40;
3: 41-58;
4: >58.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C29: Female index c

1: 15 or less;
2: 16-45;
3: 46 or more.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C30: Female index c'

1: 2.2 or less;
2: 2.3-4.1;
3: 4.2-5.5;
4: 5.6 or more.

Note: this character was used by Loof (1964), Tarjan and Baeza-Aragon (1982), Brzeski and Baujard (1997), Braasch and Schmutzenhofer (2000) and Kanzaki *et al.* (2000).

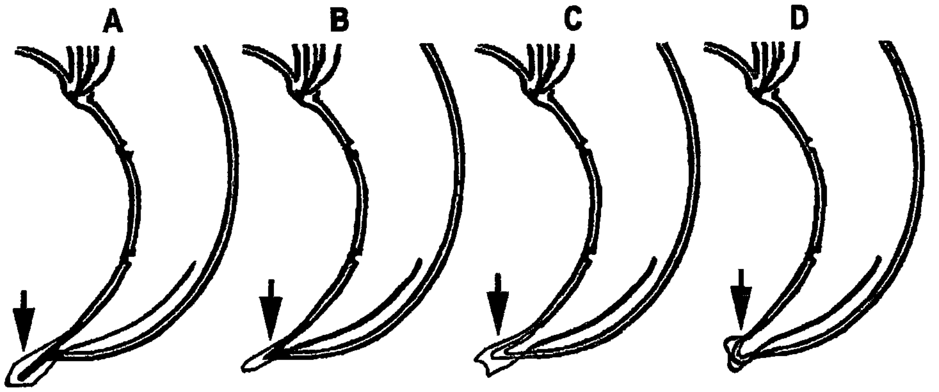

Fig. 22. *Character 26: Male tail terminus shape (lateral view). A: Mucronate; B: Pointed; C: narrowly rounded; D: Rounded.*

C31: Female index V

1: 65 or less;
2: 66-83;
3: 84 or more.

Note: this character was used by Fuchs (1937), Rühm (1956), Tarjan and Baeza-Aragon (1982) and Yin *et al.* (1988).

C32: Ratio of female postuterine branch length to vulva-anus distance (Fig. 23)

1: <0.2 (Fig. 23A);
2: 0.2-0.3 (Fig. 23B);
3: 0.31-0.69 (Fig. 23C);
4: 0.7 or more (Fig. 23D).

Note: this character was used by Baujard (1980), Braasch and Schmutzenhofer (2000) and Braasch and Braasch-Bidasak (2002).

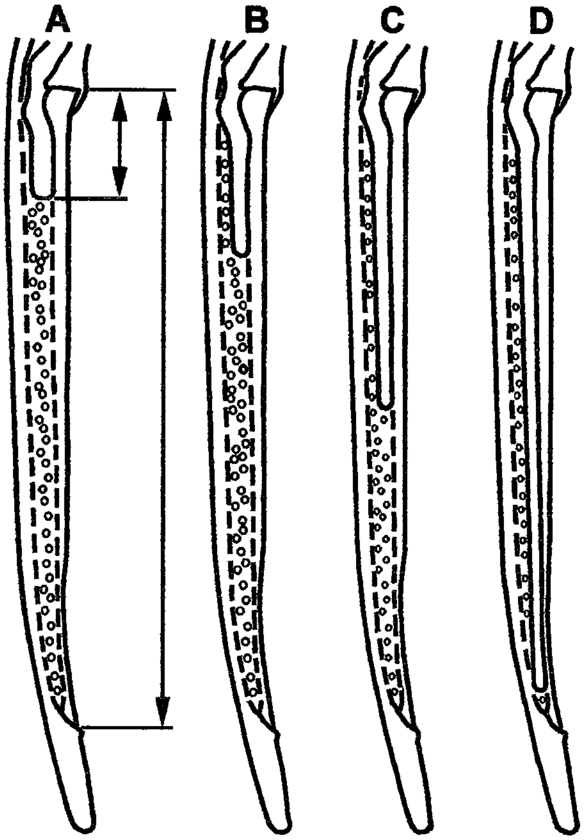

Fig. 23. *Character 32: Ratio of female genital postuterine branch length to vulva-anus distance. A: Less than 0.2; B: 0.2-0.3; C: 0.31-0.69; D: 0.7 or more. (Note: Method of measuring is shown in A.)*

NOTES ON SOME SPECIES IN THE TABULAR KEY (TABLE 1)

i) Bursaphelenchus bestiolus. Female postuterine branch length of 8-9 times body diam. was given in Massey (1974); but in the figure, the postuterine branch is 6.6 body diam. long. In Table 1 the summarised range of 6.6-9.0 is used.

ii) Bursaphelenchus borealis. J4 ectophoretic juveniles have the excretory pore anterior to the median bulb.

iii) Bursaphelenchus cryphali. Male characters are mainly given by Rühm (1956); males were not described by Fuchs (1930).

iv) Bursaphelenchus erosus. Described only from males.

v) Bursaphelenchus gonzalezi. Male characters were measured from the drawing as they were not mentioned in the original description. The spicule is 18 μm, not 13 μm long, as given in tables by Yin *et al.* (1988) and later repeated by Braasch (2001). Here the range 13-18 μm is used (Table 1).

vi) Bursaphelenchus hylobianum. According to Korentchenko (1980), the male has one pair of large precloacal papillae and two pairs of small postcloacal papillae; but according to Braasch and Braasch-Bidasak (2002), there is one pair of postanal papillae and one unpaired adanal papilla. Both possible papillae patterns are included here in a range of the character states (Table 1).

vii) Bursaphelenchus lini. In Table 1 for *B. lini* c and c′ indexes are calculated from the figures and table in Braasch (2004b) by using the end of the intestine as demarcating the beginning of the tail. This somewhat perplexing species differs from other *Bursaphelenchus* spp. in the obscure rectum and anus in females, wide stylet lumen and absence of basal knobs or thickenings of stylet. In these features *B. lini* is close to Ektaphelenchidae, although the male does have a terminal bursa.

viii) Bursaphelenchus scolyti. The length of spicule (7 μm) was calculated by Yin *et al.* (1988) from the closest scale given for the head in Massey (1974). The real scale is different, however, a fact that can be proved by calculation of the male tail length from the same figure and comparing it with the value of L/c-index in Massey's description. The real spicule length, as calculated from the drawing, is 16 μm.

ix) Bursaphelenchus seani. The male spicule is 26-27 μm long as calculated from the scale and testing the scale from tail length = L/c index. The 14 μm spicule length given by Yin *et al.* (1988) is an error.

x) Bursaphelenchus xylophilus. The disposition of the male papillae has been studied most thoroughly in this species. There are two postcloacal pairs of papillae located very together at *ca* mid-tail; one pair of precloacal papillae and one unpaired precloacal papilla (Nickle *et al.*, 1981; Mota *et al.*, 1999).

List of records, with names of natural vectors, associated plants and taxonomic notes

Table 2 gives the country by country distribution of *Bursaphelenchus* species, summarised from the records listed in the Appendix. In the Appendix, *Bursaphelenchus* species are listed alphabetically with the references for each species record listed chronologically. All available data are listed for each reference (country, vectors and their families; associated plants and their families). If data on a vector or a plant are absent they are omitted without special comment (every effort was made to ensure that the literature sources were as comprehensive and up-to-date as possible). Names of plant families are given according to Takhtajan (1987). The list includes data only on the natural vectors and plants, experimental vectors and plants being excluded.

REMARKS ON THE APPENDIX

[1] The records of *B. cocophilus* do not cover all the literature and are only intended to demonstrate the diversity of distribution and the associated vector and plant taxa.

[2] The records of *B. xylophilus* do not cover all the literature and are only intended to demonstrate the diversity of distribution and the associated vector and plant taxa. A detailed review, to be published separately, is planned.

[3] Braasch *et al.* 2001 (pp. 134-136, Figs 2, 5, Table 1) identified two females as *B. xylophilus* plus four males and 16 juveniles in a wood sample imported from Byelorussia. Molecular DNA confirmation of the species identification was not possible and a re-examination of the record is needed.

Recommended standard for species descriptions within the genus *Bursaphelenchus* Fuchs, 1937

The current research has led to the realisation of the desirability of a minimum standard for future species descriptions/redescriptions in this genus. The standard proposed herein includes characters already listed and used in keys and other taxonomic papers by the most experienced specialists in the identification of *Bursaphelenchus* species. The combination of characters in the list below is necessary in order to reliably distinguish the existing nominal species. It was shown by using the Pickey 8 software (Dianov & Lobanov, 2004), module 'Test of taxa differences', that if any four of these characters were removed, an 'unrecognisable group' of two or more species resulted. This will be described in a future publication on the computerised identification of *Bursaphelenchus* species.

In the list below, alternative character states for each qualitative character (in brackets) are separated by a slash (/). Measured characters should be expressed in μm.

General characters (common for male and female)

Cephalic annuli (indistinct under light microscope / of equal diameter / anterior annulus distinctly greater in diameter than others and offset). Excretory pore position (at nerve ring or posterior / between nerve ring and median bulb / at median bulb / anterior to median bulb). Number of lateral incisures.

Male

Body length. Stylet length. Ratios a and c. Number of pairs of male caudal papillae and their arrangement pattern relative to cloacal aperture and bursal flap. Male bursa shape, ventral view: (conical to finely pointed / oval to rounded / truncate with posterior edge curved inwards). Male tail terminus shape, lateral view: (mucronate / pointed / narrowly rounded / rounded).

Spicule

Length along arc. Ratio of spicule length along arc to its width measured posterior to rostrum (lateral view). Ratio of depth of capitulum depression/capitulum width. Ratio of spicule length (along arc) to capitulum width. Angle between line along capitulum (condylus-rostrum) and line extending the spicule end (in degrees) with an indication of the point of intersection (ventral/dorsal). Spicule structure type (species group name: *aberrans-*, *borealis-*, *eidmanni-*, *hunti-*, *piniperdae-*, *xylophilus-*group). Rostrum

Table 1. *Characters of* Bursaphelenchus *species. Numbers of characters (C1, C2, to C32) and their states are as listed in the text. N_lit = number of studied male specimens (figures and descriptions) in the literature sources. N_col = number of studied male specimens in collection material. Names:* piniperdae_piniperdae *and* piniperdae_ruehmpiniperdae, poligraphi_poligraphi *and* poligraphi_ruehmpoligraphi *are subspecies of* B. piniperdae *Fuchs, 1937 and* B. poligraphi *Fuchs, 1937, respectively.* Aphelenchoides ritzemabosi *is included as an outgroup. For* B. lini, *c and c' indexes are calculated from the figures and table in Braasch (2004b), using the end of the intestine as the anterior demarcation of the tail (no anus shown).*

Group	Species/ Character	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	N_ lit	N_ col
hunti- group	*kevini*	1	1	4	2	1	2	23	2	2	2	3	3	1	1	2	2	1	2	2	2	2	3	2	2	13	12	2	12	2	2	2	3	3	
	dongguanensis	1	2	5	234	1	2	1	2	2	1	3	3	1	1	1	2	1	1	2	2	1	3	3	3	3	2	23	4	3	1	3	34	2	
	gonzalezi	1	2	4	2	4	2	1	4	1	2	2	3	1	1	1	2	1	2	3	1	2	23	2	2	12	?	2	12	12	3	2	3	1	
	seani	1	2	4	2	3	2	1	2	2	2	2	3	1	1	2	2	2	23	2	2	1	23	2	2	1	2	2	12	12	2	2	3	2	
	hunti	1	3	4	3	1	2	1	2	1	2	1	2	1	1	2	2	1	2	2	2	2	23	1	2	?	2	2	12	1	3	2	4	1	
	fungivorus	1	3	4	3	2	2	1	2	1	2	2	3	1	2	2	2	2	2	2	2	2	23	12	2	12	2	2	123	12	3	2	3	1	
	cocophilus	1	3	5	4	23	2	1	2	2	2	2	34	1	1	1	2	1	1	1	2	1	3	3	2	2	2	2	4	1	4	2	4	2	
aberrans- group	*idius*	2	12	3	34	2	?	?	4	2	2	2	5	2	1	2	2	1	2	4	1	2	2	2	2	3	3	2	2	2	12	2	4	2	
	sinensis	2	123	3	23	4	1	2	4	2	2	2	1	2	1	12	2	1	2	4	1	2	2	12	2	2	3	2	12	2	2	2	34	3	
	elytrus	2	2	4	4	2	2	3	2	2	3	2	?	2	1	2	2	1	3	3	1	2	3	2	2	1	2	2	2	2	3	2	4	1	
	aberrans	2	23	2	2	2	1	1	4	1	5	2	3	2	1	3	2	1	12	5	1	2	2	12	2	12	23	2	23	12	2	2	3	5	
eidmanni- group	*digitulus*	3	1	2	2	2	2	1	2	2	3	2	?	2	1	2	2	1	2	2	2	2	2	2	2	23	1	2	23	12	34	3	3	1	
	steineri	3	1	23	12	4	?	1	2	2	2	2	?	2	1	4	2	1	2	3	23	1	2	1	2	1	2	2	1	1	4	2	3	1	
	erosus	3	1	3	2	1	2	?	2	2	1	2	?	2	1	3	1	1	1	2	1	1	2	1	2	2	2	?	?	?	2	?	2	1	
	teratospicularis	3	3	2	1	3	1	?	2	2	2	2	?	2	2	3	23	1	2	2	2	2	2	2	2	2	23	2	2	2	3	2	1	1	
	eidmanni	3	3	3	3	4	2	12	1	2	3	1	?	2	1	3	2	1	2	3	1	2	23	2	2	3	3	2	12	3	3	2	3	2	
	typographi	3	4	3	1	2	2	?	2	2	2	1	?	2	1	2	3	1	1	2	1	1	2	1	2	2	2	2	1	?	?	3	?	1	
borealis- group	*borealis*	4	2	23	2	23	1	1	3	2	2	2	?	1	1	2	2	1	2	23	2	2	23	12	2	3	2	2	12	12	2	2	3	4	2
	cryphali	4	23	2	2	2	2	1	3	2	2	1	?	2	1	23	2	1	2	3	1	2	3	2	2	2	2	2	3	2	2	2	3	2	
	silvestris	4	3	3	2	3	1	1	2	2	2	2	?	1	1	2	2	1	3	3	3	2	3	2	2	2	3	2	23	1	34	2	3	2	
	tusciae	4	3	3	34	23	1	1	2	12	2	2	2	2	1	2	2	1	2	3	1	2	3	2	2	3	23	2	2	2	23	2	34	6	25
	leoni	4	3	4	23	2	1	1	3	2	2	2	?	2	1	2	2	1	12	2	2	2	23	2	2	3	?	2	23	1	34	2	3	2	
xylophilus- group	*conicaudatus*	5	1	1	3	13	1	1234	4	12	1	1	?	2	1	3	2	1	3	4	1	1	23	2	2	3	?	2	2	12	23	2	34	1	
	fraudulentus	5	1	1	?	2	1	?	2	2	1	1	?	2	3	23	2	?	23	34	1	1	23	12	2	2	3	2	23	2	23	2	?	2	
	mucronatus	5	1	1	34	2	1	1	2	2	1	1	3	2	1	3	2	1	23	4	2	1	23	12	2	3	2	2	123	2	2	2	4	3	25
	crenati	5	1	3	?	23	?	12	2	1	3	2	?	2	1	23	2	1	2	3	1	2	3	2	2	3	3	2	23	2	2	2	?	2	
	baujardi	5	12	1	3	2	1	3	2	2	2	2	3	2	2	2	2	1	3	3	1	1	23	2	2	?	2	2	12	2	2	2	4	2	
	kolymensis	5	12	1	23	3	1	34	2	2	1	1	?	2	3	2	2	1	2	4	2	2	2	12	2	23	2	2	12	2	23	2	34	1	3
	xylophilus	5	14	1	4	12	1	1	2	2	1	2	3	2	3	3	2	1	23	34	1	1	23	2	2	12	2	2	23	2	2	2	4	5	50
	eroshenkii	5	23	1	3	2	2	1	2	2	2	1	4	2	1	3	2	1	2	23	12	1	23	2	2	2	2	2	23	2	12	2	3	5	9
	luxuriosae	5	3	1	3	12	1	123	2	1	1	2	3	2	3	3	2	1	3	34	1	1	23	12	2	3	2	2	2	2	23	2	3	1	
	abruptus	5	35	1	12	2	1	1	2	2	3	2	3	2	2	2	2	1	3	3	1	1	3	12	12	2	3	2	12	2	23	2	12	1	
piniperdae- group	*pinophilus*	6	1	1	34	12	1	3	123	2	2	2	3	2	1	2	2	1	2	2	2	1	23	2	2	3	2	2	23	2	2	2	34	5	20
	maxbassiensis	6	1	2	3	2	1	4	2	2	2	2	2	2	1	2	3	3	2	2	2	2	23	2	2	1	1	2	2	2	2	2	4	1	
	sutoricus	6	1	3	3	2	2	?	2	2	2	2	?	2	1	2	2	2	2	3	2	2	3	2	1	2	2	2	3	2	2	1	3	1	
	chitwoodi	6	1	3	1	3	?	12	2	2	2	2	?	2	1	2	2	1	2	2	2	2	2	2	2	2	3	2	1	12	2	2	1	2	
	ratzeburgii	6	1	4	2	3	2	12	2	1	3	2	?	2	1	2	12	1	1	2	2	2	2	2	2	3	3	2	23	2	23	2	2	2	
	tritrunculus	6	1	4	23	3	?	1	1	2	2	1	?	2	1	2	2	1	3	2	3	2	2	2	2	3	?	2	2	1	4	2	3	1	
	wekuae	6	12	2	3	3	2	?	2	2	3	2	?	2	1	2	2	1	2	2	1	1	2	2	2	1	3	2	2	2	4	2	34	1	

Table 1. *(Continued).*

Group	Species/ Character	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	N_lit	N_col
	eucarpus	6	12	3	2	2	2	1	1	2	4	2	?	2	1	2	2	1	2	2	12	2	3	2	2	3	3	2	23	2	23	2	2	2	
	pinasteri	6	12	3	3	2	1	2	2	2	2	2	2	2	1	2	2	1	12	2	2	2	2	2	2	2	2	2	23	2	2	2	3	1	
	xerokarterus	6	12	3	?	2	?	?	2	1	1	1	?	2	1	2	2	1	2	2	1	2	23	2	2	2	4	2	12	2	2	2	?	2	
	thailandae	6	12	4	23	23	2	1	2	2	1	2	3	1	1	3	2	1	12	23	1	2	2	2	2	1	?	2	2	2	23	2	3	6	
	naujaci	6	14	4	34	2	1	234	1	2	2	2	3	2	1	2	2	1	2	2	2	2	23	2	2	3	2	2	23	2	12	2	34	1	
	fuchsi	6	123	1	3	2	1	1	12	2	3	2	3	2	2	2	2	1	2	2	1	1	3	2	2	3	2	2	23	2	2	2	4	3	
	varicauda	6	134	23	23	3	1	1	2	2	2	2	?	2	1	2	2	1	2	2	1	2	23	2	2	2	2	2	12	12	2	2	34	1	
	minutus	6	2	1	1	2	2	1	2	2	12	2	3	2	1	2	1	1	1	2	12	1	1	1	2	?	2	1	1	1	2	2	2	2	
	wilfordi	6	2	2	4	2	2	?	2	2	1	2	?	2	1	1	2	1	1	2	3	2	23	2	2	2	2	2	4	2	2	2	34	1	
	eremus	6	2	3	34	1	1	?	3	2	3	2	?	2	1	2	2	1	2	2	2	2	3	2	2	2	3	2	3	2	2	2	34	2	
	georgicus	6	2	3	2	2	2	?	2	2	1	3	?	2	1	2	12	?	2	3	2	2	3	2	2	12	2	2	2	2	2	2	3	1	
	sachsi	6	2	3	?	2	?	?	2	2	3	1	?	2	2	2	2	1	2	3	2	2	2	12	2	1	2	2	2	1	2	2	?	2	
	sychnus	6	2	3	?	2	?	?	2	2	2	1	?	1	1	2	2	1	2	23	2	2	2	12	2	3	2	2	12	1	3	2	?	2	
	hylobianum	6	2	5	23	3	1	1	2	2	1	3	1	1	2	2	2	2	23	2	1	2	23	12	2	1	4	2	12	12	23	2	34	2	1
	abietinus	6	23	1	2	3	1	1	2	12	2	1	1	1	12	2	2	1	12	2	2	1	2	12	2	3	3	2	12	2	12	2	3	6	
	corneolus	6	23	1	3	2	1	1	1	1	2	3	?	2	1	2	2	1	2	2	2	2	2	2	2	1	2	2	2	2	2	2	4	1	
	paracorneolus	6	23	1	2	23	1	1	2	1	2	2	2	1	1	2	2	1	2	2	2	2	2	12	2	3	2	2	12	2	2	2	23	5	
	hunanensis	6	23	23	1	3	2	1	2	2	1	1	23	1	1	2	3	1	2	2	3	1	2	2	2	3	1	2	23	12	2	2	1	2	
	rainulfi	6	23	3	23	3	1	3	2	1	3	1	1	2	12	2	2	1	12	2	23	1	23	12	2	2	3	2	12	12	23	2	3	7	
	glochis	6	23	3	34	2	2	1	2	1	2	2	2	2	1	2	2	2	2	3	23	2	3	2	2	1	2	2	23	2	3	2	3	2	28
	poligraphi_ poligraphi	6	23	3	34	2	?	23	1	2	2	4	?	2	1	2	1	1	1	2	2	1	23	2	2	2	3	2	3	23	2	2	34	1	
	poligraphi_ ruehmpoligraphi	6	23	3	34	12	?	?	1	2	1	2	?	2	1	23	2	1	2	3	23	1	3	2	2	1	2	2	23	2	23	2	34	2	
	hofmanni	6	3	1	2	2	1	1	2	1	4	1	2	2	1	2	2	1	12	23	1	2	2	12	2	3	2	2	12	12	12	2	3	2	
	vallesianus	6	3	1	34	2	1	3	1	2	2	2	3	2	1	2	2	1	2	2	2	2	23	12	2	2	3	2	23	2	12	2	3	4	
	newmexicanus	6	3	2	34	2	1	23	2	2	2	1	?	2	1	2	2	1	3	3	2	2	3	2	2	3	4	3	23	2	23	2	3	1	
	scolyti	6	3	2	34	23	2	1	2	1	3	2	?	2	1	2	2	1	2	2	3	1	3	2	2	3	2	2	2	2	3	2	4	1	
	nuesslini	6	3	2	?	2	?	?	2	1	2	2	?	2	1	2	2	?	2	2	2	2	3	2	2	12	2	2	12	2	2	2	?	2	
	eggersi	6	3	3	3	3	2	1	2	1	2	2	2	2	1	2	2	1	23	3	2	2	23	12	2	2	2	2	2	2	23	2	3	2	
	lini	6	3	4	1	2	2	1	2	2	2	1	2	1	1	12	23	1	2	2	1	12	23	12	2	23	3	2	123	2	2	2	1	3	
	pityogeni	6	3	4	3	1	1	23	1	2	2	1	1	2	1	2	2	1	2	2	3	1	3	2	2	3	2	2	2	2	2	2	34	1	
	talonus	6	3	4	3	3	?	?	2	2	2	1	?	1	12	2	2	1	2	2	1	2	3	2	2	1	2	2	2	2	2	2	4	1	
	bestiolus	6	3	45	4	2	1	?	3	2	2	2	?	2	1	2	2	1	2	2	1	2	2	2	2	1	12	2	2	2	2	2	34	1	
	hellenicus	6	34	1	2	2	1	1	2	2	2	2	2	2	1	2	2	1	12	2	2	2	23	12	2	12	2	2	12	2	2	2	3	2	
	incurvus	6	4	2	3	2	2	12	1	2	2	1	?	2	1	2	2	1	2	2	2	2	2	1	2	3	2	2	1	2	12	2	34	1	
	piniperdae_ ruehmpiniperdae	6	4	3	3	2	2	23	2	2	1	4	?	2	1	2	2	1	2	3	3	2	3	2	2	2	2	2	2	2	2	2	34	2	
	sexdentati	6	4	3	?	1	?	?	1	2	2	2	?	2	1	2	2	1	2	2	2	2	3	2	2	2	2	2	23	2	2	2	?	2	
	piniperdae_ piniperdae	6	4	4	3	2	2	3	2	2	2	4	?	2	1	2	2	1	12	2	2	2	2	2	2	2	3	2	2	2	2	2	4	1	
	willi	6	4	45	2	4	2	2	2	2	2	1	1	2	1	1	2	1	23	4	3	2	2	2	2	3	4	2	12	1	2	2	3	1	
	Aphelenchoides ritzemabosi	2	1	3	4	4	2	1	4	2	1	2	3	2	1	23	2	2	2	4	1	2	3	2	2	4	1	2	3	2	2	2	3	3	25

shape (thorn-like / sharply conical to pointed or acute / digitate / bluntly conical to almost flattened). Shape of junction of rostrum and calomus (angular / smoothly curved). Condylus, posterior curvature (recurved posteriorly / not recurved posteriorly). Condylus shape (truncate / rounded / pointed / reduced to indistinct). Spicule tip, lateral view (with cucullus / without cucullus: sharp to angular / finely rounded to digitate / bluntly rounded to widely rounded / broadly truncate). Lamina midpoint (exceptionally broad to mitten-shaped / not exceptionally broad). Lamina dorsal line (smoothly and symmetrically curved / angular at midpoint / angular in last third or quarter).

Female

Body length. Stylet length. a, c, c′, V indexes. Vulval flap (present / absent). Vulval flap length. Ratio of female genital postuterine branch length to vulval body diameter. Ratio of female genital postuterine branch length to vulva-anus distance. Tail tip shape (mucronate / pointed / finely rounded / broadly rounded / truncate). Tail tip curvature (strongly recurved / straight to slightly curved ventrally).

Dispersal juvenile

Tail tip shape of J3/J4 ectophoretic stage (mucronate / pointed, finely rounded / broadly rounded / truncate).

Habitat

Type locality and other localities. Associated plant species (Latin name with authority). Location in plant. Associated vector species (Latin name with authority). Location of the dispersal juvenile in/on vector.

Dendrograms of general phenetic similarity

The dendrogram of general phenetic similarity (type of cluster analysis: distance; UPGMA, standard distance: mean character difference) based on Table 1 is given in Figure 24 (for all characters) and Figure 25 (spicule characters only, namely characters 1, 3, 5, 8, 10, 13-15, 18-21 in Table 1). PAUP4.0v10 software (Swofford, 2001) was used for the cluster analysis. *Aphelenchoides ritzemabosi* was used as the outgroup to root the tree.

Discussion

Clusters represent assemblages of species within the multidimensional space of the diagnostic characters, as analysed by the algorithm employed (here the general similarity algorithm has been used). If the diagnostic group (based on the combination of a few diagnostic characters) forms, either completely or partially, a separate cluster in a multidimensional space of all the important diagnostic characters, it may be concluded that these few characters were well-selected for the group diagnosis and that there is, therefore, a high probability of the group being a natural one (*i.e.*, originating from a single ancestor and morphologically distinct).

However, there are many clusters on the dendrogram and it is not possible to provide a brief and convenient taxonomic diagnosis for all of them, *i.e.*, not all of them represent natural groups. The most important additional argument to support a cluster as being a natural taxon is a specific niche for the constituent species. This niche should be different from the niches of adjacent clusters. For this reason, a dendrogram needs to be verified by niche-specific criteria (*e.g.*, systematic position of the associated plants, insects, fungi). Of course, niche parameters should be independent, *i.e.*, not included in the dataset from which the dendrogram is generated. Even if a diagnostic species group coincides generally well with its dendrogram cluster, some of its members may be more distant from the main cluster of species.

The main issue of this discussion is whether the diagnostic groups of species proposed herein are natural. From the two dendrograms (Figs 24, 25), the one based on spicule characters (Fig. 25) better reflects the natural relationships among the species. Sclerotised and complicated structures have been recommended as the basis for the analysis of relationships (Remane, 1952) and the male spicules represent the best such structures in *Bursaphelenchus* and the superfamily Aphelenchoidoidea as a whole. To verify the relationships shown in the dendrograms (Figs 24, 25), the data relating to the taxonomic position of vectors and associated plants for different *Bursaphelenchus* species were used. The list of records of natural vectors, plants and their families from the cited literature sources is given in the Appendix.

In general, the biological link between vectors of the family Scolytidae (bark beetles) and the associated plants of the family Pinaceae (the main nutrition source for both the insect and the nematode) is dominant (*i.e.*, most frequent). The 'vector-associated plant' link may be referred to as the 'transmission-associated complex' (TA complex). The task is to follow changes in the TA complex within the genus *Bursaphelenchus* at the level of the family of the vectors and associated plants. The complex

Table 2. *Country by country distribution of* Bursaphelenchus *species (summarised from records listed in the Appendix).*

	EUROPE																								ASIA							AMERICA					AFRICA	Total number of countries
	AT	BG	CH	CZ	CY	DE	ES	FI	FR	GE	GR	HR	HU	IT	LT	NO	PL	PT	RU	SE	SI	SK	TR	UK	CN	IN	RK	JP	MY	TH	TW	CA	CLA	MX	US	VE	ZA	
B. aberrans																									P*					P								2
B. abietinus	P																																					1
B. abruptus																																			P			1
B. baujardi																										P												1
B. bestiolus																																			P			1
B. borealis					P	P													P																			3
B. chitwoodi						P				P																												2
B. cocophilus																																	P	P		P		3
B. conicaudatus																												P										1
B. corneolus																																			P			1
B. crenati						P				P																												2
B. cryphali						P																P																2
B. digitulus																																				P		1
B. dongguanensis																									P													1
B. eggersi	P		P			P	P			P	P																											6
B. eidmanni						P				P												P																3
B. elytrus																																			P			1
B. eremus				P		P				P																												3
B. eroshenkii																			P																			1
B. erosus										P																												1
B. eucarpus						P				P																												2
B. fraudulentus	P					P				P			P						P																P			6
B. fuchsi																			P																			1
B. fungivorus				P		P	P																	P														4
B. georgicus										P																												1
B. glochis																	P																					1
B. gonzalezi																																				P		1
B. hellenicus						P					P							P	P						P													5
B. hofmanni	P			P		P												P			P				P													6
B. hunanensis																									P													1
B. hunti																												P										1
B. hylobianum							P											P	P											P								4
B. idius					P	P				P												P																4
B. incurvus						P				P																												2
B. kevivni																																			P			1
B. kolymensis																			P																			1
B. leoni	P				P	P	P		P		P			P				P	P																		P	10
B. lini																									P													1
B. luxuriosae																												P										1
B. maxbassiensis																																			P			1
B. minutus																										P												1
B. mucronatus	P	P		P		P	P	P	P		P			P		P	P	P	P	P			P		P		P	P		P	P	P						21
B. naujaci									P								P																					2
B. newmexicanus																																			P			1

Table 2. *(Continued).*

	EUROPE																							ASIA							AMERICA					AFRICA	Total number
	AT	BG	CH	CZ	CY	DE	ES	FI	FR	GE	GR	HR	HU	IT	LT	NO	PL	PT	RU	SE	SI	SK	TR	UK	CN	IN	RK	JP	MY	TH	TW	CA	CLA	MX	US	VE	ZA	of countries
B. nuesslini						**P**				P												P																3
B. paracorneolus						**P**													P																			2
B. pinasteri						P	P		**P**																													3
B. piniperdae	**P**					P				P												P																4
B. pinophilus						P											**P**	P																				3
B. pityogeni																																			**P**			1
B. poligraphi						**P**																P																2
B. rainulfi																													**P**									1
B. ratzeburgii						**P**				P																												2
B. sachsi						**P**																P																2
B. scolyti																																			**P**			1
B. seani																																			**P**			1
B. sexdentati	P	P			P	**P**	P			P	P			P	P			P	P																			11
B. silvestris									**P**																													1
B. sinensis																									**P**													1
B. steineri						**P**																																1
B. sutoricus										**P**																												1
B. sychnus						**P**																																1
B. talonus																																			**P**			1
B. teratospicularis					P	P	P			**P**	P	P		P				P																				8
B. thailandae																									P					**P**								2
B. tritrunculus																																			**P**			1
B. tusciae						P								**P**				P																				3
B. typographi										**P**																												1
B. vallesianus			**P**																																			1
B. varicauda																																**P**						1
B. wekuae										**P**																												1
B. wilfordi																																			**P**			1
B. willi																																			**P**			1
B. xerokarterus						**P**				P																												2
B. xylophilus																		P							P		P	P			P	P		P	**P**			8
Total species	8	2	2	4	5	30	8	1	5	20	6	1	1	5	1	1	4	10	11	1	1	7	1	1	9	2	2	5	1	4	2	3	1	2	16	3	1	

*P = present. Letters in bold indicate the country from where the species was originally described.

Countries: AT – Austria; BG – Bulgaria; CH – Switzerland; CZ – Czech Republic; CY – Cyprus; DE – Germany; ES – Spain; FI – Finland; FR – France; GE – Georgia; GR – Greece; HR – Croatia; HU – Hungary; IT – Italy; LT – Lithuania; NO – Norway; PL – Poland; PT – Portugal; RU – Russia; SE – Sweden; SI – Slovenia; SK – Slovakia; TR – Turkey; UK – United Kingdom; CN – China; IN – India; RK – South Korea; JP – Japan; MY – Malaysia; TH – Thailand; TW – Taiwan; CA – Canada; CLA – Caribbean and Latin American regions (Belize, Brazil, Colombia, Costa – Rica, Ecuador, El Salvador, Grenada, Guyana, Honduras, Panama, Peru, St Vincent, Surinam, Tobago, Trinidad, West Indies); MX – Mexico; US – United States; VE – Venezuela; ZA – South Africa.

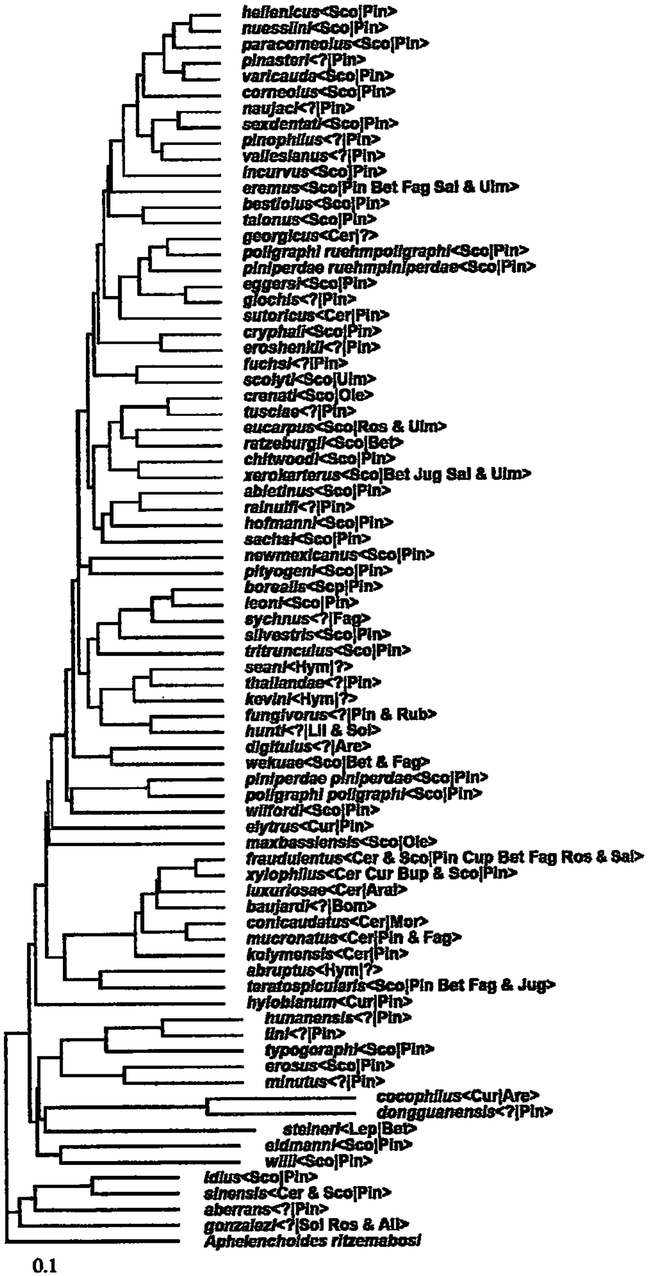

Fig. 24. *Dendrogram of general phenetic similarity (UPGMA, standard distance: mean character difference) of* Bursaphelenchus *species based on all characters (Table 1). In brackets: Vector families: Bup = Buprestidae; Cer = Cerambycidae; Cur = Curculionidae; Sco = Scolytidae; Hym = Halictidae; Lep = Sesiidae; Plant families: All = Alliaceae; Aral = Araliaceae; Are = Areaceae; Bet = Betulaceae; Cup = Cupressaceae; Fag = Fagaceae; Jug = Juglandaceae; Mor = Moraceae; Ole = Oleaceae; Pin = Pinaceae, Ros = Rosaceae; Rub = Rubiaceae; Sal = Salicaceae; Sol = Solanaceae; Ulm = Ulmaceae. Names:* piniperdae_piniperdae *and* piniperdae_ruehmpiniperdae, poligraphi_poligraphi *and* poligraphi_ruehmpoligraphi *refer to subspecies of* B. piniperdae *Fuchs, 1937 and* B. poligraphi *Fuchs, 1937, respectively.* Aphelenchoides ritzemabosi *is included as an outgroup.*

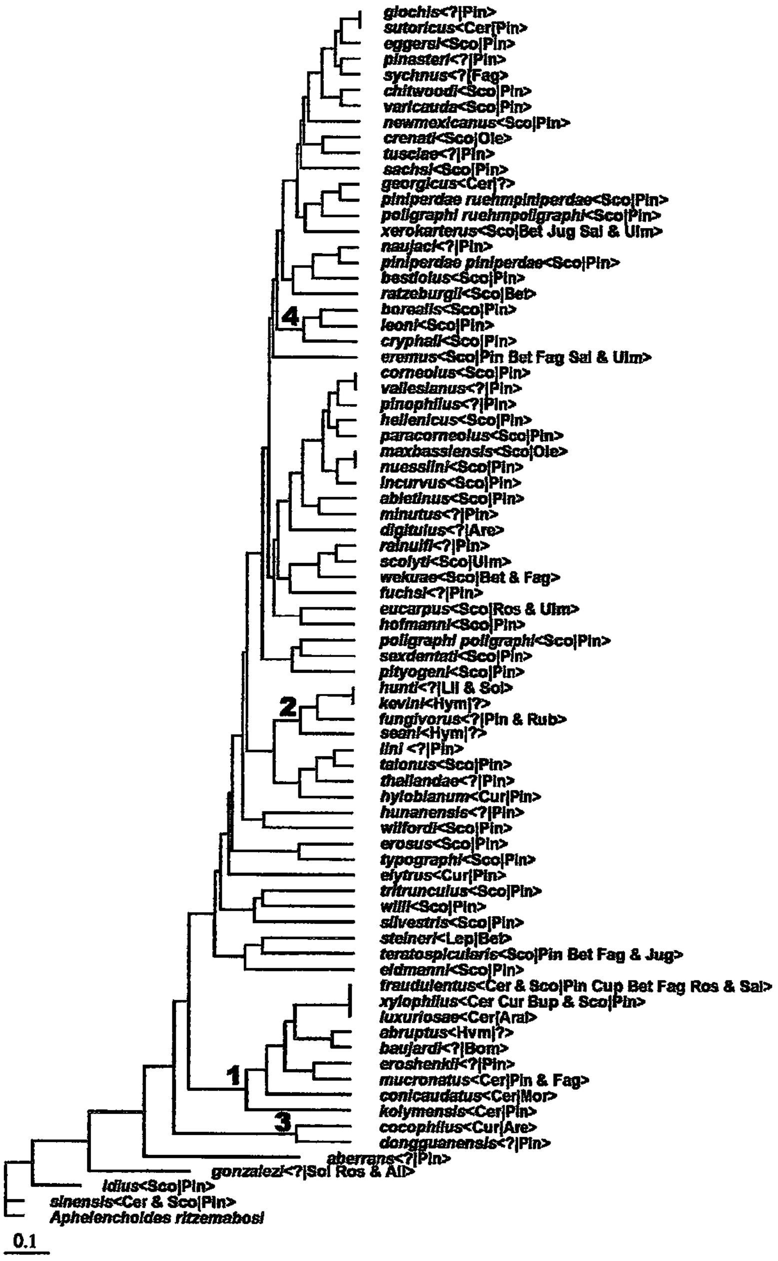

Fig. 25. *Dendrogram of general similarity (UPGMA, standard distance: mean character difference) of* Bursaphelenchus *spp., based only on spicule characters (1, 3, 5, 8, 10, 13-15, 18-21 in Table 1). In brackets: Vector families: Bup = Buprestidae; Cer = Cerambycidae; Cur = Curcullionidae; Sco = Scolytidae; Hym = Halictidae; Lep = Sesiidae. Plant families: All = Alliaceae; Aral = Araliaceae; Are = Areaceae; Bet = Betulaceae; Cup = Cupressaceae; Fag = Fagaceae; Jug = Juglandaceae; Mor = Moraceae; Ole = Oleaceae; Pin = Pinaceae, Ros = Rosaceae; Rub = Rubiaceae; Sal = Salicaceae; Sol = Solanaceae; Ulm = Ulmaceae. Clusters are numerated as: 1:* 'xylophilus' *cluster; 2:* 'hunti' *cluster; 3:* 'cocophilus' *cluster; 4:* 'borealis' *cluster. Names:* piniperdae_piniperdae *and* piniperdae_ruehmpiniperdae, poligraphi_poligraphi *and* poligraphi_ruehmpoligraphi *refer to subspecies of* B. piniperdae *Fuchs, 1937 and* B. poligraphi *Fuchs, 1937, respectively.* Aphelenchoides ritzemabosi *is included as an outgroup.*

Scolytidae-Pinaceae may be considered as primitive for the genus as it is typical for species at the root of the dendrogram, as well as for the more advanced groups in the upper part of the tree (Fig. 25).

The greatest deviation from the initial vector-associated plant combination may be seen in the *xylophilus*-group comprising *B. xylophilus*, *B. abruptus*, *B. baujardi*, *B. conicaudatus*, *B. eroshenkii*, *B. fraudulentus*, *B. kolymensis*, *B. luxuriosae* and *B. mucronatus* (cluster 1 in Fig. 25). This species-group has changed the presumed initial scolytid vector to beetles that are mainly from the family Cerambycidae. The *xylophilus*-group may therefore be considered as a 'natural' species group.

Bursaphelenchus crenati, a member of the *xylophilus*-group (in the diagnostic sense), clusters outside the main group. This species has the same shape of spicule as the other species in the group, yet lacks a cucullus. Only beetles of the family Scolytidae are known to vector this species and it may therefore be concluded that *B. crenati* is a member of the diagnostic *xylophilus*-group, but not the natural *xylophilus*-group (which is vectored by Cerambycidae). The presence of a cucullus therefore appears to be a highly significant character in the identification of this economically important group.

The *hunti*-group consists of two assemblages. One includes four species (cluster 2 in Fig. 25): *B. hunti*, *B. seani*, *B. kevini* and *B. fungivorus*, and may also be considered as a natural group. The basic TA complex of Scolytidae-Pinaceae has changed, Hymenoptera now serving as vectors and the associated plants belong to Liliaceae, Solanaceae and Rubiaceae. Another cluster (cluster 3 in Fig. 25) consists of the two rather similar species *B. cocophilus* and *B. dongguanensis*. This cluster is situated near the root of the dendrogram (Fig. 25).

The main part of the *borealis*-group (*B. borealis*, *B. cryphali*, *B. leoni*), a diagnostic group based on the posteriorly recurved condylus of the male spicule, forms cluster 4 in Figure 25. For this group the Scolytidae-Pinaceae complex is typical.

Other species-groups may be considered as purely diagnostic assemblages. In Figure 25, the *aberrans*-group is, based on the primitive characters, paraphyletic, its species being located at the root of the diagram (with TA complex Scolytidae-Pinaceae). The most numerous species-group is the *piniperdae*-group. It is undoubtedly paraphyletic and represents the majority of the genus with the exception of the above-mentioned natural groups (clusters 1-4 in Fig. 25) and the primitive paraphyletic assemblage of the *aberrans*-group. The basic Scolytidae-Pinaceae complex is typical for the *piniperdae*-group with rare changes of the vector to Cerambycidae (*B. sutoricus*, *B. georgicus*) and the associated plants to Fagaceae (*B. sychnus*).

EVOLUTIONARY TRENDS WITHIN THE TA COMPLEX

The initial TA complex of Scolytidae-Pinaceae is changeable but only rarely does the preferred vector shift to the Cerambycidae (the *xylophilus*-group) or Hymenoptera (the *hunti*-group), thereby leading to the formation of natural species-groups. In other cases the change of the vector to Cerambycidae (*B. georgicus*, *B. sutoricus*) or Lepidoptera (*B. steineri*) did not lead to the formation of natural superspecies groups, nor did the transition to other plant associations, such as: Oleaceae (*B. crenati*, *B. maxbassiensis*), Solanaceae (*B. hunti*, *B. gonzalezi*), Rosaceae (*B. gonzalezi*), Alliaceae (*B. gonzalezi*), Liliaceae (*B. hunti*), Rubiaceae (*B. wilfordi*), Ulmaceae (*B. scolyti*, *B. xerokarterus*), Betulaceae (*B. hofmanni*), Fagaceae (*B. wekuae*, *B. sychnus*), Araliaceae (*B. luxuriosae*), or Arecaceae (*B. digitulus*). It is clear that, although vector selection is changeable (Kulinich & Orlinsky, 1998), it is comparatively more important for the evolution of the genus *Bursaphelenchus* than associations with plants at the family level.

The third trophic component associated with the nematode are fungi, an association that may be of even greater significance in the origin and evolution of the genus *Bursaphelenchus* (Giblin-Davis *et al.*, 2003). However, data on the fungi species occurring in natural *Bursaphelenchus* associations are as yet insufficient for the detailed comparative analysis necessary to elucidate relationships.

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Appendix. *Records of* Bursaphelenchus *species with lists of natural vectors and associated plants.*

Species	Country	Insect vector	Associated plant	Reference	Notes
B. aberrans	China (Guangdong Province)		*Pinus massoniana* Lamb.* (Pinales: Pinaceae)	Fang *et al.*, 2002b	*Dead wood
	Thailand		*Pinus merkusi* Jungh & de Vriese* (Pinales: Pinaceae)	Braasch & Braasch-Bidasak, 2002	*Dead wood
	China (intercepted in Austria)		Load boards and pallets (tree not specified)*	Tomiczek *et al.*, 2003	*Imported wood
B. abietinus	Austria	*Pityokteines spinidens* (Reitter) (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Schmutzenhofer, 1981	
	Austria	*Pityokteines curvidens* (Germar), *P. spinidens* (Reitter), *P. vorontzovi* (Jacobson) (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Braasch & Schmutzenhofer, 2000	
B. abruptus	USA (Alabama)	*Anthophora abrupta* Say (Hymenoptera: Anthophoridae)		Giblin-Davis *et al.*, 1993	
B. baujardi	India (Haryana)		*Bombax ceiba* L. (Malvales: Bombacaceae)	Walia *et al.*, 2003	
B. bestiolus	USA (New Mexico)	*Dendroctonus adjunctus* Blandford (Coleoptera: Scolytidae)	*Pinus ponderosa* P. & C. Lawson (Pinales: Pinaceae)	Massey, 1974	
B. borealis	Russia (Magadan territory)	*Ips subelongatus* Motschulsky (Coleoptera: Scolytidae)	*Larix dahurica* Turcz. (Pinales: Pinaceae)	Korentchenko, 1980	
	Germany	*Dryocoetes autographus* Ratzeburg (Coleoptera: Scolytidae)	*Picea abies* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch *et al.*, 1999	
	Russia (Asian part; intercepted in Germany)		*Pinus sylvestris* L.* (Pinales: Pinaceae)	Braasch *et al.*, 2001	*Imported wood
	Cyprus		*Pinus brutia* Tenore (Pinales: Pinaceae)	Braasch & Philis, 2002	
B. chitwoodi	Germany	*Hylastes ater* (Fabricius) (Coleoptera: Scolytidae)	*Pinus sylvestris* L. (Pinales: Pinaceae)	Rühm, 1956	
	Georgia	*Hylastes ater* (Fabricius) (Coleoptera: Scolytidae)		Kakulia & Shalibashvili, 1976a	
B. cocophilus[1)]	Grenada, West Indies (southern Caribbean)		*Cocos nucifera* L. (Arecaceae: Cocosoideae)	Cobb, 1919	
	Caribbean and Latin American regions (Belize, Brazil, Costa-Rica, Ecuador, El Salvador, Granada, Guyana, Honduras, Mexico, Panama, St Vincent, Tobago, Trinidad, Venezuela)	*Rhynchophorus palmarum* L. (Coleoptera: Curculionidae)	*Cocos nucifera* L., *Elaeis guineensis* Jacq. (Arecaceae: Cocosoideae)	Brathwaite & Siddiqi, 1975	

Appendix. (*Continued*).

Species	Country	Insect vector	Associated plant	Reference	Notes
	Colombia and Surinam	*Rhynchophorus palmarum* L. (Coleoptera: Curculionidae)	*Elaeis guineensis* Jacq. (Arecaceae: Cocosoideae)		
	Costa Rica		*Cocos nucifera* L. (Arecales: Arecaceae)	Salazar, 1980	
	Brazil	*Rhynchophorus palmarum* L. (Coleoptera: Curculionidae)	*Elaeis guineensis* Jacq., *Oenocarpus distichus* Mart. (Arecales: Arecaceae)	Schuiling & Van Dinther, 1981	
	All countries in Central America, South America (Brazil, Colombia, Ecuador, Guyana, Peru, Venezuela); southern Caribbean (Grenada, St Vincent, Tobago, Trinidad)		*Cocos nucifera* L. (Arecales: Arecaceae)		
	Brazil, Colombia, Costa Rica, Ecuador, Guyana, Surinam, Venezuela		*Elaeis guineensis* Jacq. (Arecales: Arecaceae)	Gerber *et al.*, 1989	
	Brazil	*Metamasius* sp. (Coleoptera: Curculionidae)	*Elaeis guineensis* Jacq. (Arecales: Arecaceae)	Silva & Martins e Silva, 1991	
B. conicaudatus	Japan	*Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae)	*Ficus carica* L. (Urticales: Moraceae)	Kanzaki *et al.*, 2000	
B. corneolus	USA (New Mexico)	*Dendroctonus ajunctus* Blandford (Coleoptera: Scolytidae)	*Pinus ponderosa* P. & C. Lawson (Pinales: Pinaceae)	Massey, 1966	
B. crenati	Germany	*Hylesinus crenatus* (Fabricius) (Coleoptera: Scolytidae)	*Fraxinus excelsior* L. (Oleales: Oleaceae)	Rühm, 1956	
	Georgia	*Hylesinus crenatus* (Fabricius) (Coleoptera: Scolytidae)	*Fraxinus excelsior* L. (Oleales: Oleaceae)	Kurashvili *et al.*, 1980	
B. cryphali	Germany	*Cryphalus piceae* Ratzeburg (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Fuchs, 1930	
	Germany	*Cryphalus piceae* Ratzeburg (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Rühm, 1956	
	Slovakia	*Cryphalus piceae* Ratzeburg (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Tenkácová & Mituch, 1987, 1988	
B. digitulus	Venezuela		*Cocos nucifera* L. (Arecales: Arecaceae)*	Loof, 1964	*Skin of nut
B. dongguanensis	China (Guangdong Province)		*Pinus massoniana* Lamb.* (Pinales: Pinaceae)	Fang *et al.*, 2002a	*Dead wood

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
B. eggersi	Germany	*Hylurgops palliatus* (Gyllenhal) (Coleoptera: Scolytidae)	*Larix leptolepis* (Siebold & Zucc.), *Picea excelsa* (Lamb.), *Pinus sylvestris* L., *P. strobus* L. (Pinales: Pinaceae)	Rühm, 1956	
	Switzerland	*Hylurgops palliatus* (Gyllenhal) (Coleoptera: Scolytidae)			
	Georgia	*Hylurgops palliatus* (Gyllenhal) (Coleoptera: Scolytidae)	*Picea orientalis* (L.) (Pinales: Pinaceae)	Kakulia & Maglakelidze, 1973	
	Georgia	*Hylurgops palliatus* (Gyllenhal) (Coleoptera: Scolytidae)	*Abies* sp., *Larix* sp.; *Picea orientalis* (L.), *Pinus cedrus* L. (Pinales: Pinaceae)	Kurashvili *et al.*, 1980	
	Germany	*Hylurgops palliatus* (Gyllenhal) (Coleoptera: Scolytidae)	*Picea abies* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch *et al.*, 1999	
	Greece		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Skarmoutsos & Skarmoutsos, 1999	
	Austria	*Hylurgops palliatus* (Gyllenhal) (Coleoptera: Scolytidae)		Tomiczek, 2000	
	Spain		*Abies alba* Mill., *Pinus pinaster* Aiton, (Pinales: Pinaceae)	Escuer *et al.*, 2002	
	Spain		*Pinus pinaster* Aiton, *P. radiata* D. Don (Pinales: Pinaceae)	Abelleira *et al.*, 2003	
	Spain		*Abies alba* Mill., *Pinus pinaster* Aiton, (Pinales: Pinaceae)	Escuer *et al.*, 2004a	
	Greece		*Pinus brutia* Tenore, *P. pinaster* Aiton (Pinales: Pinaceae)	Michalopoulos-Skarmoutsos *et al.*, 2004	
B. eidmanni	Germany	*Ips typographus* L. (Coleoptera: Scolytidae)	*Picea abies* (L.), *P. excelsa* (Lamb.), *P. sitchensis* (Bong.) (Pinales: Pinaceae)	Rühm, 1956	
	Georgia	*Ips typographus* L. (Coleoptera: Scolytidae)		Kakulia, 1971	
	Georgia	*Ips typographus* L. (Coleoptera: Scolytidae)	*Abies* sp., *Larix* sp., *Picea orientalis* (L.), *Pinus cedrus* L., *P. sosnowskyi* Nakai (Pinales: Pinaceae)	Kurashvili *et al.*, 1980	
	Uzbekistan (Surkhandar'insk region)		*Beta vulgaris* L. (Caryophyllales: Chenopodiaceae)	Khaliknazarov & Khurramov, 1989*	*Doubtful record because unusual host plant and absence of description

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Slovakia	*Ips typographus* L., *I. amitinus* (Eichhoff) (Coleoptera: Scolytidae)	*Picea abies* (L.), *P. excelsa* (Lamb.), *P. sitchensis* (Bong.) (Pinales: Pinaceae)	Tenkácová & Mituch, 1987, 1991	
B. elytrus	USA (Connecticut)	*Pissodes approximatus* Hopkins, (Coleoptera: Curculionidae) *P. strobi* (Peck)	*Pinus resinosa* Soland. (Pinales: Pinaceae)	Massey, 1971b	
B. eremus	Germany	*Scolytus intricatus* (Ratzeburg) (Coleoptera: Scolytidae)		Rühm, 1956	
	Georgia	*Scolytus intricatus* (Ratzeburg) (Coleoptera: Scolytidae)	*Populus gracilis* Grossh., *Salix* sp. (Salicales: Salicaceae), *Castanea vulgaris* Hance, *Quercus iberica* Steven ex Bieb., *Q. pedunculata* Ehrh., *Q. sessiliflora* Salisb. (Fagales: Fagaceae), *Ulmus foliacea* Gilib. (Urticales: Ulmaceae)	Kurashvili *et al.*, 1980	
	Czech Republic	*Scolytus intricatus* (Ratzeburg) (Coleoptera: Scolytidae)	*Quercus* spp. (Fabales: Fagaceae)	Kubatova *et al.*, 2000	
B. eroshenkii	Russia (Primorsky territory)		*Pinus sibirica* Du Tour (Pinales: Pinaceae)*	Kolossova, 1998	*Dead wood
B. erosus	Georgia	*Orthotomicus erosus* (Woll.) (Coleoptera: Scolytidae)	*Abies* sp., *Picea orientalis* (L.), *Pinus sosnowskyi* Nakai (Pinales: Pinaceae)	Kurashvili *et al.*, 1980	
B. eucarpus	Germany	*Scolytus mali* (Bechstein & Scharfenberg) (Coleoptera: Scolytidae)	*Malus silvestris* Mill., *Pyrus communis* L. (Rosales: Rosaceae)	Rühm, 1956	
	Georgia	*Scolytus mali* (Bechstein & Scharfenberg) (Coleoptera: Scolytidae)	*Malus domestica* Borkh., *Prunus* sp., *Sorbus* sp. (Rosales: Rosaceae), Ulmaceae gen. sp. (Urticales)	Kurashvili *et al.*, 1980	
B. fraudulentus	Germany	*Cerambyx scopolii* Fuesslins (Coleoptera: Cerambycidae), *Trypophloeus granulatus* (Ratzeburg) (Coleoptera: Scolytidae)	*Populus nigra* L., *P. tremula* L. (Salicales: Salicaceae) *Prunus avium* (L.) (Rosales: Rosaceae)	Rühm, 1956	
	Georgia			Kakulia *et al.*, 1980	
	Germany		*Quercus* sp. (Fagales: Fagaceae)	Balder, 1987, 1989*	*Described as *B. mucronatus*
	Germany		*Fagus silvatica* L., *Quercus robur* L. (Fagales: Fagaceae)	Schauer-Blume, 1987*	*Described as '*B. mucronatus* like population'

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Germany		*Alnus glutinosa* (L.), *Betula pendula* Roth, *B. pubescens* Ehrh. (Betulales: Betulaceae), *Fagus sylvatica* L. *Quercus robur* L., *Q. petraea* (Mattuschka) (Fagales: Fagaceae), *Prunus avium* (L.), *P. cerasus* L. (Rosales: Rosaceae)	Schauer-Blume & Sturhan, 1989	
	Austria		*Quercus robur* L. (Fagales: Fagaceae)	Braasch *et al.*, 1995	
	Germany		*Betula pendula* Roth (Betulales: Betulaceae), *Prunus avium* (L.), *P. cerasus* L. (Rosales: Rosaceae), *Quercus* sp., *Q. robur* L. (Fagales: Fagaceae)		
	Hungary		*Quercus petraea* (Mattuschka) (Fagales: Fagaceae)		
	USA (Oregon and Washington)		*Pinus monticola* Douglas ex D. Don (Pinales: Pinaceae), *Thuja plicata* Donn ex D. Don (Pinales: Cupressaceae)*		*Dead wood
	Germany		*Picea* sp., *Pinus* sp. (Pinales: Pinaceae)	Braasch *et al.*, 1999	
	Russia (Krasnoyarsk; intercepted wood)		*Larix* sp., *Larix sibirica* Ledeb. (Pinales: Pinaceae)*	Braasch *et al.*, 2001	*Imported wood
B. fuchsi	Russia (Primorsky territory)		*Pinus koraiensis* Sieb. & Zucc. (Pinales: Pinaceae)*	Kruglik & Eroshenko, 2004	*Dead wood
B. fungivorus	UK (North Wales)		*Gardenia* sp. (Gentianales: Rubiaceae)*	Franklin & Hooper, 1962	*Buds infected by *Botrytis cinerea* in glasshouse
	Germany		Growing medium containing bark*	Braasch *et al.*, 1999	*Glasshouse
	Czech Republic		Coniferous bark	Braasch*	*As unpublished in Braasch, 2001
	Spain (Andalusia)		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Escuer *et al.*, 2002	
	Czech Republic, Germany		Growing medium containing bark	Braasch *et al.*, 2002	
	Spain		*Pinus* sp., *P. pinaster* Aiton (Pinales: Pinaceae)	Escuer *et al.*, 2004a, b	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
B. georgicus	Georgia	*Rhopalopus macropus* Germar (Coleoptera: Cerambycidae)		Devdariani *et al.*, 1980	
B. glochis	Poland		*Pinus sylvestris* L. (Pinales: Pinaceae)	Brzeski & Baujard, 1997	
B. gonzalezi	Venezuela		*Allium sativum* var. *vulgare* L. (Amaryllidales: Alliaceae), *Solanum tuberosum* L. (Solanaceae: Solanaceae)	Loof, 1964	
B. hellenicus	Greece		*Pinus brutia* Tenore (Pinales: Pinaceae)	Skarmoutsos *et al.*, 1998	
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch *et al.*, 1999	
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch *et al.*, 2000	
	Greece	*Tomicus piniperda* (L.) (Coleoptera: Scolytidae)			
	Russia (intercepted wood)		*Larix* sp. (Pinales: Pinaceae)*	Braasch *et al.*, 2001	*Imported wood
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002	
	China (Yunnan Province)		*Pinus yunnanensis* Franchet (Pinales: Pinaceae)	Dan & Yu, 2003	
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2004	
	Greece		*Pinus brutia* Tenore, *Pinus halepensis* Mill. (Pinales: Pinaceae)	Michalopoulos-Skarmoutsos *et al.*, 2004	
B. hofmanni	Germany		*Picea abies* (L.) (Pinales: Pinaceae)	Braasch, 1998	
	Germany		*Picea abies* (L.) (Pinales: Pinaceae)	Braasch *et al.*, 1999	
	Austria	*Pityokteines curvidens* (Germar) (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Braasch *et al.*, 2000; Tomiczek, 2000	
	Czech Republic		Imported coniferous wood.	Tomiczek & Braasch*	*As unpublished in Braasch, 2001
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002	
	Slovenia		*Pinus* sp. (Pinales: Pinaceae)	Urek & Sirca, 2003	
	China (Yunnan Province)		*Pinus armandii* Franchet (Pinales: Pinaceae)	Dan & Yu, 2003	
B. hunanensis	China (Hunan province)		*Pinus massoniana* Lamb. (Pinales: Pinaceae)*	Yin *et al.*, 1988	*Dead wood

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
B. hunti	Japan (intercepted by Quarantine Service, USA in Alabama)		*Lilium tigrinum* Ker. (Liliales: Liliaceae)*	Steiner, 1935	*Bulbs
B. hylobianum	Russia (Magadan territory)	*Hylobius albosparsus* Boheman (Coleoptera: Curculionidae)	*Larix dahurica* Turcz. (Pinales: Pinaceae)	Korentchenko, 1980	
	Russia (intercepted in Germany)		*Larix sibirica* Ledeb., *Pinus sylvestris* L. (Pinales: Pinaceae)*	Braasch *et al.*, 2001	*Intercepted wood
	Thailand		*Pinus merkusi* Jungh & de Vriese (Pinales: Pinaceae)*	Braasch & Braasch-Bidasak, 2002	*Dead wood
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002	
	Spain		*Pinus radiata* D. Don (Pinales: Pinaceae)	Escuer *et al.*, 2004a	
	Portugal	*Hylobius* sp. (Coleoptera: Curculionidae)	*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2004	
	China		*Pinus massoniana* Lamb. (Pinales: Pinaceae)	Wang *et al.*, 2004	
B. idius	Germany	*Pityogenes chalcographus* L. (Coleoptera: Scolytidae)	*Picea excelsa* (Lamb.) (Pinales: Pinaceae)	Rühm, 1956	
	Georgia	*Pityogenes chalcographus* L. (Coleoptera: Scolytidae)	*Pinus* sp. (Pinales: Pinaceae), *Carpinus caucasica* Grossh. (Betulales: Betulaceae), *Juglans* sp. (Juglandales: Juglandaceae), *Populus tremula* L. (Salicales: Salicaceae), *Quercus iberica* Steven ex Bieb. (Fagales: Fagaceae)	Kurashvili *et al.*, 1980	
	Slovakia	*Pityogenes chalcographus* L. (Coleoptera: Scolytidae)	*Picea abies* (L.) (Pinales: Pinaceae)	Tenkácová & Mituch, 1987; Vilagiova, 1993	
	Cyprus		*Pinus brutia* Tenore (Pinales: Pinaceae)*	Braasch*	*As unpublished data in Braasch, 2001
	Cyprus		*Pinus brutia* Tenore (Pinales: Pinaceae)	Braasch & Philis, 2002	
B. incurvus	Germany	*Dendroctonus micans* (Kugel.) (Coleoptera: Scolytidae)	*Abies alba* Mill., *Picea excelsa* (Lamb.), *P. sitchensis* (Bong.), *P. breweriana* S. Watson, *Pinus pungens* Lamb. (Pinales: Pinaceae)	Rühm, 1956	

Appendix. (*Continued*).

Species	Country	Insect vector	Associated plant	Reference	Notes
	Georgia	*Dendroctonus micans* (Kugel.) (Coleoptera: Scolytidae)	*Abies* sp., *Picea orientalis* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)	Kurashvili *et al.*, 1980	
B. kevini	USA (California, Oregon and Idaho)	*Halictus farinosus* Smith., *H. ligatus* Say (Hymenoptera: Halictidae)		Giblin *et al.*, 1984	
B. kolymensis	Russia (Magadan territory)	*Monochamus sutor* (L.) (Coleoptera: Cerambycidae)	*Larix dahurica* Turcz. (Pinales: Pinaceae)	Korentchenko, 1980	
B. leoni	France		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Baujard, 1980	
	Italy		*Pinus halepensis* Mill., *P. pinaster* Aiton, *P. pinea* L. (Pinales: Pinaceae)	Ambrogioni *et al.*, 1994	
	Italy		*Pinus halepensis* Mill., *P. pinaster* Aiton, *P. pinea* L. (Pinales: Pinaceae)	Palmisano & Ambrogioni, 1994	
	Cyprus		*Pinus brutia* Tenore, *P. pinea* L. (Pinales: Pinaceae)	Philis & Braasch, 1996	
	Cyprus		*Pinus brutia* Tenore, *P. nigra* Arnold, *P. pinea* L. (Pinales: Pinaceae)	Philis, 1996	
	Italy		*Pinus halepensis* Mill., *P. pinaster* Aiton, *P. pinea* L., *P. sylvestris* L. (Pinales: Pinaceae)	Ambrogioni & Caroppo, 1998	
	South Africa		*Pinus radiata* D. Don (Pinales: Pinaceae)	Braasch *et al.*, 1998	
	Italy		*Pinus halepensis* Mill., *P. pinaster* Aiton, *P. pinea* L., *P. sylvestris* L. (Pinales: Pinaceae)	Caroppo *et al.*, 1998	
	Germany	*Dryocoetes autographus* Ratzeburg (Coleoptera: Scolytidae)		Braasch *et al.*, 1999	
	Greece		*Pinus brutia* Tenore, *P. nigra* Arnold, *P. pinaster* Aiton, *P. radiata* D. Don (Pinales: Pinaceae)	Skarmoutsos & Skarmoutsos, 1999	
	Austria		*Pinus nigra* Arnold, *P. sylvestris* L. (Pinales: Pinaceae)	Braasch *et al.*, 2000; Tomiczek, 2000	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch*	*As unpublished data in Braasch, 2001
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Braasch*	*As unpublished data in Braasch, 2001
	Russia (Serov, intercepted wood)		*Picea* sp., *Pinus* sp. (Pinales: Pinaceae)*	Braasch *et al.*, 2001	*Imported wood
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Schönfeld *et al.*, 2001	
	Cyprus		*Pinus brutia* Tenore, *P. nigra* Arnold (Pinales: Pinaceae)	Braasch & Philis, 2002	
	Spain (Balearic Islands)		*Pinus halepensis* Mill. (Pinales: Pinaceae)	Escuer *et al.*, 2002	
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002, 2004	
	Spain		*Pinus pinea* L. (Pinales: Pinaceae)	Escuer *et al.*, 2004a	
	Greece		*Pinus brutia* Tenore, *P. halepensis* Mill., *P. nigra* Arnold, *P. pinaster* Aiton, *P. radiata* D. Don (Pinales: Pinaceae)	Michalopoulos-Skarmoutsos *et al.*, 2004	
B. lini	China (Nanjing)		*Pinus massoniana* Lamb., *P. thunbergii* Parl. (Pinales: Pinaceae)*	Braasch, 2004b	*Dead wood
B. luxuriosae	Japan	*Acalolepta luxuriosa* Bates (Coleoptera: Cerambycidae)	*Aralia elata* (Miq.) (Apiales: Araliaceae)	Kanzaki & Futai, 2003	
B. maxbassiensis	USA (North Dakota)	*Hylesinus californicus* (Swaine) (syn. *Lepersinus californicus* Swaine) (Coleoptera: Scolytidae)	*Fraxinus pennsylvanica* Marsh. (Oleales: Oleaceae)	Massey, 1971a	
B. minutus	India (Himachal Pradesh)		*Pinus wallichiana* AB Jackson (Pinales: Pinaceae)*	Walia *et al.*, 2003	*Dead wood
B. mucronatus	Japan	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Pinus densiflora* Sieb. & Zucc., *P. thunbergii* Parl., *P. pentaphylla* Mayr. (Pinales: Pinaceae)	Mamiya & Enda, 1979	

Appendix. (*Continued*).

Species	Country	Insect vector	Associated plant	Reference	Notes
	France		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Baujard *et al.*, 1979*	*Reported as *B. lignicolus*
	France		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Baujard, 1980*	*Reported as *B. lignicolus*
	China		*Cedrus deodara* (Roxb. ex D. Don), *Pinus densiflora* Sieb. & Zucc., *P. elliotti* G. Engelm., *P. massoniana* Lamb., *P. pinaster* Aiton, *P. rigida* Mill., *P. serotina* Michx., *P. taeda* L., *P. thunbergii* Parl. (Pinales: Pinaceae)	Li, 1983	
	China		Wilted tree (species not specified)	Yang, 1985	
	China		*Pinus* spp. (Pinales: Pinaceae)	Wang & Shi, 1986	
	China (Anhui province)		*Cedrus deodara* (Roxb. ex D. Don), *Pinus massoniana* Lamb., *P. taeda* L., *P. thunbergii* Parl. (Pinales: Pinaceae)	Jiang, 1988	
	Norway		*Pinus sylvestris* L. (Pinales: Pinaceae)	McNamara & Stoen, 1988	
	Korea	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Pinus* spp. (Pinales: Pinaceae)	Choi & Moon, 1989	
	Sweden	*Monochamus galloprovincialis* (Olivier), *M. sutor* (L.) (Coleoptera: Cerambycidae)	*Picea abies* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)	Magnusson & Schroeder, 1989	
	Finland	*Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae)	*Picea abies* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)	Tomminen *et al.*, 1989	
	Korea (Chinju, Chinhae)	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)		Lee *et al.*, 1990	
	Finland	*Monochamus galloprovincialis* (Olivier), *M. sutor* (L.) (Coleoptera: Cerambycidae)		Tomminen, 1990	
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch, 1991	
	Russia (Yenisei region; intercepted wood)		*Pinus sylvestris* L. (Pinales: Pinaceae)*	Braasch, 1991	*Intercepted wood (sawn timber)
	Italy	*Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae)	*Pinus pinaster* Aiton (Pinales: Pinaceae)	Palmisano *et al.*, 1992	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Russia (Primorsky Territory)		*Pinus koraiensis* Sieb. & Zucc., *P. sylvestris* L. (Pinales: Pinaceae)	Kulinich *et al.*, 1994	
	Italy		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Palmisano & Ambrogioni, 1994	
	Canada (Quebec)		*Abies balsamea* (L.) (Pinales: Pinaceae)	Braasch *et al.*, 1995	
	China (Sichuan)		*Pinus massoniana* Lamb. (Pinales: Pinaceae)		
	France		*Pinus pinaster* Aiton (Pinales: Pinaceae)		
	Finland		*Pinus sylvestris* L. (Pinales: Pinaceae)		
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)		
	Italy	*Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae)			
	Japan		*Pinus densiflora* Sieb. & Zucc. (Pinales: Pinaceae)		
	Norway		*Pinus sylvestris* L. (Pinales: Pinaceae)		
	Russia (Siberia)		*Pinus sylvestris* L. (Pinales: Pinaceae)		
	Russia		*Pinus* sp. (Pinales: Pinaceae)	Kulinich & Kolossova, 1995	
	Poland		*Pinus* sp. (Pinales: Pinaceae)	Brzeski & Slipinska, 1996	
	Russia (Far East)	*Monochamus saltuarius* (Gebler) (Coleoptera: Cerambycidae)		Eroshenko & Kruglik, 1996	
	China (Zhejiang)	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Pinus massoniana* Lamb., *P. thunbergii* Parl. (Pinales: Pinaceae)	Lai *et al.*, 1996	
	Poland		*Pinus sylvestris* L. (Pinales: Pinaceae)	Brzeski & Baujard, 1997; Brzeski & Brzeski, 1997	
	Taiwan		*Pinus taiwanensis* Hayata (Pinales: Pinaceae)	Yen *et al.*, 1997	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Italy	*Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae)	*Pinus nigra* Arnold, *P. pinaster* Aiton, *P. strobus* L., *P. sylvestris* L. (Pinales: Pinaceae)	Ambrogioni & Caroppo, 1998; Caroppo *et al.*, 1998	
	Russia		*Abies* sp., *Pinus* spp. *Picea* spp. (Pinales: Pinaceae)	Kulinich & Orlinski, 1998	
	China (Zhejiang province)		*Pinus massoniana* Lamb., *P. thunbergii* Parl. (Pinales: Pinaceae)	Wang & Yie, 1998	
	Germany	*Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae)	*Larix decidua* Mill., *Picea abies* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch *et al.*, 1999	
	Japan	*Monochamus saltuarius* (Gebler) (Coleoptera: Cerambycidae)	*Pinus densiflora* Sieb. & Zucc. (Pinales: Pinaceae)	Jikumaru & Togashi, 1999	
	Greece		*Pinus brutia* Tenore (Pinales: Pinaceae)	Skarmoutsos & Skarmoutsos, 1999	
	China (Zhejiang province)		*Pinus* spp. (Pinales: Pinaceae)	Wang *et al.*, 1999	
	Austria		*Abies alba* Mill., *Larix decidua* Mill., *Picea abies* (L.), *Pinus nigra* Arnold, *P. sylvestris* L. (Pinales: Pinaceae)	Tomiczek, 2000	
	Bulgaria		*Pinus* sp. (Pinales: Pinaceae)	Choleva*	*As *pers. comm.* in Braasch, 2001
	Czech Republic		*Pinus sylvestris* L. (Pinales: Pinaceae)	Tomiczek*	*As *pers. comm.* in Braasch, 2001
	Spain		*Pinus* sp. (Pinales: Pinaceae)	Braasch, 2001*	*Rep. EU survey (2000)
	Russia (intercepted wood)		*Larix* sp., *Larix sibirica* Ledeb., *Picea* sp., *Pinus* sp., *P. sylvestris* L. (Pinales: Pinaceae)*	Braasch *et al.*, 2001	*Imported wood
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Schönfeld *et al.*, 2001	
	Thailand		*Pinus* sp. (Pinales: Pinaceae)	Braasch & Braasch-Bidasak, 2002	
	Spain		*Pinus* spp., *P. halepensis* Mill. (Pinales: Pinaceae)	Escuer *et al.*, 2002	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002	
	France & Ukraine (intercepted in Spain)		Sawmills, imported wood*	Abelleira *et al.*, 2003	*Imported wood
	Norway		*Pinus sylvestris* L. (Pinales: Pinaceae)	Magnusson *et al.*, 2003	
	China (intercepted in Austria)		Load boards and pallets (tree not specified)*	Tomiczek *et al.*, 2003	*Imported wood
	China (Yunnan province)		*Pinus armandii* Franchet, *P. yunnanensis* Franchet (Pinales: Pinaceae)	Dan & Yu, 2003	
	Spain		*Pinus halepensis* Mill., *P. nigra* Arnold, *P. sylvestris* L. (Pinales: Pinaceae)	Escuer *et al.*, 2004a, b	
	Norway		*Pinus sylvestris* L. (Pinales: Pinaceae)	Magnusson *et al.*, 2004	
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2004	
	Greece		*Pinus brutia* Tenore (Pinales: Pinaceae)	Michalopoulos-Skarmoutsos *et al.*, 2004	
	Turkey		*Pinus nigra* Arnold (Pinales: Pinaceae)	Vieira *et al.*, 2004	
	China (Huangshan Scenic Area)	*Arhopalus rusticus* (L.), *Spondylis buprestoides* L., *Monochamus alternatus* Hope (Coleoptera: Cerambycidae)		Zhao *et al.*, 2004	
B. naujaci	France		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Baujard, 1980	
	Poland		*Pinus sylvestris* L. (Pinales: Pinaceae)	Brzeski & Baujard, 1997	
B. newmexicanus	USA (New Mexico)	*Hylurgops* sp. (Coleoptera: Scolytidae)	*Pinus ponderosa* P. & C. Lawson (Pinales: Pinaceae)	Massey, 1974	
B. nuesslini	Germany	*Pityokteines curvidens* (Germar) (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Rühm, 1956	
	Georgia	*Pityokeines curvidens* (Germar) (Coleoptera: Scolytidae)		Kakulia & Shalibashvili, 1976b	
	Slovakia	*Pityokteines curvidens* (Germar) (Coleoptera: Scolytidae)	*Abies alba* Mill. (Pinales: Pinaceae)	Tenkácová & Mituch, 1987; 1988	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
B. paracorneolus	Germany		*Picea abies* (L.), *Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch, 2000	
	Russia (intercepted wood)		*Larix sibirica* Ledeb. (Pinales: Pinaceae)*	Braasch *et al.*, 2001	*Imported wood
B. pinasteri	France		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Baujard, 1980	
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Schönfeld *et al.*, 2001	
	Spain		*Pinus* sp. (Pinales: Pinaceae)	Rep. EU survey (2000)*	*In Braasch, 2001
	Spain		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Escuer *et al.*, 2002, 2004b	
	Spain		*Pinus pinaster* Aiton, *P. pinea* L. (Pinales: Pinaceae)	Escuer *et al.*, 2004a	
B. piniperdae	Germany, The Netherlands	*Tomicus piniperda* (L.) (Coleoptera: Scolytidae)	*Pinus sylvestris* L. (Pinales: Pinaceae)	Fuchs, 1937	
	Germany	*Tomicus piniperda* (L.) (Coleoptera: Scolytidae)	*Picea excelsa* (Lamb.), *Pinus sylvestris* L., *P. montana* Mill. (Pinales: Pinaceae)	Rühm, 1956	
	Georgia	*Blastophagus* sp. (Coleoptera: Scolytidae)		Kurashvili *et al.*, 1980	
	Slovakia	*Tomicus piniperda* (L.) (Coleoptera: Scolytidae)	*Pinus sylvestris* L. (Pinales: Pinaceae)	Tenkácová & Mituch, 1987; Vilagiova & Mituch, 1991	
B. pinophilus	Poland		*Pinus sylvestris* L. (Pinales: Pinaceae)	Brzeski & Baujard, 1997	
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch*	*As unpublished data in Braasch, 2001
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas & Braasch*; Penas *et al.*, 2002	*As unpublished data in Braasch, 2001
B. pityogeni	USA (New Mexico)	*Pityogenes carinulatus* (LeConte) (Coleoptera: Scolytidae)	*Pinus ponderosa* P. & C. Lawson (Pinales: Pinaceae)	Massey, 1974	
B. poligraphi	Germany	*Polygraphus poligraphus* (L.) (Coleoptera: Scolytidae)	*Picea abies* (L.) (Pinales: Pinaceae)	Fuchs, 1937	
	Germany	*Polygraphus poligraphus* (L.) (Coleoptera: Scolytidae)	*Picea excelsa* (Lamb.) (Pinales: Pinaceae)	Rühm, 1956	
	Slovakia	*Polygraphus poligraphus* (L.) (Coleoptera: Scolytidae)	*Picea abies* (L.) (Pinales: Pinaceae)	Tenkácová & Mituch, 1987	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Germany	*Hylurgops palliatus* (Gyllenhal), *Polygraphus poligraphus* (L.) (Coleoptera: Scolytidae)	*Picea abies* (L.) (Pinales: Pinaceae)	Braasch *et al.*, 1999	
B. rainulfi	Malaysia (Peninsular Malaysia)		*Pinus caribaea* Morelet. (Pinales: Pinaceae)*	Braasch & Burgermeister, 2002	*Dead wood
B. ratzeburgii	Germany	*Scolytus ratzeburgii* Janson (Coleoptera: Scolytidae)	*Betula verrucosa* Ehrh. (Betulales: Betulaceae)	Rühm, 1956	
	Georgia	*Scolytus ratzeburgii* Janson (Coleoptera: Scolytidae)	*Betula* sp. (Betulales: Betulaceae)	Kurashvili *et al.*, 1980	
B. sachsi	Germany	*Dryocoetes autographus* Ratzeburg (Coleoptera: Scolytidae)	*Picea excelsa* (Lamb.) (Pinales: Pinaceae)	Rühm, 1956	
	Slovakia	*Dryocoetes autographus* Ratzeburg (Coleoptera: Scolytidae)	*Picea abies* (L.) (Pinales: Pinaceae)	Tenkácová & Mituch, 1987	
B. scolyti	USA (Colorado)	*Scolytus multistriatus* (Marsh.) (Coleoptera: Scolytidae)	*Ulmus americana* L. (Urticales: Ulmaceae)	Massey, 1974	
B. seani	USA (California)	*Anthophora bomboides stanfordiana* Cockerell (Hymenoptera: Anthophoridae)		Giblin & Kaya, 1983	
B. sexdentati	Germany	*Ips sexdentatus* (Boerner) (Coleoptera: Scolytidae)		Rühm, 1960	
	Georgia	*Ips sexdentatus* (Boerner) (Coleoptera: Scolytidae)	*Picea orientalis* (L.), *Pinus sosnowskyi* Nakai (Pinales: Pinaceae)	Kurashvili *et al.*, 1980	
	Georgia, Lithuania, Russia	*Ips sexdentatus* (Boerner) (Coleoptera: Scolytidae)		Vosilite, 1990	
	Italy		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Palmisano & Ambrogioni, 1994; Ambrogioni *et al.*, 1994	
	Italy		*Pinus halepensis* Mill., *P. pinaster* Aiton, *P. pinea* L. (Pinales: Pinaceae)	Ambrogioni & Caroppo, 1998; Caroppo *et al.* (1998)	
	Germany	*Tomicus piniperda* (L.) (Coleoptera: Scolytidae)	*Pinus sylvestris* L. (Pinales: Pinaceae)	Braasch *et al.*, 1999	
	Greece		*Pinus brutia* Tenore, *P. halepensis* Mill., *P. nigra* Arnold, *P. pinaster* Aiton, *P. radiata* D. Don (Pinales: Pinaceae)	Skarmoutsos & Skarmoutsos, 1999	
	Austria		*Pinus sylvestris* L. (Pinales: Pinaceae)	Tomiczek, 2000	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Bulgaria		*Pinus* sp. (Pinales: Pinaceae)	Choleva*	*As pers. comm. in Braasch, 2001
	Spain		*Pinus* sp. (Pinales: Pinaceae)	Rep. EU survey (2000)*	*In Braasch, 2001
	Cyprus		*Pinus brutia* Tenore (Pinales: Pinaceae)	Braasch & Philis, 2002	
	Spain		*Pinus* spp. (Pinales: Pinaceae)	Escuer *et al.*, 2002	
	Spain		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Abelleira *et al.*, 2003	
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002, 2004	
	Spain		*Abies alba* Mill., *Pinus pinaster* Aiton, *P. pinea* L., *P. sylvestris* L. (Pinales: Pinaceae)	Escuer *et al.*, 2004a, b	
	Greece		*Pinus brutia* Tenore, *P. nigra* Arnold, *P. pinaster* Aiton, *P. halepensis* Mill., *P. radiata* D. Don (Pinales: Pinaceae)	Michalopoulos-Skarmoutsos *et al.*, 2004	
B. silvestris	France	*Ips sexdentatus* (Boerner) (Coleoptera: Scolytidae)	*Pinus sylvestris* L. (Pinales: Pinaceae)	Lieutier & Laumond, 1978	
B. sinensis	China (wood intercepted in Austria)		*Pinus* sp. (Pinales: Pinaceae)*	Palmisano *et al.*, 2004	*Imported wood
B. steineri	Germany	*Synanthedon spheciformis* (Denis & Schiffermüller) (Lepidoptera: Sesiidae)	*Alnus glutinosa* (L.) (Betulales: Betulaceae)	Rühm, 1956	
B. sutoricus	Georgia	*Monochamus sutor* (L.) (Coleoptera: Cerambycidae)	*Pinus* sp. (Pinales: Pinaceae)	Devdariani, 1974	
B. sychnus	Germany		*Quercus pedunculata* Ehrh. (Fagales: Fagaceae)	Rühm, 1956	
B. talonus	USA (Utah)	*Dendroctonus monticolae* Hopk. (Coleoptera: Scolytidae)	*Pinus contorta* Douglas ex Loudon (Pinales: Pinaceae)	Thorne, 1935	
	USA	*Dendroctonus ponderosae* Hopk. (Coleoptera: Scolytidae)		Massey, 1974	
B. teratospicularis	Georgia	*Tomicus minor* (Hart.), (syn. *Blastophagus minor* (Hart.), *Orthotomicus proximus* Eich. (Coleoptera: Scolytidae)	*Picea orientalis* (L.), *Pinus nigra* Arnold (Pinales: Pinaceae)	Kakulia & Devdariani, 1965	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Georgia	*Orthotomicus proximus* Eich., *Taphrorychus bicolor* (Herbst) (Coleoptera: Scolytidae)	*Carpinus caucasica* Grossh. (Betulales: Betulaceae), *Juglans* sp. (Juglandales: Juglandaceae), *Pinus sosnowskyi* Nakai (Pinales: Pinaceae), *Populus tremula* L. (Salicales: Salicaceae), *Quercus iberica* Steven ex Bieb. (Fagales: Fagaceae)	Kurashvili *et al.*, 1980	
	Italy		*Pinus halepensis* Mill., *P. pinaster* Aiton, *P. pinea* L. (Pinales: Pinaceae)	Ambrogioni & Caroppo, 1998; Caroppo *et al.*, 1998	
	Greece		*Pinus brutia* Tenore, *P. halepensis* Mill. (Pinales: Pinaceae)	Skarmoutsos & Skarmoutsos, 1999	
	Croatia		*Cupressus sempervirens* L. (Pinales: Cupressaceae)	Braasch*	*As unpublished data in Braasch, 2001
	Cyprus		*Pinus brutia* Tenore (Pinales: Pinaceae)		
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)		
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)		
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Schönfeld *et al.*, 2001	
	Cyprus		*Pinus brutia* Tenore (Pinales: Pinaceae)	Braasch & Philis, 2002	
	Spain (Mallorca, Ibiza)		*Pinus halepensis* Mill. (Pinales: Pinaceae)	Escuer *et al.*, 2002	
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002, 2004	
	Spain		*Pinus pinea* L. (Pinales: Pinaceae)	Escuer *et al.*, 2004a	
	Greece		*Pinus brutia* Tenore, *P. pinaster* Aiton, *P. halepensis* Mill. (Pinales: Pinaceae)	Michalopoulos-Skarmoutsos *et al.*, 2004	
B. thailandae	Thailand		*Pinus merkusi* Jungh & de Vriese* (Pinales: Pinaceae)	Braasch & Braasch-Bidasak, 2002	*Dead wood
	China (wood intercepted in Austria)		Load boards and pallets*	Tomiczek *et al.*, 2003	*Intercepted wood

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	China (wood intercepted in Austria)		*Pinus* sp. (Pinales: Pinaceae)	Palmisano *et al.*, 2004	*Intercepted wood
B. tritrunculus	USA (Texas)	*Dendroctonus terebrans* (Olivier) (Coleoptera: Scolytidae)	*Pinus taeda* L. (Pinales: Pinaceae)	Massey, 1974	
B. tusciae	Italy		*Pinus pinea* L. (Pinales: Pinaceae)*	Ambrogioni & Palmisano, 1998	*Dead wood
	Germany		*Pinus sylvestris* L. (Pinales: Pinaceae)	Schönfeld *et al.*, 2001	
	Portugal		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Penas *et al.*, 2002, 2004	
B. typographi	Georgia	*Ips typographus* L. (Coleoptera: Scolytidae)	*Picea orientalis* (L.) (Pinales: Pinaceae)	Kakulia, 1967	
B. vallesianus	Switzerland		*Pinus sylvestris* L. (Pinales: Pinaceae)*	Braasch *et al.*, 2004	*Dead wood
B. varicauda	Canada (British Columbia)	*Dendroctonus pseudotsugae* (Hopkins) (Coleoptera: Scolytidae)		Thong & Webster, 1983	
B. wekuae	Georgia	*Trypophloeus* sp. (erroneously named as *Trypodendron sygnatum*) (Coleoptera: Scolytidae)	*Carpinus caucasica* Grossh. (Betulales: Betulaceae), *Fagus orientalis* Lipsky. (Fagales: Fagaceae)	Kurashvili *et al.*, 1980	
B. wilfordi	USA (New Mexico)	*Scolytus ventralis* (LeConte) (Coleoptera: Scolytidae)	*Abies concolor* (Gord. & Glend.) (Pinales: Pinaceae)	Massey, 1964	
B. willi	USA (New Mexico)	*Dendroctonus valens* (LeConte) (Coleoptera: Scolytidae)	*Pinus ponderosa* P. & C. Lawson (Pinales: Pinaceae)	Massey, 1974	
B. xerokarterus	Germany	*Scolytus scolytus* (Fabricius), *S. multistriatus* (Marsh.) (Coleoptera: Scolytidae)	*Ulmus campestris* L., *U. pedunculata* Foug. (Urticales: Ulmaceae)	Rühm, 1956	
	Georgia	*Scolytus scolytus* (Fabricius) (Coleoptera: Scolytidae)		Kakulia & Devdariani, 1967	
	Georgia	*Scolytus scolytus* (Fabricius), *S. multistriatus* (Marsh.) (Coleoptera: Scolytidae)	*Ulmus foliacea* Gilib., *Zelkova* sp. (Urticales: Ulmaceae), *Carpinus caucasica* Grossh. (Betulales: Betulaceae), *Juglans* sp. (Juglandales: Juglandaceae), *Populus nigra* L. (Salicales: Salicaceae)	Kurashvili *et al.*, 1980	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
B. xylophilus[2,3)]	USA (Texas, Virginia)	*Ips* sp., *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae)	*Pinus echinata* Mill., *P. palustris* Mill. (Pinales: Pinaceae)	Steiner & Buhrer, 1934	
	Japan		*Pinus densiflora* Sieb. & Zucc., *P. thunbergii* Parl. (Pinales: Pinaceae)	Mamiya & Kiyohara, 1972	
	Japan	*Acanthocinus griseus* (Fabricius), *Arhopalus rusticus* (L.), *Corymbia succedanea* (Lewis), *Monochamus alternatus* Hope, *Spondylus buprestoides* (L.) (Coleoptera: Cerambycidae)		Mamiya, 1972	
	USA (Missouri)		*Pinus nigra* Arnold, *P. sylvestris* L. (Pinales: Pinaceae)	Dropkin & Foudin, 1979	
	Japan	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Cedrus deodara* (Roxb. ex D. Don) (Pinales: Pinaceae)	Ebine, 1980	
	USA	*Arhopalus rusticus obsoletus* (Rand.), *Monochamus carolinensis* (Olivier), *M. titillator* (Fabricius), *M. scutellatus* (Say), *M. obtusus* Casey (Coleoptera: Cerambycidae)	*Larix laricina* (Du Roi), *Pinus banksiana* Lamb., *P. cembra* L., *P. clausa* (Chapman ex Engelm.), *P. contorta* var. *murrayana* (Grev. & Balf.), *P. densiflora* Sieb. & Zucc., *P. echinata* Mill., *P. elliotti* Engelm., *P. mugo* Turra, *P. nigra* Arnold, *P. palustris* Mill., *P. ponderosa* P. & C. Lawson, *P. radiata* D. Don, *P. resinosa* Soland., *P. sylvestris* L., *P. strobus* L., *P. taeda* L., *P. thunbergii* Parl., *P. virginiana* Mill. (Pinales: Pinaceae)	Dropkin *et al.*, 1981	
	USA		*Pinus elliotti* Engelm., *P. cembra* L., *P. ponderosa* P. & C. Lawson, *P. thunbergii* Parl. (Pinales: Pinaceae)	Nickle *et al.*, 1981	
	USA (Delaware)		*Pinus resinosa* Soland., *P. rigida* Mill., *P. strobus* L., *P. sylvestris* L., *P. taeda* L., *P. thunbergii* Parl., *P. virginiana* Mill. (Pinales: Pinaceae)	Adams & Morehart, 1982	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	USA	*Amniscus sexguttata* (Say), *Arhopalus rusticus obsoletus* (Rand.), *Asemum striatum* (L.), *Monochamus carolinensis* (Olivier) (Coleoptera: Cerambycidae), *Chrysobothis* sp. (Coleoptera: Buprestidae), *Hylobius pales* (Herbst), *Pissodes approximatus* Hopkins (Coleoptera: Curculionidae)	*Cedrus deodora* (Roxb. ex D. Don), *C. atlantica* (Endl.), *Larix decidua* Mill., *L. laricina* (Du Roi), *Picea glauca* (Moench), *P. pungens* Engelm., *Pinus banksiana* Lamb., *P. cembra* L., *P. clausa* (Chapman ex Engelm.), *P. contorta* var. *murrayana* (Grev. & Balf.), *P. densiflora* Sieb. & Zucc., *P. echinata* Mill., *P. elliotti* Engelm., *P. halepensis* Mill., *P. mugo* Turra, *P. nigra* Arnold, *P. palustris* Mill., *P. ponderosa* P. & C. Lawson, *P. radiata* D. Don, *P. resinosa* Soland., *P. rigida* Mill., *P. strobus* L., *P. sylvestris* L., *P. taeda* L., *P. thunbergii* Parl., *P. virginiana* Mill. (Pinales: Pinaceae)	Kondo *et al.*, 1982	
	USA		*Pinus banksiana* Lamb., *P. nigra* Arnold, *P. resinosa* Soland., *P. strobus* L. (Pinales: Pinaceae)	Wingfield *et al.*, 1982	
	China (Nanjing)		*Pinus thunbergii* Parl. (Pinales: Pinaceae)	Cheng, 1983	
	USA (Missouri)	*Amniscus sexguttata* (Say), *Arhopalus rusticus obsoletus* (Rand.), *Asemum striatum* (L.), *Monochamus carolinensis* (Olivier) (Coleoptera: Cerambycidae), *Chrysobothis* sp. (Coleoptera: Buprestidae), *Hylobius pales* (Herbst), *Pissodes approximatus* Hopkins (Coleoptera: Curculionidae)	*Pinus sylvestris* L. (Pinales: Pinaceae)	Linit *et al.*, 1983	
	Canada (Belair Provincial Forest)		*Pinus banksiana* Lamb. (Pinales: Pinaceae)	Knowles *et al.*, 1983	
	USA (Virginia)	*Monochamus titillator* (Fabricius) *Neocanthocinus obsoletus* (Olivier) (Coleoptera: Cerambycidae)	*Picea glauca* (Moench), *Pinus sylvestris* L., *P. thunbergii* Parl. (Pinales: Pinaceae)	Carling, 1984	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Taiwan (Taipei prefecture)		*Pinus luchuensis* Mayr (Pinales: Pinaceae)	Tzean & Jan, 1985	
	China (Nanjing, Jurong, Zhenjiang)		*Pinus bungeana* Zuccarini ex Endlicher, *Pinus densiflora* Sieb. & Zucc., *P. massoniana* Lamb., *P. pinaster* Aiton, *Pinus thunbergii* Parl. (Pinales: Pinaceae)	Cheng, 1988	
	USA	*Amniscus sexguttata* (Say), *Arhopalus rusticus obsoletus* (Rand.), *Asemum striatum* (L.), *Monochamus carolinensis* (Olivier), *M. marmorator* Kirby, *M. mutator* LeConte, *M. obtusus* Casey, *M. scutellatus* (Say), *M. titillator* (Fabricius), *Neacanthocinus obsoletus* (Oliver), *N. pusillus* (Kirby), *Xylotrechus sagittatus* (Germar) (Coleoptera: Cerambycidae), *Chrysobothis* sp. (Coleoptera: Buprestidae), *Hylobius pales* (Herbst), *Pissodes approximatus* Hopkins (Coleoptera: Curculionidae)		Linit, 1988	
	Japan	*Acalolepta fraudatrix* (Bates), *Acanthocinus griseus* (Fabricius), *Arhopalus rusticus* (L.), *Corymbia succedanea* (Lewis), *Monochamus alternatus* Hope, *M. nitens* (Bates), *M. saltuarius* Gebl., *Spondylis buprestoides* L., *Uraecha bimaculata* Thomson (Coleoptera: Cerambycidae)			
	Korea (Pusan)	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Pinus densiflora* Sieb. & Zucc., *P. thunbergii* Parl. (Pinales: Pinaceae)	Yi *et al.*, 1989	
	Korea	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)		Lee *et al.*, 1990	

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Nigeria*		*Pinus caribaea* Morelet., *P. oocarpa* Schiede ex Schltdl., *Pinus kesiya* Royle ex Gordon (syn. *P. khasya* Royle), *P. merkusi* Jungh & de Vriese (Pinales: Pinaceae)	Khan & Gbadegesin, 1991	*This report, published without morphological or molecular data, needs to be confirmed
	Canada (Newfoundland (Island), Nova Scotia, New Brunswick, Quebec, Ontario, Manitoba, Saskatchewan, Alberta, British Columbia)		*Abies balsamea* (L.), *Larix laricina* (Du Roi), *Picea glauca* (Moench), *P. mariana* (Mill.), *P. rubens* Sarg., *Pinus banksiana* Lamb., *P. contorta* Dougl. ex Loud., *P. ponderosa* P. & C. Lawson, *P. resinosa* Soland., *P. strobus* L., *P. sylvestris* L., *Pseudotsuga menziesii* (Mirb.) (Pinales: Pinaceae)	Bowers *et al.*, 1992	
	Mexico (Nuevo León)		*Pinus pseudostrobus* Lindl. (syn. *Pinus estevesii* (Mart.) (Pinales: Pinaceae)	Dwinell, 1993	
	Taiwan	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)		Chang *et al.*, 1995	
	Canada		*Abies balsamea* (L.), *Pinus* sp., *P. banksiana* Lamb. (Pinales: Pinaceae), *Abies* and *Picea* chips and dunnage wood	Braasch *et al.*, 1995	
	Japan		*Pinus densiflora* Sieb. & Zucc. (Pinales: Pinaceae)		
	USA		*Abies balsamea* (L.), *Larix laricina* (Du Roi), *Pinus halepensis* Mill., *P. strobus* L., *P. sylvestris* L. (Pinales: Pinaceae)		
	Canada		*Pinus banksiana* Lamb., *P. contorta* Dougl. ex Loud., *P. strobus* L. (Pinales: Pinaceae)	Kishi, 1995	
	China		*Pinus elliotti* Engelm., *P. massoniana* Lamb., *P. thunbergii* Parl. (Pinales: Pinaceae)		
	Japan	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Cedrus deodara* (Roxb. ex D. Don), *Picea excelsa* (Lamb.), *Pinus bungeana* Zucc. ex Endl., *P. densiflora* Sieb. & Zucc., *P. echinata* Mill., *P. elliotii* Engelm.,		

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
			P. engelmannii Carr., *P. greggii* Engelm. ex Parl., *P. leiophylla* Schiede & Deppe, *P. luchuensis* Mayr, *P. massoniana* Lamb., *P. muricata* Dougl. ex D. Don, P. *nigra* Arnold, *P. oocarpa* Schiede ex Schltdl., *P. palustris* Mill., *P. parviflora* Siebold & Zucc., *P. pinaster* Aiton, *P. ponderosa* P. & C. Lawson, *P. pseudostrobus* Lindl., *P. radiata* D. Don, *P. rigida* Mill., *P. strobus* L., *P. sylvestris* L., *P. taeda* L., *P. thunbergii* Parl. (Pinales: Pinaceae)		
	Korea		*Pinus densiflora* Sieb. & Zucc., *P. thunbergii* Parl. (Pinales: Pinaceae)		
	Taiwan		*Pinus luchuensis* Mayr, *P. thunbergii* Parl. (Pinales: Pinaceae)		
	USA	*Amniscus sexguttata* (Say), *Arhopalus rusticus* (L.), *Asemum striatum* (L.), *Monochamus carolinensis* (Olivier), *M. marmorator* Kirby, *M. mutator* LeConte, *M. scutellatus* (Say), *Neacanthocinus pusillus* (Kirby), *Xylotrechus sagittatus* (Germar) (Coleoptera: Cerambycidae), *Chrysobothis* sp. (Coleoptera: Buprestidae), *Hylobius pales* (Herbst), *Pissodes approximatus* Hopkins (Coleoptera: Curculionidae)	*Abies balsamea* (L.), *Cedrus deodara* (Roxb. ex D. Don), *C. atlantica* (Endl.), *Larix europaea* DC., *L. americana* Michx., *Picea canadensis* (Mill.), *P. pungens* Engelm., *Pinus banksiana* Lamb., *P. cembra* L., *P. clausa* (Chapm. ex Engelm.), *P. contorta* Dougl. ex Loud., *P. densiflora* Sieb. & Zucc., *P. echinata* Mill., *P. elliotti* Engelm., *P. halepensis* Mill., *P. mugo* Turra, *P. nigra* Arnold, *P. palustris* Mill., *P. ponderosa* P. & C. Lawson, *P. radiata* D. Don, *P. resinosa* Soland., *P. rigida* Mill., *P. strobus* L., *P. sylvestris* L., *P. taeda* L., *P. thunbergii* Parl., *P. virginiana* Mill., *Pseudotsuga douglasii* (Sabine ex D. Don) (Pinales: Pinaceae)		

Appendix. *(Continued).*

Species	Country	Insect vector	Associated plant	Reference	Notes
	Taiwan		*Pinus armandii* var. *mastersiana* (Hayata), *P. elliotti* Engelm., *P. luchuensis* Mayr, *P. patula* Schiede & Schltdl. & Cham., *P. taeda* L., *P. taiwanensis* Hayata, *P. thunbergii* Parl. (Pinales: Pinaceae)	Chang & Lu, 1996	
	Taiwan		*Pinus taiwanensis* Hayata (Pinales: Pinaceae)	Yen *et al.*, 1997	
	Korea	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Pinus densiflora* Sieb. & Zucc., *P. thunbergii* Parl. (Pinales: Pinaceae)	La *et al.*, 1999	
	Portugal (Península de Setúbal)		*Pinus pinaster* Aiton (Pinales: Pinaceae)	Mota *et al.*, 1999; Penas *et al.*, 2004	
	Portugal (Península de Setúbal)	*Monochamus galloprovincialis* (Oliver) (Coleoptera: Cerambycidae)	*Pinus pinaster* Aiton (Pinales: Pinaceae)	Sousa *et al.*, 2001	
	China	*Monochamus alternatus* Hope (Coleoptera: Cerambycidae)	*Pinus densiflora* Sieb. & Zucc., *P. massoniana* Lamb., *P. pinaster* Aiton, *P. thunbergii* Parl. (Pinales: Pinaceae)	Yang, 2004	