Occurrence, taxonomy, biology and pathogenicity of aphelenchid nematodes associated with conifers in south-eastern Australia

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A thesis submitted for the degree of

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June 2006

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Statement

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June 2006

Acknowledgements

Abstract

Australia has large plantations of exotic *Pinus radiata* conifers. This species is highly susceptible to *Bursaphelenchus xylophilus*, the pine wilt nematode, which is not found in Australia. Potentially pathogenic nematodes were isolated from several dead *Pinus* trees in Williamstown, Heidelberg and Knoxfield, suburbs of Melbourne, Victoria in 2000-2002. A survey of the above-ground nematode fauna of *Pinus* and other conifers in south-eastern Australia was undertaken. Stands of *Pinus* were surveyed in the Kuipto Forest and the South-East Region of SA; the south-west and the Gippsland region of Victoria; and the Hume region in NSW; and native *Callitris preissii* was sampled in the Murray Mallee. A total of 1140 samples from *P. radiata*, 50 from *P. pinaster* and 40 from *C. preissii* were examined. No nematodes were found in wood or young shoots of conifers except in the wood samples from diseased trees at Knoxfield and Heidelberg in Victoria. In contrast, nematodes were common in the bark samples of healthy trees.

Morphologically, extracted nematodes were classified into five trophic groups, including: aphelenchida (plant, fungal and lichen feeders), rhabditids and areolaimids (bacterial feeding), *Macrolaimus* spp. (saprophagus), tylenchids (plant feeding), and dorylaimids (bacterial and algal feeders). Aphelenchids were the most commonly found trophic group. Three genera and twelve morphospecies of aphelenchids were identified. Eight species of *Laimaphelenchus* and one putative species of *Acugutturus* appear to be new records for Australia. Descriptions of two new species, *L. preissii* and *L. australis* have been published. Three species of *Aphelenchoides* were also found. No *Bursaphelenchus* spp. were found.

Molecular studies included sequencing of the ITS region of *Laimaphelenchus preissii*, morphospecies Aphelenchid K1, and Aphelenchid H1; D2D3 fragments of 28S and 18S of *L. preissii*, morphospecies Aphelenchid K1, Aphelenchid K2, and Aphelenchid H1, *Laimaphelenchus australis*, and *Laimaphelenchus* Heidelberg; and COI of three aphelenchid morphospecies *L. preissii*, *Laimaphelenchus* Heidelberg and Aphelenchid K1. Phylogenetic analyses confirmed that *Laimaphelenchus* spp.

are new species and that the unknown aphelenchids are close to *Aphelenchoides*. None of the six isolates studied from Australia was close to *Bursaphelenchus*.

Population growth and mean doubling time of *L. preissii*, Aphelenchid K1 and Aphelenchid H1 were studied at different temperatures and on different food resources. The different species had markedly different population growth rates, which were significantly affected by temperature and food.

A study on desiccation was carried out with *L. preissii* and morphospecies Aphelenchid K1, Aphelenchid K2, Aphelenchid H1 and *Laimaphelenchus* Heidelberg. Ability to survive desiccation varied between species, and the recovery rate of the different species was significantly different.

A pathogenicity study was performed using young *P. radiata* trees in a shadehouse. No symptoms were observed following inoculation with Aphelenchid K1, Aphelenchid K2, Aphelenchid H1 and *Laimaphelenchu*s Heidelberg isolated from diseased *P. radiata* in Victoria, or *L. preissii* from native *Callitris* in South Australia.

Chapter 1: Introduction

Nematodes are a complex, diverse group of animals that occur worldwide in most environments. Many species are important parasites of plants and animals. Plant parasitic nematodes have been recognised as agricultural pests for more than fifty years (Whitehead 1998), but it is only within the past 40 or so years that nematodes have been recognised as important pathogens of trees (Sutherland and Webster 1993). Initially, nematodes were recognised as causing losses in forest nurseries and plantations, but in recent years pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970, has become one of the most serious tree diseases in the world (Sutherland and Webster 1993). Pine wilt illustrates how a disease can change forests in just a few years (Ruehle 1972).

The pinewood nematode is an important cause of disease in conifers worldwide (Mamiya 1984). The nematode is native to North America, and also occurs in Japan, southern China, Taiwan, Canada and Portugal. It is thought to have entered Japan through imported timber, and since then it has been the most serious disease of native pines in Japan (Mamiya 1984). In 1984, there were about 650,000 ha of native pines infested by pinewood nematode in Japan, about 25% of the nation's total of 2.6 x 10^6 ha of pine forest. The annual loss of timber amounted to 2 x 10^6 m^3, with the death of nearly 10 x 10^6 trees (Mamiya 1984). Pine wilt disease is now internationally recognised as the most harmful disease of the conifer forestry industry.

In 2000-2002, some symptoms similar to those of pine wilt disease were found in trees at Heidelberg Park, Knoxfield and Williamstown, suburbs of Melbourne, Victoria. A nematode tentatively identified as *Bursaphelenchus hunanensis* (Yin, Fang *et al.* 1988) was extracted from the affected pine trees (Hodda, pers comm. 2003). Although the damage was limited to a relatively small number of trees, it was a strong alert to the Australian forestry industries. It is imperative that Australia maintains effective barriers against the entry of *Bursaphelenchus* and appropriate surveillance, to ensure early detection in the event of an incursion and to

support area freedom claims that underpin phytosanitary restriction on imports and market access for exports. This cannot be achieved without baseline information on the native and naturalised nematode fauna of conifers in Australia, and development of local expertise in identification of these nematodes. The studies described in this work aimed to build this essential scientific knowledge and expertise, vital for on-going protection of Australia's softwood resource.

Chapter 2: Review of Literature

2.1 Introduction

This review of the literature includes five parts. The first part covers Australian forests, and introduces Australian softwood plantations, including species grown, distribution, economic importance and future development. The second part is about nematodes in plantation pines, and covers the finding of nematodes in conifers and studies on their biology. The third part introduces nematode taxonomy, and discusses species concepts, taxonomic problems, and aphelenchid taxonomy. The fourth part deals with the pinewood nematode *B. xylophilus*, the most serious nematode pest of pine forests. In this part, the key features of its morphological taxonomy are described and the symptoms, mechanism vectors and occurrence of pine wilt disease are discussed. Finally, the gaps in knowledge and aims of this project are addressed.

2.2 Australian Forests

The Australian forest resource has two main components – native forest, predominantly angiosperm, and a large plantation forest (Lewis *et al.* 1993). In the native forest, hardwood species predominate, and the majority of these hardwoods belong to the family Myrtaceae including the genera *Eucalyptus, Angophora, Corymbia, Syncarpia* and *Tristania* (Hall *et al.* 1970). Of the plantation forests, one type comprises broad-leaved species, predominantly eucalypt but including poplars; and other coniferous species, dominated by softwoods, especially the genus *Pinus* (Lewis *et al.* 1993). This review will focus on native and plantation conifer resources in Australia.

2.2.1 Native softwood resources

Australia's native species of conifers are few in comparison with the diversity of hardwoods so characteristic of the Australian landscape. In all, there are only about a dozen species that have been of real economic importance at some time and most of the remaining species are shrubs of little significance (Hall *et al.* 1970).

The tropical and subtropical rainforests of the Australian east coast and highlands contain kauri (*Agathis* spp.), hoop and bunya pines (*Araucaria* spp.), brown and black pines (*Podocarpus* spp.) and brush cypress pines (*Callitris macleayana*) and are considered a source of high-quality softwood for ply and mill logs (Hall *et al.* 1970).

Further inland, in lower rainfall areas, the smaller white cypress pine (*Callitris glauca*) covers extensive tracts and is an important source of timber. Its timber is unsurpassed in resisting termite attack and is used largely for house stumps, floors, scantling, posts and poles (Hall *et al.* 1970).

Other valuable native softwood species are huon pine (*Dacrydium franklinii*), celery-top pine (*Phyllocladus asplenifolius*) and King William pine (*Athrotaxis selaginoides*). These occur in the temperate rainforest of the north-west and west coast regions of Tasmania; their timbers are particularly durable for softwoods and are used for a wide range of purposes such as boat building, vat construction, joinery, turnery and bentwork. Unfortunately, none of these species is plentiful, and, because of their slow growth, it is unlikely that they will become important in the future (Hall *et al.* 1970).

In Australia, the original source of logwood, begun with the first settlement, was the native forest. As this was depleted by exploitation and clearing for agriculture, a need to conserve and supplement arose variously in the different states, depending upon the extent and type of native forest resource that occurred in them (Lewis *et al.*

1993). Therefore, from early in the 19th century, exotic softwood plantations were
planted extensively in Australia.

2.2.2 Plantation pines

Australia has actively planted pines since the latter part of the 19th century. More
than two thirds of Australia's plantation forest resources were developed by state
government forestry services. In December 2003, Australia had around 1.7×10^6 ha
of plantations, of which about 1×10^6 ha were softwood and 0.7×10^6 ha hardwood
(Kelly *et al.* 2005).

2.2.2.1 Species

Early trials testing species for plantations included softwoods, eucalypts and other
broadleaf species (Lewis *et al.* 1993). *Pinus* had significantly higher growth rates in
the trials. There was also demand for softwood supplies in Australia, and a number
of *Pinus* species were planted successfully. The major coniferous plantation
resources are *Pinus radiata, P. elliotti, P. caribaea*, and *P. pinaster*.

2.2.2.2 Distribution

In September 2000, the state distributions of Australian softwood plantations were:
New South Wales (NSW) 271,000 ha, Victoria (Vic.) 215,000 ha, Queensland (Qld)
179,000 ha, South Australia (SA) 114,000 ha, Western Australia 98,000 ha,
Tasmania 76,000 ha, Northern Territory 5,000 ha and Australian Capital Territory
15,000 ha. Among these, 948,000 ha (97% of the total) were planted with *Pinus
radiata* (ABS, 2001).

2.2.2.3 Economic importance

The Australian forest products industry has an important role in the national
economy. The industry supports hardwood and softwood sawmilling, plywood and
panels manufacturing, woodchip production and export, and production of pulp and

papers. In 1999-2000, the industry was about 1% of Gross Domestic Product, and provided employment for nearly 75,000 people (ABS 2001). The industry is particularly important in providing economic development and employment in many regions of rural Australia. For example, Kuitpo in South Australia was the site of the first plantation forests established in the Mount Lofty Ranges in 1898. Kuitpo Forest now covers an area of some 3,600 ha, of which 60% (about 2,100 ha) is softwood plantation. The plantations currently yield about 116,000 m^3 metres of timber each year, valued in excess of $12,000,000. Kuitpo is a community forest, managed for sustainable forestry production, while providing for the conservation of native flora and fauna and community use for recreation (Charlma Phillips, pers. comm. 2006). In the South East Region of South Australia, the annual economic impact of the forestry sector and its associated flow-on effects was about AU$1 billion or 30% of the gross domestic product for the area in 2001, and supported nearly 7,000 jobs or 25% of the Region's employment (Charlma Phillips, pers. comm. 2006).

2.2.2.4 Future prospects

The report "Plantation for Australia: the 2020 Vision" was released in October 1997, by the Department of Sustainability and Environment, Victoria. Vision 2020 is a strategic partnership between the Commonwealth Government, the State Governments and the forest industry (ABS, 2001). Australia aims to increase the national plantation from 1.4 x 10^6 ha in 2001 to about 3 x 10^6 ha by 2020 (Spencer 2001). The Commonwealth Government has supported the expansion of Australia's plantation resource base for many years. The expansion will be in both hardwoods and softwoods. Therefore, the exotic and native conifers will continue to play an important role in future plantations in Australia.

2.3 Nematodes in Plantation Pines and Other Conifers

Plant-parasitic nematodes occur in only two orders: Dorylaimida (Adenophorea) and Tylenchida (Secernentea). The majority of classification schemes for the aphelenchids regard them as belonging to the suborder Aphelenchina Geraert within

the order Tylenchida (Hunt 1993). However, Siddiqi (1980) and Hunt (1993) argued that the aphelenchids were significantly different from the tylenchids. Therefore, Aphelenchida also has been thought of as an independent Order. The systematics and taxonomy of the Nematoda remains controversial, and will be discussed further in 2.4.2. The focus of this section will be on nematodes found in plantation pines and other conifers.

A wide variety of plant parasitic nematodes is found associated with *Pinus* in forest nurseries, plantation and natural forests (Sutherland and Webster 1993). For instance, Sutherland and Webster (1993) reported nematodes associated with pine plantations and nursery seedlings in Belgium, Croatia, France, Germany, Italy, Japan, Portugal, Russia and USA (nematodes in pine plantations were: *Cephalenchus* sp., *Criconema* sp., *Criconemoides* sp., *Helicotylenchus* spp., *Heterodera* sp., *Hoplolaimus* sp., *Meloidodera* sp., *Meloidogyne* spp., *Paratylenchus* spp., *Pseudhalenchus* sp., *Trichodrous* spp., *Tylenchus* sp., *Tylenchorhynchus* spp., *Xiphinema* spp. and *Bursaphelenchus* spp.; and in nursery seedlings were: *Helicotylenchus* spp., *Hemicycliophora* sp., *Hoplolaimus* spp., *Longidorus* sp., *Macroposthonia* sp., *Meloidodera* sp., *Meloidogyne* sp., *Paratrichodorus* sp., *Paratylenchus* spp., *Roytylenchus* sp., *Trichodorus* spp., *Tylenchus* spp., *Tylenchorhynchus* spp., and *Xiphinema* spp.). Other authors also reported more information about the nematodes associated with conifers. Magnusson (1981) described nematodes of pines and spruces. Braasch (1978) reported on those feeding on roots of pines. Lownsbery and Lownsbery (1985) reported on conifer and broadleaf forest nematodes occurring in California, USA. Ruehle (1972) described a possible role of nematodes associated with forest tree decline. Mcleod *et al.* (1994) listed nematodes associated with *Pinus* and conifers in Australia (Table 2.1 and Table 2.2). Over all, most of the nematodes listed were found in soil, of which the genera *Paratylenchus*, *Pratylenchus*, *Tylenchorhynchus*, *Trichodorus* and *Xiphinema* are common in forest nurseries, and ectoparasitic nematodes are found far more commonly than are endoparasites (Sutherland and Webster 1993). Nematodes important to forestry are diverse in types and forms. Discussion of those feeding in the rhizosphere, and those feeding in aerial parts of pine trees, follows.

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Table 2.1 Plant feeding nematodes recorded in association with *Pinus* spp. in Australia.

Hosts	State	Nematodes
Pinus caribaea	Qld	*Pratylenchus zeae, Radopholus rectus.*
P. elliottii	Qld	*Boleodorus thylactus, Cephalenchus emarginatus, Gracilacus peperpotti, Helicotylenchus exallus, Morulaimus whitei, Paratrichodorus minor, Paratylenchus coronatus, Pateracephalanema pectinatum, Pseudhalenchus minutus, Scutellonema minutum, Tylenchus sp.*
P. radiata	Qld	*Helicotylenchus sp., Paratrichodorus porosus, Tylenchorhynchus capitatus.*
	SA	*Hemicriconemoides obtusus.*
	Vic.	*Aphelenchoides bicaudatus, Pratylenchus penetrans, Radopholus sp., Rotylenchus robustus, Rotylenchus sp. Tylenchus sp.*
Pinus sp.	NSW	*Hemicycliophora ovata, Pateracephalanema australe, Radopholus crenatus, Rotylenchulus sp., Xenocriconemella macrodora.*
	Qld	*Criconema mutabile, Hemicriconemoides brachyurus, Heterodera sp., Xiphinema americanum.*
	SA	*Xiphinema radicicola.*
	Vic.	*Rotylenchus sp., Scutellonema sp.*

SA, South Australia; NSW, New South Wales; Vic., Victoria; Qld, Queensland (Modified from Mcleod, Reay, and Smyth, 1994).

Table 2.2 Plant feeding nematodes associated with native conifers in Australia.

Hosts	States	Nematodes
Callitris columellaris	NSW	*Helicotylenchus* sp., *Hemicycliophora truncata* *Macroposthonia curvata*, *Morulaimus arenicolus* *Morulaimus whitei*, *Paralongidorus eucalypti* *Pateracephalanema imbricatum*, *Rotylenchus* sp. *Xenocriconemella macrodora,* *Xiphinema americanum*, *Xiphinema radicicola.*
	Qld	*Gracilacus* sp., *Helicotylenchus* sp. *Hemicriconemoides obtusus,* *Paralongidorus eucalypti,* *Pateracephalanema imbricatum*, *Rotylenchus* sp. *Scutellonema minutum*, *Tylenchorhynchus* sp. *Xenocriconemella macrodora*, *Xiphinema insigne*
	SA	*Hemicriconemoides insignis* *Hemicriconemoides obtusus* *Paralongidorus eucalypti*
	Vic.	*Morulaimus* sp.
C. glaucophylla	SA	*Helicotylenchus* sp., *Hemicycliophora arenaria* *Pratylenchus* sp., *Rotylenchus* sp.
C. preissii	SA	*Hemicriconemoides obtusus* *Hemicycliophora arenaria* *Hemicycliophora halophila* *Hemicycliophora tesselata* *Morulaimus arenicolus* *Neodolichodorus adelaidensis* *Paralongidorus eucalypti* *Paralongidorus sacchari* *Pateracephalanema imbricatum* *Radopholus vangundyi*, *Rotylenchus gracilidens,* *Scutellonema minutum* *Tylenchorhynchus bastulatus*

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Table 2.2-Continued

Hosts	States	Nematodes
C. preissii murrayensis	Qld	*Morulaimus* sp.
Callitris sp.	NSW	*Criconema lanxifrons*
		Hemicrionemoides insignis
		Hemicrionemoides obtusus
		Hemicycliophora truncata, Xiphinema radicicola
	SA	*Hemicriconemoides insignis*
	Tas.	*Morulaimus whitei*
Nothofagus moorei	NSW	*Blandicephalanema bossi, Helicotylenchus* sp.
		Pateracephalanema imbricatum
		Pateracephalanema pectinatum
		Trophotylenchulus clavicaudatus
	Qld	*Helicotylenchus exallus, Helicotylenchus* sp.

SA, South Australia; NSW, New South Wales; Vic., Victoria; Qld, Queensland; Tas., Tasmania (Modified from Mcleod, Reay, and Smyth, 1994).

2.3.1 Nematodes inhabiting the rhizosphere of trees

Nematodes inhabiting the rhizosphere of trees can be divided into two groups (Ruehle 1972). The first includes myceliophagous nematodes feeding on mycorrhizae. For example, a species of *Aphelenchoides* is associated with mycorrhizae of southwestern American tree species and *Aphelenchus avenae* feeds on various mycorrhizal fungi and inhibits formation of mycorrhizae on red pine. The second group includes parasitic nematodes feeding directly on roots, such as the species of *Cephalenchus* and *Tylenchorhynchus* commonly occurring in nursery soils and around seedling roots. *Cephalenchus emarginatus*, prevalent in nursery soils in Canada and the United Kingdom, feeds and reproduces on several species of seedlings grown in eastern Canada (Sutherland and Webster 1993).

Inhibition of mycorrhizal formation by myceliophagous nematodes could indirectly contribute to root disease, since mycorrhizal symbionts protect roots from attack by soil pathogens (Marx and Davey 1969). Myceliophagous nematodes that damage mycorrhizae may reduce the beneficial effect of fungal symbionts and become important in the health and growth of trees in areas of marginal rainfall and fertility (Ruehle 1972).

2.3.2 Nematodes of aerial parts of pine trees

In comparison, the diversity of nematodes parasitic on the aerial parts of pine trees, where the environment is more variable, may actually be less than that of nematodes in the rhizosphere. However, given the economic importance of above ground nematodes of pines, they have deservedly attracted more attention. Ruehle (1972) suggested that the nematodes parasitic in forest trees above ground were usually limited to tropical regions. Normally, it is hard to find nematodes in the aerial parts, including wood, of healthy trees, but they can be easily found in weak, dying, diseased and dead trees. A number of nematodes has been found in aerial parts of pine trees and described. These include genera such as *Bursaphelenchus, Laimaphelenchus, Ektaphelenchus, Cryptaphelenchoides, Ektaphelenchoides,* and

Aphelenchoides (Hunt 1993). In this group, the nematodes normally have some relationship with insects, which act as vectors for them. According to the relationship between nematodes and their insect hosts, above ground nematodes can be placed in three groups: nematodes associated with beetle galleries; nematodes with alternating insect parasitic and mycetophagous free-living cycles in pine trees; and nematodes ecto-phoretically associated with insects and having mycetophagous free-living cycles in pine trees.

2.3.2.1 Nematodes associated with beetle galleries

Worldwide, bark beetles are among the most destructive insects in forests (Berryman 1974). In the western USA, the beetle genera *Dendroctonus* and *Ips* are the most destructive scolytids of coniferous forests and can destroy a large forest in a short period of time (Kaya 1984). In trees attacked by bark beetles, firstly the beetle's galleries can damage and greatly devalue the timbers, and secondly the beetles may introduce other potentially pathogenic micro-organisms to the trees, including nematodes and blue-stain fungus. Nematodes, such as *Contortylenchus* (Hunt and Hague 1974), *Bovienema* (Massey 1960) and *Parasitaphelenchus* (Rühm 1956) have been studied. These nematodes are associated with beetle galleries at different stages, but are all endoparasitic within the beetle for part of their life cycle.

2.3.2.2 Nematodes endoparasitic in insects and having mycetophagous free-living cycles in pine trees

These nematodes have a free-living cycle in pine trees. Both a tylenchid nematode (Ogura and Kosaka 1991) and *Deladenus siricidicola* (Bedding 1972) have been studied. These nematodes are endoparasitic in the insect's haemocol for part of their life-cycle but are free living in the pine tree to complete it. *Deladenus siricidicola* is successfully used for biological control of the wood wasp, *Sirex noctilio*, a serious pest *Pinus radiata* in Australia and New Zealand (Bedding 1972). A tylenchid nematode has been found in the reproductive organs of the Japanese pine sawyer, *Monochamus alternatus*, and may be a candidate as a biological control agent of the beetle (Ogura and Kosaka 1991).

2.3.2.3 Nematodes ectophoretically associated with insects and having mycetophagous free-living cycles in pine trees

Some nematodes only associate with insects as vectors to carry them to a new environment. These nematodes are said to have a phoretic association with insects. *Bursaphelenchus* is typical of this group of nematodes. Many *Burspahenchus* spp. are ectophoretic on insects, such as scolytid and cerambycid beetles and anthophorid and halictid bees. A few are endophoretic in insects, such as the *Bursaphelenchus* sp. associated with the nitidulid beetle *Urophorus humeralis*, and *B. abruptus* associated with dagger bee *Anthoophora abrupta* (Gibin-Davis 1985; 1993). The pinewood nematode, *B. xylophilus*, is an endoparasitic nematode vectored by beetles (Mamiya 1984; Giblin-Davis 1993). Due to its economic importance, extensive studies on *B. xylophilus* have been conducted in Japan, USA and China in the past thirty years, and more detail on this nematode is given in Section 2.5.

2.4 Nematode Taxonomy

The nematodes are a taxonomically, ecologically and geographically diverse group. The Phylum Nematoda is well delineated, and separate from all other organisms. However, the evolutionary relationships of the group with other organisms are far from clear (Inglis 1983; Hodda 2000) and the systematics of the group is controversial (Inglis 1983; Maggenti 1991b; De Ley and Blaxter 2002).

In higher level taxonomy, the Phylum Nematoda may consist of two classes (Adenophorea and Secernentea) and twenty-four orders. This classification is found in many textbooks, but has never been universally accepted (De Ley and Blaxter 2002). For example, Filipjev (1934) suggested that the nematodes comprise not less than eleven orders; Chitwood (1937) argued for two classes (Adenophorea and Secernentea); Goodey (1963) recognised ten orders; Inglis (1983) postulated that the Phylum Nematoda consisted of at least three classes (Rhabditea, Enoplea and Chromadorea) and twenty-five orders; Maggenti (1991) argued for two classes and eighteen orders; Lorenzen (1981; 1994) considered the free-living nematodes and argued for two classes of the Phylum; and Andrassy (1984) for two classes and

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eight orders. More recently, De Ley and Blaxter (2002) published a tentative system for nematode classification based on molecular evidence, which consists of two classes (Enoplea and Chromadorea) and twenty-one orders including two orders *Incertae sedis*. As De Ley and Blaxter (2002) pointed out, "it is probably even more true now than ever before, that nematode systematics is inherently prone to controversy and instability."

In lower level taxonomy, however, nematode classification is challenged by the extraordinary diversity of species and the difficulty of using morphological characters for species-level identification. What species are, and how they should be delimited in nature, has been a problem for biologists since the advent of taxonomy (Nadler 2002), and is far from resolved for biological research. In order to understand some of the difficulties for nematode taxonomy, the species concepts and problems are addressed in Section 2.4.1. In particular, aphelenchid taxonomy is discussed in Section 2.4.2.

2.4.1 Species concepts and problems

In the past, various species concepts have been recognised by nematologists; including the Linnean, biological, evolutionary, and phylogenetic species concepts (Adams 1998). Debate about what a species actually is, and how to define it, is ongoing. The Linnean, or typological morphospecies concept, delimits species as groups of organisms with the most overall similarity (Mayr 1963). The biological species concept (Mayr 1942) recognises species as groups of interbreeding natural populations that are reproductively isolated. The Evolutionary Species Concept (Simpson 1961; Wiley 1978) defines a species as "… a single lineage of ancestral-descendent populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Adams 1998). In contrast with the Linnean and biological species concept, the Evolutionary Species Concept was the first to realise the necessity of understanding evolutionary history in making rational statements about species by requiring that lineage and fates of lineages be identified (Adams 1998). The Phylogenetic Species Concept recognises species as the smallest units reflecting phylogenetic history that can be analysed by

cladistic methods (Rosen 1978, 1979; Nelson and Platnick 1981; Cracraft 1983, 1989; Nixon and Wheeler 1990).

Adams (1998) also pointed out that: "As long as the boundaries between populations and species have exhibited variations, systematists have at some point had to rationalise a way of distinguishing between the two. In the absence of any objective methodology within the Linnean paradigm, proponents of alternative species concepts have tried to establish objective solutions to the species problem (the problem of not being able to objectively identify just exactly what is or is not a species). Yet, in every case all current species concepts at some level fail to satisfy the objectives of taxonomy. If reproductive incompatibility is a requirement of species, how can unisexual species or fossils be accounted for? If species are based solely on the similarities and differences between populations, exactly how similar or different must they be?"

Realising this species problem, Myers (1952) pointed out that "definitional species concepts will always fail because taxonomic statements about species are predictions of future events, and the complex interactions within and among populations and species in the future are difficult to predict." Adams (1998) calls these failed predictions "predictive systematic errors". Thus, three possible errors existed for distinguishing a species in research. Type I error occurs when the taxonomist predicts more species than actually exist, type II error predicts fewer species than actually exist, and type III error occurs when a depiction of phylogenetic relationships among species is incongruent with recovered evolutionary history (Adams 1998). In practice, in terms of their potential to lead to misleading conclusions and to impede nematode research, type III errors are the most troublesome, followed by type II, and finally type I (Adams 1998).

2.4.2 Aphelenchid taxonomy

As mentioned in Section 2.3, the aphelenchs are regarded as the suborder Aphelenchina Geraert, 1966 in the Order Tylenchida Siddiqi, 1980 in most classification schemes (Andrássy 1976; Luc *et al.* 1987; Maggenti *et al.* 1987;

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Nickle and Hooper 1991). However, Siddiqi (1980) argued that the aphelenchs were sufficiently different from the tylenchs to be regarded as a separate order, based on a combination of morphological, biological and phylogenetic grounds. Hunt (1993) also said that Aphelenchida is sufficiently distinct in both morphology and certain biological aspects to justify the rank, but neither he nor Maggenti (1991b) accept the phylogenetic arguments of Siddiqi (1980). Blaxter *et al.* (1998) reported molecular evidence supporting separation of the Orders Tylenchida and Aphelenchida, but in a later classification DeLey and Blaxter (2002) combined both with rhabditids in the Order Rhabditida, and reduced the aphelenchs to the level of Superfamily Aphelenchoidea. Here, the classification according to Hunt (1993) will be used.

Hunt (1993) described the Aphelenchida as a moderately large order of nematodes containing two superfamilies, eight families, eleven subfamilies and thirty genera. Aphelenchs have a stylet for feeding and a very prominent median bulb in the oesophagus. They are cosmopolitan. Some are associated with insects, and may be ecto- or endoparasites, or merely use the insect as transport. Others are associated with plants, as root, stem or leaf parasites, and some are pathogenic to the plant. Still others are associated with fungi, and are free-living. Two families, Aphelenchoididae Skarbilovich, 1947 (Paramonov, 1953) and Parasitaphelenchidae Ruehm, 1956 (Siddiiqi, 1980), that contain many nematodes associated with conifers, will be highlighted in this literature review.

2.4.2.1 Aphelenchoididae Skarbilovich, 1947 (Paramonov, 1953)

Aphelenchoididae is the most taxonomically diverse family in the Order Aphelenchida. Most species currently recognised are in the ubiquitous genus *Aphelenchoides*. Aphelenchoidids are characterised by having short stylets with small to large basal swellings and a narrow lumen. The vermiform females have a functional anus and rectum, and lack elongate tails, and males may or may not have a rudimentary bursa. Some members are economically important as parasites of plants, and others of cultivated mushrooms. Other species cause the reduction of plant yields only under certain circumstances. Some species can cause diseases by

interacting with other micro-organisms (Hunt 1993; Hodda 2000). However, most species in this family are thought to be innocuous (Hunt 1993; Hodda 2000).

Aphelenchoides is a large, reasonably distinct genus of worldwide distribution. According to Hunt (1993), the genus contains 138 species and 16 species *inquirendae vel incertae sedis*. Others list over 180 species (Nickle 1992; Liu, Wu *et al.* 1999). Many nominal species are inadequately characterised for reliable recognition and the genus is in urgent need of a major revision (Hunt 1993). The majority of species appear to be free-living and are found in soil, decaying plant material, galleries of wood-boring beetles etc. However, a few species have been reported as ecto- and endoparasites of plants. The major plant-parasitic species in the genus include *A. bessyi* Christie, 1942 which causes 'white-tip' disease of rice, *A. fragariae* (Ritzema Bos 1890) Christie, 1932 and *A. ritzemabosi* (Schwartz 1911) Steiner, 1932, which have a wide host range (Hunt 1993). Moreover, a number of *Aphelenchoides* spp. have been reported from conifers in the USA, which include *A. rhytium* (Massey 1971), *A. conophthori* (Massey 1971), *A. hylurgi* (Massey 1974), *A. pityokteini* (Massey 1974), an *Apehelenchoides* sp. of the *A. parietinus* (Bastian 1865) Steiner, 1932 group, and *A. resinosi* (Kaisa *et al.* 1995).

2.4.2.2 Parasitaphelenchidae Ruehm, 1956 (Siddiqi, 1980)

Parasitaphelenchidae consist of two subfamilies (Hunt 1993). Parasitaphelenchinae have the fourth stage juvenile endoparastic in the insect haemocoel, a posterior vulva (85-90%), spicules partially fused, and the tail is not strongly recurved in the male. Bursaphelechinae have ectophoretic third stage dauerlarvae (exceptionally endophoretic), the vulva is more anterior (70-80%), the spicules are usually separate and the male tail is strongly recurved. The latter family is dominated by the genus *Bursaphelenchus*, characterised by having short stylets with the conus and shaft of similar length, small to large basal swellings and narrow lumens; stout to very slender vermiform females with a functional anus and rectum and tails of variable length, and males with or without a rudimentary bursa (Hunt 1993; Hodda 2000).

All known *Bursaphelenchus* spp. are ectophoretic or endophoretic on insects, phytoparasitic on palms or conifers, endoparasitic on insects, or free-living and mycetophagous. Two species, *B. cocophilus* (Cobb 1919) Baujard, 1989 and *B. xylophilus* are major economic pathogens of plants, and several others may be pathogens with limited distribution or pathogenic effects only under certain conditions (Hunt 1993; Mamiya 1999; Braasch and Schmutzenhofer 2000; Hodda 2000; Magnusson *et al.* 2004). Details on *Bursaphelenchus xylophilus* are presented in the following section.

2.5 The Pine Wood Nematode *Bursaphelenchus xylophilus*

2.5.1 Background

Pine wood nematode was first described as *Aphelenchoides xylophilus* by Steiner and Buhrer in 1934. It was largely ignored until Nickle transferred it to *Bursaphelenchus* (Nickle 1970). When Mamiya and Kiyohara (1972 a) extracted the causal nematode of pine wilt disease in Japan, they described it as *B. lignicolus*. Subsequently, Nickle *et al.* (1981) re-examined specimens of *B. lignicolus* and synonymised it with *B. xylophilus*. *Bursaphelenchus xylophilus* is the cause of pine wilt disease. It is thought to be native to North America, and was introduced to Japan early last century. Since then, it has also spread to China, South Korea and Taiwan and recently to Portugal (Sousa *et al.* 2001).

2.5.2 Pine wilt disease

2.5.2.1 Symptoms

Bursaphelenchus xylophilus is known as the 'pine wilt' or 'pine wood' nematode because of the symptoms it causes in susceptible *Pinus* species (Hunt 1993). Wilt diseases are less common in gymnosperms than in angiosperms. To date, only two wilt diseases of pines have been reported - one caused by blue-stain fungi of the genus *Ceratocytsis* (Basham 1970) and the other by the nematode *B. xylophilus*

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(Mamiya 1983). Symptoms begin to develop during summer or autumn, depending upon the time of infection. The first evidence of the disease requires microscopic examination and is the reduction and stoppage of resin (oleoresin) flow within the trees (Mamiya 1983). Following this, wilting symptoms begin to occur. Wilting may be confined to portions of diseased trees or affect entire plants. The colour of the needles changes from green to yellow and finally to reddish brown. Needles on dead trees remain attached through the winter and into the following year (Lambe *et al.* 1984).

2.5.2.2 Mechanism of disease

The mechanism of pine wilt disease has been investigated worldwide, especially in Japan and America. Togashi and Arakawa (2003) reported that three transmission pathways of *B. xylophilus* are recognised (Fig. 2.1). The primary mode of transmission is the phoretic fourth-stage dauer larva of the pine wood nematode which occurs during maturation feeding by the beetles (Mamiya and Enda 1972). During this, nematodes leave the beetle host and enter a maturation feeding wound on a healthy young pine twig. The nematodes then feed on plant cells within the pine host, inducing a lethal wilt. The secondary mode of transmission occurs when nematodes enter a dying tree or recently cut log through the host beetle's oviposition sites (Edwards and Linit 1992) and feed on fungi in dead wood. The third transmission pathway is during the mature male beetle's search for females via wounds such as oviposition wounds on the bark (Arakawa and Togashi 2002).

Although the transmission pathway of the nematode is well known, the reason it causes wilt disease is still not completely understood. Kiyohkara and Tokushige (Kiyohara 1984) demonstrated that nematode inoculation resulted in cessation of oleoresin exudation in tested trees long before they showed external symptoms. Sasaki *et al.* (1984) observed blockage of water conductivity in tracheids of pine seedlings possibly caused by cessation of resin flow, three days after nematode inoculation. The hypothesis of Myers (1988) was that the tree's death was induced by an innate hypersensitive defence mechanism. When the pine wood nematode invasion begins, they rapidly migrate in low numbers throughout the tree tissues. The hypersensitive reaction results in the release of phenolics, synthesis of toxins

and phyto-alexins during the catabolism of oleoresin and storage products, and the compartmentalisation of xylem and other tissues, followed by flooding of tracheids with oleoresin and toxic substances. The occurrence of plant cell death before nematode populations increase and spread through wood tissues indicates that some biochemical factors are involved in the pathological reactions of pine tissues (Myers 1988). Oku *et al.* (1979; 1980) pointed out that some metabolites produced by the pinewood nematode or associated bacteria had toxic effects on a pine tree. A number of chemicals have been isolated and identified from the infected pine trees, such as benzoic acid, catechol, dihydroconiferyl alcohol, and 8-hydroxycarbotanacetone (Kondo *et al.* 1982).
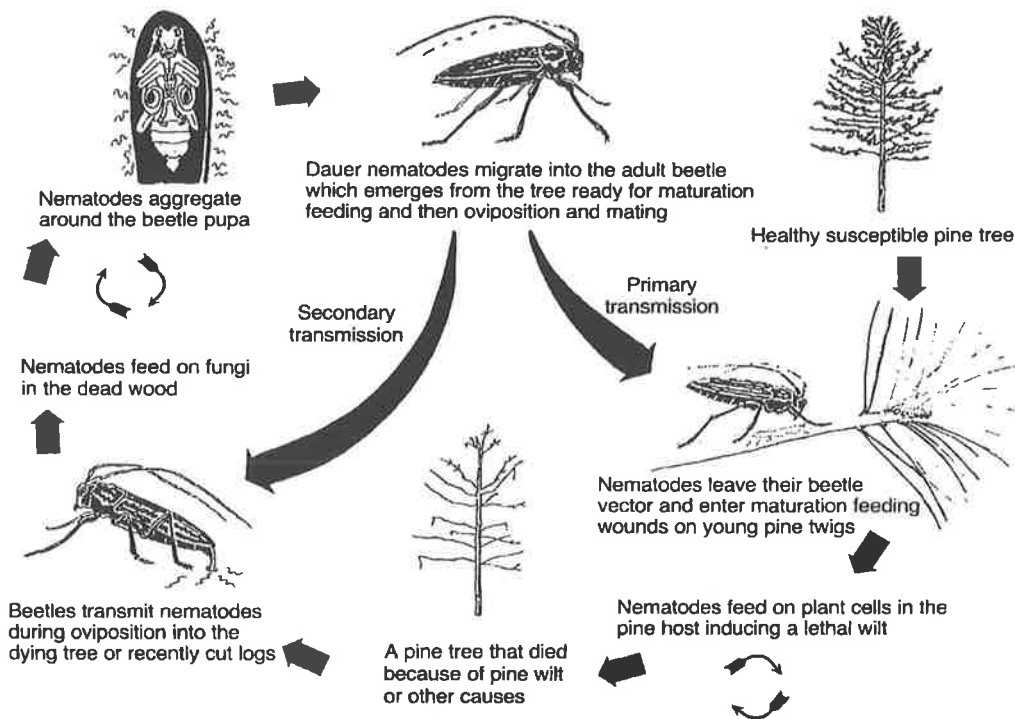
Fig. 2.1 The life cycle of *Bursaphelenchus xylophilus* (Giblin-Davis, 2000).

In addition, environmental factors affect nematode transmission and the mechanism by which dauer juveniles move onto and into *Monochamus* vectors. Stamps and Linit (2001) suggested that the amount of neutral storage (NS) lipid in the

nematodes was a modifier of nematode response to beetle- and tree-produced volatiles. Their results showed that nematodes with lowest NS lipid content were attracted to myrcene, a pine volatile, while nematodes with highest NS lipid content were attracted to toluene, a beetle cuticular hydrocarbon (Stamps and Linit 2001).

2.5.2.3 Vectors

Beetles act as vectors of *B. xylophilus* (Sutherland and Webster 1993). Dauer juveniles are transported to new host trees both on the body surface and within the tracheal system of several beetle species. Linit (1988) has reported that twenty one species in the Cerambycidae can act as vectors. They are *Acaloptera fraudatrix, Acanthocinus griseua, Amniscus sexguttatus, Arhoplus rusticus, A. rusticus obsoletus, Asemum striatum, Corymbia succedanea, Monochamus alternatus, M. carolinensis, M. marmorator, M. mutator, M. obtusus, M. scutellatus, M. titillator, M. nitens, M. saltuarius, Neacanthocinus obsoletus, N. pusillus, Spondylis busprestoides, Uraecha bimaculata* and *Xylotrechus saggitatus. Chysobothris* spp. (Buprestidae) and *Hylobius pales* and *Pissodes approximatus* (Curculionidae) are also known to carry pinewood nematode dauer juvenile on emergence from nematode-infected trees. However, only insect species within the genus *Monochamus* carry a high mean number of *B. xylophilus* dauer juveniles per adult beetle. Five species (four North American and one Japanese) of *Monochamus* are known to transmit dauer juveniles to new host trees (Linit 1988). The most common vector in Japan is *M. alternatus*, and in the USA *M. carolinensis* (Linit 1988). Recently, *M. galloprovincialis* has been identified as a vector in Portugal (Sousa *et al.* 2001).

2.5.2.4 Occurrence

The first occurrence of pine wilt disease in Japan dates back to 1905, and since then infested areas have spread progressively along the coastal areas of south western Japan, and inland areas of low elevation (Mamiya 1983). The infestation spread from only one location in Japan before 1930 to 34 prefectures through the 1940s, and to 45 of the 47 prefectures by the 1970s. During the 1930s, the annual loss of pine timber rose from 30,000 m³ to 200,000 m³. During the 1940s losses were

estimated at 400,000 m³. The heaviest loss of timber was recorded in 1979; approximately 2.4 x 10⁶ m³. In 1979, the pine wood nematode was first reported in the USA (Dropkin and Foudin 1979). Subsequently, extensive surveys revealed widespread occurrence of pine wood nematode throughout the country (Dropkin *et al.* 1981). In 1982, the first case of *B. xylophilus* was found in Jiangsu Province in China, now it has occurred in seven provinces (Yang 2003). *Bursaphelenchus xylophilus* was introduced into Taiwan in 1985, and Korea in 1988 (Mamiya 1987; Enda 1989). Most recently, it was found in Portugal (Mota *et al.* 1999).

2.5.2.5 Hosts and tolerance

The hosts of *B. xylophilus* include twenty species of *Pinus* and seven other conifers in North America (Robbins 1982). Mamiya (1983) investigated the resistance or susceptibility of various pine species to pine wilt disease. Twenty pine species are resistant and nineteen are susceptible. The resistant pine species are *P. banksiana, P. brutia, P. bungeana, P. caribaea, P. contora, P. echinata, P. elliotti, P. excelsa, P. halepensis, P. massoniana, P. palustris, P. pungens, P. resinosa, P. rigida, P. strobus, P. tabulaeformis, P. taeda, P. taiwanensis, P. rigida* x *P. taeda,* and *P. thunbergii* x *P. massoniana,* and the susceptible species are *P. densiflora, P. engelmannii, P. koraiensis, P. leiophylla, P. luchuensis, P. monticola, P. mugo, P. muricata, P. nigra, P. oocarpa, P. pentaphylla, P. pinaster, P. ponderosa, P. radiata, P. rudis, P. strobiforms, P. sylvestris, P. thunbergii* and *P. yunnanensis.* From a host tolerance test, Mamiya (1983) concluded that pine species vary in susceptibility to nematode infection. Some species had strong immunity to pine wilt disease. These include *P. taeda* and *P. elliottii*, which have been observed at various locations in Japan, healthy but surrounded by heavily damaged *P. densiflora* or *P. thunbergii* forests (Mamiya 1983). However, *P. radiata* and *P. pinaster* are highly susceptible to pine wilt disease (Mamiya 1983).

2.5.3 Taxonomy of pine wood nematodes

2.5.3.1 Morphological taxonomy

The key descriptive features of *B. xylophilus* were listed by Hunt (1993). Female: vulva posterior, with the anterior lip over-hanging to form a flap. Tail subcylindroid with a broadly rounded terminus, mucron usually absent, but some populations, especially from Japan, have a short 1-2 μm mucron. Male: spicules large, strongly arcuate so that prominent transverse bar is almost parallel to the body axis when the spicules are retracted. The distal tip of each spicule is expanded into a disc-like structure, named the cucullus by Yin *et al.* (1988). Seven caudal papillae are present; comprising one pair adanal, a single preanal ventromedian papilla and two postanal pairs near the tail spike and just anterior to the start of the bursa (Hunt 1993).

Identification of *B. xylophilus* is difficult because of its morphological similarity to *B. mucronatus* (Mamiya and Enda 1979). Mamiya and Enda (1979) described *B. mucronatus*, a closely related species with a strongly mucronate tail. Differentiation of *B. xylophilus* and *B. mucronatus* on morphological grounds alone is problematic and the two species were even considered conspecific (Hunt 1993). However, biochemical and cross-hybridization studies confirmed their separate identity (Webster *et al.* 1990; Abad *et al.* 1991; Riga *et al.* 1992).

2.5.3.2 Molecular approaches to taxonomy

Molecular biology techniques, especially DNA-based techniques, have brought a revolution in the biological sciences. For example, DNA-based methods require little biological material, thus allowing parallel identification of the same organism by classical methods. Moreover, DNA analysis does not require fresh material and tissue can be fixed in many kinds of media, e.g., ethanol, sodium chloride. Therefore, molecular techniques were developed rapidly for use in phylogenetic and evolution studies in nematodes and other animals. DNA-based techniques are sufficiently sensitive to distinguish species within *Drosophila* (Dowsett and Young

1982), *Caenorhabditis* (Emmons *et al.* 1979), *Trypanosoma* and *Brugia* (Majiwa and Webster 1987). Over the last decade, a number of molecular methods have been employed in studies on taxonomy, evolution and identification of nematodes. For example, Webster *et al.* (1990) used DNA probes to differentiate isolates of the pinewood nematode species complex. Judith and Matthew (1993) used DNA fingerprinting and polymerase chain reaction (PCR) to detect and identify *Bursaphelenchus* species.

Ribosomal DNA (rDNA) is useful for classifying eukaryotes at various taxonomic levels, because of its high sequence polymorphism (Iwahori *et al.* 1998). The structure of the rDNA cistron contributes to its wide applicability. The rDNA cistron is divided into domains that evolve at different rates and thus this region has been used to address diagnostic and evolutionary problems at different levels of divergence (Powers *et al.* 1997).

The internal transcribed spacer (ITS) region, located between the repeating array of nuclear 18S small subunit ribosomal RNA (SSU) and 28S large subunit ribosomal RNA (LSU) genes, is a versatile genetic marker. Non-coding regions such as internal transcribed spacers (ITS) are variable because they are not translated, which means that they are free from selection pressure. Therefore, the ITS regions of rDNA can be used for comparison at or below the species level. Using ITS in nematode identification has become common in the past decade (Ferris *et al.* 1993; 1994; Campbell *et al.* 1995; Chilton *et al.* 1995; Ferris *et al.* 1995; Gasser and Hoste 1995; Hoste *et al.* 1995; Zijlstra *et al.* 1995; Fallas *et al.* 1996; Cherry *et al.* 1997; Zijlstra *et al.* 1997; Zheng *et al.* 2003).

Among coding regions in rDNA, the D2/D3 expansion segments (part of the LSU) have evolved the most rapid changes. This region is readily amenable to the determination of DNA sequences. It has proved useful for resolving closely related taxa (Al-Banna *et al.* 1997; Nadler and Hudspeth 1998; Duncan *et al.* 1999; Kanzaki and Futai 2002; Nadler 2002).

By contrast, the SSU rRNA genes are used for phylogenetic studies across the phylum (Fitch *et al.* 1995; Liu *et al.* 1997; Aleshin *et al.* 1998; Blaxter *et al.* 1998;

Nadler and Hudspeth 1998; De Ley *et al.* 2002; Dorris *et al.* 2002; Floyd *et al.* 2002; Kanzaki and Futai 2002; Nadler 2002).

2.5.3.2.1 DNA extraction

Extraction of DNA from nematodes is the first step in using the molecular taxonomic approach. Optimisation of DNA extraction and amplification is essential to permit analysis of a small number of nematodes. Retrieving adequate nematode DNA material is very important. Methods of extraction of DNA from *Bursaphelenchus* have been developed (Iwahori *et al.* 1998). Due to their small size, genomic DNA extraction normally needs a large amount of nematode suspension (Iwahori *et al.* 1998). Recently, a new technique was developed, which uses a single nematode sample to extract DNA (Iwahori *et al.* 2000). This technique is very convenient and applicable for the molecular biological analysis of nematodes.

2.5.3.2.2 PCR amplification and Restriction fragment length polymorphism (RFLP) analysis

Ribosomal DNA sequences of some isolates of *Bursaphelenchus* species are available in GenBank. Primers were selected to amplify ribosomal DNA containing the internal transcribed spacer 1 (ITS1), 5.8S rDNA, and the internal transcribed spacer 2 (ITS2) regions in *B. xylophilus* (Ferris *et al.* 1993; Iwahori *et al.* 2000). The amplification was carried out with a PCR machine and specific reaction conditions. After the PCR products were generated, restriction enzymes were used to digest the PCR product at 37°C overnight. The DNA fragments were separated by electrophoresis with a dye marker solution in a polyacrylamide gel in Tris-acetate ETDA (TBE) buffer. The gel was stained with ethidium bromide, and the bands observed under UV light (Iwahori *et al.* 2000). The enzyme digestion patterns are different in different species, and while they can clearly discriminate between different species they do not show differences among isolates of each species (Iwahori *et al.* 1998).

2.5.3.2.3 DNA sequence comparison and phylogenetic analysis

In recent years, phylogenetic analyses of nematodes using ribosomal RNA have refined our understanding of nematode evolution (Blaxter *et al.* 1998). PCR-RFLP and DNA sequences comparisons were used to estimate the genetic relationships among four isolates of *B. xylophilus* nematodes, one *Aphelenchus*, and one *Aphelenchoides* species (Iwahori *et al.* 1998). To date, more than 300 sequences of *B. xylophilus* which include 18S, ITS, 28S and mt COI have been deposited into GenBank, and sequence comparison was conducted using the computer program GENETYX-MAC (Iwahori *et al.* 1998).

With the development of bioinformatics, a number of programs have been developed and used for phylogenetic analysis. Maximum Parsimony (MP), Neighbour-joining (NJ), and Maximum Likelihood (ML) methods (Gibin-Davis *et al.* 2003) have been used with PAUP* (Swofford 1998; 2002), MEGA (Kumar *et al.* 1993), and PHYLIP (Felsenstein 1993) to infer phylogenies to study entomophilic nematode biodiversity and cospeciation. In addition, Giblin-Davis *et al.* (pers. comm. 2006) have also tested phylogenetic hypotheses by using winning sites (Prager and Wilson 1988), Templeton (1983), Kishino-Hasegawa (1989) tests and bootstrapping (Felsenstein 1985; Sanderson 1989; Hillis and Bull 1993; Sanderson 1995; Hillis *et al.* 1996; Huelsenbeck *et al.* 1996). The growth of molecular taxonomy techniques has led to testing usage of the DNA "barcode", specific short DNA sequences for the identification of all nematode species (Powers 2004). Furthermore, linkages with morphological, molecular and ecological data are being established on NemATOL (Powers 2004), the nematode-specific Tree of Life database (http://nematol.unh.edu).

2.5.4 Significance of *Bursaphelenchus xylophilus* to Australia

2.5.4.1 Threat

Pine wilt disease is thought to be the most destructive disease of pine forests in the world. *Bursaphelenchus xylophilus*, the causal agent of the disease, is recognised as the highest-ranking pest for forests in many countries. The European and Mediterranean Plant Protection Organisation (EPPO) has placed the nematode on the A1 list of quarantine pests and recommended that untreated softwood products not be imported from North America, Japan, China and elsewhere where the nematode occurs (Sutherland and Webster 1993). In Australia, as mentioned in Section 2.2, most softwood pine plantations comprise exotic species, such as *P. radiata, P. elliottii, P. caribaea* and *P. pinaster*. *Pinus radiata* and *P. pinaster* are especially susceptible to the pine wilt disease. Therefore, if pinewood nematode was to infest Australian pine plantations, the consequences would be devastating for the forest and wood products industry, and also serious for the Australian economy.

2.5.4.2 Incident

In February 2000, an exotic nematode (suspected to belong to the genus *Bursaphelenchus*) was detected in wood samples taken from a dying *Pinus halepensis* tree in Williamstown, near the Melbourne docklands. The species of nematode extracted from the tree was tentatively identified as *Bursaphelenchus hunanensis* (Mike Hodda, pers. comm. 2003). Further work showed that the extracted organisms comprised several forms of nematodes (Mike Hodda, pers. comm. 2003). One of these was thought to be in the *Bursaphelenchus* or *Ektaphelenchus* genus and the other was thought to be a *B. hunanensis* or closely related species. In 2002, the same symptoms were observed in pines dying at Heidelberg Park and Knoxfield in Melbourne. More than ten *P. radiata* trees were infested by a pine wilt-like disease, and the suspected causal nematode was again extracted from the wood samples (David Smith, pers. comm. 2005). *Pinus radiata* is known to be susceptible to pine wilt disease. Surprisingly, the first Australian

case of a disease suggestive of pine wilt disease occurred in *P. halepensis*, which is considered resistant to *B. xylophilus*. These incidences indicated a distinct possibility of the introduction of a pine wilt nematode to Australia.

2.5.4.3 Response

Due to a lack of information on above-ground nematodes of conifers in Australia, it was difficult to determine the cause of the first case of pine wilt-like disease. After extraction of nematodes from affected trees, the species and its status as an endemic or exotic nematode was uncertain. This resulted in delays, which could have been critical if the pine deaths were caused by *B. xylophilus*. In 2002, the Standing Committee on Forestry agreed to share costs between the Commonwealth Government (50%) and State Governments (50% in combination) for an interim eradication program. This response program includes removal and destruction of infected trees as a precautionary measure, a public awareness campaign and further surveys to delimit the extent of affected pine trees in other parts of Melbourne and Australia.

2.5.4.4 Future Plans

In 1999, the Administration of Exit and Entry Inspection and Quarantine of the People's Republic of China reported that many cases of *B. xylophilus* had been found in wooden packing materials imported from the United Sates and Japan (Jingwu Zheng, pers. comm. 2003). In order to prevent further entry of *B. xylophilus* to Europe, the EPPO has funded a project to survey *B. xylophilus* in the whole of the European Union, and has also set up a Diagnostic Protocol for regulation of *B. xylophilus*. The intercepts reported from China have shown that Australia should be seriously concerned about entry of this devastating pest nematode. The current incidence of nematodes in soft wood plantations should also be investigated to establish baseline data on the nematode fauna of conifers in Australia, both native and exotic.

2.6 Summary

Clearly, the pinewood nematode is a devastating pest of pine plantations around the world. The incidents of pine disease in Melbourne in 2000 and 2002 alerted the Australian pine industry to the serious threat posed by *Bursaphelenchus*. Questions requiring urgent answers include: Are the observed incidents actually pine wilt disease? Are the recovered nematodes *Bursaphelenchus* species? Are the recovered nematodes pathogens to *P. radiata* trees?

A large area of pine plantation already exists in Australia, and by 2020, this is expected to be expanded more than three times the current area. Thus, it is necessary to investigate what nematodes occur in the aerial parts of conifers in order to build baseline information of nematodes in Australian pine plantations. Therefore the aims of my study were:

- To survey the above ground nematode fauna of *Pinus* and related conifers in south-eastern Australia,

- To describe and characterise any *Bursaphelenchus* spp. detected and morphologically similar taxa collected, and

- To examine the biology and pathology of the aphelenchid species detected.

Chapter 3: Survey of Conifers for Nematodes

3.1 Introduction

Pinewood nematode *Bursaphelenchus xylophilus* has caused severe damage to the pine plantations in Japan, China, and South Korea. More recently, it has been detected in Portugal (Evans *et al.* 1996; Mota *et al.* 1999), changing the earlier view of Europe as an area free from this pest. The Standing Committee on Plant Health of the European Union has since obliged each member state to survey their territories for pinewood nematode (Magnusson *et al.* 2000). However, to date, very limited systematic survey work has been performed in pine forests around the world. Mostly survey samples were taken from wood imports, wood from diseased, declining and dead trees, pine tree stumps and potential vector insects such as beetles (Massey 1974; Mota *et al.* 1999; Magnusson *et al.* 2000; Michalopoulous-Skarmoutsos *et al.* 2003).

As mentioned in Chapter 2.1, there were about 1×10^6 ha of pine plantations in Australia in 2002, and more will be established in the future. Among them, *Pinus radiata* is the dominant species, and is known to be susceptible to *B. xylophilus* (Mamiya 1983). Such an extensive area of pine monoculture creates opportunity for rapid spread of pinewood nematode if it is introduced, and puts the Australian softwood industry at great risk.

There has been limited research on nematodes in Australian pine plantations, particularly on the distribution of above-ground nematodes. McLeod and co-workers (McLeod *et al.* 1994) compiled a list of plant nematodes in Australia, in which the nematodes were listed by plant and genus. Nematodes associated with some Australian native conifers and exotic *Pinus* species were included. However, the list did not provide adequate information about the nematodes associated with pine trees, no voucher material was available for many of the nematodes, and it gave no detailed descriptions of the nematodes listed. To provide more information about distribution of pine nematodes in Australian forests and to indicate possible

relationships between pines and particular nematodes, a systematic survey was carried out in south-eastern Australia.

3.2 Materials and Methods

3.2.1 Survey in Kuitpo Forest

In September 2003, stands of *Pinus* were surveyed at Kuipto (Fig. 3.1, Table 3.1, Fig. 3.2), South Australia (SA). To give a representative sample, five compartments were selected with establishment years of 1948, 1962, 1985, 1986 and 2000, providing a range of plantation age and a representative range of topography. A "W" transect method was used and 50 trees were sampled in each compartment. A total of 200 *P. radiata* and 50 *P. pinaster* trees were sampled.

In four compartments, both bark and wood samples were collected from 1.5 m above the ground on the south-eastern side of the tree. In the compartment planted in 2000, only twig samples could be collected because the trees were small. The south-eastern side was selected as it has the least exposure to the sun and desiccation, and might therefore support greater numbers of nematodes. A borer (5 mm diameter) was used for wood sampling and an axe for bark sampling. Samples were placed in an insulated container for transport back to the laboratory, and kept at 16°C until extraction. Trunk circumference (at 1.5 m) and tree condition were recorded for each tree. Sampled trees were marked by yellow waterproof paint to facilitate re-sampling, if needed. The geographic location of each compartment was recorded using a Global Positioning System (GPS, Garmin 12XL, USA).

3.2.2 Survey of native pine, *Callitris* spp.

For comparative purposes, native *Callitris* woodland in SA was also sampled in September 2003. Two areas were selected: a) roadside stands near Burdett, and b) the Tailem Bend Forest Reserve (Fig. 3.1, Fig. 3.2). Forty *Callitris preissii* trees from six sites were sampled. For the roadside population 5 trees at 4 sites several

kilometres apart were sampled. In the Reserve, 10 trees were sampled on a single transect on either side of a main access road. In addition, a sample was collected from trees on the roadside at Tutye, Victoria in May 2003. Sampling, data collection, sample handling was as described in Section 3.2.1 and Section 3.3.1.
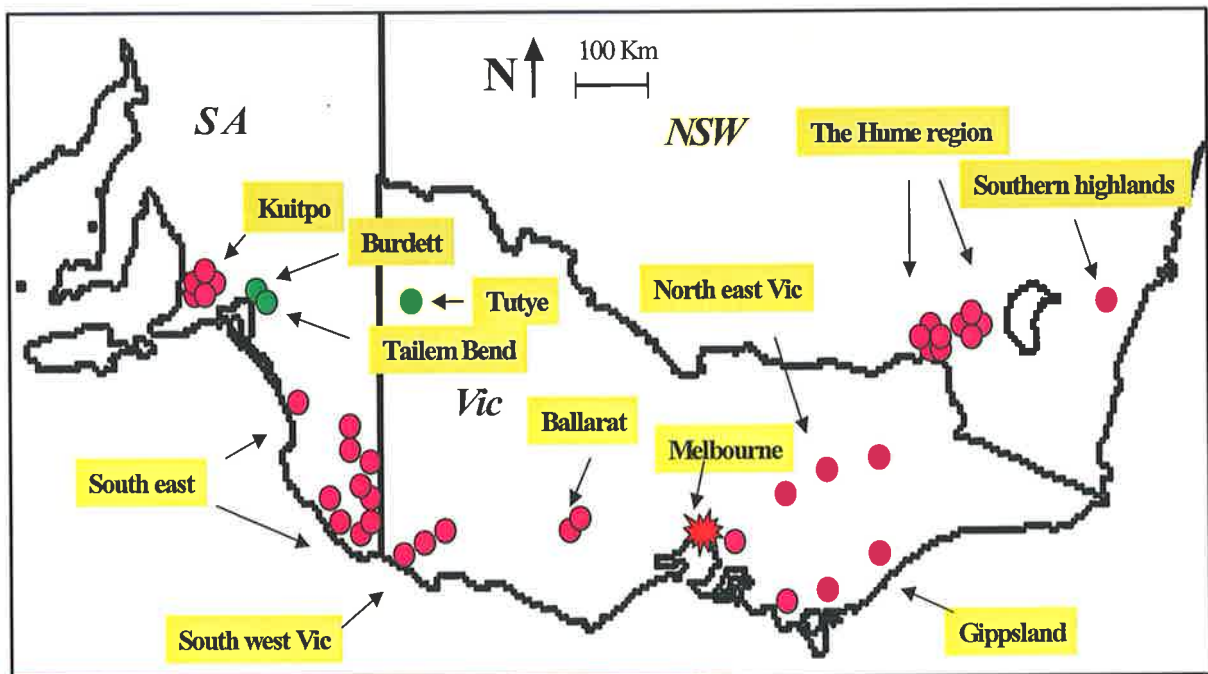
Fig. 3.1. Map showing sites in southeast Australia surveyed for nematodes associated with conifers. Pink circles, *Pinus radiata* forests; green circles, *Callitris* spp.; red star, diseased *P. radiata* at Knoxfield and Heidelberg, Victoria. 39 sites in total, 1240 bark samples, 400 wood samples.

Table 3.1. Sites from which samples were collected in the South-East of Australia.

Locations	State	Species	Trees	Survey Date	Latitude	Longitude	Plantation Year	Rainfall (mm)	MinT °C	MaxT °C
Kuitpo	SA	Pr	50	04.10.2003	35°14'	138°42'	1985	854	8.7	20.6
Kuitpo	SA	Pr	50	06.10.2003	35°93'	138°41'	1986	854	8.7	20.6
Rocky Creek	SA	Pr	50	11.10.2003	35°08'	138°44'	1961	854	8.7	20.6
Rocky Creek	SA	Pr	50	12.10.2003	35°08'	138°44'	2000	854	8.7	20.6
Rocky Creek	SA	Pp	50	19.10.2003	35°08'	138°45'	1948	854	8.7	20.6
Burdett	SA	Cp	20	06.11.2003	34°59'	139°22'	mixed	376	9.5	22.4
Tailem Bend	SA	Cp	20	06.11.2003	35°19'	139°23'	mixed	376	9.5	22.4
Kongorong East	SA	Pr	50	14.04.2004	37°57'	140°35'	1950	704	10.2	17.9
Near Nelson	SA	Pr	50	14.04.2004	37°46'	140°35'	1952	704	10.2	17.9
Near Nelson	SA	Pr	50	14.04.2004	38°00'	140°57'	1977	704	10.2	17.9
Tarpeena	SA	Pr	50	15.04.2004	37°36'	140°48'	1985	712	8.8	20.9
Penola South	SA	Pr	50	15.04.2004	37°25'	140°54'	1987	711	8.8	20.9
Windy Hill NFR	SA	Pr	50	16.04.2004	37°43'	140°33'	1965	787	8.7	19.1
Comaum Forest	SA	Pr	50	16.04.2004	37°13'	140°53'	1970	711	8.8	20.9
Mount Burr	SA	Pr	50	16.04.2004	37°33'	140°26'	1989	787	9	19.5
Glenbumie	SA	Pr	50	17.04.2004	37°51'	140°53'	1968	775	8.7	19.1
Noolook	SA	Pr	50	18.04.2004	37°01'	139°48'	1981	668	10.9	18.1
Dartmoor	Vic.	Pr	20	03.04.2005	37°37'	140°17'	1978	797	7.4	19.2
Casteron	Vic.	Pr	20	03.04.2006	37°37'	140°17'	1978	660	8.3	19.9
North Dartmoor	Vic.	Pr	20	03.04.2005	37°48'	141°12'	1990	797	7.4	19.2
Ballarat	Vic.	Pr	20	05.04.2005	37°27'	143°51'	1986	699	7.1	17.3
Ballarat	Vic.	Pr	20	05.04.2005	37°24'	143°53'	1989	699	7.1	17.3
Narbethong	Vic.	Pr	20	06.04.2005	37°30'	145°38'	1985	1020	8	19.2
Longford	Vic.	Pr	20	06.04.2005	37°10'	147°2'	1985	607	8.4	20.2
Briagolong	Vic.	Pr	20	06.04.2005	37°52'	147°12'	1990	607	8.4	20.2
Churchill	Vic.	Pr	20	06.04.2005	38°19'	146°26'	1990	607	8.4	20.2
Myrtleford	Vic.	Pr	20	07.04.2005	36°34'	146°44'	1975	905	6.6	21.7
Benalla	Vic.	Pr	20	07.04.2005	36°49'	145°54'	1980	671	8.7	22
Stanley*	Vic.	Pr	20	08.04.2005	36°22'	146°45'	1980	951	7.9	18.5
Green Hill S F	NSW	Pr	20	09.04.2005	35°24'	148°06'	1983	1221	5.9	16.9
Green Hill S F	NSW	Pr	20	09.04.2005	35°24'	148°06'	1989	1221	5.9	16.9
Green Hill S F	NSW	Pr	20	09.04.2005	35°33'	148°05'	1975	1221	5.9	16.9
Bago S F	NSW	Pr	20	09.04.2005	35°33'	148°05'	1990	1413	4.2	15.3
Carabost S F	NSW	Pr	20	09.04.2005	35°39'	147°45'	1970	1077	5.6	17.3
Billapaloola S F	NSW	Pr	20	10.04.2005	35°14'	148°23'	1985	1510	6.5	19.2
Red Hill S F	NSW	Pr	20	10.04.2005	35°10'	148°23'	1992	1077	6.5	19.2
Billapalooa S F	NSW	Pr	20	10.04.2005	35°09'	148°26'	1985	1510	5.6	17.3
Wee Jasper S F	NSW	Pr	20	10.04.2005	35°10'	148°29'	1990	1510	6.5	19.2
Penrose S F	NSW	Pr	20	10.04.2005	34°37'	150°12'	1970	804	8.8	23.5

Pr: *Pinus radiata*; Pp: *Pinus pinaster*; Cp: *Callitris preissii*; Trees: Number of trees sampled; Rainfall: Annual average in millimetre; Min T: Minimum annual average temperature; Max T: Maximum annual average temperature. *: Burnt site; S F: State Forest; NFR: Native Forest Reserves. Source: The Commonwealth Bureau of Meteorology (www.bom.gov.au).
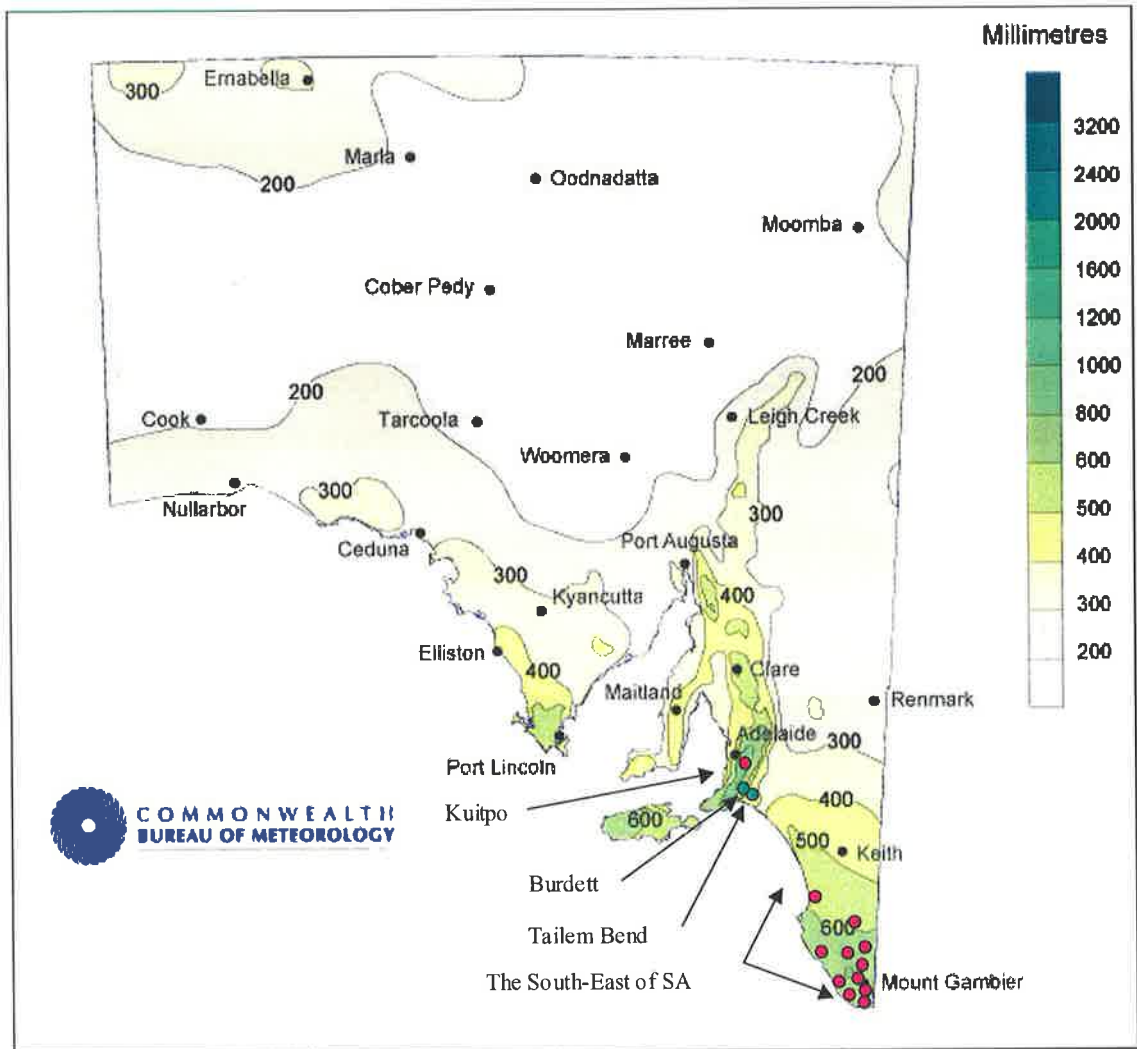
Fig. 3.2. Map showing annual average rainfall of South Australia and sites surveyed for nematodes associated with conifers. Pink circles, *Pinus radiata* forests; green circles, *Callitris* spp. Source: The Commonwealth Bureau of Meteorology (www.bom.gov.au).

3.2.3 Survey in South-East Region of South Australia

In April 2004, stands of *Pinus* were surveyed in forests in the South-East Region of SA (Fig. 3.1). To give a representative sample, ten compartments were selected with establishment years of 1950, 1952, 1965, 1968, 1970, 1977, 1981, 1985, 1987 and 1989. The compartments selected covered the north to south and east to west spread of plantations in the region and were representative of variation in rainfall zones and growing conditions (See Table 3.1 and Fig. 3.2). The methods of sample and data collection, and handling, were as described in Section 3.2.1, except that a different method for selection of trees was used. Two parallel rows of trees were selected and 50 trees were sampled in each compartment, giving a total sample of 500 *P. radiata* trees. Given the lack of nematodes in wood collected earlier from pines at Kuitpo Forest, wood was only collected from 20% of the trees sampled in each compartment.

3.2.4 Survey in Victoria and New South Wales

In April 2005, stands of *P. radiata* were surveyed in Vic. and NSW. In Vic., twelve compartments established between 1975 and 1990 were selected. One of them, the Stanley Plantation, was burnt in 2004. In NSW, ten compartments established between 1970 and 1990 were selected (Table 3.1, Fig. 3.1). The compartments selected covered the north to south and east to west spread of plantations in Vic. and the Tumut region, and were representative of variation in rainfall zones and growing conditions (See Table 3.1, Fig. 3.3. and Fig. 3.4). The survey methods were as described in Section 3.2.3, but only 20 trees were sampled in each compartment and no wood samples were collected (given the lack of nematodes in wood collected earlier from pines at both Kuitpo Forest and the South-East Region of SA). Two parallel rows of trees were selected and 20 trees were sampled in each compartment, giving a total sample of 440 *P. radiata* trees.
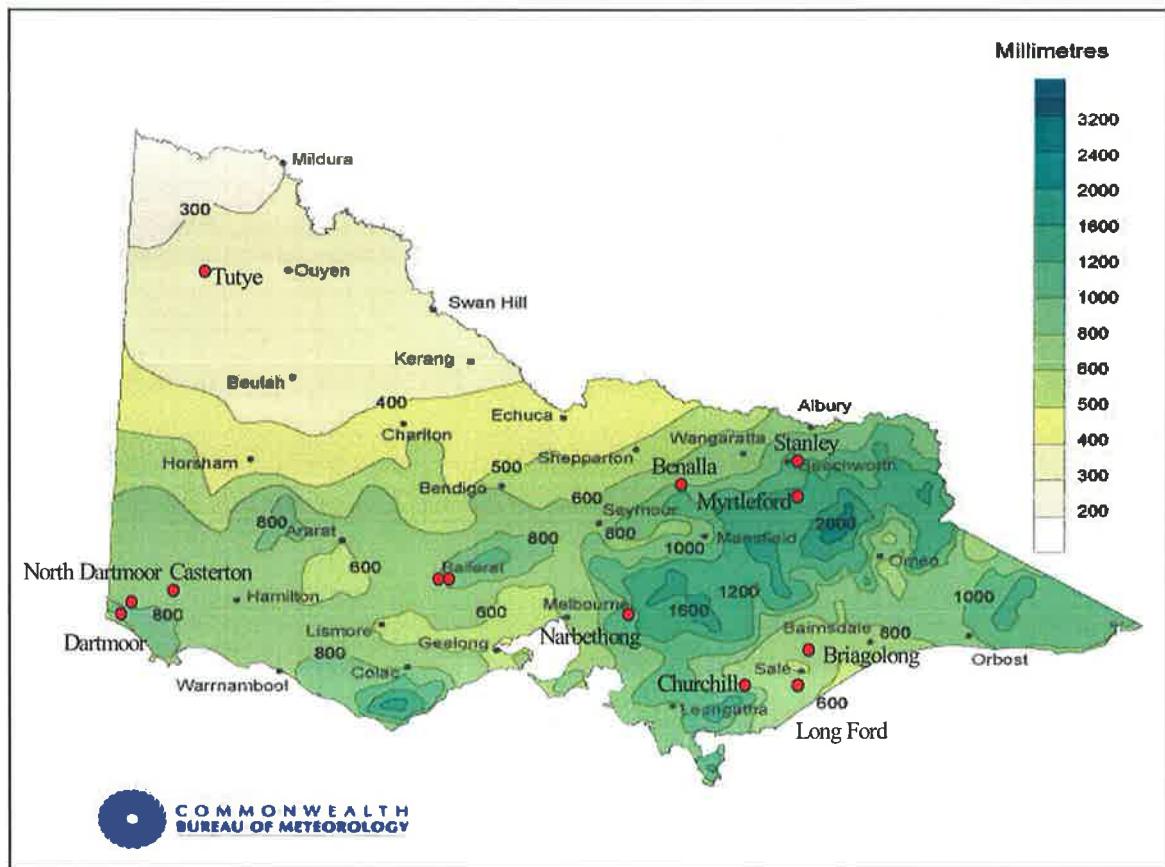
Fig. 3.3. Map showing annual average rainfall of Victoria and sites surveyed for
nematodes associated with conifers. Pink circles, *Pinus radiata* forests. Source:
The Commonwealth Bureau of Meteorology (www.bom.gov.au).
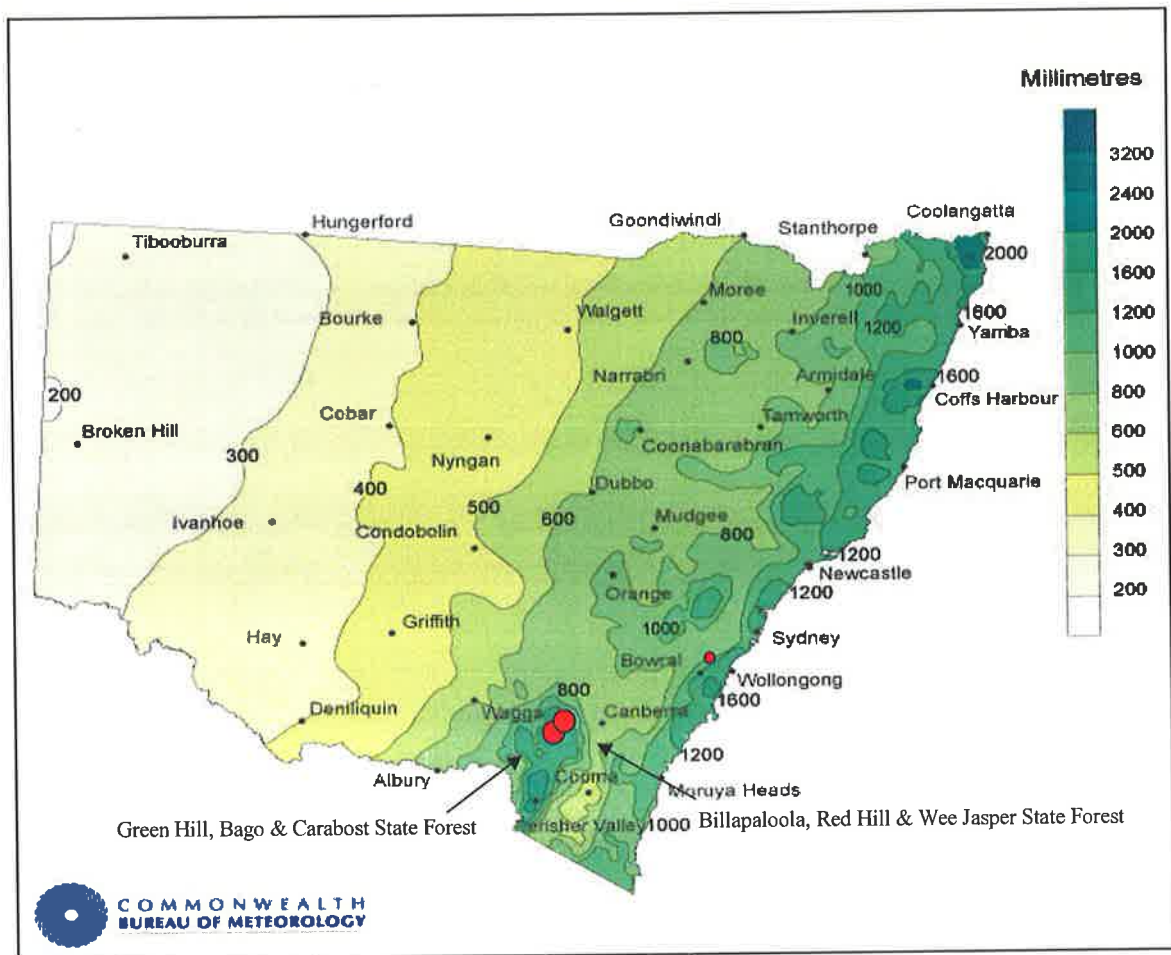
Fig. 3.4. Map showing annual average rainfall of NSW and sites surveyed for nematodes associated with conifers. Pink circles, *Pinus radiata* forests; Green circle, *Callitris* sp. Source: The Commonwealth Bureau of Meteorology (www.bom.gov.au).

3.2.5 Samples from diseased trees in Melbourne

In February and May 2003, wood chip samples were collected from the trunk of diseased *Pinus radiata* trees in Knoxfield and Heidelberg, Victoria. The wood chips were sealed in plastic bags and transported to Adelaide, and stored in a 10-16°C temperature room until used.

3.2.6 Sample data analyses

Correlation analysis, regression analyses and analyses of variance (ANOVA) were performed using GenStat Release 8.2 (PC/Windows 2000). Data analysed was the proportion of trees at each site from which the particular nematode group was collected.

3.3 Extraction, Fixing and Mounting

3.3.1 Nematode extraction and scoring

Nematodes were extracted using a misting cabinet (Bohmer and Weil 1978). The frequency of misting was once every 10 min for 15 s. Both bark and wood samples (20-30 g for each sample) were cut into small pieces (about 10 x 10 x 5 mm), for extraction. After 48 h in the mister, tubes were used to collect about 10 ml of the nematode suspension water and were left in the laboratory on the bench for about 1 h to let the nematodes settle. The volume of water was reduced to 3 ml by suction, taking care not to resuspend the nematodes. The nematodes were then resuspended, and the total volume was transferred to a glass block and examined under a dissecting microscope at 16X and 40X magnification. Occasionally, a few nematodes were mounted in 20 µl water on a glass slide and examined under a compound microscope at 100X to 400X magnifications. Nematodes were classified into broad categories based on morphology to Order and/or trophic group (see Fig. 3.5), and scored on a presence/absence basis. Thus, nematodes with a stylet and a

large median bulb were classified as aphelenchids (plant, fungal and lichen feeders); with a relatively strong stylet and a less obvious median bulb as tylenchids (plant parasites and lichen feeders); with a narrow stoma and three-part oesophagus as rhabditids and areolaimids, probably *Plectus* sp., (bacterial feeders); and with a variable stoma and two part oesophagus as dorylaimids (probably bacterial and algal feeders). A fifth group of nematodes had a wide, stirrup-shaped stoma with rhabdious and an apparent two part oesophagus and were initially identified as mononchids. However, when representatives of this group were examined at 100X, they were seen to have a three-part oesophagus, rhabdions jointed near the middle, and the hooked tail tip characteristic of the panagrolaimid *Macrolaimus*. They were described as probable saprophages by Goodey (1963). These nematodes were so distinctive that they are classified as putative *Macrolaimus* and considered here as a separate group.

3.3.2 Fixing and mounting

Established methods (Seinhorst 1966) were used to heat kill and fix the nematodes for permanent mounting on glass slides. Details of the method used follow. After the initial examination and classification, the nematodes were again allowed to settle and the volume of water in the glass block was reduced to 1 ml. Nematodes were killed and fixed by adding 1 ml hot 3% formalin to each glass block, and then left to harden for at least 2 weeks. If not processed directly for permanent mounting, the killed nematodes were stored in fixative in scintillation bottles. To determine the identity of aphelenchid nematodes and some other morphotypes, samples were processed to glycerol and mounted on glass slides as described by Davies and Giblin-Davis (2004). Nematodes were examined using interference contrast microscopy. About 250 specimens of 12 morphotypes were mounted.

3.5 Results

In the survey, about 1200 samples were collected and the areas sampled represented more than 60% of the pine plantations in Australia. No nematodes were found in the wood samples or young shoots of *P. radiata* except in the wood samples from

diseased trees at Knoxfield and Heidelberg in Vic. In contrast, nematodes were commonly found in the bark samples of healthy trees.

3.5.1 Proportion of trees colonised by nematodes

The percentage of trees that contained nematodes in the bark is shown for each sampling region in Fig. 3.5. The proportion of *Pinus* trees with nematodes ranged from 80 to 98%, with the exception of Stanley, where only 25% of trees had nematodes recovered from the bark. This was not unexpected because the Stanley Plantation had been burnt in the recent past and most trees were dead.

As in the *Pinus* plantations, bark samples from trees in the *Callitris preissii* stands in SA had a high proportion with nematodes (98%).

3.5.2 Nematode trophic groups in each region

The extracted nematodes were classified into five trophic groups (Fig. 3.6), including: Aphelenchida Siddiqi, 1980 (plant, fungal and lichen feeders), Rhabditida (Oerley 1880) Chitwood, 1933 and Areolaimida de Coninck and Schuurmanns Stekhoven, 1933 (bacterial feeding), putative *Macrolaimus* Maupas, 1900 (saprophagus), Tylenchida Thorne, 1949 (plant feeding) and Dorylaimida (de Man, 1976) Pearse, 1942 (bacterial and algal feeders). Among these, three genera and twelve morphospecies of aphelenchids were found.

The overall ratios of nematode groups collected from each forest region are shown in Fig. 3.6. Aphelenchid nematodes formed 59 to 86% of the population in all but one region. Aphelenchids were the most commonly found nematodes present in all samples taken from *Pinus* trees in SA, Vic. and NSW. However, aphelenchids did not dominate in bark collected from *Callitris* (Fig. 3.6), where they were found in only 28% of the trees sampled.

In South Australia, nematodes from five trophic groups were collected from *Pinus* trees at Kuitpo, Rocky Creek and the South-East Region. Only three trophic groups were collected from native pines at Tailem Bend. In Victoria, nematodes of four trophic groups were extracted from forests at Dartmoor, Ballarat, Gippsland and North Vic. In NSW, nematodes from three trophic groups were found in bark sampled from Tumut forest and Penrose forest.

3.5.3 Nematode trophic groups and environmental associations

With the exception of the 2000 planting in SA and the burnt Stanley plantation in Vic., the number of nematodes associated with *Pinus* trees was not obviously affected by the age or species of *Pinus* trees. Correlation analysis of the proportion of the five nematode groups and their host species and ages, latitude and longitude, annual average rainfall, minium and maximum temperatures and the highest temperature were performed. The simple linear regression results showed that the proportion of putative *Macrolaimus* was significantly affected by the latitude of its hosts (Fig. 3.7a, $P<0.001$). The percentage of putative *Macrolaimus* present in trees decreased very significantly from north to south. Similarly, the other bacterial feeding nematodes were also significantly affected by latitude of their hosts (Fig. 3.7b, $P=0.001$), increasing from south to north ($P=0.001$).

The quadratic regression analysis of putative *Macrolaimus* and longitude is shown in Fig. 3.8. The percentage of the nematodes present in trees was affected significantly by the longitude ($P=0.008$).

Other nematode groups had no significant relationship to latitude and longitude.

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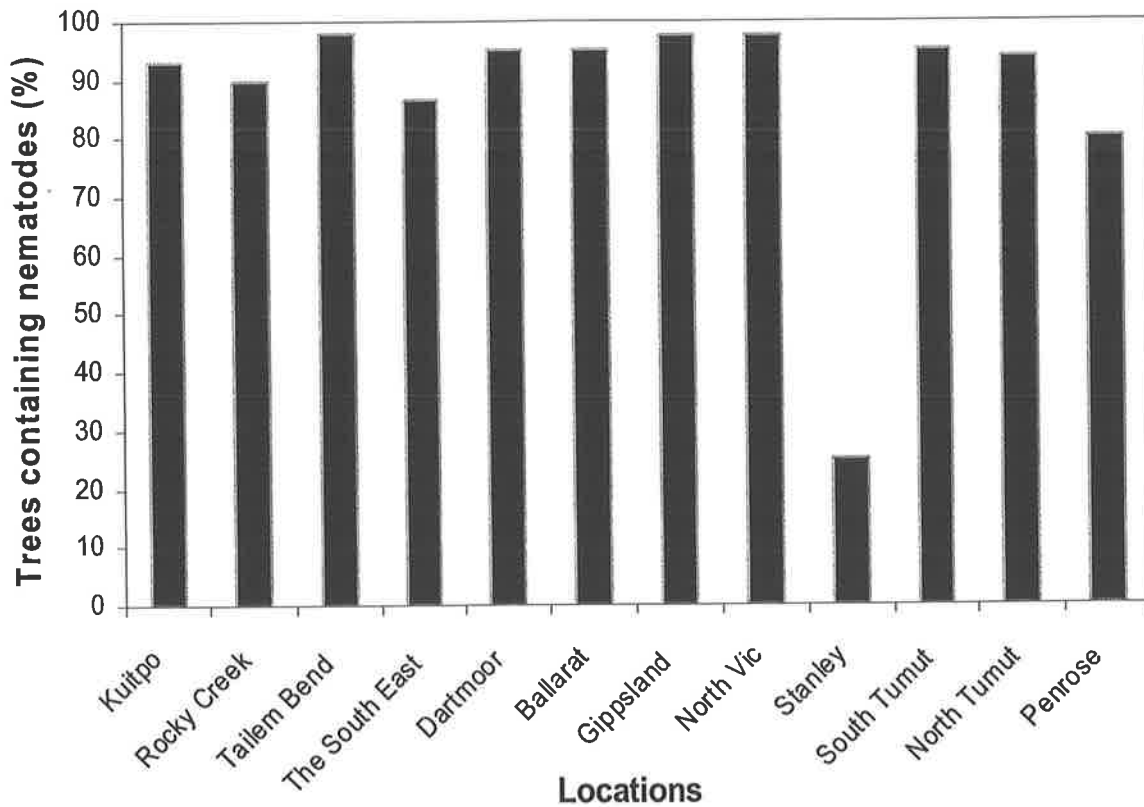
Fig. 3.5. The percentage of trees with bark containing nematodes. Sites: names of the forest locations. Note: Tailem Bend, native pine tree *Callitris preissii*; Stanley, burnt trees; The South East includes 10 forest compartments, Kongorong, two near Nelson, Tarpeena, Penola South, Windy Hill NFR, Comaun Forest, Mount Burr, Glenburnie and Noolook; Kuitpo includes 2 forest compartments; Rocky Creek includes 3 forest compartments; North Vic. includes 4 forest compartments, Narbethong, Myrtleford and Benalla; Gippsland includes 3 forest compartments, Longford, Churchill and Briagolong; Ballarat includes 2 compartments; South Tumut includes 5 forest compartments, three in Green Hill State Forest, Bago and Carabost State Forest; North Tumut includes 4 forest compartments, two in Billapaloola, Red Hill and Wee Jasper.

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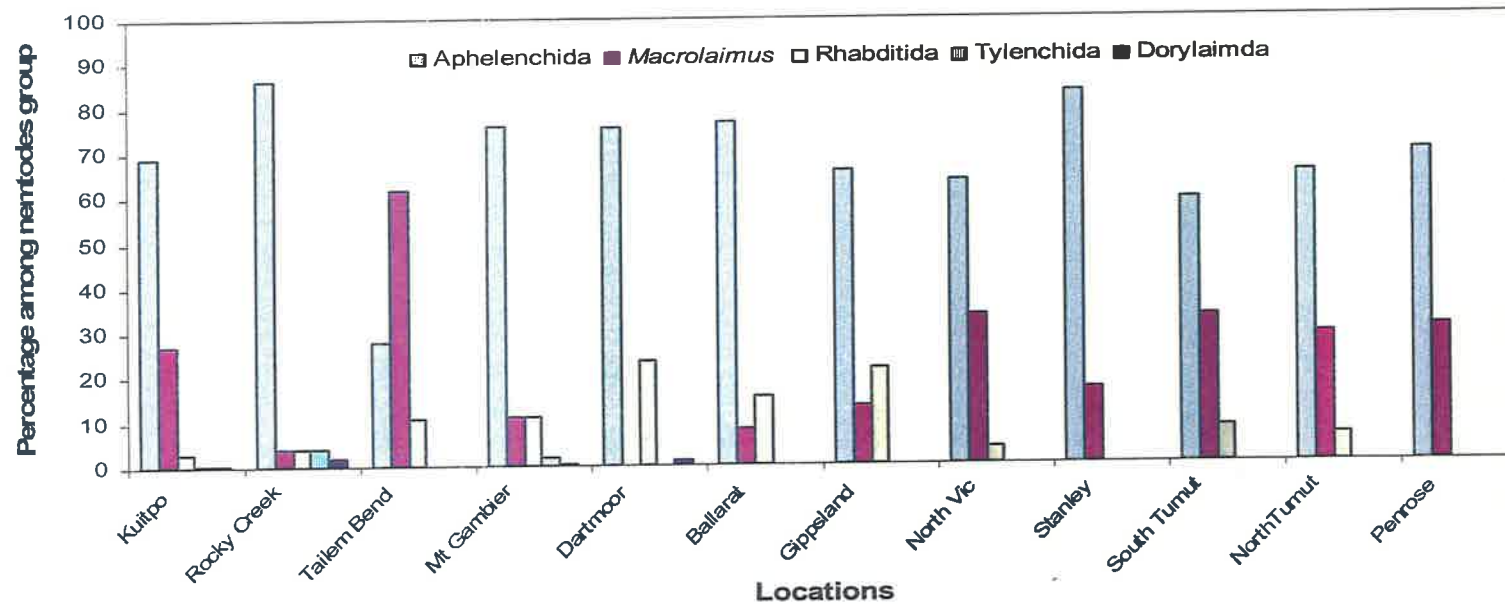
Fig. 3.6. The proportion of nematodes in each of five groups (Aphelenchida, *Macrolaimus*, Rhabditida, Tylenchida and Dorylaimida), collected from various conifer platantions in southeastern Australia from 2003 to 2005. Sites compriesd healthy *Pinus radiata* except at Tailem Bend, which was native *Callitris preissii,* and Stanley which was burnt *P. radiata.* The South Esat includes 10 forest compartments, Kongorong, two near Nelson, Tarpeena, Penola South, Windy Hill NFR, Comaun Forest, Mount Burr, Glenburnie and Noolook; Kuitpo includes 2 forest compartments; Rocky Creek includes 3 forest compartments; North Vic.includes 4 forest compartments, Narbethong, Myrtleford and Benalla; Gippsland includes 3 forest compartments, Longford, Churchill and Briagolong; Balarat includes 2 compartments; South Tumut includes 5 forest compartments, three in Green Hill State Forest, Bago and Carabost State Forest; North Tumut includes 4 forest compartments, two at Billapaloola, Red Hill and Wee Jasper.

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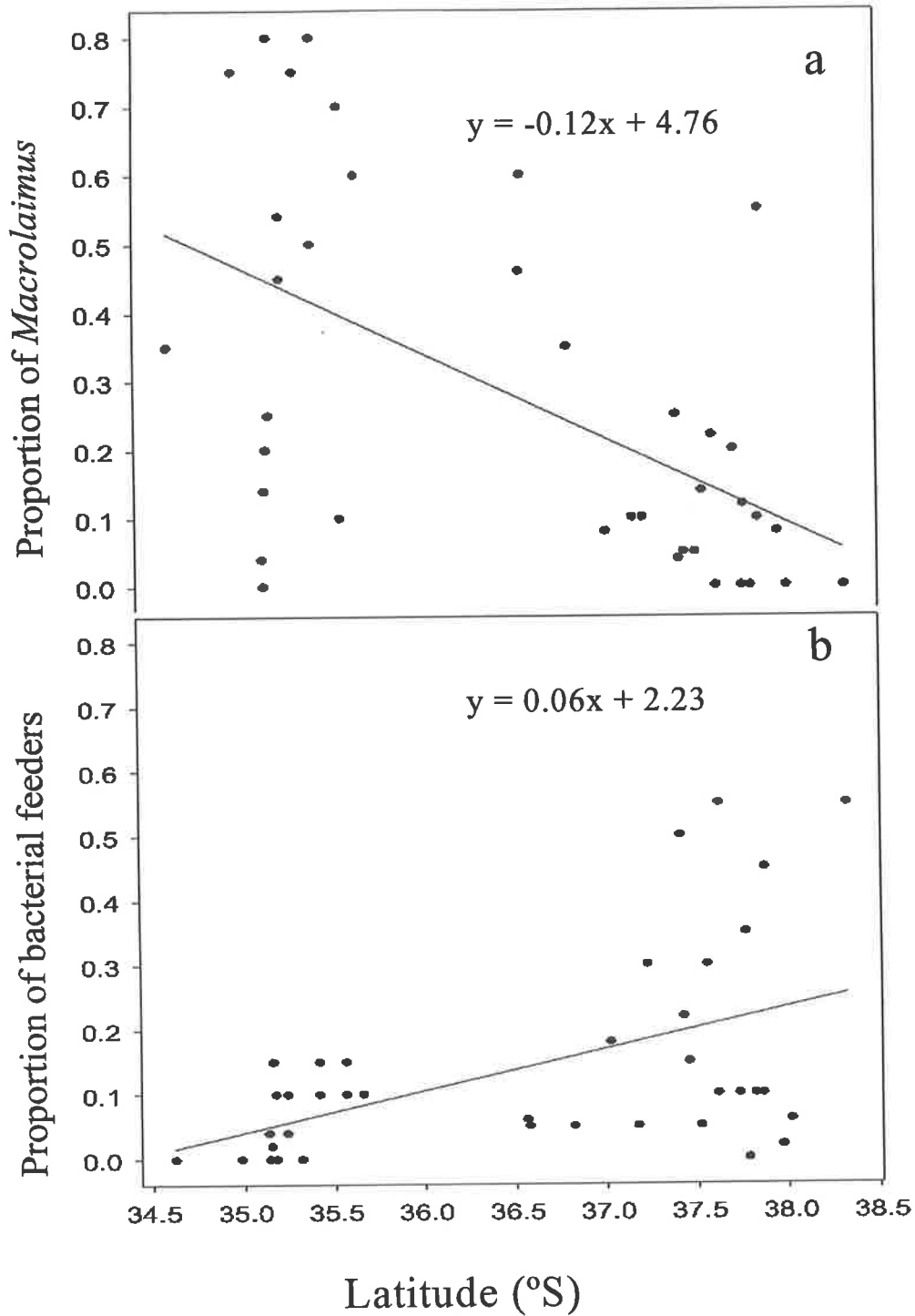
Fig. 3.7. Simple linear regression analyses of (a) putative *Macrolaimus*, and (b) other bacterial feeders nematodes against latitude. P(a) < 0.001; P(b) = 0.001.
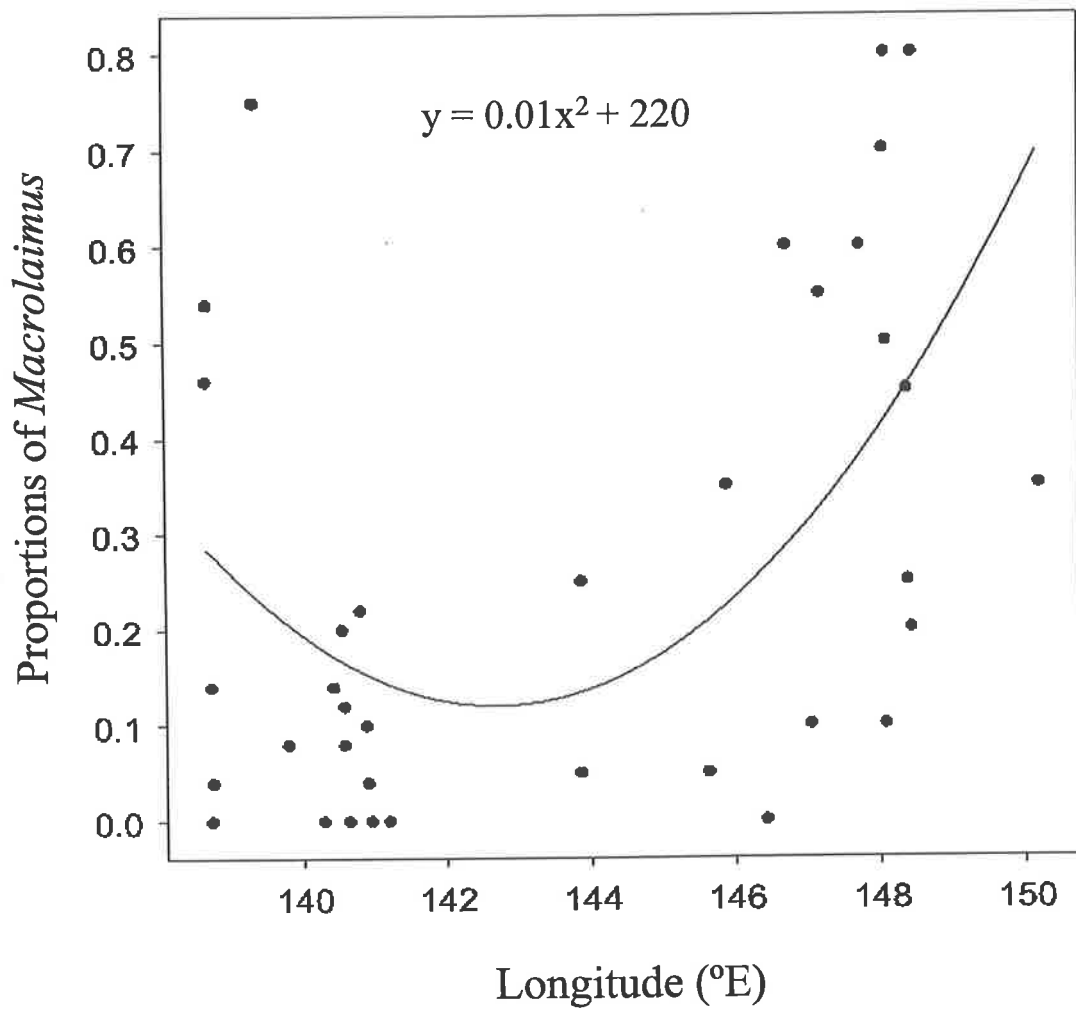
Fig. 3.8. Quadratic regression analysis of putative *Macrolaimus* against longitude. P = 0.008.

3.6 Discussion and Conclusions

This survey covered more than 60% of the pine plantations in Australia, and spanned distances of more than 1000 km. Therefore, the characteristics, e.g., age of the trees, and the environments of the pine plantations varied from place to place, including the latitude and longitude, the annual rainfall and temperatures (see Table 3.1). Results of the survey showed that the aphelenchid nematodes found in bark, i.e., above the ground, are diverse. Four morphospecies were commonly collected from Kuitpo forest, and three from forests of the South-East Region of SA, Vic. and NSW. In addition, three morphospecies of aphelenchids were frequently present in pine trees from all places surveyed. These are discussed in detail in Chapter 4.

Five trophic groups of nematodes were identified in the survey, based on stomal and oesophageal morphology. From Fig. 3.6, it is clear that aphelenchids were predominant (were most frequently found) in all the *Pinus* forests surveyed. These included a number of *Laimaphelenchus* spp. (not previously recorded in Australia, see Chapter 4). No obvious *Bursaphelenchus* spp. was detected. Although Ridely and Bain (2001) reported that *Bursaphelenchus* was found in an exotic pine in Melbourne, this was not supported by a detailed description. The widespread distribution indicates that most of the aphelenchids found are likely to be feeding on lichens or fungi attached to bark, rather than to be insect parasites. Nematodes parasitic on insects would be expected to have a more patchy distribution than was seen. However, some might have phoretic associations with insects.

In order to obtain nematodes for molecular work and biological studies, five marked locations were re-sampled. These included Kuitpo, Burdett, Knoxfield, Heidelberg and Tutye in NSW. Nematodes were extracted from all re-sampled trees, including six samples from *C. preissii* near Burdett, six samples from *P. radiata* at Christmas Hill in Kuitpo Forest, and four samples from diseased trees at Knoxfield and Heidelberg. However, unfortunately, the particular nematode sought from Kuitpo (a possible *Laimaphelenchus* sp.) and the nematode from Tutye were not recollected. The reasons for this failure to re-collect are unclear. Nematode populations may

change with seasonal conditions and may be difficult to find if present at low densities. Since the re-collection was carried out in a different season from the initial sampling, time of sampling may have been a factor affecting the presence of the nematodes. Sampling was limited, and only a small part of the bark of a tree was collected. If the particular nematodes sought have a patchy distribution on the bark of a tree, they may not have been present in the particular pieces of bark collected.

In the USA and Europe, nematodes are commonly associated with bark beetles, either as parasites or phoretically (Rühm 1956; Massey 1974; Kaya 1984). These include tylenchids, aphelenchids, diplogasterids, rhabditids, dorylaimids, and mononchids. In Australia, *Contortylenchus grandicollis* has been found with *Ips* (Gibb and Fisher 1986). Several *Ips* specimens were collected from the burnt bark of the pine trees in Stanley, and *Ips* galleries were observed in *P. radiata* trees at Burnside, SA, but no tylenchids were associated with them. Mononchids were mentioned by Rühm (1956), who reported finding *Mononchus papillatus* in the old mould of galleries of *Hylurops ligniperda*, and *Prionchulus muscorum* in the mould of *Dryocoetes autographus* in Germany. However, the relative density (percentage) of mononchids found was not mentioned (Rühm 1956). Kaya (1984) also listed thirteen families of nematodes associated with bark beetles including Mononchidae. Here, no mononchids were isolated from bark or wood samples of either *Pinus* or *Callitris*.

In the last decade in the European Union, including Norway, Greece, Germany, and Portugal, nematode surveys have been extensively conducted in coniferous forests (Magnusson *et al.* 2000; Michalopoulous-Skarmoutsos *et al.* 2003; Magnusson *et al.* 2004) with the particular aim of determining the *Bursaphelenchus* fauna. Comparing the survey results between Australia and Norway (Magnusson *et al.* 2004), similarities and differences are observed. Firstly, three groups of nematodes, Rhabditida, Aphelenchida and Tylenchida, were recorded in both countries, but no Dorylaimida were recorded in Norway; secondly, the proportion of trees colonised by nematodes was similar, 92% of trees sampled in Australia contained nematodes and 94% in Norway; thirdly, the most common group of nematodes found in

Australian pine plantations was the aphelenchids (88%) whereas the microbivorous nematodes (87%) were the most common group in Norway. In addition, *Laimaphelenchus* was the most geographically widespread aphelenchid found in Australian pine plantations and no definitive *Bursaphelenchus* was recorded in Australian survey, but *Aphelenchoides* was the most frequently recovered genus in Norway, and *Bursaphelenchus* was recorded in 13 samples. Moreover, no *Monochamus* beetles and only a few *Ips* were found in the survey in Australia, but 81% of pines sampled had signs of *Monochamus* activity in Norway.

The population of trees with putative *Macrolaimus* decreased from north to south (P<0.001), whereas the other bacterial feeders increased from south to north (P=0.001). Although the regression analysis between them was not significant (P=0.237), the populations of these two groups of nematodes may have some interaction. As saprophages, *Macrolaimus* may compete with other bacterial feeders, and suppress their populations. However, in this survey, the actual numbers of nematodes were not counted, but rather their presence or absence was recorded. Given this, it is difficult to comment on the population composition changes. In addition, quadratic regression analysis indicated that the proportion of putative *Macrolaimus* present in the pine bark samples collected was also significantly affected by longitude (Fig. 3.8, P=0.008). In fact, this significance probably reflects the way in which the survey was carried out, i.e., its geography. While the direction for sampling was generally from SA to Victoria and NSW, i.e., from west to east, samples were also collected from north to south (from Adelaide in SA to the South-East Region), then from south to north again (from near Melourne in Vic north to NSW). Therefore, the analysis could reflect a north to south to north effect, such as climate.

Nematodes present in native pine, *Callitris,* were compared with those from exotic *Pinus* spp. The putative *Macrolaimus* and other bacterial feeding nematodes predominated in bark from the native pine, but fungal feeders predominated in the bark from exotic pine. The reasons for this could be: 1) The native trees have been growing in Australia for millions of years, and the exotic pine for less than 200 years. A smaller diversity of nematodes would be expected from exotic pines in countries where they are not endemic, as the ecosystems of their countries of origin

and Australia are different. 2) The sample of native pines was small (40 trees) compared with more than 1000 samples of exotic pine. If large numbers of samples had been collected from the native pine, the proportion of nematode composition might have changed. 3) Bark from the two conifer groups may support different populations of lichens, fungi and bacteria, which would be reflected in the populations of nematodes feeding on them.

From this survey of nematode distribution in Australian pines, it can be concluded that five trophic groups are present, and that aphelenchids are the predominant group in SA, Vic. and NSW pine forests. No nematodes were isolated from the wood samples, and no *Bursaphelenchus* were found.

Chapter 4: Morphological Studies

4.1 Introduction

Records of aphelenchids from conifers in Australia are few. Mcleod *et al.* (1994) listed a number of plant nematodes in conifers in Australia, of which *Aphelenchoides bicaudatus* was the only species of aphelenchids, but the information about this nematode is limited, and no Australian voucher material is available. Moreover, Ridley *et al.* (2001) reported in a New Zealand forestry newsletter that a *Bursaphelenchus* sp. was found in a dying pine (*Pinus halepenisis*) in Australia, but did not mention any description of the species. Therefore, there is no previous work on aphelenchids from Australian conifers with which the work described here can be compared.

As discussed in Chapter 2.4, nematode classification schemes have been controversial for decades (Inglis 1983; Maggenti 1991; De Ley *et al.* 2002). There has been considerable debate about whether the aphelenchs should be regarded as a) the suborder Aphelenchina Geraert, 1966 under the order Tylenchida Thorne, 1949 (Andrássy 1976; Luc *et al.* 1987; Maggenti *et al.* 1987; Nickle and Hooper 1991), or b) an independent Order Aphelenchida Siddiqi, 1980 or c) a Superfamily Aphelenchoidea Fuchs, 1937 (Thorne, 1949) under the orders Rhabiditida (Oerley 1880) Chitwood, 1933 and Areolaimida de Coninck and Schuurmanns Stekhoven, (De Ley and Blaxter 2002). Debate will undoubtedly continue, and will include not only evidence from morphological and biological studies, but also evidence from sequencing of DNA (refer to Chapter 5).

To understand the diversity of nematodes associated with bark and wood of pine trees, the species of nematodes present have to be determined. Indentification of nematodes described as species of *Aphelenchoides* Fischer, 1894 requires highly specialised knowledge, and access to a large literature source and specimens for adequate diagnoses. Such species determinations are outside the scope of this thesis, and hence nematodes collected here and belonging to the genus *Aphelenchoides* are described only as morphospecies. Morphospecies are taxa readily identified by

morphological differences distinct to individuals (Oliver and Beattie 1996). Use of morphospecies was recommended, for example, for surveys of invertebrates producing information on biodiversity, particularly where there are groups that are little known and/or taxonomically difficult. Oliver and Beattie (1996) compared lists of invertebrates produced by taxonomic specialists and non-specialists from large samples of insects, and found that both obtained consistent results. Some *Laimaphelenchus* Fuchs, 1937 taxa are also described only as morphospecies.

In order to determine the aphelenchids found in conifers in the survey, it was necessary to identify them accurately to genus level, at least. Even this was not always possible, with some nematodes having features belonging to more than one genus. The classification system used in this thesis is the one developed by Hunt (1993).

4.2 Materials and Methods

4.2.1 Collection of specimens

Bark and wood were collected from several pine plantations located in South Australia (SA), Victoria (Vic.) and New South Wales (NSW). They were also collected from native pine *Callitris preissii* growing in the road reserve at Burdett, in SA, from the Tailem Bend Forest Reserve SA, and from *Callitris* sp. near Tutye in NSW. Methods of extraction from wood and bark are as described in Sections 3.2 and 3.3.

4.2.2 Culturing species and morphospecies

Nematodes collected from trees at Burdett, Knoxfield and Heidelberg were surface sterilised in 1% streptomycin for 10 min, washed 3 times with sterilised water, and transferred to cultures of *Botrytis cinerea* Pers. on potato dextrose agar (PDA, Difco Laboratories, Detroit, USA). Plates were incubated at 25°C, and were sub-cultured monthly. Nematodes were washed off the plates with water, and killed and fixed using hot 3% formalin, and left to harden for at least 2 weeks.

4.2.3 Processing specimens for measurements

Nematodes sharing a common morphology were hand-picked from suspensions, and killed and fixed in hot 3% formalin.

All nematodes were processed to glycerol, and mounted on glass slides, as described by Davies and Giblin-Davis (2004). Nematodes were examined using interference contrast microscopy. Drawings and measurements were made from material mounted in glycerol, using a camera lucida. *Laimaphelenchus* species and morphospecies were measured in full, and three were described in full, but not all measurements were made for other morphospecies. Measurements included body length, stylet length, body width, tail length, tail, oesophageal length, body width at anus, position of vulva. Body width and width of lateral fields was measured at mid-length. Total body length was measured along the mid-line. Spicules were measured from the top of the condylus to the spicule tip (Braasch and Schmutzenhofer 2000). De Man's ratios were determined; respectively:

a = total body length / greatest body width

b = total body length / distance from anterior end to start of oesophageal glands

b' = total body length / distance from anterior end to base of oesophageal glands

c = total body length / tail length

c' = tail length / width at anus

m = conus / stylet length x 100%

MB = distance from anterior end of body to centre of median bulb / divided by length of oesophagus x 100%

V = anterior end to vulva / total body length x 100%

T = length of testis from cloaca to end or flexure / total body length x 100%

All measurements are given in micrometers (μm).

4.2.4 Scanning electron micoscopy

Nematodes were prepared for scanning electron microscopy (SEM) by modification of the method described by Heegaard *et al.* (1986). Nematodes fixed in formalin were washed in three changes of water purified by reverse osmosis (RO). They were then immersed in a 0.05% solution of Tween 20, and sonicated for 60 s, using setting 4 on a GS UP 50 H sonication probe. The detergent was removed from the nematodes by three washes in filtered RO water, and they were then post-fixed and stained with 2% osmium tetroxide for an hour. The nematodes were then washed three times in filtered RO water. They were subsequently dehydrated through an ethanol series, with 20-30 min. in each stage (30, 70, 80, 90, 95% ethanol, and then two changes of 100% ethanol). After this, a 1:1 solution of absolute ethanol and hexamethyldisilazane (HMDS) was added, and left for 30 min. This was replaced with 100% HMDS, which was allowed to evaporate slowly overnight in a fume cupboard. For mounting of the dry nematodes for SEM examination, a piece of human hair was placed on a sticky disc on a stub. Nematodes were then placed at right angles at intervals along the hair, with some with the head and others the tail balanced on it. Stubs were coated with 3 nm of platinum, and viewed using a Philips XL30 Field Emission scanning electron microscope.

4.3 Results

Three genera were identified from the bark and wood samples of coniferous stands from the south-eastern of Australia using the key of Hunt (1993). These were *Laimaphelenchus*, *Aphelenchoides*, and a putative *Acugutturus*. Where sufficient numbers of nematodes were collected, descriptions and diagnosis of different morphospecies of the genera were made. In total, twelve species will be introduced in this Chapter, including three fully described species and nine morphospecies. Ten were collected from cultivated pine trees, and the other two species from the native conifer *Callitris*. More species were present in the samples than have been described here. Only morphospecies commonly observed in the extracts were collected and mounted for examinations.

4.3.1 *Laimaphelenchus* species and morphospecies collected from the bark of *Pinus* and *Callitris* trees

According to Hunt (1993), *Laimaphelenchus* is a small genus in the subfamily Aphelenchoidinae. *Laimaphelenchus* contains two species groups where the females have or do not have a vulval flap. They are usually recorded from moss, algae and lichens on trees, particular conifers, or in the tunnels of wood-boring beetles. The genus has several distinguishing characters (Hunt, 1993). These include:

1) Nematodes are of medium size, dying strongly ventrally arcuate to C-shape when heat relaxed.

2) Lateral fields have three or four incisures.

3) Cephalic region low, rounded, offset.

4) Stylet fairly well developed with distinct basal swelling.

5) Procorpus cylindrical, leading to a well developed rounded to rounded-rectangular median bulb with strong valve plates.

6) Vulva posterior, typically at about 60-70% of the body length.

7) Anterior vulval lip forms a flap over the genital opening.

8) Vagina usually directed anteriorly and with a cuticular annulus where it joins the uterus.

9) Genital tract monoprodelphic, outstretched. Developing oocytes in a single row. Post-uterine sac well developed and extending about halfway to the anus.

10) Conoid tail, tapering to a distinctive, offset terminus, bearing four pedunculate tubercles with fringed margins.

11) Male spicules elongate, curved. Apex broadly rounded and rostrum triangular and pointed. Three pairs of caudal papillae: one pair situated pre-anally; one pair adanal and the other about mid-way to the tail tip. Tail similar in shape to that of the female and also bearing fringed tubercles.

Descriptions of species and morphospecies

4.3.1.1 *Laimaphelenchus australis* **(Nematoda: Aphelenchina) from exotic pines,** *Pinus radiata* **and** *P. pinaster,* **in Australia**

This description has been published (Zhao *et al.* 2006), see full paper in Appendix A.

Measurements   See Table 4.1

Material examined

Holotype

Male, Nelson, Victoria, Australia (37°48' S, 141°12' E). Taken from a sample of bark from *P. radiata*. Coll. Zhao Zeng Qi, 3 April 2005. It has been deposited in the Australian National Insect Collection (ANIC). The number is 113.

Paratypes

Waite Nematode Collection (WNC 2346 and 2366), The University of Adelaide, slide numbers 004653-004669, 004670, 004673, 004674, 004676-004678. Seventeen males and 22 females were examined. Taken from five locations including: Kuitpo and Mt Gambier, SA; Dartmoor and Ballarat, Vic.; and Tumut, NSW.

Description

Female. Short, relatively stout (ratio a range 24.2-31.6) nematodes; habitus ventrally arcuate, with curvature more pronounced in posterior region (Fig. 4.1: 2). Cuticular debris from a previous moult retained on posterior body in some specimens. Body annules about 0.8 μm wide at mid-body. Lateral fields with 4 incisures (Fig. 4.1: 6 &10), occupying about 15% of body width, not areolate, extending to origin of tubercle.

Table 4.1 Morphometric data for *Laimaphelenchus australis* (measurements in μm ± s.d.)

	Holotype male	Paratype males		Paratype females	
		Mean ± s.d.	Range	Mean ± s.d.	Range
n	1	12		17	
L	427.7	385.2±34.4	300.8-411.5	404.9±31.4	371.5-459.2
a	27.8	27.4±3.1	22.7-31.5	26.4±1.9	24.2-31.6
b	7.3	7.3±0.7	6.6-8.4	7.6±0.6	6.8-9.0
b'	2.8	3.1±0.3	2.4-3.8	3.1±0.2	2.8-3.4
c	12.6	12.6±0.7	11.1-13.7	13.6±1.4	11.9-17.6
c'	2.4	2.7±0.3	2.2-3.4	3.14±0.4	2.3-3.8
V or T	67.4	56.6±6.3	43.2-66.4	68.9±6.5	50-83.9
MB	34.3	37.8±4.2	30.5-43.3	35.5±2.3	32.4-39.9
m	46.7	45±3.9	40-46.4	45±3.2	42.9-46.7
Anterior end to valves of median bulb	52.3	46.4±3.8	40-46.4	46.7±2.8	41.5-51.5
Oesophagus length	152.3	124.4±18.9	92.3-169.2	131.9±9.5	115.4-58.5
Stylet length	11.5	11.2±0.5	10-11.5	11.0±0.8	9.2-12.3
Head width	6.2	6.2±0.4	5.4-6.9	6.1±0.5	5.4-6.9
Head height	2.3	2.4±0.4	1.5-3.1	2.4±0.3	1.9-3.1
Tail length	33.8	30.9±3.0	25.1-36.9	30.1±3.9	25.4-38.5
Anus to vulva	-	-	-	93.8±8.1	79.2-108.5
Anterior end to vulva	-	-	-	279.3±36.8	198.5-369.2
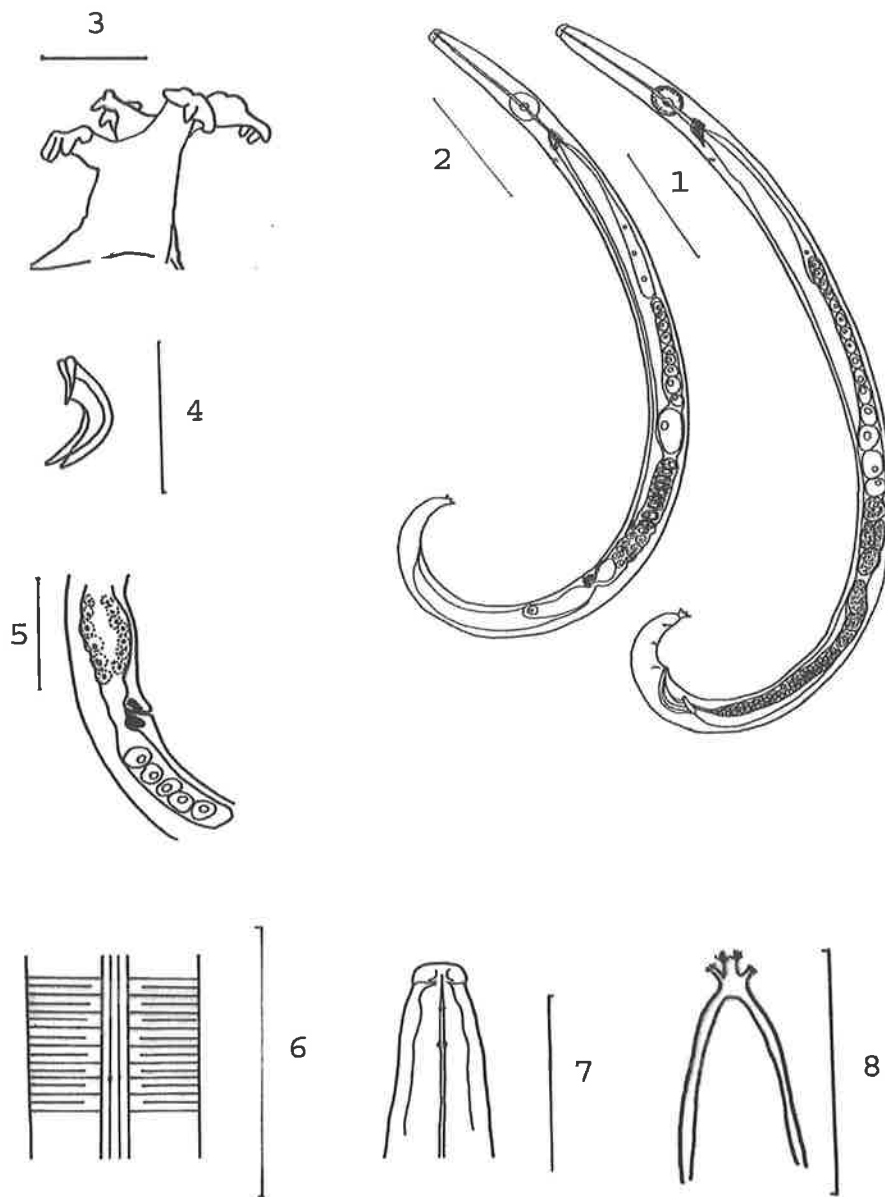
Fig. 4.1 *Laimaphelenchus australis*

1. Male; 2. Female; 3. Tail tip showing pedunculate tubercles; 4. Spicules;
5. Vulva; 6. lateral field; 7. Head & stylet; 8. Tail. Scale bars = 50 μm, 1, 2;
2 μm, 3; 25 μm, 4, 5, 6, 7, 8.
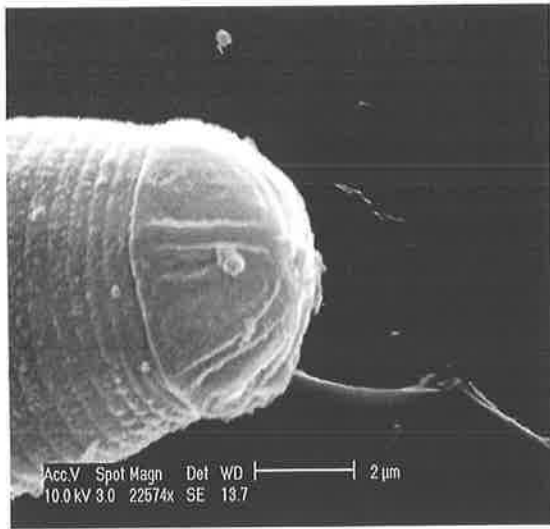
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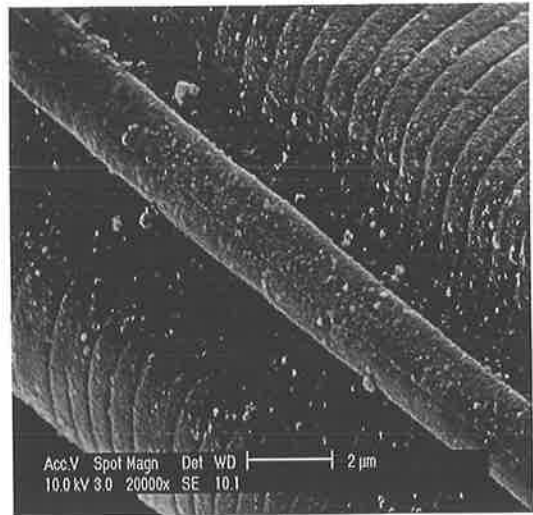
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Fig. 4.1 *Laimaphelenchus australis*

9. Female head; 10 Incisures; 11. Female tail.
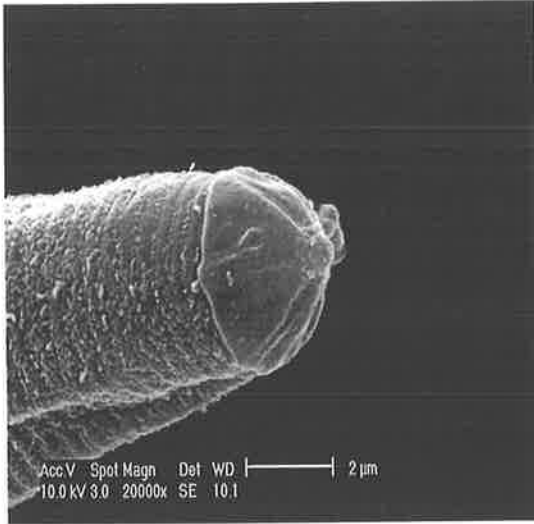
12
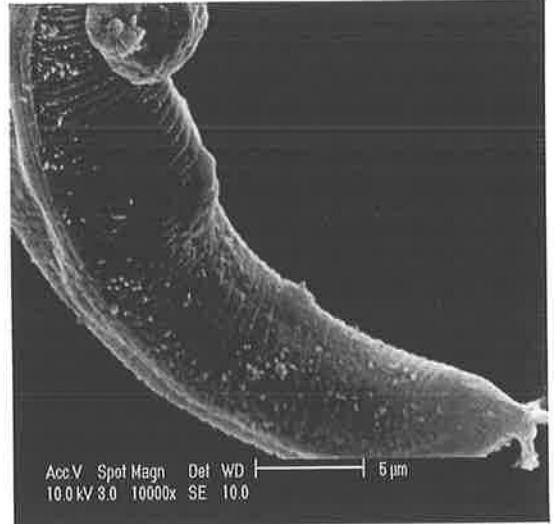
13
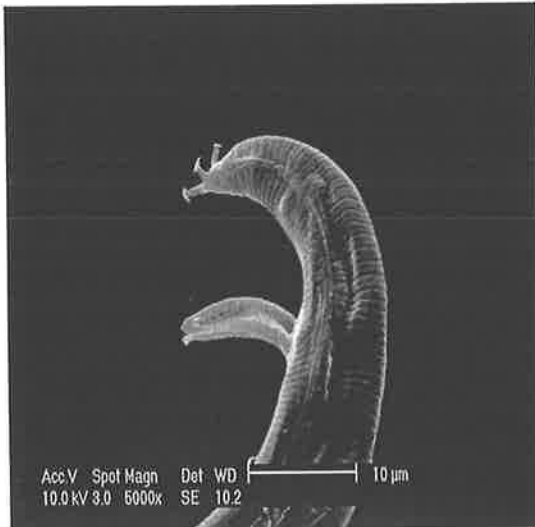
14

Fig. 4.1 *Laimaphelenchus australis*

12. Male head; 13. Male papillae; 14. Male tail & spicule.

Cephalic region rounded, offset, slightly wider than body at base (Fig. 4.1: 7). With SEM, labial area appears hemispherical, no labial disc, smooth, oral aperture flattened (Fig. 4.1: 9). Cephalic region with six labial sectors of equal width, amalgamated, separated by double, well-developed ribs. Cephalids not seen. Stylet slender with distinct basal swellings, 9.2-12.3 μm long.

Median bulb rounded to oval, 11.5-14.6 μm long, and 9.2-12.3 μm wide; with crescentic valves in the middle, located 41.5-51.5 μm from the anterior end, length 3.1-4.6 μm, width 2.7-3.8 μm. The nerve ring is located about one body width anterior to the excretory pore at point where lumen of the intestinal tract widens.

Excretory pore conspicuous, about one and a half body widths posterior to median bulb, 71-85 μm from anterior end. Hemizonid not seen.

Oesophageal glands overlap intestine on dorsal side, extending for 115-148 μm. Oesophago-intestinal junction 1-1.5 body widths posterior to base of bulb. Reproductive system with outstretched ovary with oocytes in a single row; spermatheca filled with sperm cells; vagina sloping slightly towards anterior, not distally sclerotised. Post vulval uterine sac 22-45 μm long, occupying 22-41% of distance from vulva to anus; containing few cells. Vulva without anterior flap; in some specimens appears slightly protruding (Fig. 4.1: 5).

Tail conoid, ventrally curved, with a single offset terminus, bearing 3-4 pedunculate tubercles, each ending with 4-6 finger-like protrusions (Fig. 4.1: 8 & 11).

Male. Morphology similar to that of female (Fig. 4.1: 1 & 2). Testis outstretched, spermatocytes in one single column. Spicules paired, dorsal limb 22-27 μm long, ventral limb 13-17 μm long, from distal to proximal end 15-19 μm long and 17-21 μm measured along median line; rosethorn-shaped, with prominent capitulum and broad rostrum with bluntly rounded tip (Fig. 4.1: 4 & 14). No gubernaculum present. Caudal papillae located at three positions: first pair preanal subventral, second pair postanal subventral, at about 40-45% of distance between cloaca to tail tip, and third pair at about 75-80% of distance between cloaca to tail tip (Fig. 4.1:

13 & 14). Tail conoid, ventrally curved, with a single offset terminus, bearing 3-4 pedunculate tubercles, ending with 4-6 finger-like protrusions (Fig. 4.1: 14).

Discussion

This is the second record of the genus from Australia, and the first record of *Laimaphelenchus* from the widely grown, commercial forest trees, *P. radiata* and *P. pinaster*. *Laimaphelenchus australis* occurred widely in bark samples of *Pinus radiata* and *P. pinaster*, but not in the wood. This suggests that it feeds on fungi or lichens growing on the bark. No insects appeared to be associated with it on the host pine trees.

Diagnosis and relationships are presented in Appendix A, in the proof copy of the paper describing *L. australis*.

4.3.1.2 *Laimaphelenchus* Morphospecies Heidelberg (Nematoda: Aphelenchina) from *Pinus radiata* in Victoria, Australia

Measurements   See Table 4.2

Material examined

Paratype

Ten males and 12 females were examined. Taken from a culture on *B. cinerea* fungus, stored in the Waite Nematode Collection (WNC), slides with number WNC 2438.

Description

Female. Long, slender (ratio a range 28-40) nematodes; habitus slightly ventrally arcuate, with curvature more pronounced in posterior region (Fig. 4.2: 2). Cuticle appears tessellated in some specimens. Body annules 0.8 μm wide at mid-body.

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Lateral fields with 3 incisures, occupying about 20% of body width, not areolate, extending to origin of tubercle (Fig. 4.2: 5 & 9).

Cephalic region rounded, offset, not wider than body at base (Fig. 4.2: 3 & 8). SEM shows a clear labial disc, not divided by ribs, with a demarcation between labial disc and postlabial disc area (Fig. 4.2: 8). Anterior cephalid a few micrometres anterior to level of conus base; posterior cephalid at level of stylet knobs.

Median bulb rounded to oval, 9-11 μm long, and 12-14 μm wide. Nerve ring located near the excretory pore at the point where the lumen of the intestinal tract widens.

Excretory pore conspicuous, about one body width posterior to median bulb, 76-90 μm from anterior end of body. Hemizonid not seen.

Oesophageal glands overlap intestine on dorsal side, extending for 114-162 μm. Reproductive system with outstretched ovary with oocytes in a single row; conspicuous spermatheca filled with sperm cells; vagina sloping slightly towards anterior, not distally sclerotised (Fig. 4.2: 6). Post vulval uterine sac 10-47 μm long, occupying 10-26% of distance from vulva to anus; containing many cells without prominent nuclei. Vulva without anterior flap; in some specimens appears slightly protruding (Fig. 4.2: 9 &10).

Tail conoid, ventrally curved, with a single offset tubercle covered by 20-30 knob-like appendages, seen only with SEM (Fig. 4.2: 11).

Male. Morphology similar to that of female (Fig. 4.2: 1). Testis reflexed in all specimens examined, the reflexed part 40-80 μm long, about 15% of testis length; developing germ cells arranged in single file at the anterior end of the testis, usually in double file in the mid-part, and in single file in the distal end; area with double file occupying about 60% of the testis. Spicules paired, 15-16 μm long from distal to proximal end; rosethorn-shaped, with prominent capitulum and rostrum broad with bluntly rounded tip (Fig. 4.2: 4). Two small protrusions appear on the ventral side, 2 μm from the distal end of the spicule (Fig. 4.2: 14). No gubernaculum

Table 4.2 Morphometric data for *Laimaphelenchus* Morphospecies Heidelberg. (measurements µm ± s.d.)

	Holotype	Allotype	Paratype males		Paratype females	
			Mean ± s.d.	Range	Mean ± s.d.	Range
	1	1	10		12	
L	681	738	672±69.4	566.7-752.4	750±77.3	533.3-895.2
a	38.0	39.6	39.0±2.7	34.3-44.7	39.9±2.4	28.6-40.5
b	10.2	11.9	10.8±1.3	8.9-13.18	12±1.3	8.6-14.2
b'	4.8	3.5	5.1±0.6	4.5-6.1	5.3±0.5	4.7-6.5
c	19.1	17.2	18.8±2.7	15.2-24.2	18.6±2.7	10.7-22.3
c'	2.7	3.8	2.9±0.5	2.2-3.6	3.9±0.7	3.0-4.9
V/T	52.5	67.7	58.1±8.2	50.0-73.4	69.8±2.05	67.9-75.0
MB	57.1	55	55±6.5	42.9-64.3	56.15±2.4	52.4-59.5
m	37.5	42.9	41.5±2.4	40.0-42.9	43.9±3.0	38.5-46.7
Anterior end to valves of median bulb	57.1	55	55±6.5	42.9-64.3	56.15±2.4	52.4-59.5
Oesophagus length	66.7	61.9	62.6±6.0	52.4-71.4	62.6±2.8	57.1-66.7
Stylet length	11.9	10.4	10.5±0.5	9.3-10.4	10.7±0.8	9.7-12.7
Head width	6.0	6.0	5.5±0.4	5.2-6.0	5.8±0.3	5.2-6.0
Head height	3.0	2.2	2.1±0.3	1.5-2.2	2.3±0.4	1.9-3.0
Tail length	35.7	42.9	36.2±4.4	28.6-42.9	41.1±6.5	33.3-47.6
Anus to vulva	-	200.0	-	-	184.7±30.2	114.3-228.6
Anterior end to vulva (μm)	-	500.0	-	-	522.6±48.3	433.3-609.5
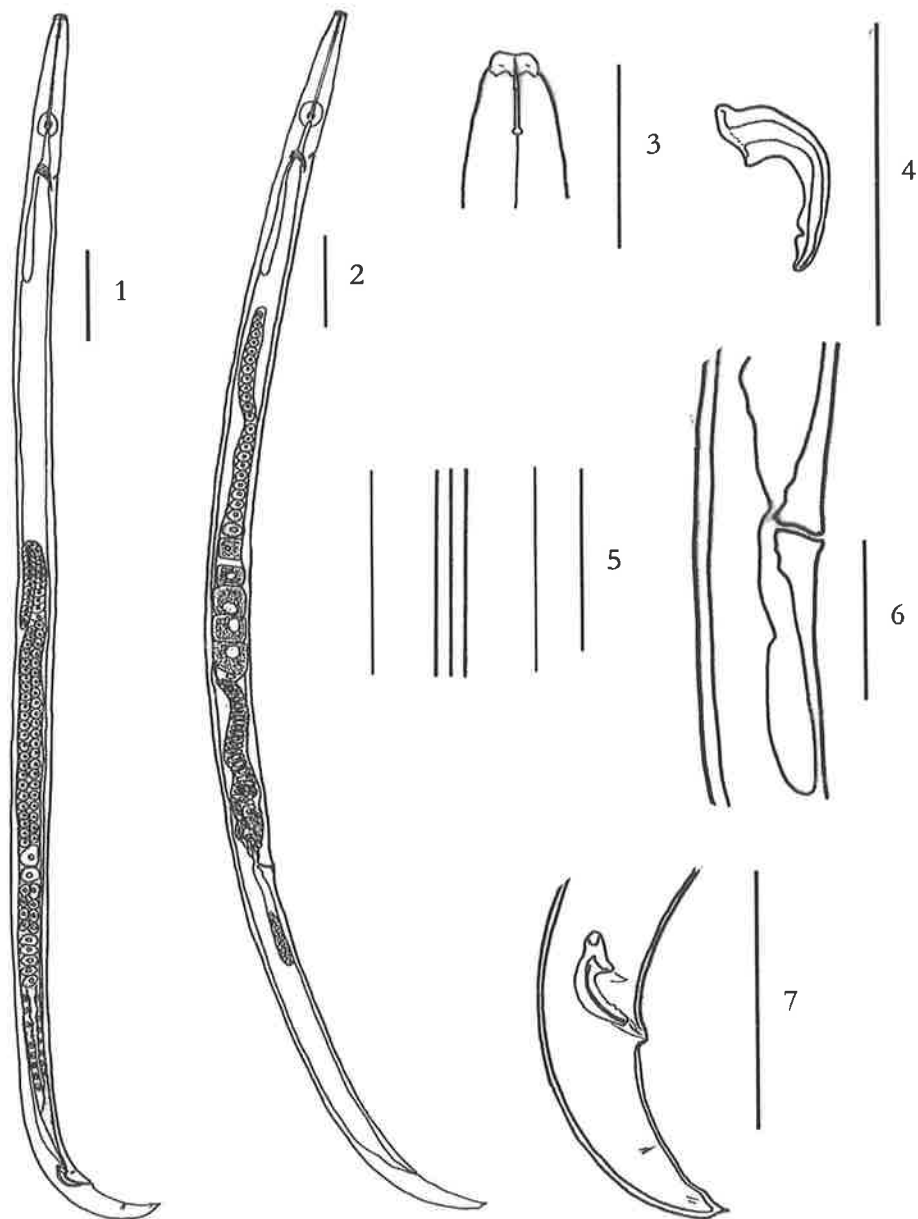
Fig. 4.2 *Laimaphelenchus* Morphospecies Heidelberg

1. Male; 2. Female; 3. Male head; 4. Spicules; 5. Lateral field;
6. Vulval region; 7. Male tail. Scale bars = 50 μm, 1, 2, 7;
25 μm, 3, 4, 5, 6.
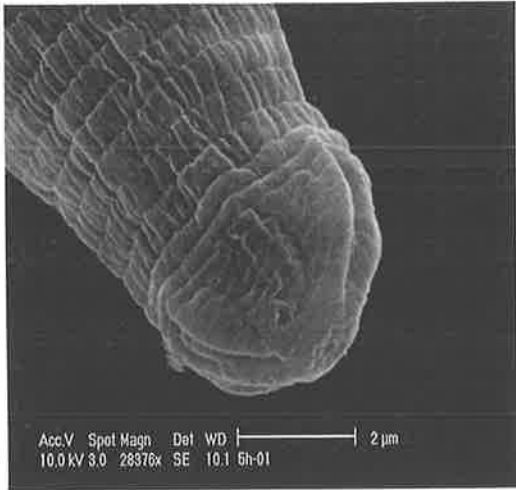
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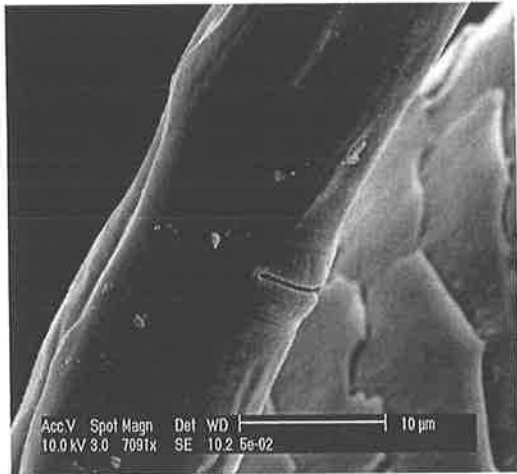
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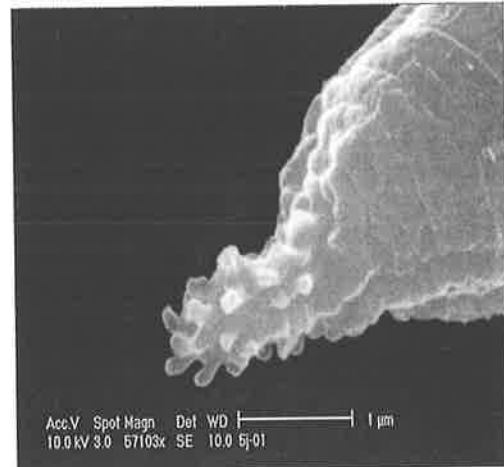
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Fig. 4..2 *Laimaphelenchus* Morphospecies Heidelberg

8. Female head; 9. Female anus; 10. Vulva; 11. Female tail.

12
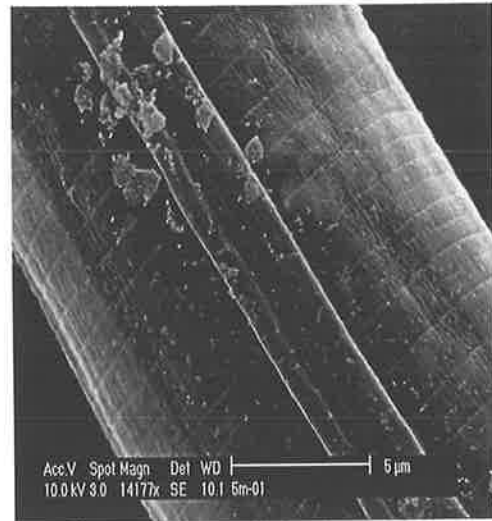
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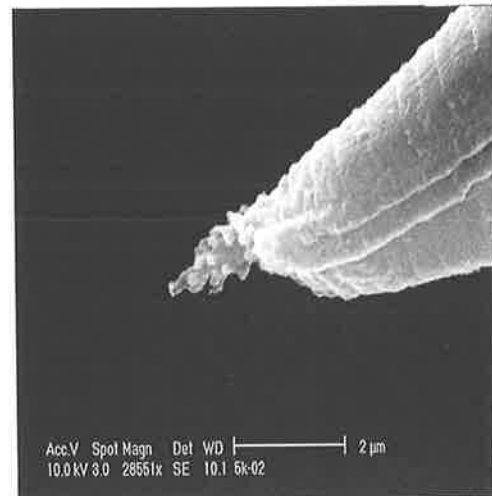
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Fig. 4..2 *Laimaphelenchus* Morphospecies Heidelberg

12. Male head; 13. Incisures; 14. Papillae; 15. Male tail.

present. Caudal papillae located at three positions: one pair preanal at level of spicule capitulum; one pair at about 60% of distance between cloaca and tail tip, and one single papilla on the tail tip before terminus (Fig. 4.2: 7).

Tail conoid, bearing single terminus, with about 20-30 knob-like appendages (seen only with SEM), including a prominent one at the tip (Fig. 4.2: 15).

Type locality and habitat

Culture of nematodes from wood chips collected from the trunk of *P. radiata* growing in Heidelberg, Victoria, Australia (37°45' S, 145°04' E). Collected by David Smith on 23 May 2003.

Diagnosis

Laimaphelenchus Morphospecies Heidelberg (*Laimaphelenchus* Heidelberg) is characterised by a distinct tail shape with an offset terminus covered by 20-30 knob-like appendages in both sexes; three lines in the lateral field; offset head; two pairs of subventral caudal papillae, one pair preanal, one pair at about 60% of distance between cloaca to tail tip, and one single papilla on the tail tip just before the terminus.

Relationships

The genus *Laimaphelenchus* contains two groups of species, one with female having a vulval flap and one without (Baujard 1981; Hunt 1993). *Laimaphelenchus* Heidelberg belongs to the second group, bringing its members to six. The other species in this group are *L. pannocaudus* Massey 1966, *L. phloesini* Massey 1974, *L. pini* Baujard 1981, *L. patulus* Swart 1997 and *L. australis* Zhao, Davies, Riley and Nobbs 2006.

Females of *Laimaphelenchus* Heidelberg (533-895 µm) are close to *L. pannocaudus* Massey 1966 (850-940 µm); *L. penardi* (Steiner 1914), Filipjev and Schuurmans

Stekhoven 1941, (573-800 μm); *L. deconincki* Elmiligy and Geraert 1971, (690-770 μm); *L. pensobrinus* Massey 1966, (610 μm); *L. cocuccii* Doucet 1992, (570-740 μm); *L. unituberculus* Bajaj and Walia 2000, (690-800 μm) and *L. helicosoma.* Peneva and Chipev 1999, (619 μm) in body length. They are larger than other described species; *L. patulus* Swart 1997, (450-530 μm); *L. phloeosini* Massey 1974, (430-510 μm), *L. pini* Baujard 1981, (350-470 μm), and *L. australis* Zhao *et al.*, 2006, (372-459 μm), and shorter than *L. preissii* Zhao *et al.* 2006, (1007-1386 μm).

Laimaphelenchus Heidelberg is separated from *L. penardi, L. deconincki, L. pensobrinus, L. cocuccii, L. unituberculus,* and *L. helicosoma* by the absence of a vulval flap in the latter, and is similar to *L. pannocaudus, L. phloesini, L. pini, L. patulus* and *L. australis.* The post-uterine sac of *Laimaphelenchus* Heidelberg is similar in length to that of *L. phloesini, L. pini, L. patulus* and *L. australis*, but it is shorter than that of *L. pannocaudus, L. penardi* and *L. preissii.* The vagina is surrounded by a relatively thick cuticularised tube, in which it is similar to *L. deconincki, L. cocuccii* and *L. unituberculus*, but it differs from them in having no vulval flap. The tail tip differs from that of all described species except *L. preissii*, in that it has one offset tubercle with about 20-30 knob-like appendages that can only be seen with SEM, but it differs from *L. preissii* by the structure of the projections, which are smaller. The labial disc is clear, and with a demarcation between labial disc and postlabial disc area. This differs from that of *L. patulus*, where there is with no clear demarcation between labial and postlabial disc area (Swart 1997). *Laimaphelenchus* Heidelberg also differs from *L. cocuccii*, which lacks a labial disc.

Males of *Laimaphelenchus* Heidelberg (567-752 μm) are similar to the female in body length. The spicule shape is similar to that of *L. patulus.* However, it has two small protrusions on the ventral side about 2 μm from the distal end, which differs from spicules of all other species. Three groups of subventral caudal papillae are present; one pair preanal, and a second pair at about 60% of the distance from the cloaca to the tail tip. A single papilla is at the tail tip, before the tubercle. The knob-like appendages on the tail terminus are smaller than found in any other described species. The labial plate of the male is similar to the female.

Discussion

This is the third record of the genus *Laimaphelenchus* from Australia, and the second from the common commercial forest tree, *Pinus radiata*. *Laimaphelenchus* Heidelberg was found in a rotting wood sample of *Pinus radiata*. It was not found in non-rotten samples collected earlier from the same tree. Part of the sample was stored in a sealed plastic bag in a constant temperature room at 16°C. After 6 months, a second extraction of the sample was made. At this time, the wood looked wet and it appeared more rotten than the earlier sample did. We suspect that *Laimaphelenchus* Heidelberg did not originate from the wood of the tree. It is possible that it comes from the bark of the tree, and that the wood sample was contaminated during collection. It was cultured successfully on *B. cinerea*, suggesting that it feeds on fungi or lichens. No insects appeared to be associated with it on the host pine trees.

According to the general description of *Laimaphelenchus* (Hunt 1993), three pairs of caudal papillae are present in the male: a preanal pair, an adanal pair and a pair midway to the tail tip. In *Laimaphelenchus* Heidelberg, there is a pair of (or a single) preanal papillae, one pair present at about 60% of the distance between the cloaca and tail tip, and one single (or a pair of) caudal papillae near the tail tip. A pair of caudal papillae near the tail tip occurs commonly in the genus (Baujard 1981; Swart, 1997), appearing in *L. patulus, L. pannocaudus, L. phloeosini, L. pini, L. penardi* and *L. pensobrinus* and *Laimaphelenchus* Heidelberg.

The diagnosis of *Laimaphelenchus* in Hunt (1993) describes the tail as conoid, tapering to a distinctive, offset terminus, bearing four pedunculate tubercles with fringed margins. However, *Laimaphelenchus* Heidelberg, *L. preissii* and *L. unituberculus* have a single offset terminus to the tail, bearing many small projections, rather than four pedunculate tubercles with fringes. Thus, the structure of the terminus is variable between species of *Laimaphelenchus,* but all have knob-like projections on the tail. Sequencing of LSU, SSU and COI genes (Chapter 5; Ye and Giblin-Davis, unpublished data) has confirmed that, despite these morphological differences, these nematodes do indeed belong to the genus

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Laimaphelenchus. Although the single terminus of *Laimaphelenchus* Heidelberg and *L. preissii* is obvious with the light microscope, it cannot be seen in detail without SEM. These nematodes provide a good example of the difficulties associated with the taxonomy of the aphelenchids based on light microscopy alone, as examination with SEM is needed to detect the knob-like projections on the tail that define them as *Laimaphelenchus*.

4.3.1.3 *Laimaphelenchus preissii* (Nematoda: Aphelenchina) from native pine *Callitris pressii* in South Australia

This description has been published (Zhao *et al.* 2006), see full papear in Appendix B.

Measurements   See Table 4.3

Material examined

Holotype

♂, Burdett, South Australia. Taken from nematode culture on *Botrytis cinerea* fungus. It has been deposited to ANIC. The number is 112.

Paratype

Twenty males, 26 females and 54 juveniles have been examined. They were taken from nematode culture on *B. cinerea* fungus. Slides, numbered WINC 004569-004597, are stored in the WNC.

Description

Female. Long, slender (ratio a range 39-57) nematodes; habitus ventrally arcuate, with curvature more pronounced in posterior region (Fig. 4.3: 1). Body annules 1.5 µm wide at mid-body. Lateral fields with 4 incisures, occupying about 30% of body width, not areolate, extending to origin of tubercle (Fig. 4.3: 5 & 10).

70

Cephalic region rounded, offset, clearly wider than body at base (Fig. 4.3: 7). SEM shows a clear labial disc, not divided by ribs, no clear demarcation between labial disc and postlabial disc area (Fig. 4.3: 9). Anterior cephalid at level of conus base; posterior cephalid a few micrometres behind stylet knobs.

Median bulb rounded to oval, 14.7-16.2 μm long, 12.5-14.0 μm wide. The nerve ring is located near the excretory pore at the point where the lumen of the intestinal tract widens.

Excretory pore conspicuous, about 1.5 body widths posterior to nerve ring, 100-114 μm from anterior end. Hemizonid not seen.

Oesophageal glands variable, usually one dorsal but may be two lobes (one small ventral and one large dorsal), overlap of intestine on dorsal side extending for 164-200 μm.

Reproductive system with outstretched ovary with oocytes in a single row; conspicuous spermatheca filled with sperm cells; vagina sloping towards anterior, not distally sclerotised. Post vulval uterine sac 86-157 μm long, occupying one third to one half of distance from vulva to anus; containing many cells with prominent nuclei. Vulva with well developed anterior vulval flap, posterior lip about twice the width of anterior (Fig. 4.3: 4 & 11).

Tail conoid, ventrally curved, with one broad tubercle with about 10 projections (seen only with SEM), including a prominent one at the tip (Fig. 4.3: 8 & 12).

Male. Morphology similar to that of female (Fig. 4.3: 2). Testis outstretched; developing germ cells in single file. Spicules paired, 22-28 μm long, rosethorn-shaped, with prominent capitulum and rostrum broad with bluntly rounded tip (Fig. 4.3: 3). No gubernaculum. Two pairs of caudal papillae present, one pair adanal, subventral; second pair subventral at about 60% of distance to tail tip (Fig. 4.3: 6). Lateral fields extending to tail tip, expanded to form small caudal alae, visible only with SEM (Fig. 4.3: 15).

Table 4.3 Morphometric data for *Laimaphelenchus preissii* sp. nov. (measurements μm ± s.d.)

	Holotype	Allotype	Paratype males		Paratype females	
			Mean ± s.d.	Range	Mean ± s.d.	Range
n	1	1	20		26	
L	971	1071	1088±60.8	1000-1218	1185±74	1007-1386
a	51.0	56.3	45.3±5.9	36.7-51.0	48.9±4.9	39.3-57.1
b	12.8	12.5	13.5±1.4	10.9-15.6	14.5±1.5	11.8-17.8
b'	6.2	5.1	5.8±1.0	5.0-7.7	6.1±0.5	5.5-6.9
c	22.7	20.5	25.4±4.3	17.6-34.7	28.3±5.8	19.1-39.3
c'	2.7	4.7	2.3±0.4	1.6-3.2	2.9±0.6	1.8-4.5
V/T	59.8	68.9	63.2±6.1	55.5-71.7	69.8±1.5	66.5-71.3
MB	42.4	36.4	41.85±4.5	37.0-47.8	37.8±3.4	33.3-43.5
m	47.1	41.2	45±1.3	43.3-47.1	43.6±3.2	38.9-46.9
Anterior end to valves of median bulb	66.7	76.2	83.3±13.7	71.4-104.8	70.8±4.7	64.3-78.6
Oesophagus length	157.1	209.5	200±30.6	152.4-233.3	188.1±14.0	164.3-200.0
Stylet length	12.7	12.7	13.6±0.9	11.2-16.7	13.6±0.7	11.9-14.9
Head width	6.7	7.5	7±0.4	6.7-7.5	6.8±0.2	6.7-7.1
Head height	2.6	2.6	2.7±0.4	2.2-3.0	2.6±0.4	2.2-2.9
Tail length	42.9	52.4	43.9±7.1	32.1-57.1	43.7±9.1	32.1-64.3
Anus to vulva	-	281.0	-	-	314.1±24.1	271.4-364.3
Anterior end to vulva	-	738.1	-	-	827.9±57.1	707.1-1000.0
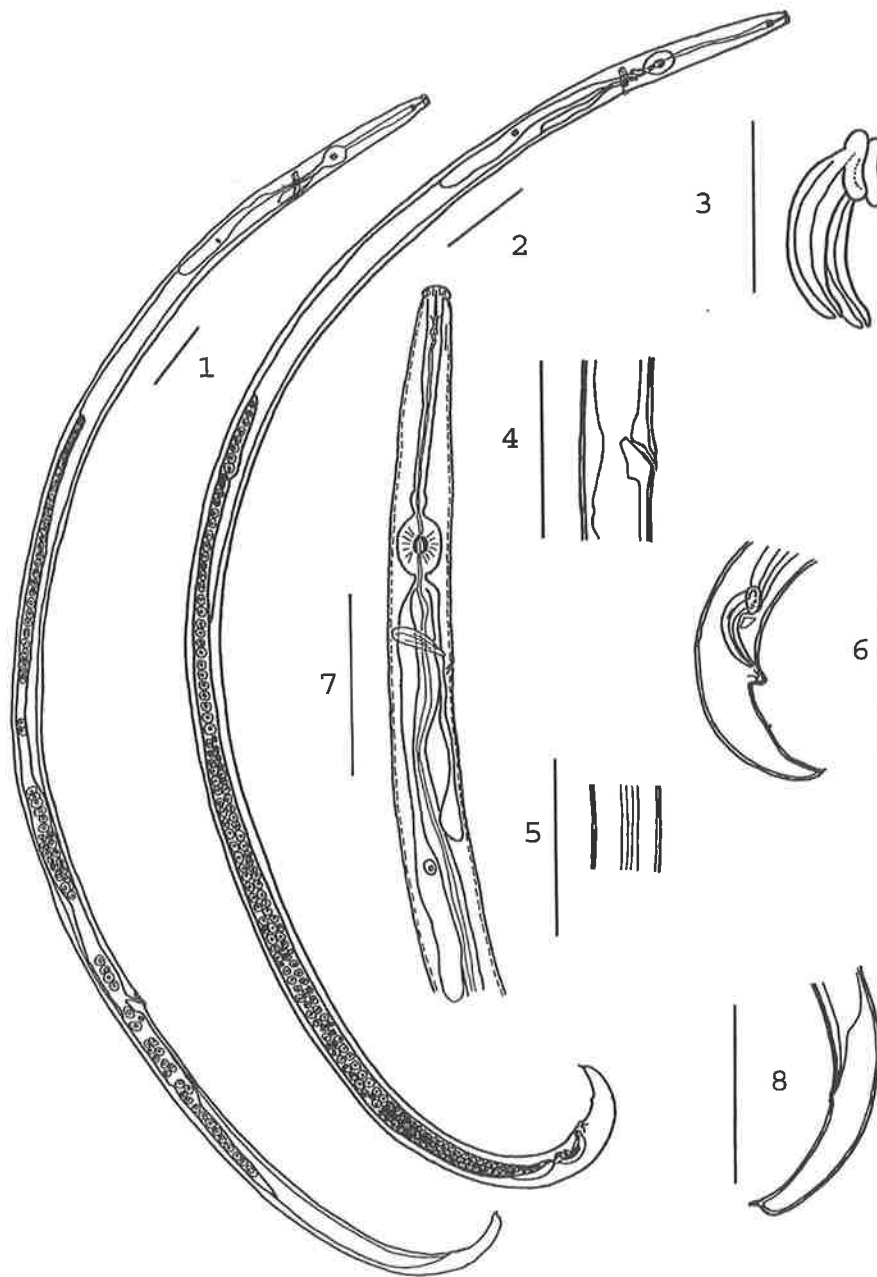
Fig. 4.3 *Laimaphelenchus preissii*

1. Female; 2. Male; 3. Spicules; 4. Vulval region; 5. Lateral field;
6. Male tail; 7. Female head; 8. Female tail. Scale bars = 50 μm, 1,
2, 4, 5, 6, 8; 25 μm, 3; 10 μm 7.

9
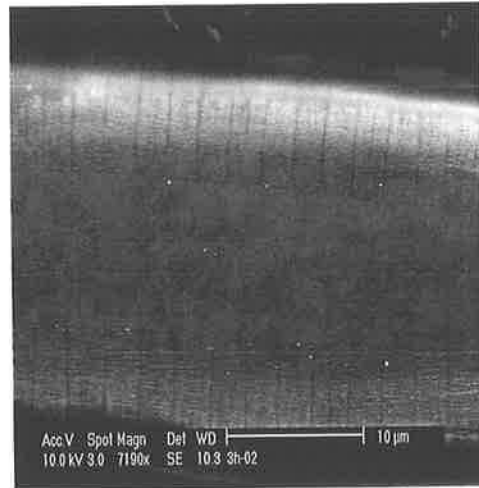
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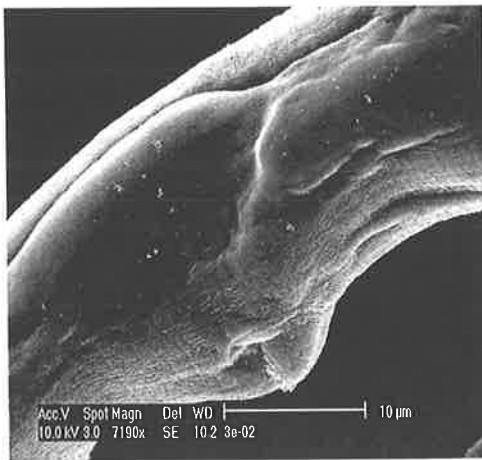
11

12

Fig. 4..3 *Laimaphelenchus preissii*

9. Female head; 10. Incisures; 11. Vulval flap; 12. Female tail.
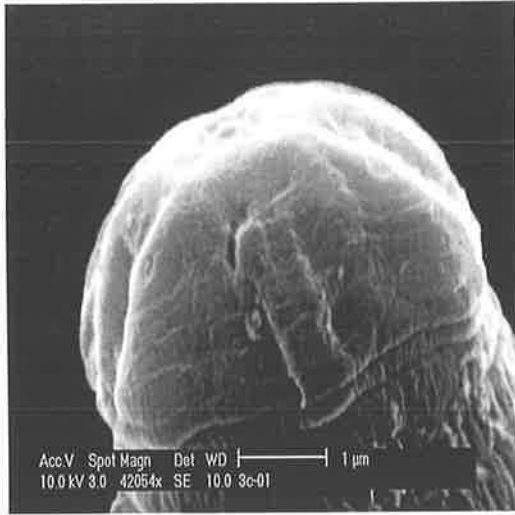
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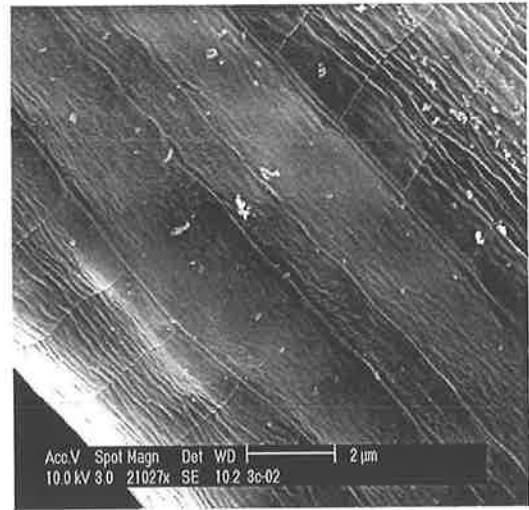
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15
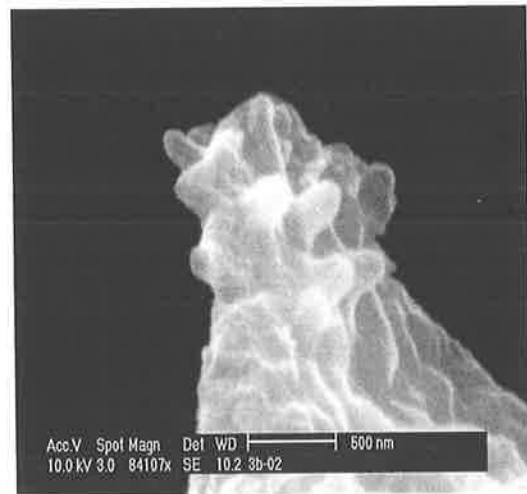
16

Fig. 4.3 *Laimaphelenchus preissii*

13. Male head; 14. Incisures; 15. Bursa & Papillae ; 16. Male tail.

Tail conoid, bearing single dorsoventrally flattened tubercle, with about 20 projections (seen only with SEM), including a prominent one at the tip (Fig. 4.3: 16).

Juveniles. The range of lengths for the juvenile stages were: J2: 286-429 μm (n =18); J3: 500-714 μm (n =18); J4: 786-1071 μm (n =18).

Type locality and habitat

Bark on trunk of *C. preissii* growing on roadside at Burdett, SA (35°98' S, 139°36' E). Collected by Z. Zhao on 6 November 2003.

Discussion

This is the first record of the genus from Australia native pine *Calitris preissii*. Full discussuion, diagnosis and relationships are presented in Appendix B, in the proof copy of the paper describing *L. preissii*.

4.3.1.4 *Laimaphelenchus* Morphospecies Tutye

Material examined

Tutye, New South Wales, Australia. Taken from a sample of bark from *Callitris* sp. from roadside trees. Coll. K. Davies, 20 September 2003. Two males and 4 females were examined. These are stored in the Waite Nematode Collection (WNC) with number 2335.

Measurements

Females: (n=4): L=1203±57.9 (1262-1135); a=61.5±4.9 (54.6-65.6); b=12±2.8 (9.0-14.6); b'=6.0±0.2 (5.7-6.2); c=26.2±2.8 (23.5-29.8); c'=3.8±0.3 (3.4-4.1); V=67.5±1.3 (66.4-69.3); MB=37.2±1.6 (35.2-38.5); m=42.4±1.3 (41.2-44.1); anterior end to valves of median bulb=75.0±2.2 (73.1-76.9); oesophagus length =201.9±3.8 (200-207.7); stylet length =13.8±1.1 (13.1-15.4); head width=6.8±0.2

6.5-6.9); head height=2.9±0.5 (2.3-3.5); tail length=46.2±3.1 (42.3-50); anus to vulva=342.3±31.7 (307.7-369.2); anterior end to vulva=811.5±41.9 (753.8-853.8).

Males: (n=2): L=1050±70.7 (1000-1100); a=53.6±5.1 (50.0-57.2); b=13.7±0.9 (13.0-14.3); b'=5.6±1.3 (4.6-6.5); c=21.8±0.2 (15.2-24.2); c'=2.7±0.2 (2.6-2.9); MB=36.5±6.2 (32.1-40.9); T=68.9±5.9 (32.1-40.9); m=37.4±10 (30.3-44.4); anterior end to valves of median bulb =69.2±0 (6.9); oesophagus length =192.3±32.6 (169.2-215.4); stylet length=13.3±0.8 (12.7-13.8); head width =6.7±0.3 (6.5-6.9); head height=2.3±0 (2.3); Tail length=48.1±2.7 (46.2-50.0).

Description

Female. Long, slender (ratio a range 55-66) nematode; habitus slightly ventrally arcuate, with curvature more pronounced in posterior region (Fig. 4.4: 2). Body annules 1.4 μm wide at mid-body, finely annulated. Lateral fields with 4 incisures, occupying about 20% of body width, ribbon-like. Cephalic region rounded, offset, knob-like (Fig. 4.4: 3). Stylet 13-15 μm long. Median bulb rounded to oval. Nerve ring located anterior to excretory pore, at point where lumen of intestinal tract widens. Excretory pore conspicuous, about 1-1.5 body widths posterior to basal median bulb. Hemizonid 5 annules behind excretory pore. Oesophageal gland with one dorsal lobe; overlap of intestine extending for 200-207 μm. Reproductive system outstretched with oocytes in a single row; inconspicuous spermatheca, vagina sloping anteriorly (Fig. 4.4: 5). Post vulval uterine sac occupying one-third of distance from vulva to anus; containing many cells. Vulva at c. 66-69% with well-developed anterior flap; posterior lip about three times width of anterior. Tail conoid, ventrally curved, with a broad tubercle with many tiny projections, difficult to see with light microscope (Fig. 4.4: 6 & 7).

Male. Morphology similar to that of female (Fig. 4.4: 1). Stylet 12 μm long. Testis outstretched; developing germ cells in single file at the anterior part, one or two or many files at the posterior part. Spicules paired, rosethorn shape, with prominent capitulum and rostrum broad, with slight flair anterior to tip, tip flattened (Fig. 4.4: 4). No gubernaculum present. Two pairs of caudal papillae present, one pair adanal, subventral; second pair subventral at about 50% of distance to tail tip. Tail

Fig. 4.4 *Laimaphelenchus* Morphospecies Tutye

1. Male; 2. Female; 3. Male anterior region; 4. Male tail & spicules
5. Female vulval flap. Scale bars = 50 µm, 1, 2, 3, 4, 5.

Fig. 4.4 *Laimaphelenchus* Morphospecies Tutye

6. Male tail; 7. Male tail

conoid without a bursa. Tail tip with single conoid tubercle, with many tiny projections difficult to see with light microscope.

Remarks

Laimaphelenchus morphospecies Tutye (*Laimaphelenchus* Tutye) was isolated from native *Callitris* near Griffith, NSW. This nematode is very similar to *L. preissii* except that the male lacks a bursa. Attempts to recollect it failed. Due to the limited sample, only two SEM images were obtained and molecular analysis was not carried out. Because of its tail structure, it is considered here as a species of *Laimaphelenchus*. It differs from all described *Laimaphenchus* species by its extremely long body size. Geographically, the area in which *Laimaphelenchus* Tutye was found is distant from the collection area of *L. preissii*. More SEM pictures and molecular data would aid in determining the relationships of these nematodes.

4.3.1.5 *Laimaphelenchus* Morphospecies Kuitpo

Material examined

Kuitpo, South Australia (39°93.641' S, 138°41.265' E). Taken from a sample of bark from *Pinus radiata*. Collected Zhao Zeng Qi, 10 March 2003.

Ten males and 12 females were examined. These are stored in the WNC with number 2343.

Measurements

Females: (n=12): L=907±54.9 (785.7-981.0); a=53.9±2.5 (49.1-58); b=11.3±0.9 (9.7-12.9); b'=4.3±0.6 (3.1-5.4); c=24.6±5.5 (17.5-36.2); c'=3.9±1.5 (2.1-8.0); V=70.2±3.5 (66.7-79.5); MB =34.4±5.0 (25.4-44.4); m=44.9±2.8 (40.6-50.0); anterior end to valves of median bulb=71.8±5.9 (66.7-81.0); oesophagus length=212.7±33.6 (171.4-300.0); stylet length =11.2±0.8 (10.4-12.7); head width=6.3±0.5 (5.2-6.7); head height=2.0±0.3 (1.5-2.2); tail length=38.3±7.9 (28.6-

52.4); anus to vulva=240.9±21.9 (200.0-281.0); anterior end to vulva=636.5±57.3 (547.6-738.1).

Males: (n=10): L=856±52.9 (809.5-752.4); a=53.1±3.4 (48-58.3); b=11.2±0.8 (9.9-12.4); b'=4.8±0.3 (4.3-5.1); c=22.5±2.5 (17.5-26.9); c'=2.6±0.2 (2.2-3.0); MB=38.3±4.3 (31.3-44.3); T=58.8±4.1 (52.0-63.4); m=43.6±2.1 (40.0-42.9); anterior end to valves of median bulb = 68.3±8.6 (57.1-81); oesophagus length =180.0±8.7 (167.2-192.5); stylet length=11.5±0.7 (10.8-12.7); head width=5.9±0.3 (5.2-6.3); head height=2.1±0.4 (1.5-2.6); tail length =38.3±3.6 (33.3-38.1).

Description

Female. Long, slender (ratio a range 49-58) nematode; habitus slightly ventrally arcuate, with curvature more pronounced in posterior region (Fig. 4.5: 1). Body annules 1.9 μm wide at mid-body, finely annulated. Lateral fields with 4 incisures, occupying about 24% of body width, ribbon-like. Cephalic region rounded, offset, knob-like. Stylet 11-13 μm long. Median bulb rounded to oval. Nerve ring at point where lumen of intestinal tract widens. Excretory pore conspicuous, about one body width posterior to basal median bulb, at the level of nerve ring. Hemizonid not seen. Oesophageal gland with one dorsal lobe overlapping intestine for 171-300 μm. Reproductive system outstretched with oocytes in a single row; inconspicuous spermatheca, vagina sloping anteriorly (Fig. 4.5: 4). Post vulval uterine sac occupying one-third to one-half of distance from vulva to anus; containing many cells with prominent nuclei. Vulva at about 66-79% with well-developed anterior flap; posterior lip about three times width of anterior. Tail conoid, ventrally curved, with a broad tubercle with many tiny projections (hard to see with light microscope), including a prominent one at the tip.

Male. Morphology similar to that of female (Fig. 4.5: 2). Stylet 12-13 μm long. Testis outstretched; developing germ cells in single file at the anterior part, one or two or many columns at the posterior part. Spicules paired, rose-thorn shape, with prominent capitulum and rostrum broad, with slight flair, tip flattened (Fig. 4.5: 3). No gubernaculum present. Two pairs of caudal papillae present, one pair adanal, subventral; second pair subventral at about 60% of distance to tail tip. Tail conoid

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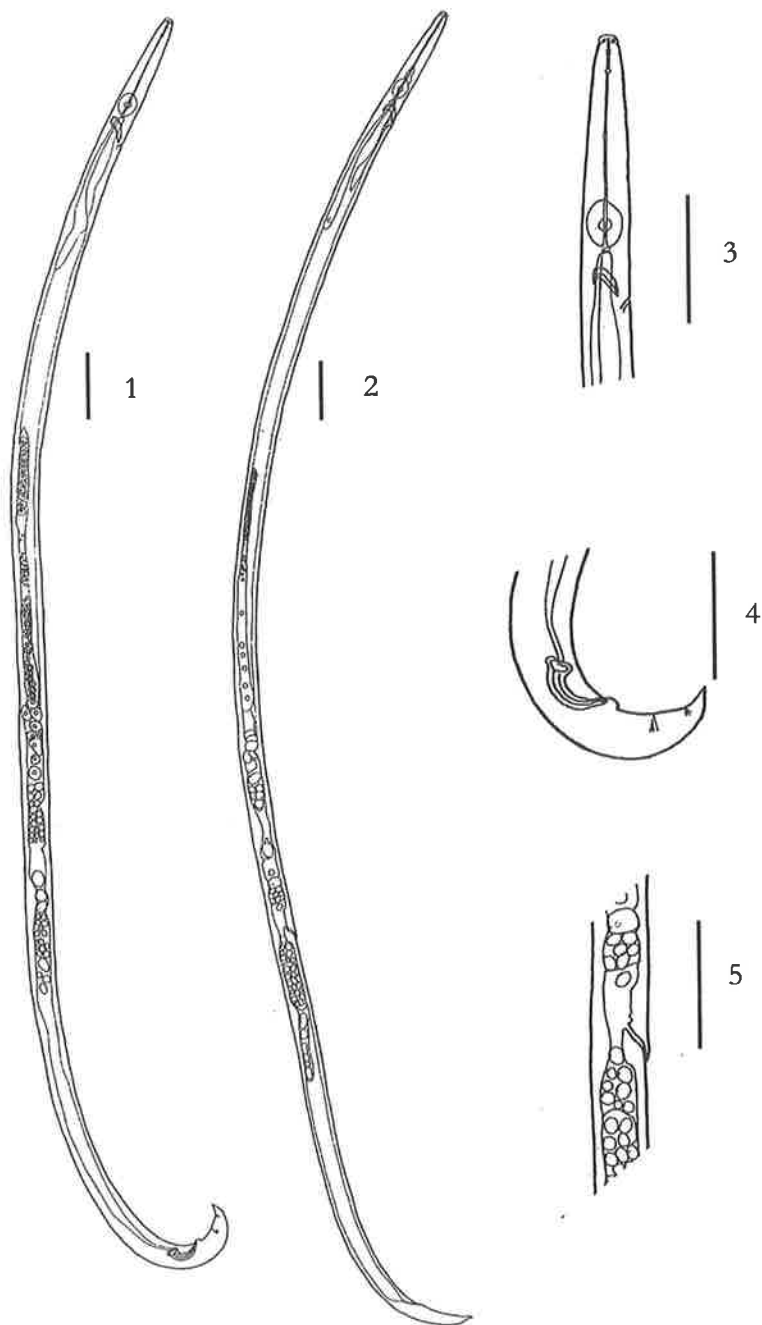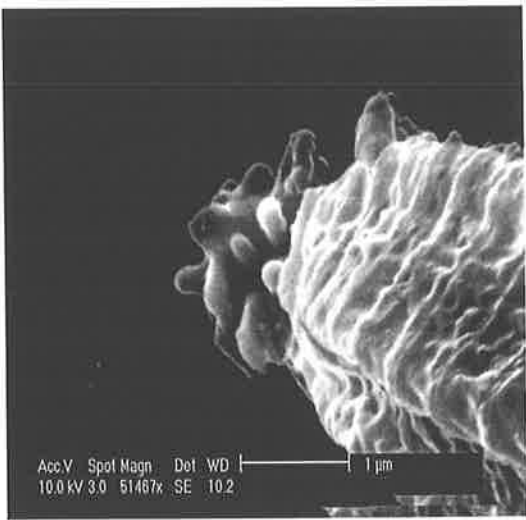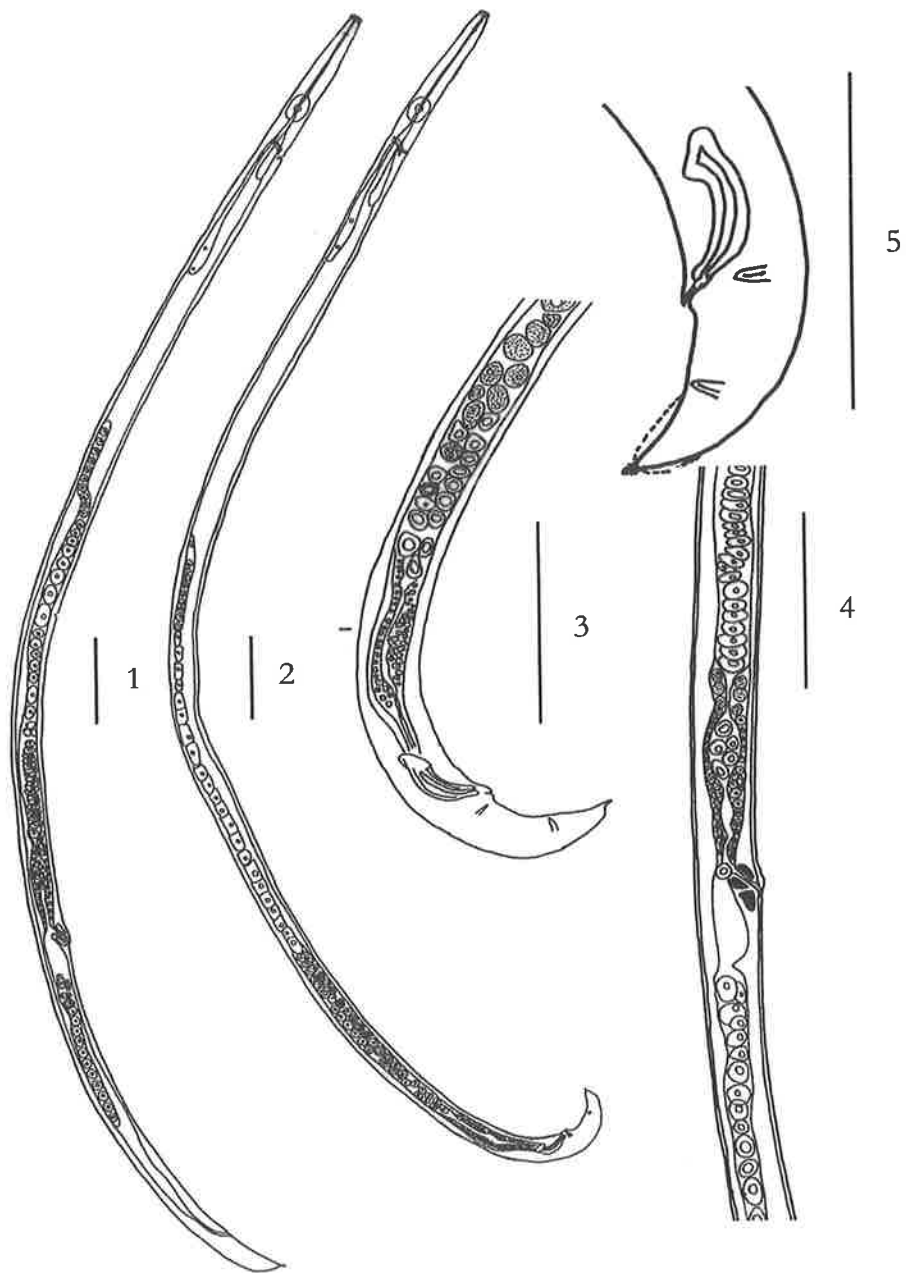Fig. 4.5 *Laimaphelenchus* Morphospecies Kuitpo

1. Female; 2. Male; 3. Male spicules & papilae; 4. Female vulval region;
5. Male tail & bursa. Scale bars = 50 μm, 1, 2, 3, 4, 5.

6
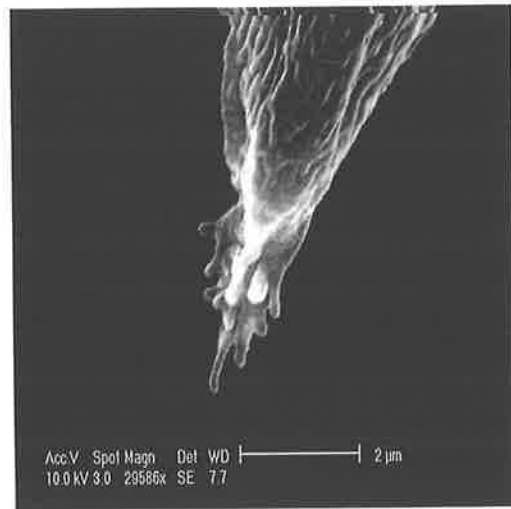
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Fig. 4.5 *Laimaphelenchus* Morphospecies Kuitpo

6. Male head; 7. Male tail

with a bursa (Fig. 4.5: 5 & 7). Tail tip with single conoid tubercle, with many tiny projections difficult to see with light microscope.

Remarks

Laimaphelenchus morphospecies Kuitpo (*Laimaphelenchus* Kuitpo) was isolated from a sick tree in a pine plantation at Kuitpo, South Australia. Because the tail of these males has a bursa, and females have a vulval flap, it was first thought likely to be *Bursaphelenchus* sp. Many attempts to recollect it have failed. The reason for that is unclear. However, molecular data for *L. preissii* and *Laimaphelenchus* Heidelberg showed that they were closer to the genus *Laimaphelenchus* than to *Bursaphelenchus* (Chapter 5). Given its tail structure, the nematode is here considered as a species of *Laimaphelenchus*. *Laimaphelenchus* Kuitpo is close to *L. preissii* in having a bursa, and to *Laimaphelenchus* Tutye in having a vulval flap and in body shape. However, its a ratio and vulval position differ from those of *L. preissii* and *Laimaphelenchus* Kuitpo, and *Laimaphelenchus* Tutye lacks a bursa. SEM pictures and ribosomal DNA sequences are needed to see the relationships of these forms of *Laimaphelenchus*.

4.3.1.6 *Laimaphelenchus* Morphospecies Noolook

Material examined

Collected from bark of *Pinus radiata* in forest at Noolook, the South-East of SA. Two males and six females were examined. These are stored in the WNC with number 2409, Collected Zhao Zeng Qi, 10 March 2004.

Measurements

Female: (n=6): L=629±106 (557.1-761.9); a=44±6.6 (38.1-49.5); V=71±1.8 (67.2-71.9); stylet length=11±0.44 (10.8-11.5); head width =4.8±0.2 (4.62-5); head height =1.8±0.2 (1.8-0.2); tail length=40±2.7 (38.1-42.9); anus to vulva =159±36 (2.9-4.2); anterior end to vulva =444±77 (381-533).

Male: (n=2): L=526 (505-547); a=44; stylet length=10.4 (10-10.8); head width=5.0; head height =1.9; spicule=15.8 (15.4-16.2); tail length=30.3 (29-32).

Description

Female. Medium size, slender (ration a range 38-50) nematode; habitus slightly ventrally arcuate, with curvature more pronounced in posterior region (Fig. 4.6: 2). Body finely annulated. Lateral field incisures not seen with light microscope. Cephalic region rounded, offset, knob-like. Stylet 10.8-11.5 μm long. Median bulb rounded, oval. Intestine begins immediately behind bulb. Nerve ring located near excretory pore, at point where lumen of the intestinal tract widens. Excretory pore conspicuous, about one body width posterior to median bulb, anterior to the nerve ring. Hemizonid not seen. Oesophageal gland with two lobes, overlapping intestine on dorsal side. Reproductive system with ovary with oocytes in a single column; inconspicuous spermatheca; vagina sloping anterior, not sclerotised distally (Fig. 4.6: 2). Post vulval uterine sac 65.4 μm long, occupying one-third of distance from vulva to anus; containing few or many sperm cells. Vulva at about 71%. Tail conoid, ventrally curved, with a broad tubercle with several tiny projections (just visible with light microscope).

Male. Morphology similar to that of female (Fig. 4.6: 1). Stylet 10-10.8 μm long. Testis outstretched; developing germ cells in one or two or many colums depending on region of reproductive system. Spicules paired, 15.4-16.2 μm long, rosethorn-shape, with prominent capitulum and rostrum broad and a small condylus (Fig. 4.6: 5). No gubernaculum present. Two pairs of caudal papillae present, one pair adanal, subventral; second pair subventral at about 50% of distance to tail tip. It is possible that there is a third pair just in front of the tail tip, but SEM is needed to confirm this. Tail conoid and tail tip with a small single conoid tubercle, with a few tiny projections difficult to see with light microscope.

Remarks

Laimaphelenchus morphospecies Noolook (*Laimaphelenchus* Noolook) is similar to *Laimaphelenchus* morphospecies Nelson (*Laimaphelenchus* Nelson, Fig. 4.7) in body size and general morphology, but the tail structures are different. SEM

Fig. 4.6 *Laimaphelenchus* Morphospecies Noolook

1. Male; 2. Female; 3. Male head; 4. Female tail;
5. Male spicules & papillae. Scale bars = 50 μm, 1,2, 4, 5; 25 μm, 3.

pictures were not made for either morphospecies, but the differences in tail shape are quite distinctive from the light microscope. The tubercle of the *Laimaphelenchus* Noolook is thin with a few tiny projections, but *Laimaphelenchus* Nelson is broader and crown-like. The excretory pore is anterior to the nerve ring in *Laimaphelenhus* morphospecies Noolook, but posterior to it in *Laimaphelenchus* morphospecies Nelson. *Laimaphelenchus* Noolook is also similar to *Laimaphelenchus* morphospecies Mt Gambier (*Laimaphelenchus* Mt Gambier, Fig. 4.8), but the body size and tail structures are clearly different between the two species. *Laimaphelenchus* Mt Gambier has tail shape resembling that of a cone with projections. The body size is greater for *Laimaphelenchus* Mt Gambier than for *Laimaphelenchus* Noolook. There are two pairs of papillae for *Laimaphelenchus* Noolook, and there may be a third pair in front of the tail tip. In contrast, there are two pairs of papillae for *Laimaphelenchus* Mt Gambier and three for *Laimaphelenchus* Nelson. These three morphospecies can be discriminated by their body size, male papillae and tail structures and position of the excretory pore. Because all three were collected from the South-East of SA, SEM pictures and ribosomal DNA sequences are needed to determine their relationships.

4.3.1.7 *Laimaphelenchus* Morphospecies Nelson

Material examined

From bark of *Pinus radiata* in forest near Nelson, the South-East of SA. Five males and five females were examined. These are stored in the WNC, with numbers 2361 and 2365. Collected Zhao Zeng Qi, 10 March 2004.

Measurement

Female: (n=5): L=548±64.3 (481.0-638.1); a=38±5.0 (32.5-46.1); V=65.4±2.9 (64.4-68.6); stylet length=11.7±1.0 (10.8-13.1); head width=5.4±0.0; head height =2.3±0.0; tail length=33.3±6.7 (23.8-42.9); anus to vulva=156.2±26.6 (123.8-195.2); anterior end to vulva =358.1±0.9 (309.5-428.5).

Male:(n=5): L=455±46.4 (381.0-504.8); a=39.2±5.2 (33.7-46.9); stylet length=10.9±0.3 (10.8-11.5); head width=5.2±0.3 (4.6-5.4); head height=2.3±0.0; tail length=25.7±4.3 (19.0-28.6); spicules=15.8±1.0 (15.4-17.7).

Description

Female. Medium to long nematodes, 480-640 µm long (ratio a range 33-46). Body shape straight to ventrally arcuate when heat-relaxed, with most curvature in the posterior region (Fig. 4.7: 2). Cuticle finely annulated. Lateral field with 2 incisures, occupying 20% body width. Cephalic region rounded, off-set, clearly wider at base than the following body. Stylet slender, 10.8-13.1 µm long, with small basal knobs higher than wide. Median bulb spherical to ovoid with central valve plates. Oesophageal gland well developed, overlapping on dorsal side of intestine. Nerve ring one body width behind bulb; excretory pore posterior to nerve ring. Anus and rectum present. Vulva at 65% body length; vulval lips indistinct. Reproductive system monoprodelphic, outstretched. Developing oocytes in one column. Post-vulval uterine sac present, with sperm; about 40% of distance from vulva to anus. Tail conoid; terminus tube-like with truncate tip carrying several small projections, crown-like (Fig. 4.7: 4).

Male. Similar features to female, but with tail strongly curved ventrally when heat-relaxed (Fig. 4.7: 1). Spicules thorn-shaped, paired, separate. Rostrum rounded, small condylus present (Fig. 4.7: 5). Three pairs caudal papillae present; one pair adanal, one pair subterminal at mid-tail length and one pair just in front of the tail tip. Tail conoid; terminus tube-like with truncate tip with small projections as above (Fig. 4.7: 5). Bursa absent.

Remarks

Same as in Section 4.3.1.6. In addition to the collection from south-eastern South Australia, this nematode was also found at some sites in Victoria. Projections on the tail tip suggest this morphospecies is a *Laimaphelenchus*. The crown-like tail structure is distinct from all *Laimaphelenchus* species mentioned in this Chapter. Under the light microscope, the tail structure of *Laimaphelenchus* morphospecies
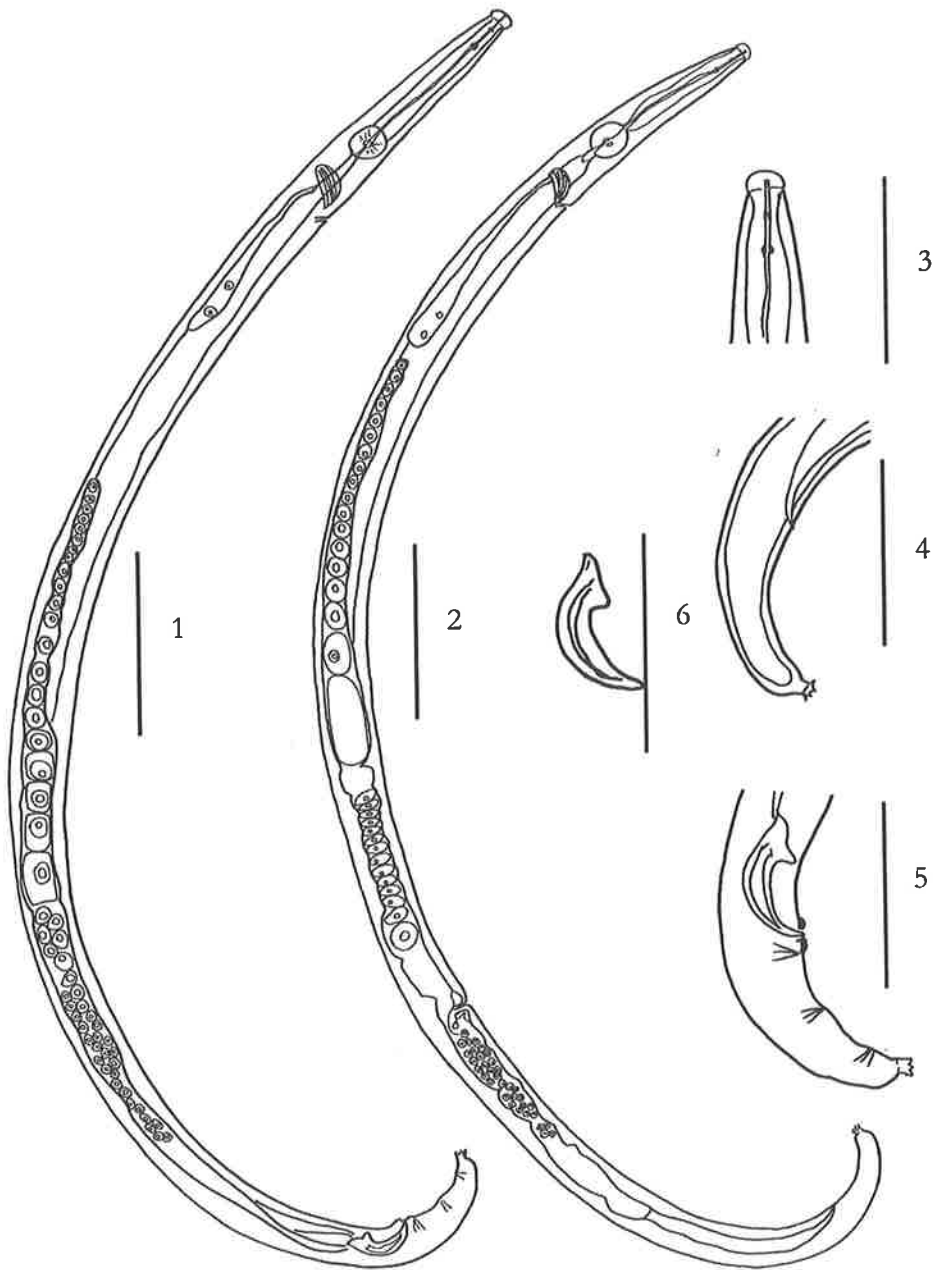
Fig. 4.7 *Laimaphelenchus* Morphospecies Nelson

1. Male; 2. Female; 3. Male head; 4. Female tail; 5. Male papillae
6. Spicules. Scale bars = 50 μm, 1, 2, 3, 4, 5, 6.

Nelson is closer to that of *L. australis,* but they differ in body size and the numbers of the projections on the tail tip. However, SEM pictures and ribosomal DNA sequences are needed to investigate their relationships.

4.3.1.8 *Laimaphelenchus* Morphospecies Mt. Gambier

Material examined

Collected from bark of *Pinus radiata* in forests at Christmas Hill, Kuitpo Forest, in the South-East of SA and Vic, and Penrose Forest in NSW. Stored in the WNC with number 2361. Collected Zhao Zeng Qi, 10 March 2004.

Although these nematodes were collected several times, and were morphologically distinct, good fixed specimens were rare, and hence only one male and one female were examined.

Measurement

Female: (n=1): L=854; a=52.9; V=68.5; Anterior end to valves of median bulb=71.8; Oesophagus length=69.2; Stylet length=13.8; Head width=6.15; Head height=1.5; Tail length=34.6; Anus to vulva=258; Anterior end to vulva=585.

Male: (n=1): L=881; a=52; Stylet length=12.3; Oesophagus length=75.4; Head width=5.4; Head height=1.5; Spicule=22; Tail length=45.4.

Description

Female. Long, slender (ratio a 53) nematode; habitus slightly ventrally arcuate, with curvature more pronounced in posterior region (Fig. 4.8: 2). Body finely annulated. Lateral fields with 2 incisures, occupying about 20% of body width, ribbon-like. Cephalic region rounded, offset, knob-like. Stylet 14 μm long. Median bulb rounded, oval. Intestine begins immediately behind bulb. Nerve ring located near excretory pore, at anterior of intestine. Excretory pore inconspicuous. Hemizonid not seen. Oesophageal gland with one dorsal lobe with three nuclei. Reproductive system with reflexed ovary with oocytes in a single column; vagina sloping anterior, sclerotised distally. Post vulval uterine sac 23 μm long, occupying

90
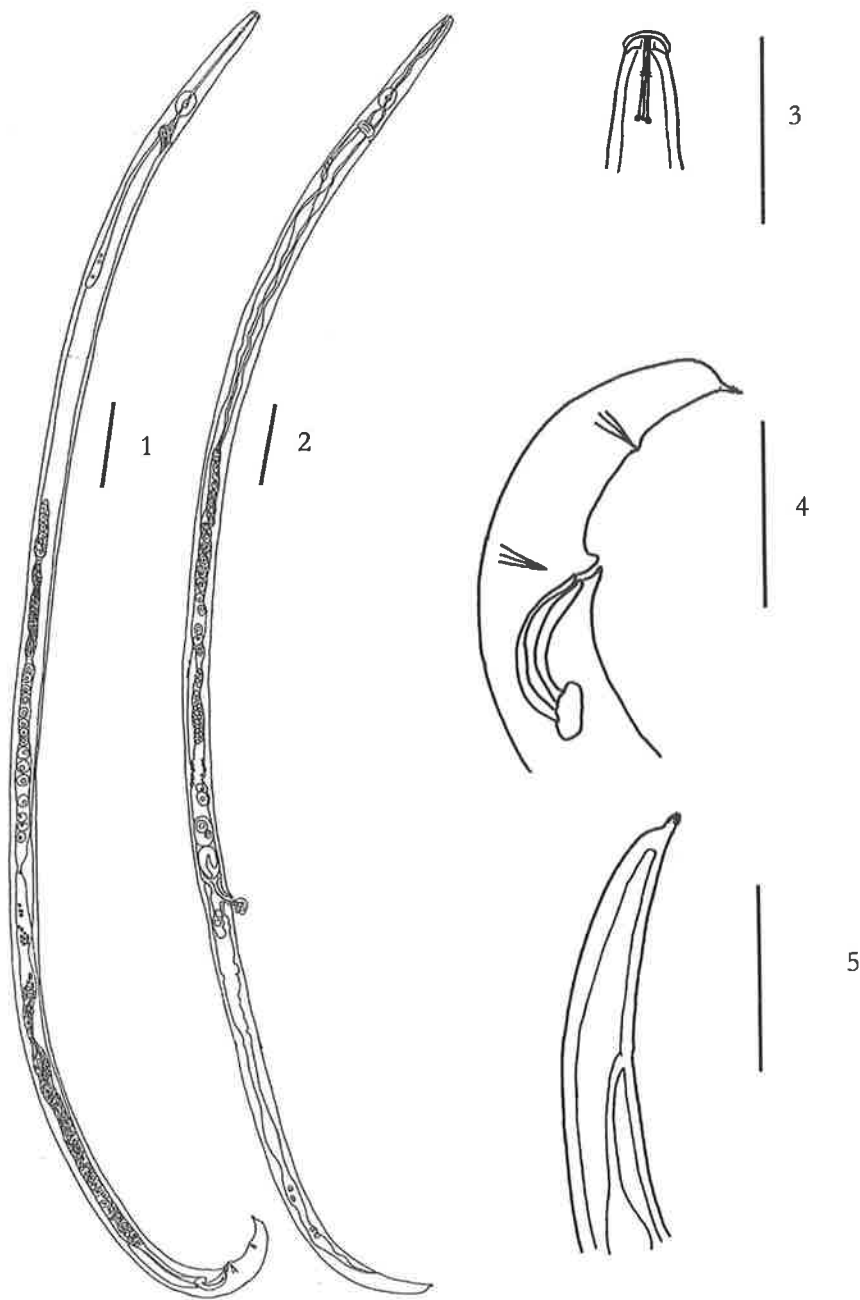
Fig. 4.8 *Laimaphelenchus* Morphospecies Mt Gambier

1. Male; 2. Female; 3. Male head; 4. Male spicules & papillae;
5. Female tail. Scale bars = 50 μm, 1, 2, 3, 4, 5.

one-tenth distance from vulva to anus; containing few cells. Vulva at about 70% with well-developed anterior flap; posterior lip about twice width of anterior. Tail conoid, ventrally curved, with a broad tubercle with several tiny projections, hard to see with light microscope (Fig. 4.8: 5).

Male. Morphology similar to that of female (Fig. 4.8: 1). Stylet 12 μm long (Fig. 4.8: 3). Testis outstretched; developing germ cells in one or two or many columns depending on region of reproductive system. Spicules paired, rosethorn shape, with prominent capitulum and rostrum broad (Fig. 4.8: 4). No gubernaculum present. Opening of cloaca has well developed anterior lip overlapping the posterior, latter is about twice width of former. Two pairs of caudal papillae present, one pair adanal, subventral; second pair subventral at about 60% of distance to tail tip. Tail conoid without a bursa. Tail tip with single conoid tubercle, with a few tiny projections difficult to see with light microscope.

Remarks

Laimaphelenchus Mt Gambier was collected from Christmas Hill, Kuitpo Forest, in the South-East Region of SA, and from Penrose Forest, NSW. Although the frequency of the nematodes was not great in the collections, they are distinctive, and easily recognised by their relatively long size and typical conoid tail. The tail shape is similar to that of *Laimaphelenchus* Heidelberg, *L. preissii*, and *Laimaphelenchus* Kuitpo, but the males differ from *L. preissii*, and *Laimaphelenchus* Kuitpo by lacking a bursa, and from *Laimaphelenchus* Heidelberg by lacking a preanal papilla. As mentioned in Section 4.3.1.6, *Laimaphelenchus* Mt Gambier also has some similarities to *Laimaphelenchus* Noolook and *Laimaphelenchus* Nelson. SEM pictures and ribosomal DNA sequences are needed to investigate their relationships.

4.3.2 *Aphelenchoides* morphospecies collected from pines

The main characteristics of the genus *Aphelenchoides* are given in Hunt (1993). These include:

1) Heat relaxed females die straight to ventrally arcute but the males assume a walking-stick like shape with the tail region sharply curled ventrally.

2) Cephalic region usually rounded and slightly offset.

3) Stylet slender, with basal knobs or swellings, often about 10-12 μm long and usually less than 20 μm. Procorpus cylindrical, leading to a well developed ovoid or spherical median bulb with central valve plates.

4) Oesopageal gland lobe well developed and lying dorsal to the intestine.

5) Genital tract monoprodelphic, typically outstretched, but may reflex. Developing oocytes in one or more rows. Post-uterine sac usually present and often containing spermatozoa, but may be absent.

6) Tail conoid with a variable terminus which may be bluntly or finely rounded, digitate or bifurcate or with a ventral projection. One or more mucrons of various shapes may be present.

7) Spicules thorn-shaped, paired and separate. The rostrum and apex are usually well developed, but may be almost absent. Typically there are three pairs of caudal papillae, one pair adanal, one pair subterminal and the other in between. Bursa absent.

As mentioned in Section 2.4.2.1, *Aphelenchoides* is a large, reasonably distinct genus of worldwide distribution. It is found within the taxonomically diverse family Aphelenchoididae. Hunt (1993) listed 155 species including 16 species *inquirendae vel incertae sedis* and over 180 species were accounted for by others (Nickle 1992; Liu, Wu *et al.* 1999). Of these, many nominal species are inadequately characterised for reliable recognition or poorly described and the genus is in urgent need of a major revision (Hunt 1993). In addition, descriptions have been published in about 6 different languages, making it more difficult to adequately compare what has been found here with published descriptions (Mike Hodda, pers. com. 2006). From molecular studies (see Chapter 5), it is clear that the nematodes described below belong to *Aphelenchoides*, which is probably paraphyletic. Due to the problematic nature of this genus, nematodes belonging to the *Aphelenchoides* are described here only as morphospecies and diagnoses have not been attempted. The morphospecies are described below.

4.3.2.1 *Aphelenchoides* Morphospecies Aphelenchid K1

Material examined

Nematodes extracted from wood chips from dead *Pinus radiata* at Knoxfield, Vic. Twenty-eight males and 16 females were examined. These are stored in the WNC with number 2329, Collected David Smith, May. 2003.

Measurement

Female: (n=16): L=633.8±137.5 (404.8-823.8); a=30.5±5.1 (23.9-40.3); b=10.1±1.6 (6.8-11.9); c=16.3±3.3 (10.4-23); c'=4.1±1.2 (2.0-6.0); V=71.3±5.3 (53.0-75.5); stylet length =12.4±1.7 (9.7-15.7); tail length=41.9±11.1 (23.3-66.7); anus to vulva =140±33.5 (91.0-183.8).

Male: (n=28): L=611.8±79.6 (433.3-714.2); a=28.3±2.3 (23.6-33.6); b=10.1±0.9 (8.8-11.5); c=17.4±1.9 (13.1-21.4); c'=2.8±0.3 (2.4-3.2); stylet length=11.4±1.1 (8.2-13.4); tail length=35.4±4.6 (26.2-42.9); spicules=17.4±1.3 (14.2-19.8).

Description

Female. Medium to long nematodes, 634 μm long (ratio a range 23.6-33.6) (Fig. 4.9: 1). Body shape straight to ventrally arcuate when heat-relaxed, with most curvature in the posterior region. Cuticle finely annulated. Lateral field with 2 incisures, occupying 30% body width (Fig. 4.9: 9). Cephalic region rounded, offset, obviously wider at base than the following body, with 6 annules, and a clear cephalic disc (Fig. 4.9: 8). Stylet slender, 12.4 μm long, with small basal knobs. Median bulb spherical to ovoid with central valve plates. Oesophageal gland well developed, one or two lobes, one dorsal and one ventral to intestine. Nerve ring half body width behind bulb; excretory pore posterior to nerve ring. Anus and rectum present. Vulva at 71% body length; vulval lips flat, indistinct (Fig. 4.9: 7 & 10). Reproductive system monoprodelphic; outstretched with prominent spermatheca. Developing oocytes in one column. Post-vulval uterine sac present, also with

Fig. 4.9 *Aphelenchoides* Morphospecies Aphelenchid K1

1. Female; 2. Male; 3. Male lateral field; 4. Male head; 5. Female tail;
6. Male spicules & tail; 7. Vulval region. Scale bars = 50 μm, 1, 2, 3, 4, 5, 6, 7.
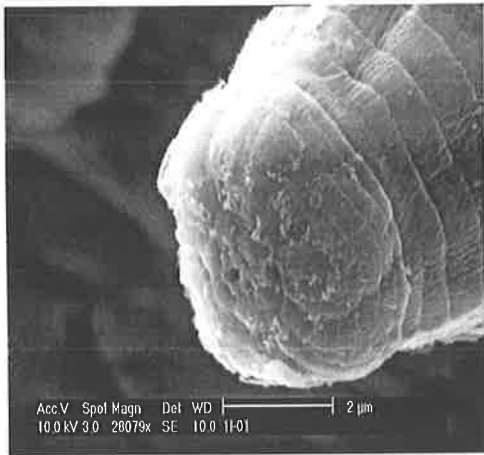
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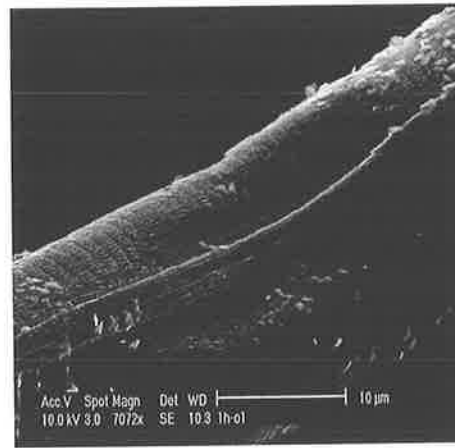
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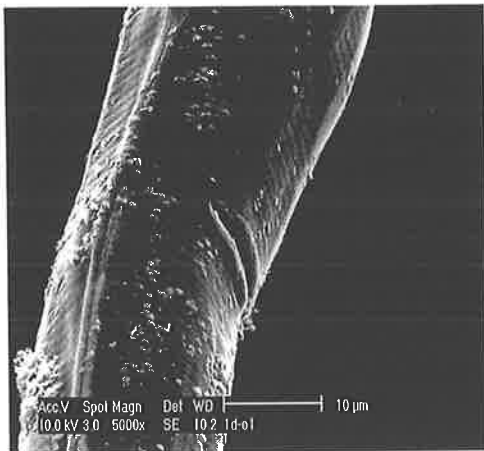
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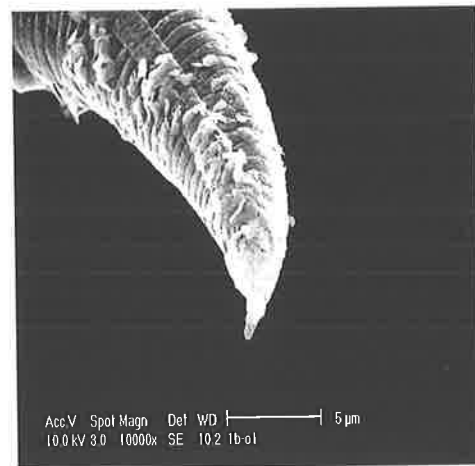
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Fig. 4.9 *Aphelenchoides* MorphospeciesAphelenchid K1

8. Female head; 9. Incisures; 10. Vulva ; 11. Female tail.
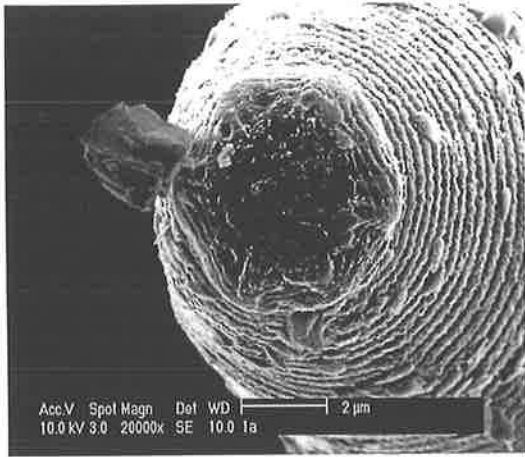
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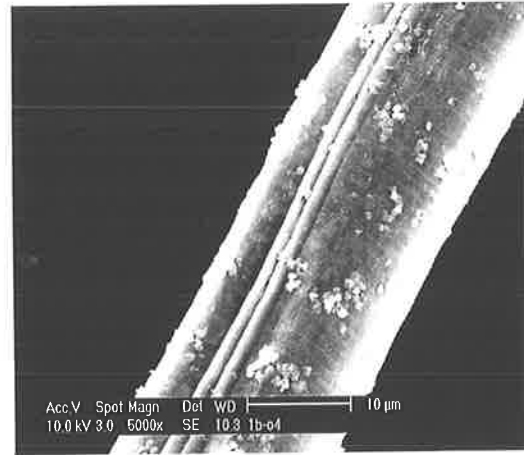
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Fig. 4.9 *Aphelenchoides* Morphospecies Aphelenchid K1

12. Male head; 13. Incisures; 14. Cloaca & Papillae; 15. Male tail.

sperm; occupying about 30% of distance from vulva to anus. Tail conoid; terminus with a mucron (Fig. 4.9: 5 & 11).

Male. Similar features to female, tail not strong hooked ventrally when heat-relaxed (Fig. 4.9: 2). Spicules thorn-shaped, paired, separate (Fig. 4.9: 6). Rostrum rounded, condylus absent. Two pairs caudal papillae present; one pair adanal, one pair subventral at mid-tail length. Tail conoid; terminus bluntly rounded with a mucron (Fig. 4.9: 15). Bursa absent.

Remarks

Aphelenchoides Morphospecies Aphelenchid K1 (Aphelenchid K1) was one of the first two nematodes extracted from the diseased wood chips from Knoxfield, Victoria. In the early stages of this study, it was considered as a putative *Bursaphelenchus* species following the finding of *B. hunanensis*. Hence it was examined in detail and successfully cultured in the laboratory. *Aphelenchoides* Morphospecies Aphelenchid K2 (Aphelenchid K2), *Aphelenchoides* Morphospecies Aphelenchid H1 (Aphelenchid H1) and some bacterial feeding nematodes were also recovered from the diseased wood chips. SEM images and molecular data of Aphelenchid K1 were obtained. Despite lacking the strongly recurved male tail thought to be typical of *Aphelenchoides* (Hunt 1993), Aphelenchid K1 is closer to the genus *Aphelenchoides* than to *Bursaphelenchus*, lacking the vulval flap and bursa. This is supported by molecular data (Chapter 5).

4.3.2.2 *Aphelenchoides* Morphospecies Aphelenchid K2

Material examined

Nematodes extracted from wood chips from dead *Pinus radiata* at Knoxfield, Vic. Five males and 5 females were examined. These are stored in the WNC with number 2436. Collected by David Smith, May 2003

Measurement

Female: (n=5):L=370±21.6 (344.6-390.8); a=23.2±0.9 (22.1-24.5); V=74.4±1.9 (77-80); stylet length=9.7±1.2 (7.7-10.8); head width=5.4; head height=1.5; tail length=31; anterior end to vulva=287±21 (264-312).

Male: (n=5): L=263±20 (235-285); a=27±1.4 (25-28); stylet length=8.6±1.1 (6.9-10); head width=4.6; head height=2.3; spicule=14.8±1 (13.1-15.4); tail length =24±1.3 (23-26).

Description

Female. Small, relatively stout nematodes, 345-309 µm long (ratio a range 22.1-24.5). Body shape straight to ventrally arcuate when heat-relaxed, with most curvature in the posterior region (Fig. 4.10: 1). Cuticle finely annulated. Lateral field with 3 incisures, occupying about 10% body width (Fig. 4.10: 4). Cephalic region rounded, not off-set, cap-like, not obviously wider at base than the following body (Fig. 4.10: 3). Stylet slender, 7.7-10.8 µm long, with small basal knobs. Median bulb spherical to ovoid with valve plates posterior to half way. Oesophageal gland well developed, two lobes, overlapping on dorsal side of intestine. Nerve ring one body width behind bulb; excretory pore one body width posterior to nerve ring. Anus and rectum absent. Vulva at 74% body length; vulval lips slightly protruding (Fig. 4.10: 5 & 6). Reproductive system monoprodelphic; outstretched with prominent spermatheca. Developing oocytes in several rows. Post-vulval uterine sac absent. Tail conoid; terminus bluntly rounded with or without a small mucron (Fig. 4.10: 6).

Male. Similar features to female, but with tail strongly ventrally arcuate, with posteror tightly curved when heat-relaxed (Fig. 4.10: 2 & 7). Spicules mitten-shaped, paired, separate. Rostrum prominent. Apex well developed. One single and three pairs papillae present; one single pre-anal; one pair adanal, two pairs subterminal close to tail tip. Tail conoid; terminus with a small mucron. Rudimentary bursa present; only apparent with SEM (Fig. 4.10: 8 & 9).
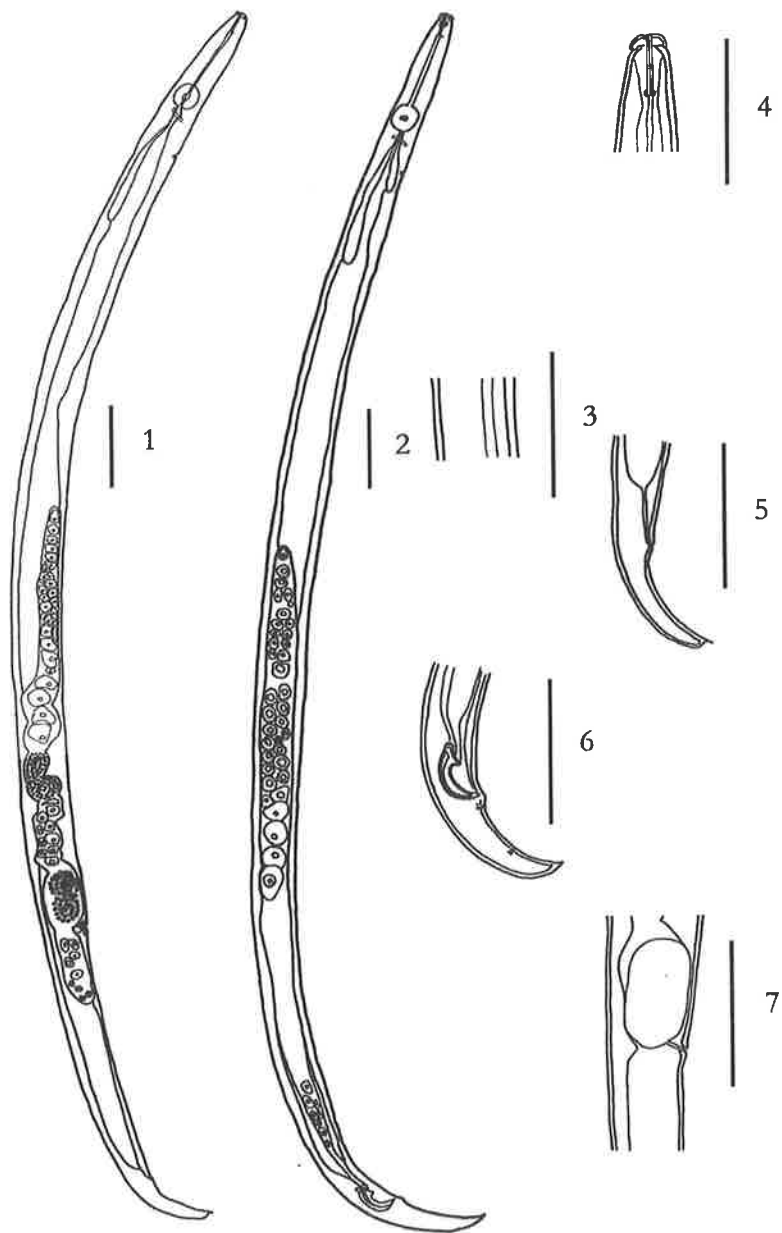
Fig. 4.10 *Aphelenchoides* Morphospecies Aphelenchid K2

1. Female; 2. Male. Scale bars = 50 μm, 1, 2.
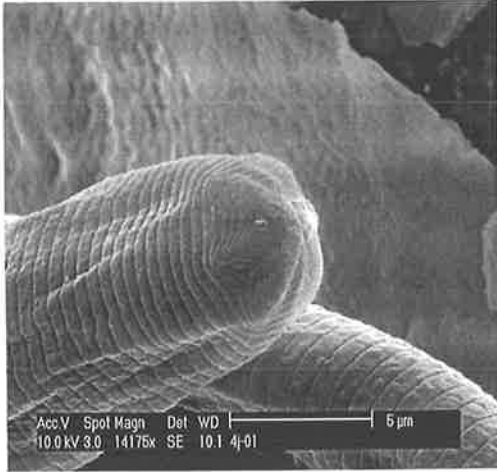
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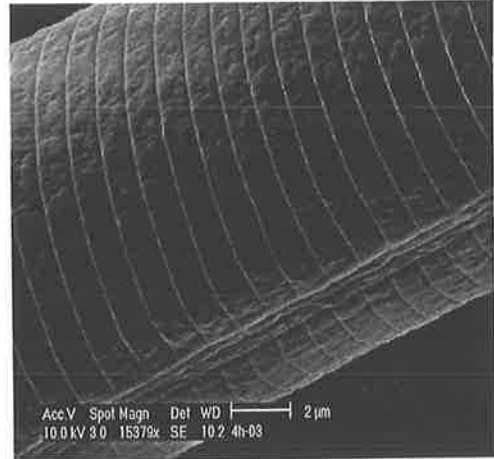
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Fig. 4.10 *Aphelenchoides* Morphospecies Aphelenchid K2

3. Female head; 4. Incisures; 5. Vulva; 6. Female tail.

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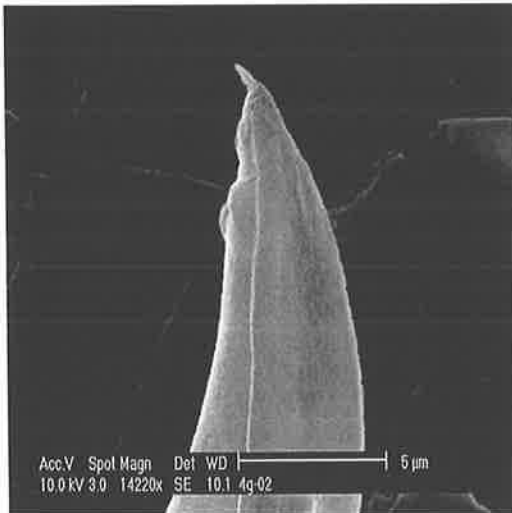
Fig. 4.10 *Aphelenchoides* Morphospecies Aphelenchid K2

7. Male head; 8. Bursa (arrowed) & Papillae; 9. Tail tip &
Papillae.

Remarks

Aphelenchid K2 was isolated from the samples that also yielded Aphelenchid K1. Initially it was thought to be the same as Aphelenchid H1, because of morphological similarity under the light microscope. However, it differs from Aphelenchid H1 by absence of an anus in the female, smaller body size, one single and three pairs of papillae and in molecular data. The absence of an anus suggests that these nematodes could be an insect associate, possibly belonging to the genus *Ekaphelenchoides* or *Cryptaphelenchoides*. It differs from *Ektaphelenchoides* in being smaller, having a stylet with knobs, a single and 3 pairs (not 2) of caudal papillae, and lacking a spicate process on the tail tip. It appears close to *Cryptaphelenchoides* but differs from it in having a small oesophageal gland, a more posterior excretory pore, a shorter intestinal diverticulum in the female, a single and 3 pairs (not just 3) of caudal papillae, and a mucron on the tail tip. From sequencing of 18S, Aphelenchid K2 appears close to *Seinura* (Chapter 5), but it is morphologically distinct from that genus. Aphelenchid K2 has a shorter oesophageal gland than *Seinura*. The female lacks an anus, and the shape of the male spicule differs from that of *Seinura*. The tail of the female is similar to that of *Aprutides*, but the excretory pore in K2 is more posterior. Aphelenchid K2 has been cultured in the laboratory on *B. cinerea*. From SEM images and molecular data (Chapter 5), Aphelenchid K2 is closer to the genus *Aphelenchoides* than to *Bursaphelenchus*, even though it has a small bursa. Details of molecular analyses are presented in Chapter 5.

4.3.2.3 *Aphelenchoides* Morphospecies Aphelenchid H1

Material examined

Nematodes extracted from wood chips from dead *Pinus radiata* at Heidelberg, Victoria. Twenty-four males and 16 females were examined. These are stored in the WNC with number 2330. Collected by David Smith, May 2003.

Measurement

Females: (n=16): L=367.8±60.8 (267.1-452.4); a=24.6±2.8 (23.8-29.5); b=9.0±1.8 (6.2-14.2); c=14.9±3.5 (8.1-20.2); c'=3.4±0.7 (2.1-5.0); V=76.1±2.5 (73.2-80.5); stylet length=8.7±1.5 (5.1-11.2); tail length=25.8±6.5 (22.4-43.3); anus to vulva =59.9±12.1 (42.7-87.1).

Males:(n=24): L=277.4±16.7 (252.8-315.1); a=28.1±3.1 (20.9-33.1); b=10.1±1.3 (7.5-13.2); c=14.9±2.1 (11.7-20.3); c'=2.3±0.4 (1.6-3.5); stylet length =8.4±1.0 (5.3-9.7); tail length =19.0±2.9 (13.4-26.4); spicules=11.8±0.9 (10.2-13.4).

Description

Female. Small, relatively stout nematodes, 267-452 µm long (ratio a range 23.8-29.5). Body shape straight to ventrally arcuate when heat-relaxed, with most curvature in the posterior region (Fig. 4.11: 1). Cuticle finely annulated. Lateral field with 4 incisures, occupying 22% body width (Fig. 4.11: 9). Cephalic region rounded, off-set, sightly wider at base than the following body, cap-like, with 7 annules and clear labial disc (Fig. 4.11: 8). Stylet slender, 5.1-11.2 µm long, with small basal knobs. Median bulb spherical to ovoid with central valve plates. Oesophageal lobes relatively small, one dorsal and one ventral to intestine. Nerve ring one body width behind bulb; excretory pore posterior to nerve ring. Anus and rectum present. Vulva at 76% body length; vulva a simple slit, vulval lips flat, indistinct (Fig. 4.11: 6 & 9). Reproductive system monoprodelphic; outstretched with prominent spermatheca. Developing oocytes in several rows. Small post-vulval uterine sac present, with a few sperm. Tail conoid; terminus narrowly rounded (Fig. 4.11: 5 & 11).

Male. Similar features to female, but with tail strongly ventrally arcuate when heat-relaxed (Fig. 4.11: 2). Spicules mitten-shaped, paired, separate (Fig. 4.11: 3). Rostrum prominent, apex well developed. One single and two pairs papillae present: one single pre-anal, one pair pre-anal and one pair subterminal in front of tail tip (Fig. 4.11: 4 & 14). Tail conoid; terminus narrowly rounded. Rudimentary

Fig. 4.11 *Aphelenchoides* Morphospecies Aphelenchid H1

1. Female; 2. Male; 3. Spicules; 4. Male tail; 5. Female tail;
6. Vulval region; 7. Male head. Scale bars = 50 μm, 1, 2, 5, 6, 7;
10 μm, 3; 25 μm, 4.

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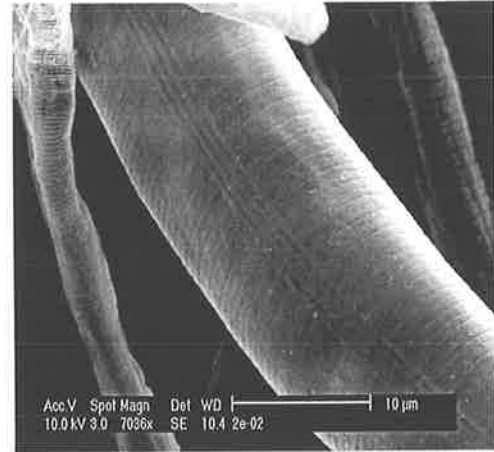
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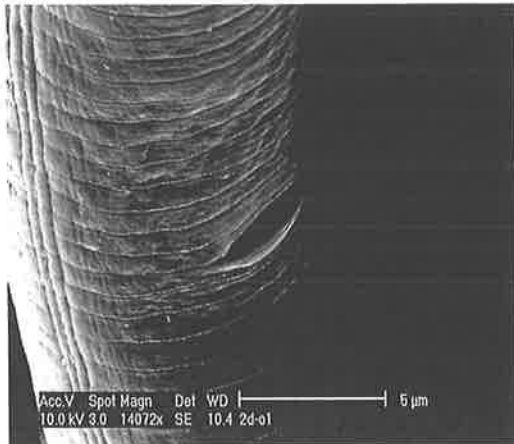
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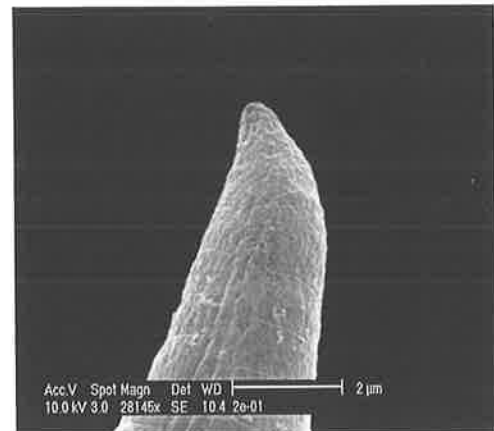
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Fig. 4.11 *Aphelenchoides* Morphospecies Aphelenchid H1

8. Female head; 9. Incisures; 10. Vulva ; 11. Female tail.

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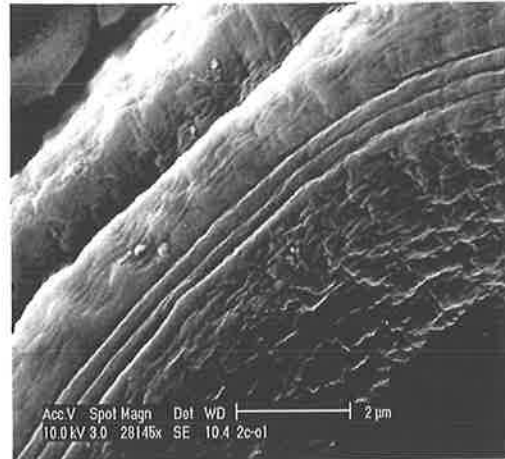
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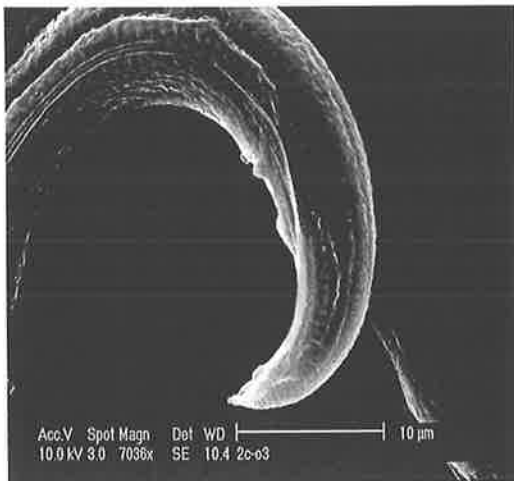
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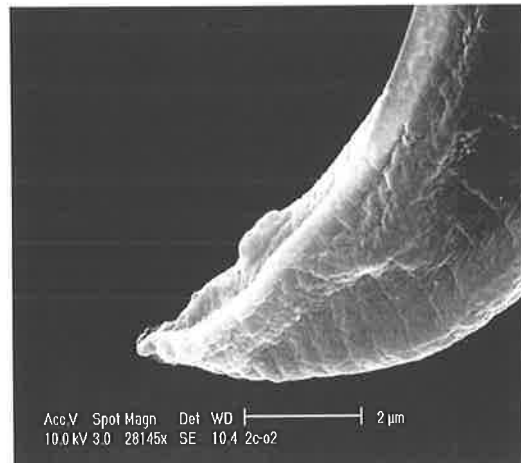
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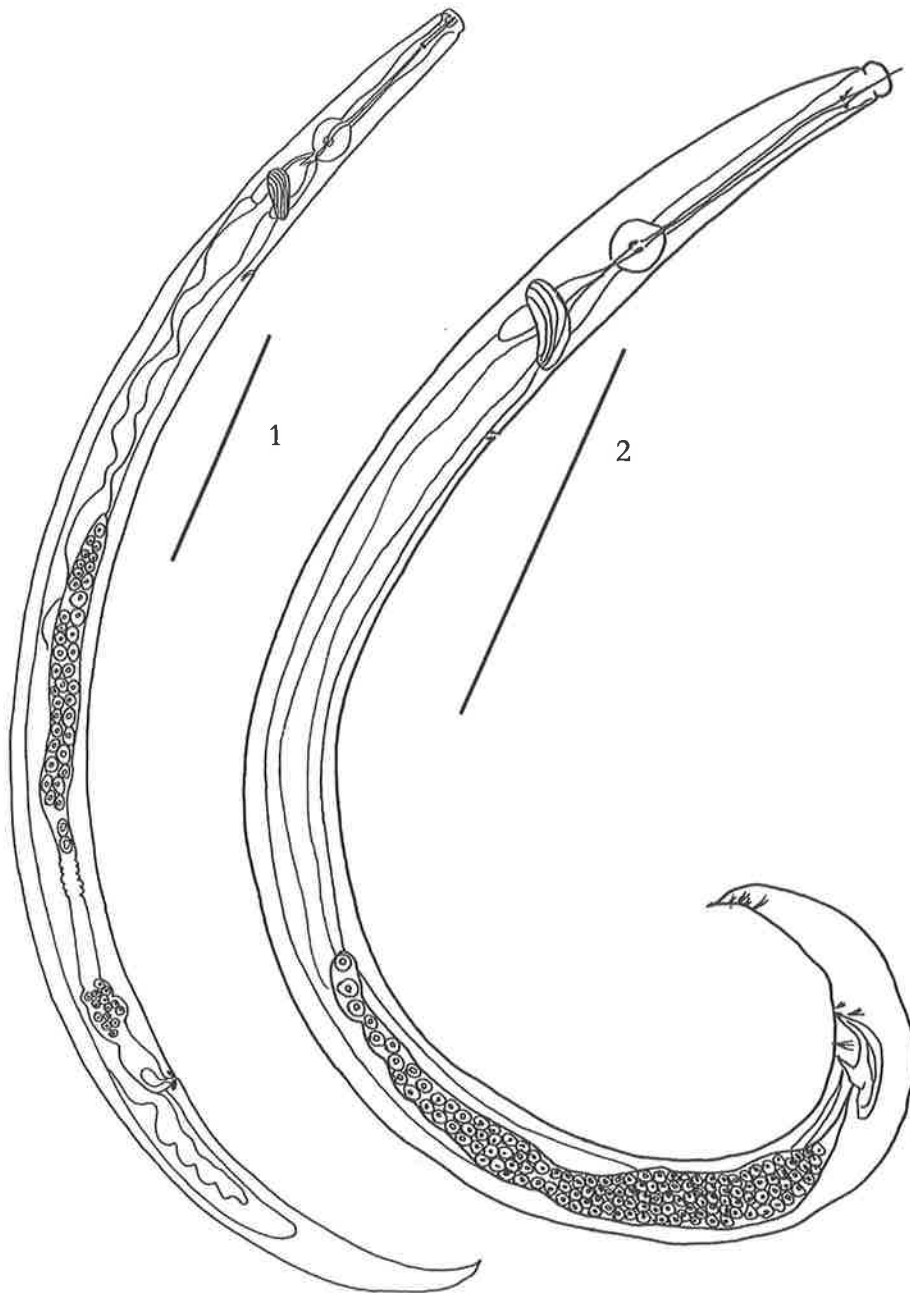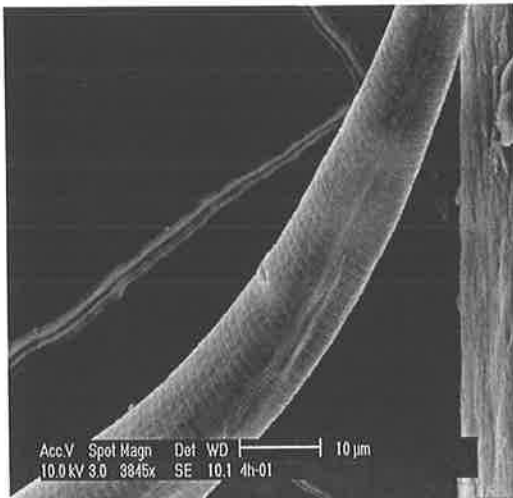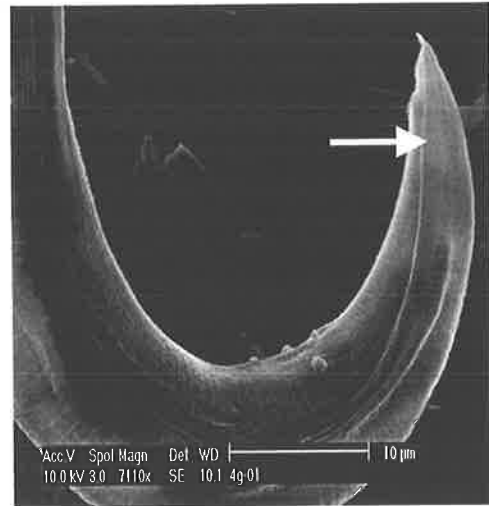
Fig. 4.11 *Aphelenchoides* Morphospecies Aphelenchid H1

12. Male head; 13. Incisures; 14. Bursa & Papillae ; 15. Male tail.

bursa present, arising near cloaca and running to tail tip, visible only with SEM (Fig. 4.11: 15).

Remarks

Aphelenchid H1 was one of two isolates extracted from wood chips from a diseased tree at Heidelberg, Victoria. At the beginning of this project, it was considered as a putative *Bursaphelenchus* species because of its association with the unusual pine disease. Thus, it was examined in detail and successfully cultured on agar plates. Later, it was also isolated from a wood sample from Knoxfield, together with Aphelenchid K1. SEM images and molecular data of aphelenchid morphospecies H1 were obtained. Morphologically, in having a small bursa, this nematode is close to *Bursaphelenchus*. However, it has a low (not high), offset cephalic region, its oesophageal gland is smaller, the excretory pore is more posterior, it lacks a vulval flap, and it has a short (not long) post-vulval uterine sac. In addition, molecular data suggests that it is genetically distant from *Bursaphelenchus* (Chapter 5), but closer to *Laimaphelenchus* and *Aphelenchoides*. However, it lacks knobs, tubercles and fingers on the tail, suggesting that it is not *Laimaphelenchus*. Hence, it has been assigned to *Aphelenchoides*. It is clearly different from Aphelenchid K1 in having a small body size, in spicule shape and in having one single plus 2 pairs of papillae. It is closer to Aphelenchid K2, but differs by having an anus and in molecular data. Details of molecular analysis are presented in Chapter 5.

4.3.3 Putative *Acugutturus* morphospecies collected from Knoxfield pines

Acugutturus Hunt 1980 belongs to the superfamily Acugutturinae (Hunt 1993). Its main characteristics (Hunt 1993) are:

1) They are medium-sized nematodes (0.6-0.9 mm).
2) The lateral field has a single incisure.
3) The cephalic region is offset, rounded and rather knob-like, and the stylet is very long (50-60 μm), slender and needle-like, and lacks basal knobs or swellings. The conus is at about 75% of the length.

4) The procorpus is slender, cylindrical and reflexed to allow for the protrusion of the stylet.

5) The rectum and anus are very indistinct or apparently absent in the females.

6) There is no post-vulval sac.

7) In males, the spicules are separate, rosethorn-shaped, with a prominent apex and rostrum. A sclerotised, gubernaculum-like structure is situated ventral to the spicule tips. There are two pairs of caudal papillae, one pre-cloacal and the other near the tail tip.

8) The known species are ectoparasites of insects (moths or cockroaches).

Descriptions of morphospecies

4.3.3.1 Putative *Acugutturus* sp.

Material examined

Nematodes extracted from wood chips from dead *Pinus radiata* at Knoxfield, Victoria. These are stored in the WNC with number 2437. Collected David Smith, May 2003.

Measurements

Female: (n=5): L=504±107 (438-629) µm; a=36.8±6.8 (26.1-44.4); V=73.8±0.4 (73.2-74.2); Oesophagus length=63.1±11.1 (47.6-76.2) µm; Stylet length=17±1.2 (16-19) µm; Head width=6.2 µm; Head height=2.3 µm; Anterior end to vulva=371±78.8 (267-467) µm.

Description

Female. Medium-sized, slender, 440-630 long (a range 26-44); excretory pore posterior to median bulb (Fig. 4.12: 1). Arcuate to C-shape when heat relaxed. Cuticle finely annulated and lateral fields not seen. Cephalic region offset, rounded, knob like (Fig. 4.12: 2 & 3). Stylet about 18 µm long, slender and needle like. Conus forms about 40% of length and shaft lacks basal knobs or swellings.
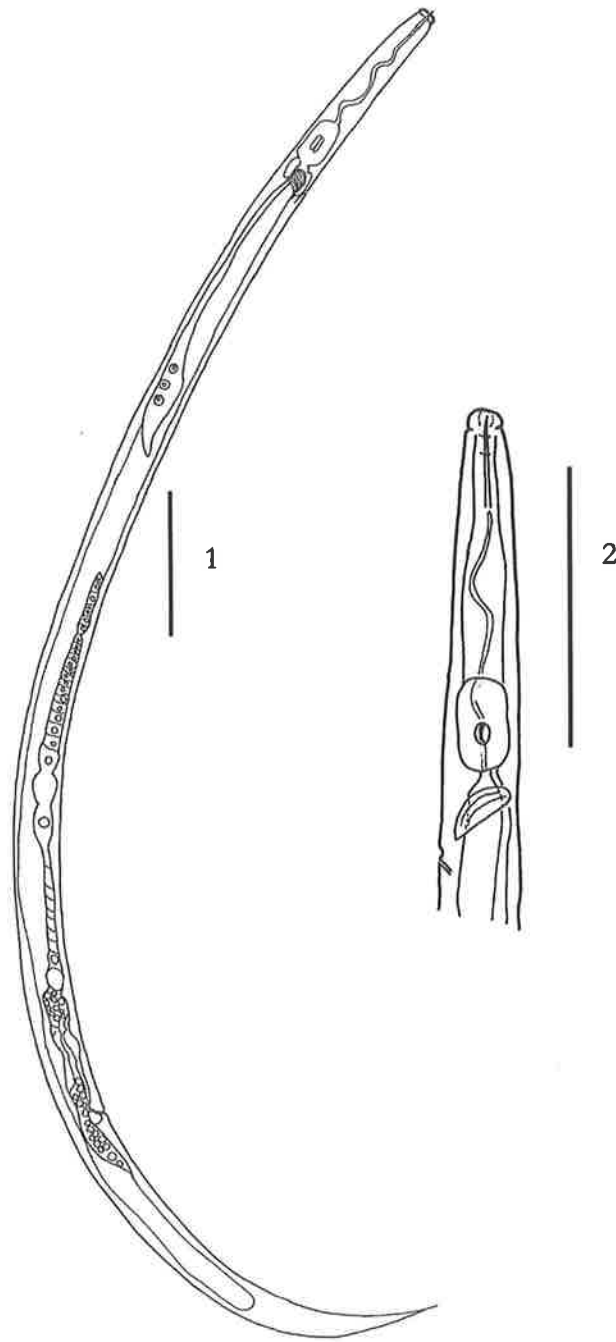
Fig. 4.12 Putative *Acugutturus* sp.

1. Female; 2. Female head. Scale bars = 50 μm, 1, 2.
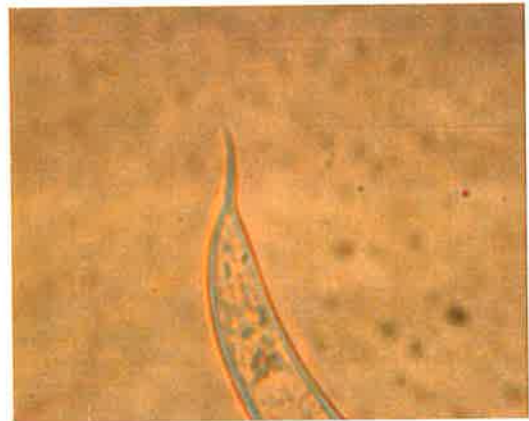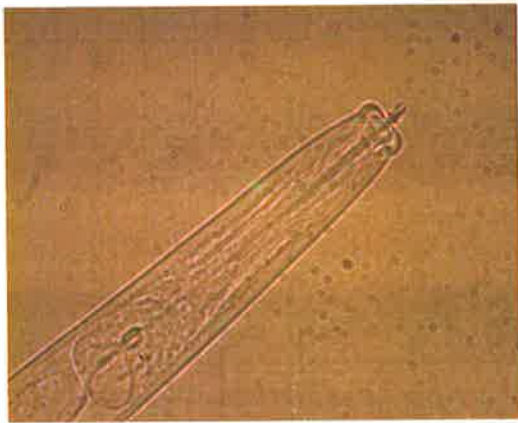
Fig. 4.12 Putative *Acugutturus*

4. Female head & stylet; 5. Female tail.

Prominent attachments for muscles on proximal part of stylet. Procorpus slender, cylindrical, sinuous to allow for stylet protrusion. Median bulb strong, sub-rectangular (not ovoid), and with valve plates central or in posterior half. Oesophageal gland lobe dorsal, well developed, overlapping oesophagus for about 8 body widths, with three prominent nuclei in the swollen tip. Hemizonid immediately anterior to excretory pore. Nerve ring just posterior to median bulb, just anterior to excretory pore. Rectum and anus apparently absent. Vulva posterior (about 73%) with slightly protuberant lips. Vagina at right angle to body, not sclerotised. Genital tract monoprodelphic, outstretched; developing oocytes in a single row. Post vulval uterine sac present; about 1.5 body widths long. Tail slender, conoid, ventrally arcuate, ending a fine point (Fig. 4.12: 5).

Male. Not seen.

Remarks

David Smith (pers. com., December 2005) reported that the pine tree from which the samples with ?*Acugutturus* were taken had many cockroaches under the bark. Given that cockroaches are known hosts of *Acugutturus* (Hunt 1993), this suggests that the nematodes could have been associated with the cockroaches.

However, the nematode named here as a putative *Acugutturus* species does not fully fit Hunt's description of the genus. The stylet is much shorter (18 instead of 50-60 long), the conus is at about 40% (not 75%) of stylet length, and the intestine was not filled with yellowish fluid (haemolymph). This suggests that these nematodes are not ectoparasites of insects. A post-vulval uterine sac is present here but not in *Acugutturus* after Hunt (1993). The absence of rectum and anus does suggest that the nematodes are insect associates (Hunt 1993). Until males are found, no firm decision can be made on the genus to which these nematodes belong.

4.4 Discussion and Conclusions

In summary, 12 morphospecies of nematodes have been studied here. Of these, 3 are new species, 5 are morphospecies of *Laimaphelenchus*, 3 are putative morphospecies of *Aphelenchoides* and one is a putative *Acugutturus*. Both *Laimaphelenchus* and *Acugutturus* are first records from Australia, and represent a contribution to our knowledge of the world distributions of these genera. *Laimaphelenchus* was previously recorded from every continent except Australia (Hunt 1993, Swart 1997, Peneva & Chipev 1999). *Acugutturus* was recovered from the West Indies (Hunt, 1993).

As mentioned earlier, there is considerable debate about the taxonomy of the aphelenchids, both at higher and genus levels. Because of this debate, problems occurred with the identification of species here. For example, the genus of *Laimaphelenchus* is a small group, and even adding the three new species described here, it has only 12 species. However, the most distinctive feature of this genus is its tail structure, and this is variable from species to species. If a worker only refers to the description given in Hunt (1993), it is very difficult to judge to what genus some of these new collections belong. Repeatedly, nematodes examined here had one or more morphological features that, when keyed out using Hunt (1993), placed them in different genera. For example, the presence of a bursa in an aphelenchoidid nematode means that it keys out to *Bursaphelenchus*. However, molecular analyses of these nematodes (see Chapter 5) placed them at considerable genetic distance from *Bursaphelenchus*. Unfortunately, not enough aphelenchid nematodes have been studied either morphologically or at the molecular level (GenBank contains relatively few sequences from aphelenchids) to allow simple, unequivocal taxonomic determination of these nematodes. While morphology of an animal is most important for taxonomic determination, DNA can be a tool to aid identification and can help in clearing up taxonomic questions. On the other hand, *Aphelenchoides* is a large genus, with so many species that it is difficult to compare new with existing species. Even more problematic, the genus also contains many invalid species; making it more difficult to describe new species, even when molecular data confirming their species status is available. The diagnosis of the

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genus *Laimaphelenchus* should be expanded to include the morphological features, particularly of the tail, observed in the forms described here, and the genus *Aphelenchoides* needs to be revised (Hunt 1993). In order to clarify the taxonomy of morphospecies described in this Chapter, more SEM pictures and possible molecular data are needed.

As discussed in Chapter 3, conifers in Australia seem to have a more limited nematode diversity than in Europe and USA (Ruhm 1956, Massey 1974, Kaya 1984). This could be because *Pinus,* the genus most commonly sampled here, was introduced to Australia relatively recently. In contrast, many species of conifers are endemic in Europe and USA. Nematodes have been able to adapt to them. In this study, only 40 *Callitris* trees were sampled, so we can not actually say much about diversity of endemic Australian species of nematodes. They could be limited by the geographic distribution of *Callitris*, which tend to grow in hot dry climates. Only limited numbers of species of nematodes may have adapted to these climates.

As mentioned in Section 4.3, more species were present in the samples collected from the survey work than have been described. Of the 12 species of nematodes commonly found in the survey and studied here, 5 species were extracted from the few diseased trees sampled from Knoxfield and Heidelberg. This indicates that it is likely that the diversity of nematodes on healthy trees is less than in diseased trees. The reasons for this could be a) that healthy trees have strong defence ability to protect themselves against nematode attack; b) that suitable food resources are limited in the healthy trees, and that they are not a suitable environment for nematodes. Further collecting of nematodes from sick, dying and dead trees, of both exotic pine plantations and from endemic conifers, could be used to compare the diversity of nematodes associated with each.

Chapter 5: Molecular Studies

5.1 Introduction

Two types of gene sequences are suitable for polymerase chain reaction (PCR)-based species resolution; one is comprised of middle-repetitive nuclear sequences, such as the ribosomal genes, the other includes mitochondrial gene sequences, which occur as multiple copies due to the presence of many mitochondria in each cell. The ribosomal DNA (rDNA) is a component of the middle repetitive DNA of the nuclear genome, and the presence of multiple copies of these genes in the genome facilitates PCR amplification from single juvenile or adult nematodes (Powers *et al.* 1997). Sequencing of this region has been used to address diagnostic and evolutionary problems at different levels of divergence (Powers *et al.* 1997). The internal transcribed spacer (ITS), located between the repeating array of nuclear 18S small subunit ribosomal RNA (SSU) and 28S large subunit ribosomal RNA (LSU) genes, is a versatile genetic marker, widely used in nematode identification (Ferris *et al.* 1993; 1994; Campbell *et al.* 1995; Chilton *et al.* 1995; Ferris *et al.* 1995; Gasser and Hoste 1995; Hoste *et al.* 1995; Zijlstra *et al.* 1995; Fallas *et al.* 1996; Cherry *et al.* 1997; Zijlstra *et al.* 1997; Zheng *et al.* 2003).

The availability of broad-range primers for amplification of a fragment of cytochrome oxidase subunit I (COI) from diverse invertebrate and vertebrate phyla means that this gene sequence is a useful target for species identification in animals (Folmer, Black *et al.* 1994). Mitochondrial DNA (mtDNA) sequences of animals evolve at a faster rate (Kanzaki and Futai 2002) than comparable nuclear genes and are suited for discriminating closely related individuals (Courtright *et al.* 2000). In contrast, the LSU rRNA gene has been useful for resolving closely related taxa (Nadler 1992; Al-Banna *et al.* 1997; Nadler and Hudspeth 1998; Duncan *et al.* 1999; Kanzaki and Futai 2002). The SSU rRNA gene is useful for phylogenetic analysis across the phylum (Fitch *et al.* 1995; Liu *et al.* 1997; Aleshin *et al.* 1998; Blaxter *et al.* 1998; Kampfer *et al.* 1998; Nadler and Hudspeth 1998; De Ley *et al.* 2002; Dorris *et al.* 2002; Floyd *et al.* 2002). Therefore, sequence analysis of PCR amplified ribosomal ITS region, D2-D3 fragment of LSU and the full length of small subunit of the ribosomal gene have enabled the development of simple and

convenient methods for nematode molecular taxonomy and for the discovery of cryptic species within morphologically defined groups.

This Chapter reports the detailed analysis of six Australian nematodes, taxonomically relevant to this project, using molecular techniques. The work presented here was performed by myself in conjunction with my American collaborators, Prof. R. M. Giblin-Davis, and Dr Ye Weimin (SSU and mtCOI sequences were done by my collaborators, ITS sequence by myself, and D2D3 sequences by both myself and our collaborators). Near-full length SSU, partial LSU and partial mtCOI sequences were used to compare closely related aphelenchid nematodes. The ITS region was used for ITS-RFLP (restriction fragment length polymorphism) patterns to discriminate morphologically similar species. Sequences from six nematode species, *Aphelenchoides* sp. Aphelenchid K1, Aphelenchid K2, Aphelenchid H1, *Laimaphelenchus* Heidelberg, *Laimaphelenchus preissii* and *Laimaphelenchus australis*, were analysed and compared.

5.2 Materials and Methods

5.2.1 Nematode samples and DNA extraction

Nematode samples were collected from several locations in Australia (Table 5.1), and reared on cultures of the fungus *Botrytis cinerea* on potato dextrose agar plates. They were hand-picked from the plates, and some were added to 1M NaCl before being shipped to the Nematology Laboratory at Fort Lauderdale Research and Education Centre, University of Florida, for subsequent DNA extraction. Total genomic DNA from multiple nematodes of each culture was extracted using worm lysis buffer containing proteinase K (Williams *et al.* 1992). DNA extracts were stored at -20°C until used as PCR template.

Table 5.1 Nematode species, sample number and collection localities.

Species	Sample No.	Locality	Plant host
Laimaphelenchus Heidelberg	395	Heidelberg, Victoria, Australia	*Pinus radiata*
Aphelenchid K2	396	Knoxfield, Victoria, Australia	*Pinus radiata*
Aphelenchid K1	466	Knoxfield, Victoria, Australia	*Pinus radiata*
Laimaphelenchus preissii	467	Burdett, South Australia, Australia	*Callitris preissii*
ApAphelenchid H1	468	Heidelberg, Victoria, Australia	*Pinus radiata*
Laimaphelenchus australis	753	Nelson, Victoria, Australia	*Pinus radiata*

5.2.2 Polymerase chain reaction (PCR)

Primers for ITS amplification were forward primer Bur18SF1 (5'
TCTCATGAACGAGGAATTCCAAG 3') and reverse primer Bur28SR1 (5'
CCTCCGCTAAATGATATGCTTAAG 3') designed by myself. Primers for LSU
amplification were forward primer D2A (5' ACAAGTACCGTGAGGGAAAGT 3')
and reverse primer D3B (5' TGCGAAGGAACCAGCTACTA 3') (Nunn 1992).
Primers for mtCOI amplification were forward primer COI-F1 (5'
CCTACTATGATTGGTGGTTTTGGTAATTG 3') and reverse primer COI-R2 (5'
GTAGCAGCAGTAAAATAAGCACG 3') (Kanzaki and Futai 2002). Primers for
SSU amplification were forward primer 18SF-Burs (5'
ATGCATGTCTAAGTGGAGTATTATA 3') and reverse primer 18SR-Burs (5'
CTACGGCTACCTTGTTACGACTTTT3') designed by Weimin Ye, or forward
primer 18S-G18S4 (5' GCTTGTCTCAAAGATTAAGCC 3') and reverse primer
18S-18P (5' TGATCCWKCYGCAGGTTCAC 3') (De Ley *et al.* 2002; Dorris *et al.*
2002).

For ITS, the 25-µl PCR contained 1X *Tag* DNA polymerase reaction buffer, 3.1
mM MgCl$_2$, 0.2 mM each dNTP, 1.8 µM each of forward and reserve primers, 1 unit
of *Tag* (Promega Corporation, NSW, Australia) and 2-µl DNA template. For LSU
and mtCOI, the 50-µl PCR contained 1X *Taq* DNA polymerase incubation buffer,
2.5 mM MgCl$_2$, 0.2 mM each dNTP, 0.4 µM each of forward and reverse primers,
1.5 units of AmpliTaq (Applied Biosystems, Foster City, Ca, USA), and 2-µl of
DNA template. For SSU, the 25-µl PCR mixture contained 1X *Taq* DNA
polymerase incubation buffer, 2.5 mM MgCl$_2$, 0.2 mM each dNTP, 0.63 µM each
of forward and reverse primers, 0.5 units of DyNAzyme (MJ Research, Inc.,
Waltham, Md, USA), and 1-µl of DNA template. The thermal cycling program for
ITS was as follows: denaturation at 95°C for 3 min, followed by 30 cycles of
denaturation at 94°C for 60 s, annealing at 55°C for 45 s, and extension at 72°C for
45 seconds. A final extension was performed at 72°C for 10 min. The thermal
cycling program for LSU and COI was as follows: denaturation at 95°C for 5 min,
followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C (LSU) or
51°C (COI) for 45 s, and extension at 72°C for 2 min. A final extension was

performed at 72°C for 10 min. The thermal cycling program for SSU was as follows: denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 42°C for 30 s, and extension at 72°C for 3 min. A final extension was performed at 72°C for 10 min.

5.2.3 PCR product purification

PCR products were purified by running electrophoresis on both 1.2% agarose gel (Promega Corporation, NSW, Australia) and 1.5% SeaPlaque® GTG® low melting agarose gel (FMC BioProducts, Rockland, Me, USA). The band of the correct size was excised using QIAquick gel purification kit (Qiagen, Clifton Hill, Vic, Australia and Valencia, Ca, USA).

5.2.4 Cloning of PCR products

The purified ITS and 18S DNA fragments were cloned into plasmids vector (pGEM® - T-Easy vector system). The plasmids were transformed into high efficiency competent cells (JM109) and colonised onto lura bertani agar (LB) plates according to the instructions of the manufacturer (Promega Corporation, NSW, Australia).

5.2.5 Plasmid DNA preparation

The method used for the plasmid DNA preparation was taken from Li (2002). For mini-preparations of plasmid DNA, the alkaline lysis method according to Sambrook et al. (Sabrook et al. 1989) was employed with slight modifications. Before the phenol: chloroform extraction, an RNase digestion step was introduced. RNase (DNase free) was added to a final concentration of 20 µg/ml and the sample was incubated for 37°C for 20 min. The phenol : chloroform extraction was only conducted when higher quality DNA was required.

When higher yields of pure plasmid DNA were needed, the Qiaprep Miniprep plasmid DNA purification kits (Qiagen, Clifton Hill, Vic, Australia) were used according to the supplier's protocols.

5.2.6 Sequencing

DNA sequencing of cloned nematode rDNA was performed with M13 reverse and forward primers using in an Applied Bio System 373 sequencer (USA). Some PCR products were sequenced in both directions using PCR primers for direct sequencing. DNA sequencing was performed by CEQ 2000 DNA Analysis System (Beckman Coulter, Fullerton, Ca, USA) following manufacturer's protocols. The sequences will be deposited into GenBank database.

5.2.7 ITS-RFLP analysis

This RFLP (Restriction fragment length polymorphism) analysis was carried out as described by Zheng *et al.* (Zheng, Subbotin *et al.* 2003) with slight modification. Briefly, 2-5 µl of each PCR product of the ITS region were digested with one of the following restriction enzymes, *Alu*I, *Hinf*I, *Rsa*I, *Sac*I and *Taq*I in the buffer stipulated by the manufacturer (Promega Corporation, Australia). The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis (110V, 40 min), stained with ethidium bromide, visualised under indirect UV light, and pictured.

5.2.8 Sequence alignments and phylogenetic inferences

DNA sequences were aligned by ClustalW (http://workbench.sdsc.edu, Bioinformatics and Computational Biology group, Dept. Bioengineering, UC San Diego, CA). The 5.8 S sequence pair distance of similarity matrix was used TreeTop-Phylogenetic Tree Prediction (htt://genebee.msu.su). The model of base substitution in the SSU, LSU and COI sets were evaluated using MODELTEST (Posada and Crandall 1998; Huelsenbeck and Ronquist 2001). The Akaike-

supported model, the log likelihood (lnL), the Akaike information criterion (AIC), the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates are listed in Table 5.3 and were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001) running the chain for 10×10^6 generations and setting the "burnin" at 1,000. Weimin Ye and Giblin-Davis used MCMC (Markov Chain Monte Carlo) methods within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon 1999) using 50% majority-rule. The maximum parsimony (MP) method was performed using the heuristic search with stepwise-addition options to determine the most parsimonious tree. Neighbour-joining (NJ) analysis (Saitou and Nei 1987) was conducted using the HKY85 (Hasagawa, Kishino *et al.* 1985) distance option. Sites with missing data or gaps were treated as missing characters for all analyses. The robustness of the parsimony and NJ trees was tested using the bootstrap method (Felsenstein 1985). All bootstrap values are based on a thousand replicates.

5.3 Results

5.3.1 ITS analysis

5.3.1.1 Sequences of Internal Transcribed Space

Sequences of the ITS rRNA gene of *L. preissii* (900 bp), Aphelenchid K1 (805 bp) and Aphelenchid H1 (721 bp) were made. Detail of the sequences is given in Appendix D.

5.3.1.2 ITS-RFLP pattern analyses

The amplification of the ITS1-5.8S-ITS2 and flanking genes yield for each isolate was approximately 720-900 bp. ITS-RFLP analyses of nematode isolates from different locations are shown in Fig. 5.1, Fig. 5.2 and Fig. 5.3; and analyses of nematode isolates from different trees at the same location are shown in Fig. 5.3 and Fig. 5.4.

RFLP of the ITS region revealed that *Alu* I, *Hinf* I and *Sac* I generated distinct patterns between Aphelenchid K1 and Aphelenchid H1, Aphelenchid K1 and *Laimaphelenchus* Heidelberg. *Alu* I, *Hinf* I and *Rsa* I generated distinct patterns between Aphelenchid K1, Aphelenchid K2 and Aphelenchid H1. These showed that the four isolates from Knoxfield and Heidelberg are different species (Fig. 5.1, Fig. 5.2 and Fig. 5.3).

Conversely, the same patterns were obtained from *Alu* I, *Hinf* I and *Rsa* I with the PCR products of nematodes isolated from tree No. 3 and tree No. 12 at Knoxfield (Aphelenchid K1) and indicated that they are the same species (Fig. 5.4). Similarly, the same patterns were also obtained from *Alu* I, *Hinf* I, *Rsa* I and *Taq* I with the PCR products of nematodes isolated from trees No. 2 and No. 22 from pine plantations at Mount Gambier, which showed that they are the same species (Fig. 5.5).

5.3.1.3 5.8 S sequences of *Laimaphelenchus preissii*, Aphelenchid K1 and Aphelenchid H1 comparisons

The distance matrix of the 5.8S sequences of 15 nematodes is shown in Table 5.2. When comparisons were made between pairs of nematodes, *L. preissii* and Aphelenchid H1 (0.456), Aphelenchid K1 and Aphelenchid H1 (0.539), Aphelenchid H1 and *A. avenae* (0.615), *A. fragariae* and Aphelenchid H1 (0.550) had the highest distance. The numbers in brackets above represent the distance between each pair of nematodes. This analysis showed that none of the Australian isolates is genetically close to *Bursaphelenchus* spp.

5.3.2 Characterisation of the nucleotide data

Sequences were made of 1673-1730 bp from the SSU rRNA gene, 682-779 bp from the LSU rRNA gene (partial sequences of *L. preissii*, Aphelenchid K1, Aphelenchid H1, *L. australis* are given in Appendix E), and 627 bp from the mitochondrial COI gene. The COI gene showed no nucleotide length polymorphism in any of the

Table 5.2 5.8 S sequence pair distance of similarity matrix using TreeTop-Phylogenetic Tree Prediction

Species & Accession Number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Bursphelenchus fraudulentus AM 179517	1	0.000	0.132	0.013	0.024	0.035	0.122	0.100	0.190	0.035	0.013	0.264	0.154	0.445	0.242	0.470
B.hofmannii Am 180516	2	0.132	0.000	0.144	0.133	0.122	0.057	0.078	0.146	0.122	0.144	0.286	0.154	0.467	0.264	0.416
B.xylophilus AM 179515	3	0.013	0.144	0.000	0.011	0.022	0.109	0.087	0.177	0.022	0.000	0.251	0.166	0.454	0.230	0.457
B.mucronatus AM 179514	4	0.024	0.133	0.011	0.000	0.033	0.098	0.076	0.188	0.033	0.011	0.262	0.177	0.465	0.240	0.468
B.conicaudatus AM 179513	5	0.035	0.122	0.022	0.033	0.000	0.087	0.087	0.155	0.000	0.022	0.230	0.144	0.454	0.208	0.436
B.seani AM 157745	6	0.122	0.057	0.109	0.098	0.087	0.000	0.066	0.155	0.087	0.109	0.251	0.144	0.476	0.230	0.414
B. sexdentati AM 160661	7	0.100	0.078	0.087	0.076	0.087	0.066	0.000	0.155	0.087	0.087	0.273	0.188	0.476	0.251	0.446
B. abruptus AB 067756	8	0.190	0.146	0.177	0.188	0.155	0.155	0.155	0.000	0.155	0.177	0.242	0.146	0.489	0.221	0.427
B. conicaudatus AB 067757	9	0.35	0.122	0.022	0.033	0.000	0.087	0.087	0.155	0.000	0.022	0.230	0.144	0.454	0.208	0.436
B. mucronatus AY 347916	10	0.013	0.144	0.000	0.011	0.022	0.109	0.087	0.177	0.022	0.000	0.251	0.166	0.454	0.230	0.457
Aphelenchoides fragariae AF 119049	11	0.264	0.286	0.251	0.262	0.230	0.251	0.273	0.242	0.230	0.251	0.000	0.199	0.550	0.043	0.488
Laimaphelenchus preissii	12	0.154	0.154	0.166	0.177	0.144	0.144	0.188	0.146	0.144	0.166	0.199	0.000	0.456	0.177	0.416
Aphelenchid H1	13	0.445	0.467	0.454	0.465	0.454	0.476	0.476	0.489	0.454	0.454	0.550	0.456	0.000	0.539	0.615
Aphelenchid K1	14	0.242	0.264	0.230	0.240	0.208	0.251	0.251	0.221	0.208	0.230	0.043	0.177	0.539	0.000	0.455
Aphelenchus avenae AF 119048	15	0.470	0.416	0.457	0.468	0.436	0.446	0.446	0.427	0.436	0.457	0.488	0.416	0.615	0.455	0.000

Note: AM, AB, AY, AF: Nematodes accession number in GenBank.
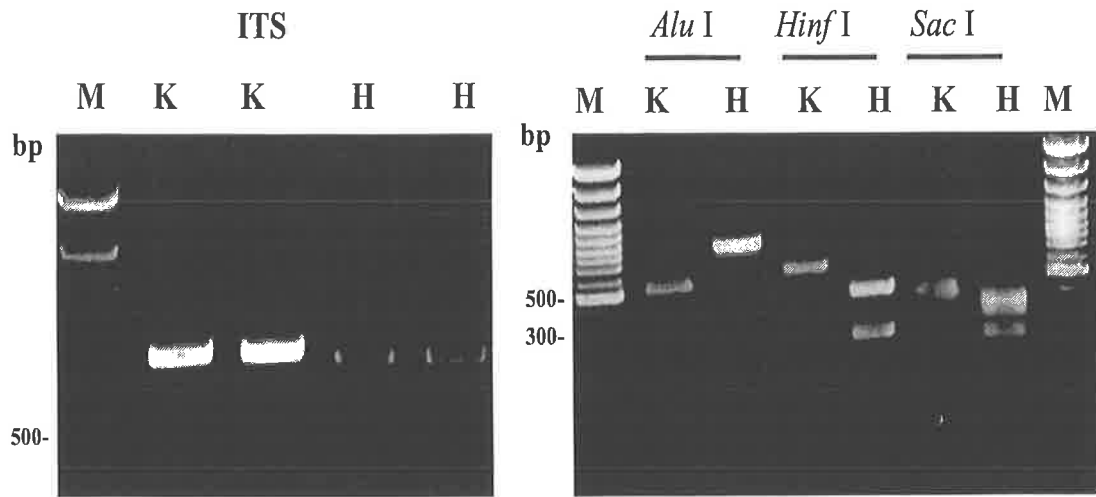
Fig. 5.1 ITS-RFLP analysis of nematodes from two locations.
K: Aphelenchid K1 extracted from Knoxfield
H: Aphelenchid H1 extracted from Heidelberg
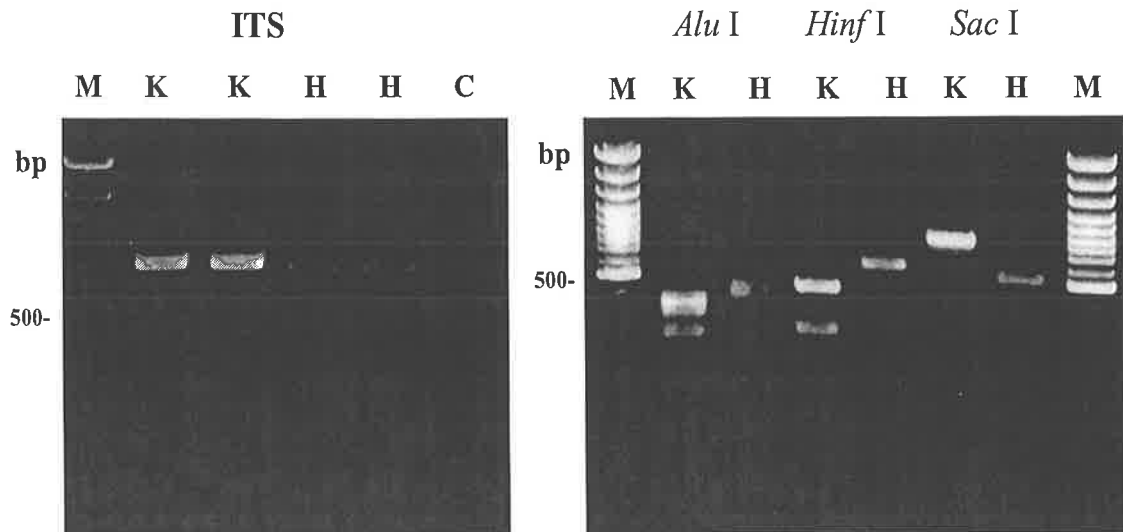
Fig. 5.2 ITS-RFLP analysis of nematodes from two locations
K: Aphelenchid K1 extracted from Knoxfield
H: *Laimaphelenchus* Heidelberg extracted from Heidelberg
C: Water control

ITS
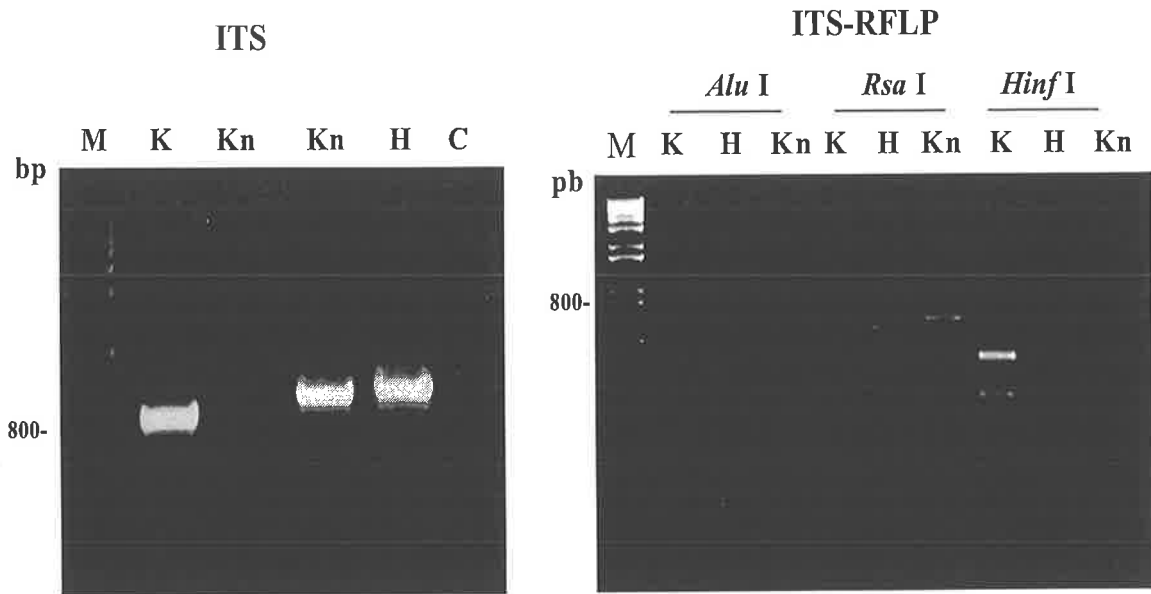
ITS-RFLP

Fig. 5.3 ITS-RFLP analysis of nematodes from two locations.
K: Aphelenchid K1 extracted from Knoxfield
Kn: Aphelenchid K2 extracted from Knoxfield tree No 3
H: Aphelenchid H1 extracted from Heidelberg
C: Water control

ITS
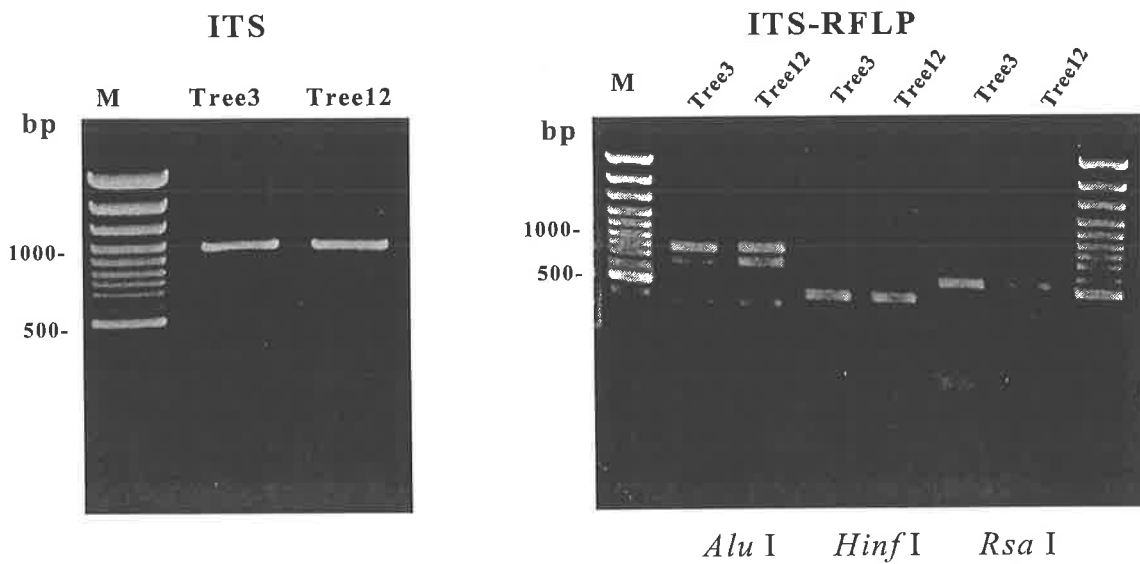
ITS-RFLP

Fig. 5.4 ITS-RFLP analysis of nematodes from two trees
Tree3: Nematode extracted from Knoxfield
Tree12: Nematode extracted from Knoxfield

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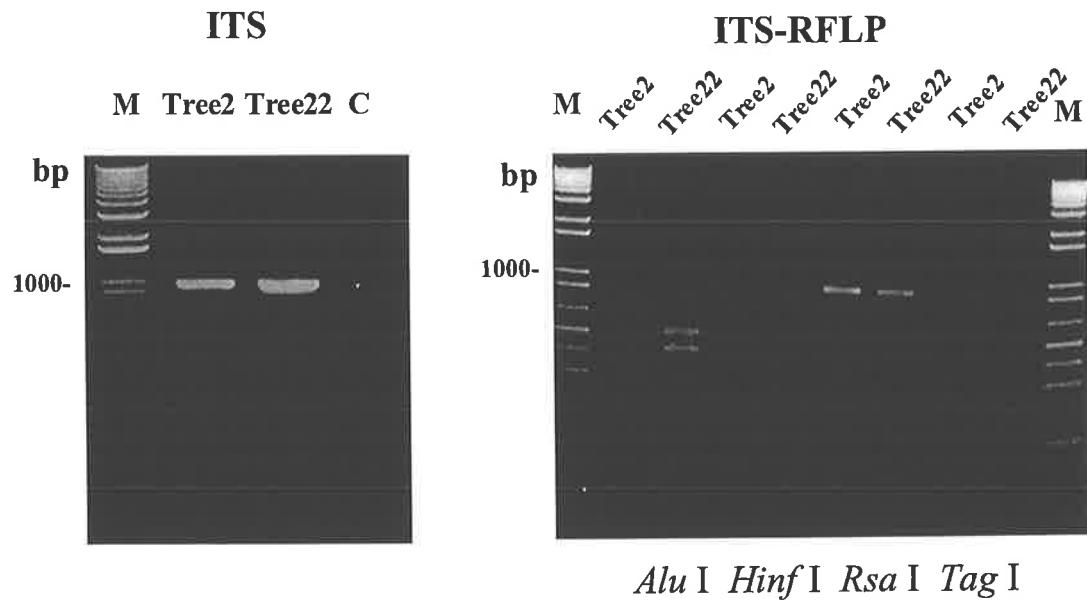
Fig. 5.5 ITS-RFLP analysis of nematodes from two trees at one location.
Tree2: Nematode extracted from Mount Gambier
Tree22: Nematode extracted from Mount Gambier
C: Water Control

nematode species studied. Table 5.3 summarises the levels of sequence variability in all taxa. The mitochondrial COI gene was A + T rich (67.6% A + T and 32.4% G + C) and had a base usage with an excess of T and a deficit of C (Table 5.3). The ribosomal gene SSU had about equal representation of A, G and T, but less C. The LSU had a higher content of G and T than A and C. Of the three genes, LSU was the most variable and informative and SSU was the most conserved with 54.3% constant characters. The COI gene had a higher Ti/Tv ratio than the two ribosomal genes.

5.3.3 Phylogenetic relationships inferred from SSU, LSU and COI sequences

The trees generated by NJ, MP and Bayesian analyses showed no significant conflict in branching order and support level. Thus only Bayesian trees were used

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to show the results of the phylogenetic relationships inferred by SSU (Fig. 5.6), LSU (Fig. 5.7) and COI (Fig. 5.8) separately.

The consensus tree inferred from SSU (Fig. 5.6) indicated: 1) the monophyly of all 5 Australian isolates studied and other reference aphelenchids with a posterior probability of 100%, 2) monophyly of all *Bursaphelenchus* species from many countries with posterior probability of 99, and none of the 5 Australian isolates is inside this clade, 3) *L. preissii* (467) and Aphelenchid H1 (468) are in one clade, 4) *Laimaphelenchus* Heidelberg (395) and Aphelenchid K1 (466) are in one clade, 5) Aphelenchid K2 (396) is close to *Seinura*, 6) *Aphelenchoides* species are paraphyletic, and 7) *Laimaphelenchus* species are paraphyletic.

The consensus tree inferred from LSU (Fig. 5.7) indicated: 1) the monophyly of all 5 Australian isolates studied and other reference aphelenchids with a posterior probability of 100, 2) the deep level of phylogenetic relationships was not resolved in Aphelenchids, 3) the *Bursaphelenchus* species were in several monophyletic clades, but none of the 6 Australian isolates studied was inside any of those clades, 4) *L. preissii* (467) and Aphelenchid H1 (468) are in one clade, and are close to *L. australis* (753) but with a low posterior probability, 5) *Laimaphelenchus* Heidelberg (395) and Aphelenchid K1 (466) are in one clade, together with *Aphelenchoides fragariae* and *Laimaphelenchus*, 6) Aphelenchid K2 (396) is by itself, 7) *Aphelenchoides* species are paraphyletic, and 8) *Laimaphelenchus* species are paraphyletic.

The consensus tree inferred from COI (Fig. 5.8) indicated: 1) the monophyly of all 3 Australian isolates studied and other reference aphelenchids with a posterior probability of 97, 2) the deep level of phylogenetic relationships was not resolved in aphelenchids, 3) the *Bursaphelenchus* species were in several monophyletic clades, and none of the 3 Australian isolates was inside these clades, 4) *L. preissii* (467) is close to *Aphelenchoides besseyi* and 5) *Laimaphelenchus* Heidelberg (395) and Aphelenchid K1 (466) are in one clade, together with *Aphelenchoides fragariae*, but a posterior probability of only 88.

Table 5.3 Sequence comparison and model test results in 3 loci among aphelenchid nematode species.

Parameter	SSU	LSU	COI
Size (bp)	1673-1730	712-779	627
A	26.0%	20.6%	26.1%
C	19.8%	20.1%	11.9%
G	26.7%	32.3%	18.3%
T	27.5%	27.1%	41.5%
Constant characters and (percentage)	975 (54.3%)	273 (34.3%)	285 (45.2%)
Parsimony-uninformative characters and percentage	179 (10.0%)	91 (11.4%)	72 (11.4%)
Parsimony-informative characters and percentage	643 (35.8%)	431 (54.2%)	273 (43.3%)
Model selected	GTR+I+G	GTR+I+G	GTR+I+G
-lnL	14261	11083	7481
AIC	28542	22187	14982
Proportion of invariable sites	0.3	0.2	0.3
Gamma distribution shape parameter	0.5	0.7	0.4
R(a) [A-C]	1.3	0.7	2.2
R(b) [A-G]	2.8	2.2	15.3
R(c) [A-T]	1.2	0.9	3.4
R(d) [C-G]	0.8	0.5	16.8
R(e) [C-T]	4.6	3.9	35.4
R(f) [G-T]	1	1	1
Ts/Tv	3.5	4.1	4.3

Provided by R. M. Giblin-Davis and Ye, Weimin (unpub. data).
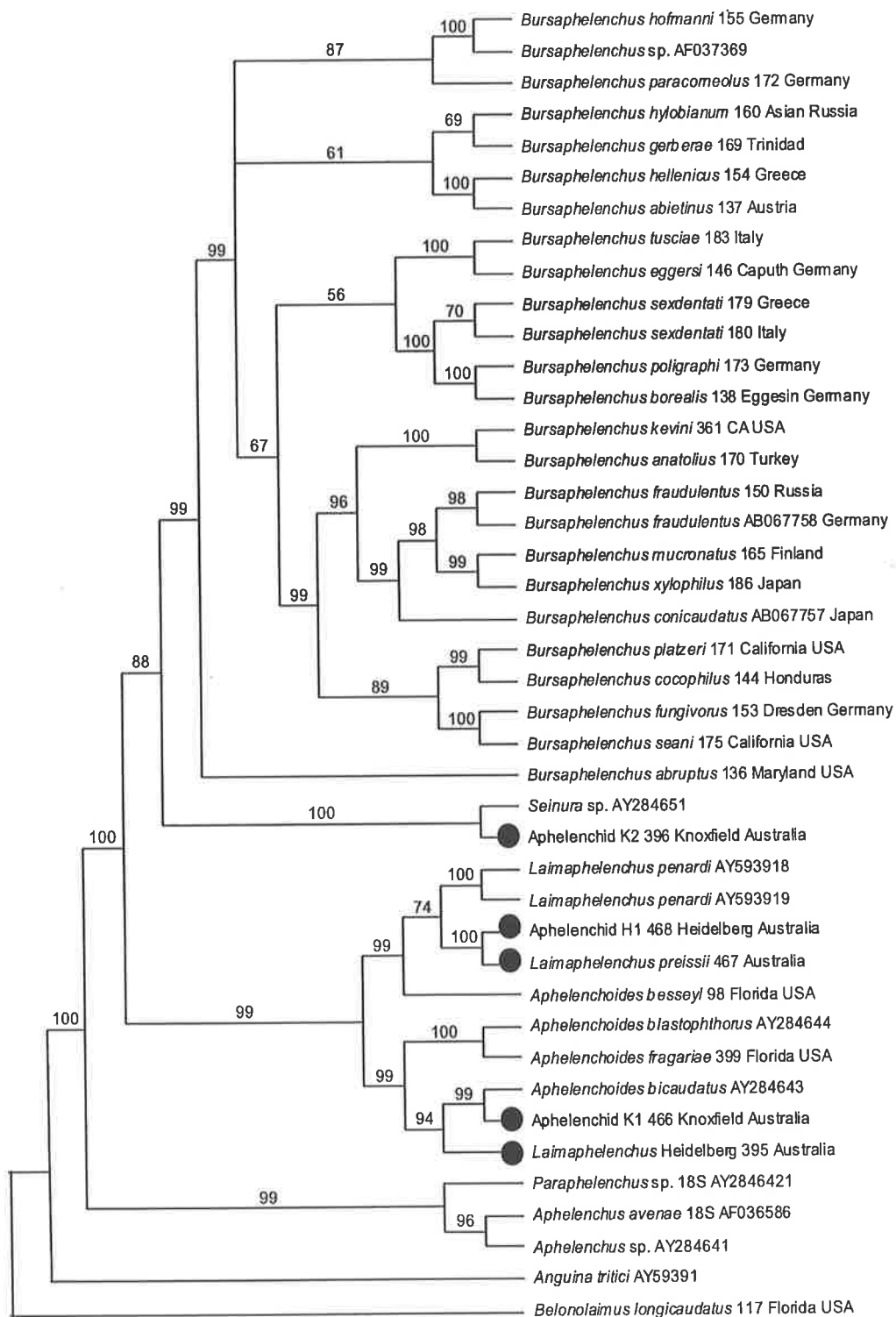
Fig. 5.6 Bayesian tree inferred from SSU DNA sequences. Provided by R. M. Giblin-Davis and Ye, Weimin (unpub. data).●, nematodes from this study. The numbers give the probability support for individual branches.

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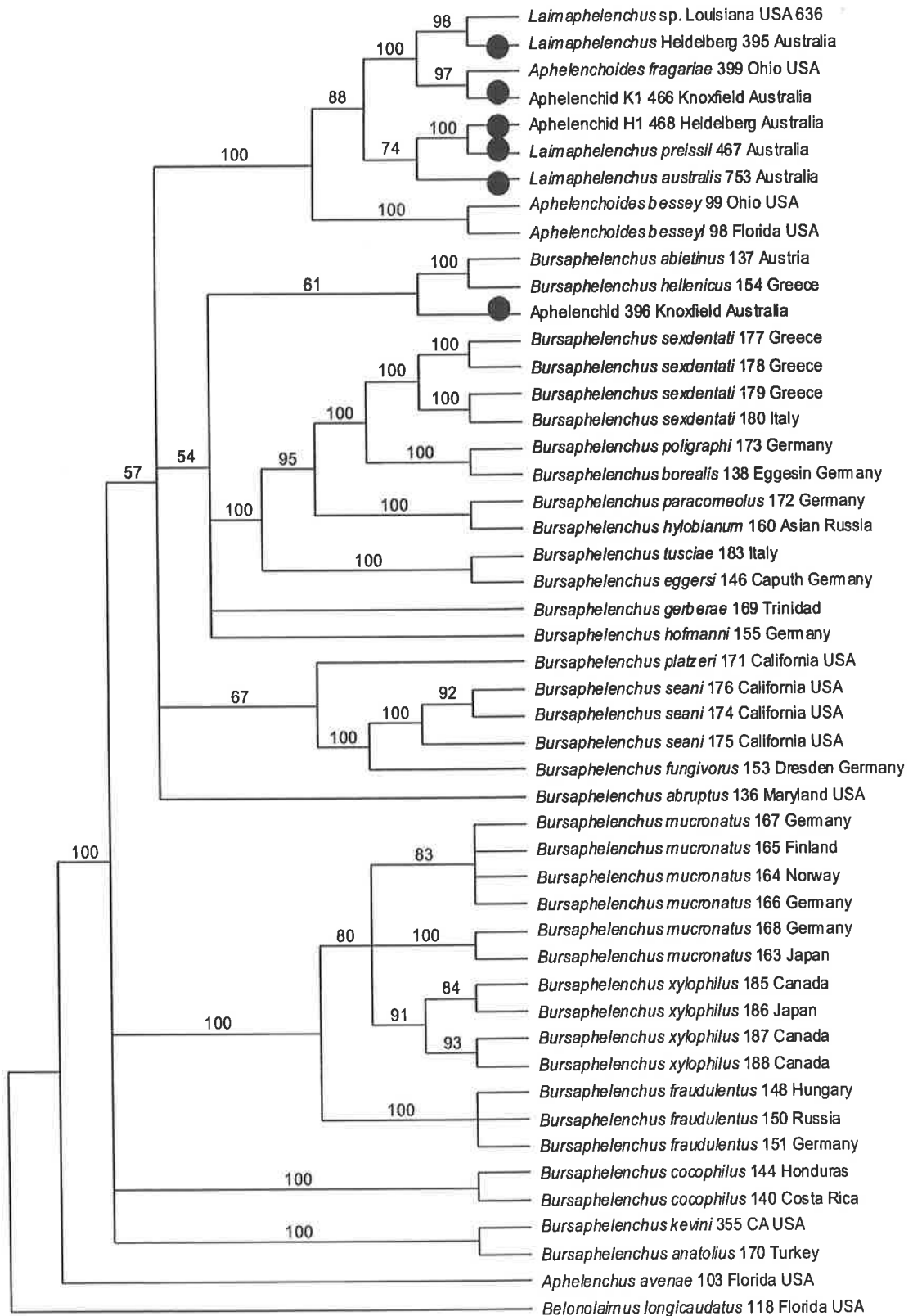
Fig. 5.7 Bayesian tree inferred from LSU gene DNA sequences. Provided by R. M. Giblin-Davis and Ye, Weimin (unpub. data). ●, nematodes from this study. The numbers give the probability support for individual branches.

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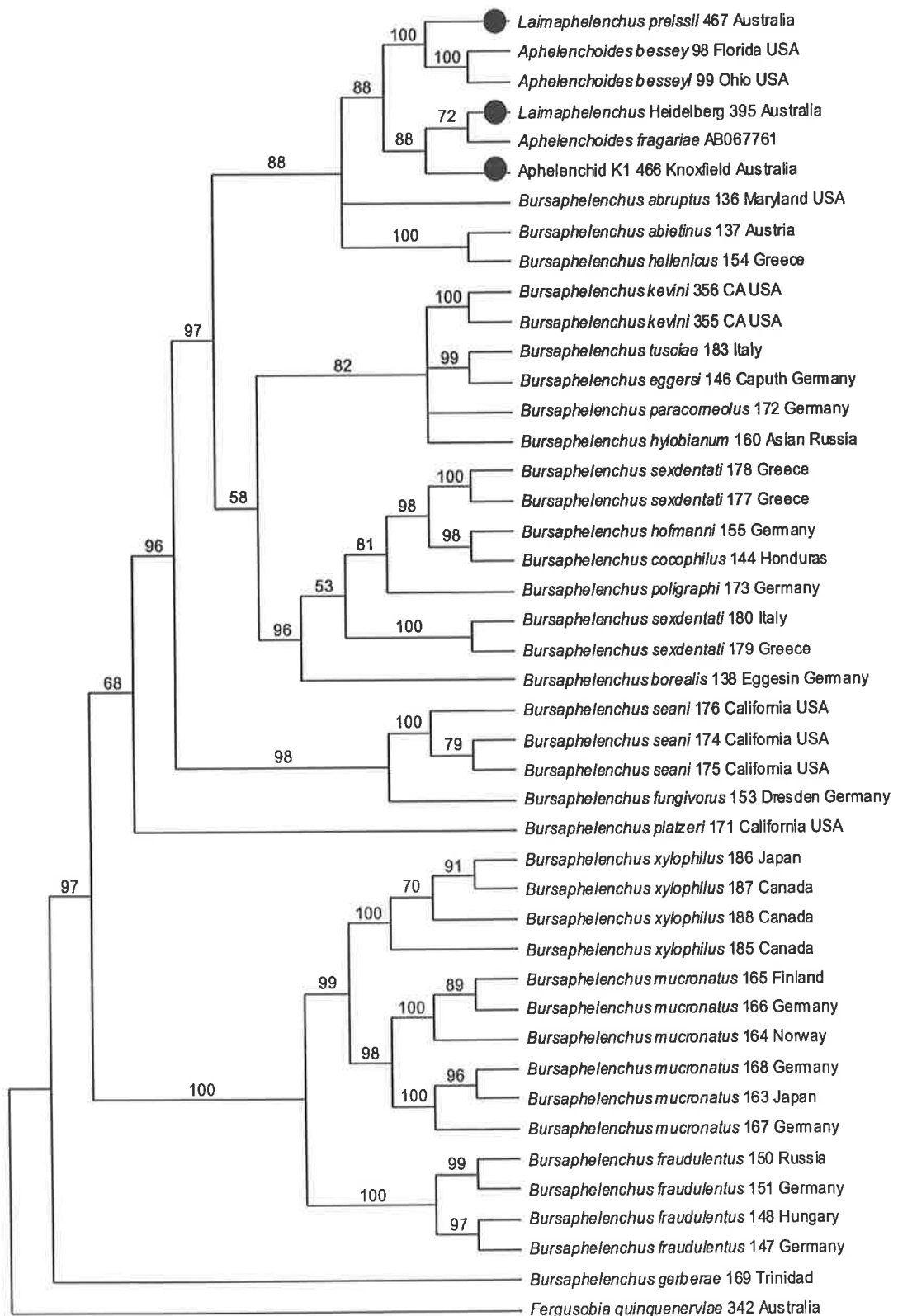
Fig. 5.8 Bayesian tree inferred from COI gene DNA sequences. Provided by R. M. Giblin-Davis and Ye, Weimin (unpub. data). ●, nematodes from this study. The numbers give the probability support for individual branches.

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5.4 Discussion and Conclusions

Molecular differentiation of *Laimaphelenchus*, *Aphelenchoides*, *Bursaphelenchus* and *Aphelenchus* can be seen in the phylogenetic trees presented here (Fig. 5.6 and Fig. 5.7). The analyses confirmed that most nematodes in this study belong to the super-family Aphelenchoidioidea (Hunt, 1993). The phylogenetic tree gives support to Hunt's morphological differentiation of superfamilies Aphelenchoidea (*Aphelenchus*) and Aphelenchoidoidea (*Bursaphelenchus, Aphelenchoides* and *Laimaphelenchus*). It also supports Hunt's separation of the families Parasitaphelenchidae (*Bursaphelenchus*) and Aphelenchoididae (*Aphelenchoides, Laimaphelenchus*).

Morphologically, the nematode *L. preissii* from native *Callitris* trees at Burdett, SA is similar to the genus *Bursaphelenchus* (it has a bursa and a vulval flap), and its characters appear basal to most described *Bursaphelenchus* species. However, the LSU and SSU phylogenetic analyses demonstrated that specimens from Burdett are genetically distinct from species of *Bursaphelenchus* collected from Europe and North and South America. The sequence data indicated that this species is actually close to those of the genus *Laimaphelenchus*. On the other hand, COI phylogenetic tree suggested that *L. preissii* is genetically close to *A. besseyi*.

Apparent paraphyly of the *Aphelenchoides* and *Laimaphelenchus* has strengthened the argument about the current difficulties of determining genus identity. The whole group of *Aphelenchoides* is in urgent need of revision, and many more species should be sequenced. Morphologically, *Aphelenchoides* and *Laimaphelenchus* may be a conserved group, with overlap of characters.

The ITS-RFLP method is quick and useful for discriminating species. It can be used as a quick approach to identify whether two or more species are the same or not without extensive background information on nematode taxonomy based on morphology. Attempts were made to use the full ITS1-5.8S-ITS2, ITS1 and ITS2 sequences for comparison with other sequences respectively. Not surprisingly, there was too much variation in ITS1 and ITS2 regions to enable this and instead

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the 5.8 S was region used in this study. The 5.8S matrix results showed that *Laimaphelenchus preissii*, Aphelenchid K1 and Aphelenchid H1 are not close to *Bursaphelenchus*. This is consistent to the findings that were obtained using SSU, LSU sequences (from Giblin-Davis and Weimin Ye, pers. comm. 2006).

In conclusion, nematode molecular taxonomy is at an early stage with very limited amounts of sequence data compared with the large amount of information available from morphological taxonomy. As Giblin-Davis (pers. comm. 2006) pointed out, the molecular taxonomy of aphelenchids can be likened to a patchwork quilt. Each time a new sequence is added to the tree, the tree topography will change because we are at the beginning with very limited data. This means that definitive statements about taxonomic relationships cannot be made at present. As more phylogenetic data becomes availiable, paraphyly can be exposed and attention given to the morphological features that are convergent (e.g. tail type) and to other characters that are phylogenetically relevant and show true homology. From the molecular data, none of the 6 Australian isolates studied here belongs to the genus *Bursaphelenchus*. The data obtained supports the morphological taxonomy presented in Chapter 4. Sequences of SSU of *L. australis* are needed to confirm its deeper relationship to the genus *Laimaphelenchus*.

Chapter 6: Growth Studies

6.1 Introduction

Aphelenchids are mostly free-living and mycetophagous nematodes, found worldwide in soil and decaying plant material, associated with lichens, on bark and in the tunnels of wood-boring beetles (Hunt 1993). *Aphelenchus* and *Aphelenchoides* are the most abundant and widespread genera of aphelenchids (Hunt 1993).

Australia is isolated, and the driest continent in the world (excluding Antarctica). In the south-east of Australia, temperatures may be high for relatively long periods in a typical summer. Therefore, nematodes both in pine plantations and in native pine trees have to be able to tolerate the hot summer, or to avoid it via some sort of diapause or dormancy in at least one life stage. However, the biology of the majority of the nominal species of aphelenchids is not known in detail (Hunt 1993).

Studies on the life cycles of some nematodes have been conducted. For example, in the natural forest ecosystem, *Bursaphelenchus xylophilus* is most commonly vectored as a specialised dauer larval stage (Mamiya 1972; 1983). While conditions remain favourable, *B. xylophilus* populations persist in a propagative cycle of four larval stages and adults. At 25 °C, the life cycle of *B. xylophilus* is completed in 4-5 days, the nematode population is rapidly built up, and the nematodes migrate throughout the tree (Ishibashi and Kondo 1977; Mamiya 1984). Dispersal third and fourth stage juveniles appear after infected trees have been dead several months (Ishibashi and Kondo 1977). The dispersal J3 is able to survive long periods without feeding (Ishibashi and Kondo 1977), and the J4's are adapted to survive desiccation (Thong and Webster 1991). Only adults and juveniles of the propagative cycle were recorded from a population of *B. xylophilus* in wood chips maintained at 25-40°C (Dwinell 1986). The short life cycle and rapid population growth of *B. xylophilus* probably contribute to its pathogenicity.

The work described in this Chapter was conducted to examine the biology of the nematodes found in Australian pines, particularly those found in diseased trees in Victoria. Nematode population growth and mean doubling time was determined to see if there was any correlation with potential pathogenicity of the nematodes studied (Chapter 8). In addition, it is considered important to include both molecular and ecological data on nematodes in NemATOL (Powers 2004), and the information obtained here will be added to the database.

Two experiments were conducted to examine nematode replication under different temperatures and on different food sources. The population growth and mean doubling time of three nematodes, Aphelenchid K1, Aphelenchid H1 and *L. preissii* were assessed at different temperatures on *B. cinerea*. In addition, Aphelenchid K1 was grown on three different fungi, and its multiplication and mean population doubling time were measured on these varying food sources.

6.2 Materials and Methods

6.2.1 *In vitro* multiplication at different temperatures

The multiplication of three species of nematodes was measured at a series of temperatures, selected to represent the range the nematodes would be likely to be exposed to in the field. For nematodes *L. preissii* and K1, 5 male and 5 female nematodes of each species were inoculated on to respective potato dextrose agar (PDA, Difco Laboratories, Detroit, USA) plates, containing a fungal mat of *B. cinerea*. Fifteen plates were inoculated. The plates were incubated at 10, 15, 20, 25 or 32°C, with three plates at each temperature. For Aphelenchid H1, 5 male and 5 female nematodes were inoculated onto 18 respective PDA plates, each with *B. cinerea*. These plates were incubated at 10, 15, 20, 25, 28 and 32°C, with three plates at each temperature.

Nematodes from plates were harvested after 30 days for *L. preissii*, 11 days for K1 and 13 days for H1. The time for nematode harvest was determined by observation of the plates to avoid the nematodes over-growing the fungus. An electrical food

blender (Atomix, Townson and Mercer, UK) was used to extract the nematodes from the plates. The contents (PDA+fungus+nematode) of individual plates were put into the blender with 200-300 ml water, and homogenised. One millilitre of nematode suspension was then pipetted into a Doncaster counting dish and counted under a dissecting microscope.

6.2.2 *In vitro* multiplication on different fungi

The nematode K1 was grown on different fungi, and multiplication on these was observed. Four PDA+*B. cinerea* plates, 4 PDA+*Monilinia fructicola* plates and 4 PDA+*Rhizoctonia solani* plates were prepared, and 100 nematodes (with approximately equal numbers of both sexes) were inoculated on to each. The plates were incubated at 25°C temperature. For comparison, 2 nematodes (one female and one male) were also inoculated on to four plates each of the three fungi. After 9 days, nematodes from the plates were harvested (harvest time was determined by observation to avoid nematodes over-growing the fungus). The nematodes were harvested and counted as described in Section 6.1.2.1.

6.2.3 Fungal isolation

Small pieces of wood from the diseased *Pinus radiata* trees at Knoxfield and Heidelberg, Victoria (Vic.), were selected for isolation of fungi to test their suitability for nematode culture. The wood chips were washed in ethanol (100%), cut into small pieces, placed on PDA plates (with 500 ppm streptomycin), and incubated at 25°C for 7 days. Fungi were purified by subculture on to fresh PDA plates.

6.2.4 Data analysis

Analyses of variance were performed using GenStat Sixth Edition with General Analysis of Variance. Data was transformed (base 10 logarithm) for production of

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Figs 6.1, 6.3 and 6.4; and (base 2 logarithms) for production of Figs 6.2, 6.5 and 6.6. Means of the numbers of nematodes were compared using analysis of variance. Where significant differences were detected between the means, the Least Significant Difference (LSD) method was used.

6.3 Results

6.3.1 *In vitro* multiplication at different temperatures

The final numbers of *L. preissii*, Aphelenchid K1 and Aphelenchid H1 after culture at different temperatures are shown in Fig. 6.1. All the nematodes tested were significantly affected by incubation temperature, particularly Aphelenchid K1 and Aphelenchid H1 (p<0.001). Aphelenchid H1 did not multiply at 10°C after 13 days incubation and *L. preissii* did not multiply at 32°C after 30 days incubation. The optimal temperatures for multiplication of *L. preissii*, Aphelenchid K1 and Aphelenchid H1 were about 20, 25 and 28°C respectively. At the optimal temperature, Aphelenchid H1 had the highest population numbers, followed by Aphelenchid K1, and *L. preissii* had the lowest population number. *Laimaphelenchus preissii* had slow growth at all tested temperatures, except at 32°C; but showed a strong ability to tolerate low temperature. In comparison, Aphelenchid K1 and Aphelenchid H1 had faster rates of growth but a poor ability to tolerate low temperature.

Aphelenchid K1 and Aphelenchid H1 had a low multiplication at 10°C (Fig. 6.1), but it increased sharply at the higher temperatures to 25 and 28°C. Whereas, *L. preissii* had small population increase from 10 to 15°C, but numbers increased rapidly from 15 to 20°C. All three nematodes had a similar number at 15°C, but after different periods of incubation. The multiplication of Aphelenchid K1 and *L. preissii* at their optimal temperatures (25°C for K1 and 20°C for *L. preissii*) was significantly greater than at 10, 15, 20 and 32°C. However, the multiplication of Aphelenchid H1 at its optimal temperature (28°C) was significantly different to that at 10, 15, 20 and 32°C, but similar to that at 25°C.

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Given that the nematodes were cultured for different periods of time, the mean population doubling time of each nematode at different temperature was calculated:

Mean population doubling time=Inoculation period /[$\log_2$ (Pf)-$\log_2$ (Pi)]

and is shown in Fig. 6.2. The mean time for the population to double of all three nematodes was significantly affected by temperature (Aphelenchid K1, P<0.001; Aphelenchid H1, p<0.001; *L. preissii*, P=0.004). At their respective optimal temperatures, Aphelenchid K1 and Aphelenchid H1 had the same mean population doubling time (0.9 d) whereas it was 7.3 d for *L. preissii*. The mean population doubling time of *L. preissii* dropped sharply from 15 to 20°C and then rose quickly from at 20 to 25°C (Fig. 6.2). In contrast, Aphelenchid H1 and Aphelenchid K1 have similar shapes; a wide, open, flat curve from 20 to 32°C. The mean doubling time was not recorded for Aphelenchid H1 and Aphelenchid K1 at 10 °C and for *L. preissii* at 32°C due to their respective low and high temperature intolerance. The mean doubling time was not significantly different between 20 and 25°C for Aphelenchid K1; 20, 25 and 28°C for Aphelenchid H1; and 10 and 15°C for *L. preissii*.

6.3.2 *In vitro* multiplication on different fungi

After culture on different fungi at 25°C, the final numbers of Aphelenchid K1 are shown in Figs. 6.3 and 6.4. The multiplication of Aphelenchid K1 was significantly affected by the fungus on which it was grown (Fig. 6.3, P = 0.004 and Fig. 6.4. P < 0.001). In the experiment where 100 Aphelenchid K1 were inoculated on to different fungi, it had high multiplication on *B. cinerea*, moderate multiplication on *M. fructicola*, and lower multiplication on *R. solani*. The multiplication of Aphelenchid K1 was similar on *B. cinerea* and *M. fructicola* but both were significantly greater than on *R. solani*.

The mean population doubling times of Aphelenchid K1 cultured on the three fungi at 25°C are shown in Figs. 6.4 and Fig. 6.5. Not surprisingly, the doubling time was also significantly affected by different fungi (Fig. 6.5, P = 0.003 and Fig. 6.6, P < 0.001). In the experiment where the initial number of Aphelenchid K1 was 100, the
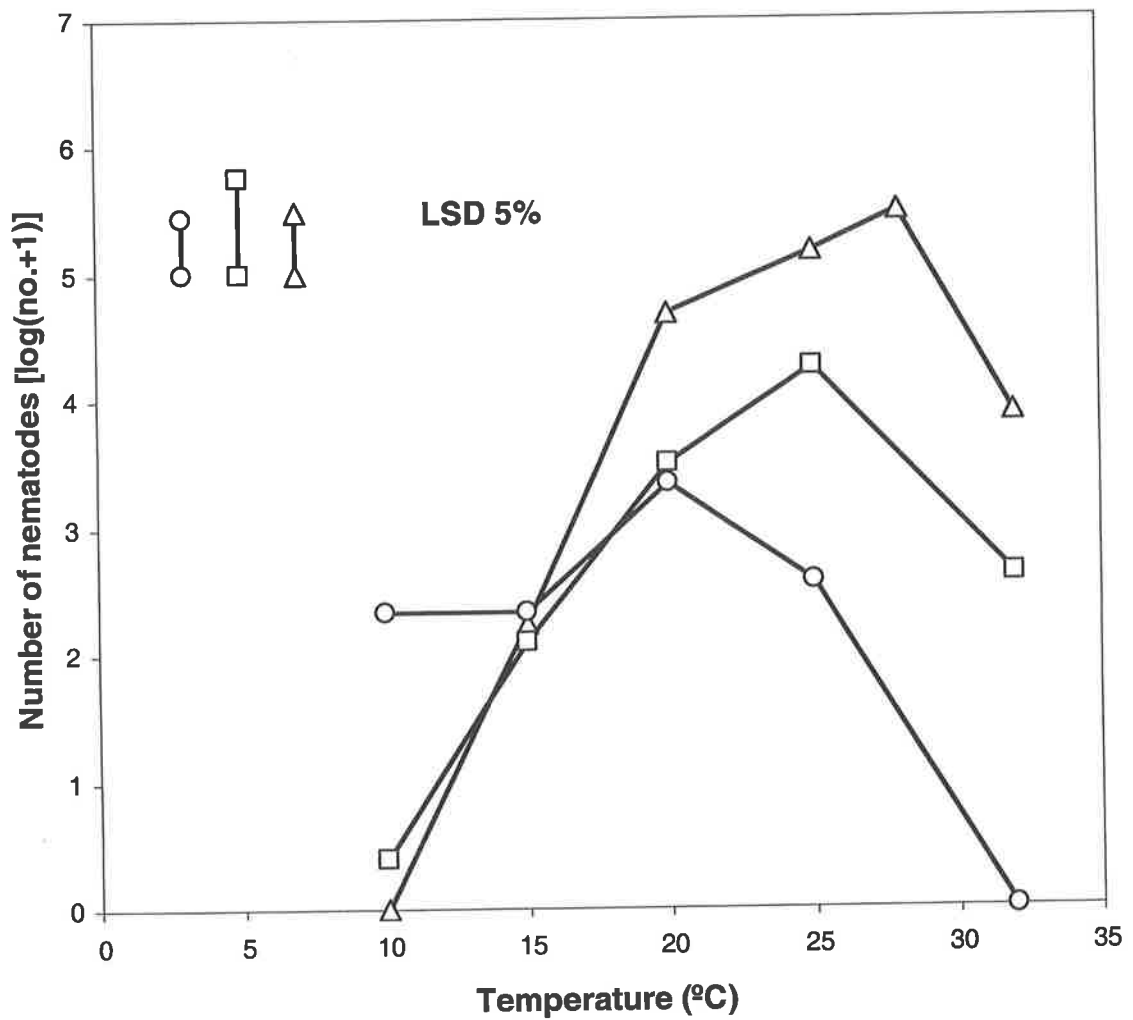
Fig. 6.1. Mean population numbers produced by 5 males and 5 females of *Laimaphelenchus preissii* (O 30 days incubation), Aphelenchid K1 (□ 11 days incubation) and Aphelenchid H1 (Δ 13 days incubation) on *B. cinerea* at different temperatures. LSD 5%: *L. preissii*=0.43, K1=0.75, H1=0.49.
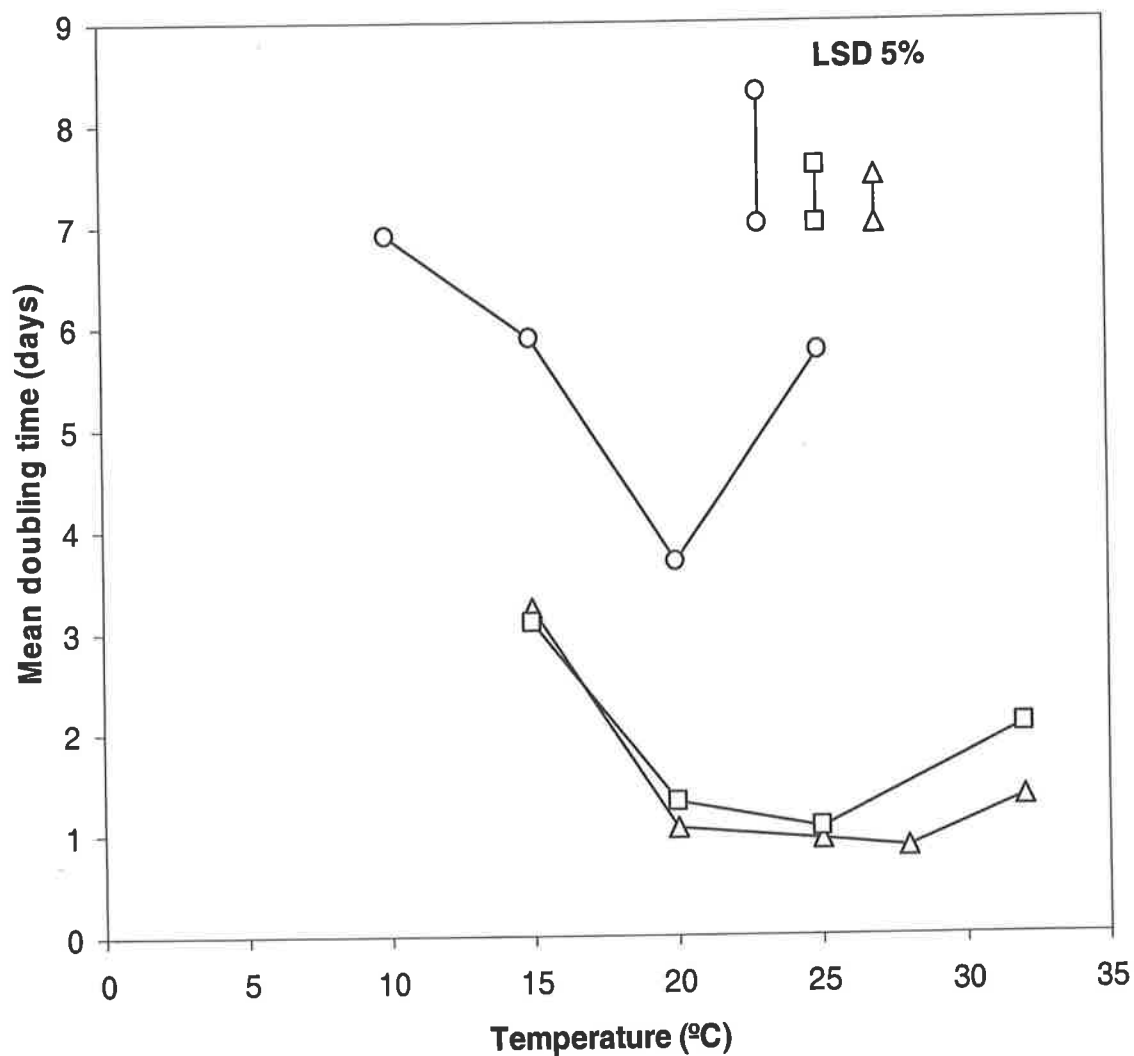
Fig. 6.2. Mean population doubling time [Inoculation period/log2 (Pf)-log2 (Pi)] of

Laimaphelenchus preissii (O 30 days incubation), Aphelenchid K1 (□ 11

days incubation) and Aphelenchid H1 (Δ 13 days incubation) on *B. cinerea*

at different temperatures; LSD 5%, *L. preissii*=1.30, K1=0.57, H1=0.46.

The numbers of Aphelenchid K1 and Aphelenchid H1 were not defined at

10°C; *L. preissii* was not defined at 32°C.

population doubling time was 0.97 d on *B. cinerea*, 1.09 d on *M. fructicola*, and 56 d on *R. solani*. The mean population doubling time of Aphelenchid K1 was significantly shorter on *B. cinerea* and *M. fructicola* than on *R. solani,* but it did not differ significantly between *B. cinerea* and *M. fructicola.*

The results obtained in the two experiments were similar. In the experiment where one male and one female were inoculated on to different fungi, Aphelenchid K1 had the best growth on *B. cinerea*, followed by *M. fructicola* and the lowest growth on *R. solani* (Fig. 6.4). The mean population doubling time on *B. cinerea, M. fructicola* and *R. solani* was 1.20, 1.36 and 1.82 d respectively (Fig. 6.6).

Observation of the experimental plates showed that both *B. cinerea* and *M. fructicola* were fully lysed by Aphelenchid K1, and that *B. cinerea* was damaged more quickly than *M. fructicola*. In comparison, fungal mats of *R. solani* were only partially lysed by Aphelenchid K1, and the nematode did not grow well.

6.3.2 Fungal isolation

A species of *Trichoderma* (identification based on structure of hyphae and spore morphology) and two unknown fungi were isolated from the wood samples. Aphelenchid K1 and Aphelenchid H1 were inoculated on to all the three fungi on PDA plates. The nematodes were produced on the two unknown fungi in small numbers. However, they did not multiply on the *Trichoderma* sp. As this was a preliminary experimental observation and growth was poor, no further attempt at identification of the fungi was made.

6.4 Discussion and Conclusions

6.4.1 *In vitro* multiplication at different temperatures

Much research has been done on development of nematodes on differing food sources and under differing conditions. For example, Giblin-Davis and Kaya (1984) reported that host, temperature and media had additive effects on the growth of *Bursaphelenchus seani*. The nematodes were grown on *Monilinia fructicola* at
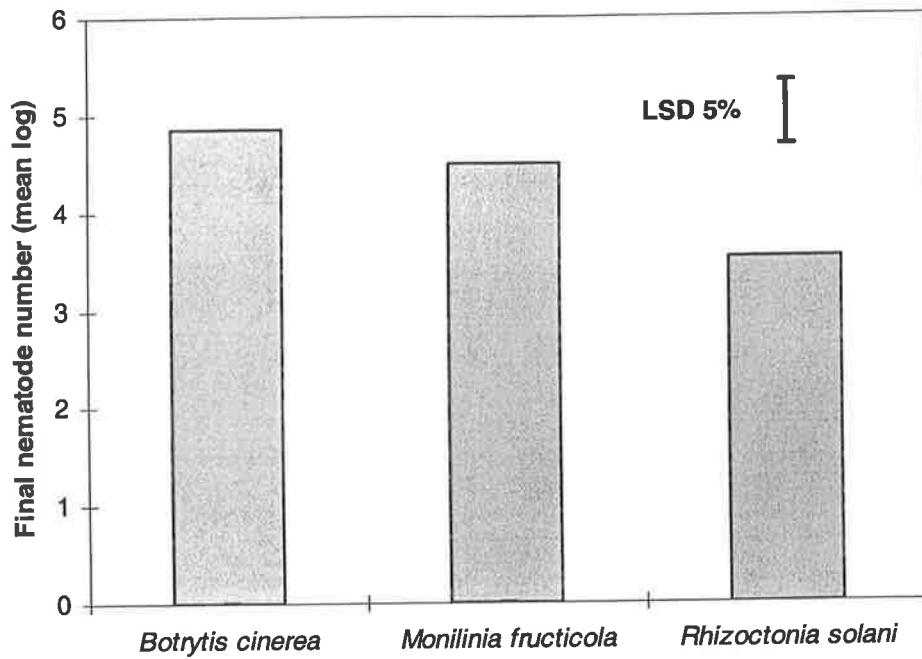
Fig. 6.3. Multiplication of Aphelenchid K1 on different fungi at 25°C for 9 days following inoculation of 100 nematodes per plate. LSD 5%=0.66.
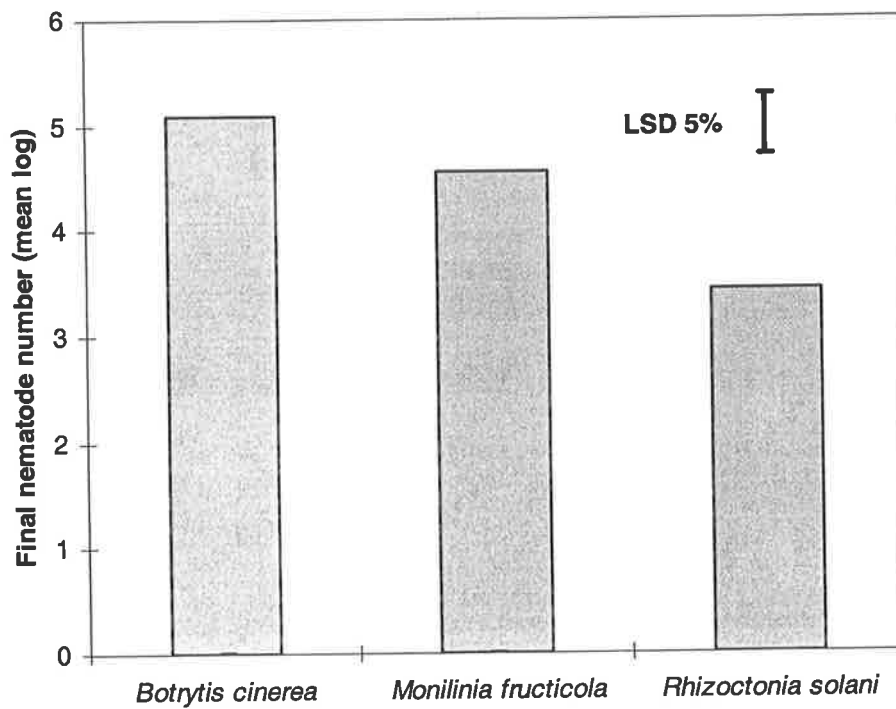
Fig. 6.4. Multiplication of Aphelenchid K1 on different fungi at 25°C for 19 days following inoculation of one male and one female per plate. LSD 5%=0.57.

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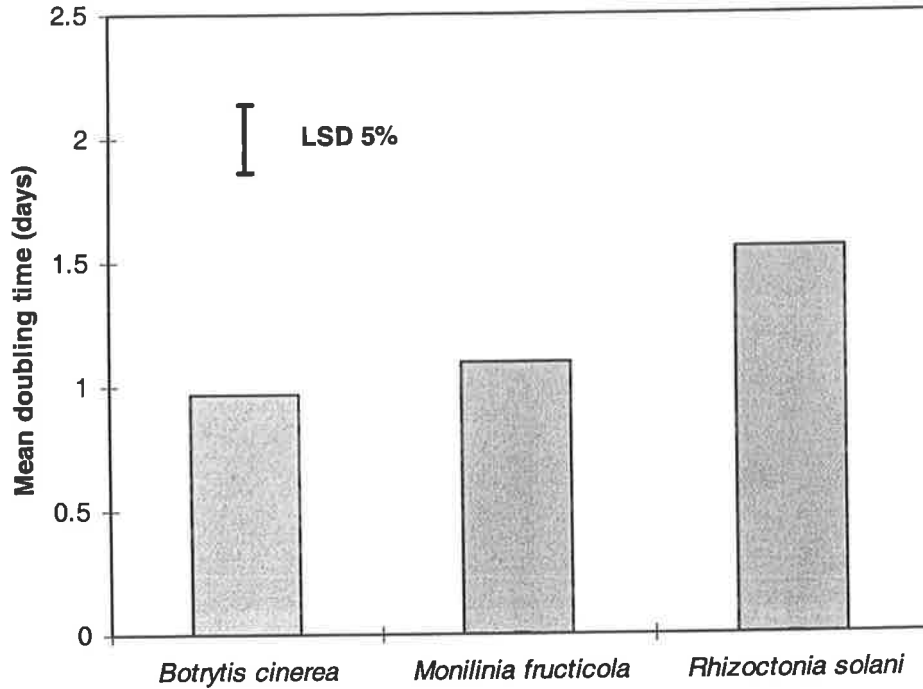
Fig. 6.5. Mean population doubling time [Inoculation period/{log2 (Pf)-log2 (Pi)}] of Aphelenchid K1 on different fungi at 25°C for 9 days following inoculation of 100 nematodes per plate. LSD 5%=0.28.
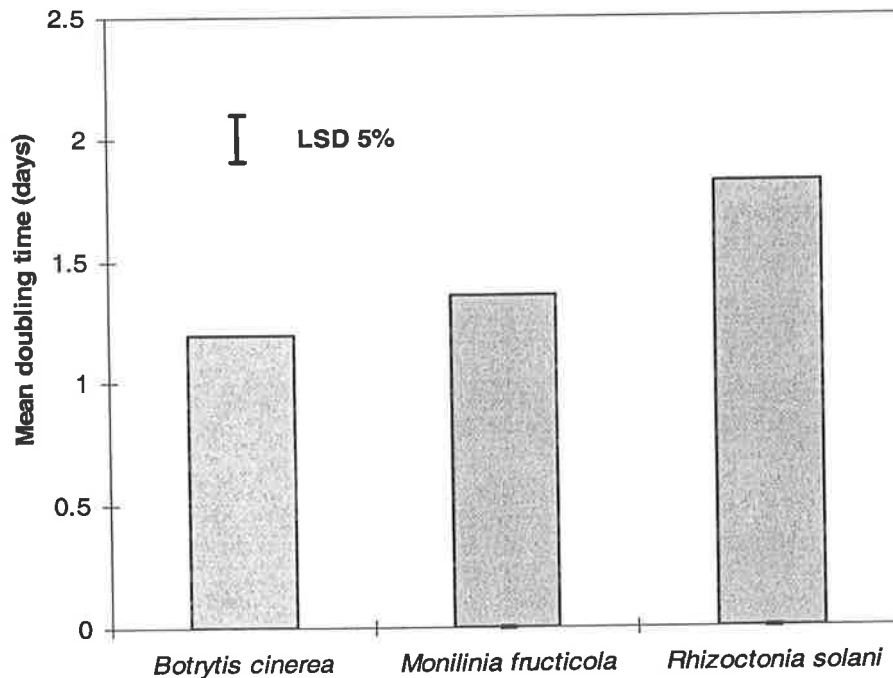
Fig. 6.6. Mean population doubling time [Inoculation period/{log2 (Pf)-log2 (Pi)}] of Aphelenchid K1 on different fungi at 25°C for 19 days following inoculation of one male and one female per plate. LSD 5%=0.19.

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seven different temperatures (9, 15, 20, 25, 30, 33°C), and mean generation time (J2 to J2) of *B. seani* was significantly affected. No development occurred at 9 and 36°C, but at 25°C dauer juveniles (J3) were found after four weeks. The results presented here show clearly that temperature significantly affected the multiplication rates of all three nematodes tested. *Laimaphelenchus preissii* had slow growth and the longest mean population doubling time (7.3 d), followed by Aphelenchid K1 (0.9 d), and Aphelenchid H1 (0.85 d) had the fastest growth and shortest mean population doubling time. No development of Aphelenchid H1 occurred at 10°C, or of *L. preissii* at 32°C. Dauer juveniles were not observed for any of the three nematode species, suggesting that they are unlikely to be insect associates.

Due to a lack of incubator space, the multiplication rates of the nematodes, *L. preissii* and Aphelenchid K1, were not tested at 28°C. This was not a problem with *L. preissii*, as its multiplication rate decreased at 25°C and higher temperatures. However, with Aphelenchid K1, the highest mean multiplication occurred at 25°C and declined at 32°C, and it is possible that multiplication could have been higher at intermediate temperatures. Therefore, to determine the optimal temperature more accurately experiments with near optimal temperatures would be needed.

Trudgill and Philips (2006) discussed various reproductive strategies of nematodes. Some are large and have long life cycles with low rates of population increase; others are relatively small, and have short life cycles and potentially higher reproductive rates. From the morphological study in Chapter 4, the three species of nematodes tested have different body sizes. *L. preissii* is about 1000 μm, K1 is about 600 μm and H1 is about 300 μm long. From this experiment, it seems that the body size in the aphelenchids tested is correlated with their multiplication rate. Nematodes with long body size had long mean doubling time, and vice versa.

Rainfall and temperature would be expected to play an important role in the nematode multiplication rate in the field. As mentioned in Chapter 3, these three nematode species were isolated from three different locations, and the Burdett, SA site is hotter and drier than the two sites in Melbourne (see Chapter 3). It would be expected that *L. preissii* is adapted to higher optimal temperature for multiplication

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and more tolerance to dry conditions. However, the optimal temperatures for multiplication of *L. preissii* is lower (20°C) than that for K1 (25°C) and H1 (28°C). This could be because Burdett is dry in the summer, and *L. preissii* probably only reproduces in the winter months when it is wet. However, the nematodes isolated from Melbourne may be able to develop throughout the year.

6.4.2 *In vitro* multiplication on different fungi

Nematodes have a wide range of food preferences, and knowledge of their feeding habits is essential to understand their biology and role in an ecosystem (Ikonen 2001). Fungal feeding, or hyphal-feeding (Yeates 1998), nematodes feed on many different species of fungi, including saprophytic, pathogenic and mycorrhizal fungi growing in the rhizosphere (Freckman and Caswell 1985; Giannakis and Sanders 1989; Ruess and Dighton 1996). The fungi *B. cinerea* and *M. fructicola* are commonly used for culturing aphelenchid nematodes in the laboratory. However, the optimal fungus for nematode development may vary depending on the species of nematodes. Giblin-Davis and Kaya (1984) tested *B. seani* on eighteen fungi, of which fourteen supported reproduction of the nematode. Of these, the plant pathogenic fungi, *M. fructicola*, *B. cinerea* and *B. allii*, and the insect pathogenic fungi, *Beauveria bassiana* and *Ascosphaera apis* were suitable for *B. seani* growth and reproduction. Very small numbers of *B. seani* developed on *Trichoderma* sp. A species of *Trichoderma* was also isolated from the diseased wood sample collected from Knoxfield and Heidelberg, Victoria (Section 6.3.2). As found with *B. seani*, Aphelenchid K1 and Aphelenchid H1 failed to grow on the fungus.

This experiment showed that Aphelenchid K1 reproduced differentially when given different fungi to feed on. It had better reproduction on *B. cinerea* than on *M. fructicola* and *R. solani* at 25°C. A number of papers have reported that the populations of fungal-feeding nematodes are affected by their food sources. For instance, Ikonen (2001) tested *Aphelenchoides bicaudatus* and *Aphelenchus avenae* on six different fungi, and noticed differences in population growth patterns on the fungal species. Kondo *et al.* (1982) tested *B. xylophilus* on three fungi, *Ceratocystis ips*, *Diplodia pini* and *Trichoderma* sp., isolated from diseased trees. The nematode

did not lyse all three fungi, and produced a small population on *C. ips* and *D. pini,* and did not reproduce on *Trichoderma* sp.

In conclusion, the nematodes tested had different optimal replication temperatures, mean population doubling time and probably food preferences. Aphelenchid H1 and Aphelenchid K1 had similar temperature sensitivity and reproduction activities. *Laimaphelenchus preissii* is quite different to those two nematodes, possibly because of their different hosts and locations. The environmental conditions to which they are adapted may play a major role in these differences.

Aphelenchid H1 and Aphelenchid K1 were both present in the diseased pine tree at Knoxfield. Both species have been successfully cultured on *B. cinerea*. It is possible that they compete with each other in the field. However, nothing is known of their respective food requirements / preferences. While each nematode has similar optimal temperatures and times for doubling of mean populations, subtle factors may operate to regulate potential competition between them. For example, Aikawa *et al.* (2006) have shown that the population structure of *B. xylophilus* within a single tree varied both with the virulence level of the nematode populations transmitted to the tree and their transmission order.

Environmental conditions other than temperature may affect these nematodes differently, and may be important for potential competition. While no dauer larvae have been observed in cultures of the three nematodes, nothing is known of their ability to survive desiccation. This is considered in the next chapter.

Chapter 7: Desiccation Studies

7.1 Introduction

Many nematode species are able to enter anhydrobiosis (a form of cryptobiosis, induced by loss of water from the living nematode). The ability to survive desiccation appears to be intrinsic in many genera, particularly insect parasitic nematodes with an endo- or ectophoretic dauer larva stage (Hunt 1993). In some aphelenchid genera, the nematodes have a specific life stage able to enter anhydrobiosis. Examples include *Ektaphelenchus* and *Cryptaphelenchoides*, in which the immature female is the resistant stage. The genera *Aphelenchoides* and *Laimaphelenchus* are known for their ability to become anhydrobiotic (Hunt 1993). For example, *A. besseyi* may survive in the desiccated state for several years on rice seeds (Todd and Atkins 1958).

Currently, there are two theories about nematode survival of unfavourable conditions. The first theory was put forward by Van Gundy (1965) and Cooper and Van Gundy (1971), and was based on the absence of metabolism of an organism. Cryptobiosis was said to occur when no metabolism can be measured, and was regarded as the most resistant state a nematode can achieve. If a nematode had a lowered, but detectable, metabolism, it was said to be in a state of dormancy or quiescence. Anhydrobiosis, cryobiosis and osmobiosis are descriptive terms used to define the environmental stress inducing nematodes to proceed from the active to the cryptobiotic state. The second theory on nematode survival does not consider the presence or absence of metabolism as a criterion for cryptobiosis. Crowe and Cooper (1971) defined cryptobiosis on the basis of the structural integrity of the organism. If its structural integrity remained intact, an organism was capable of resuming the active state. Crowe and Cooper argued that the structural integrity of a true cryptobiote remains organised and intact even when exposed to environmental extremes.

In nematodes, arrest of development in response to adverse environmental factors can occur at any time of the year and may be experienced by some or all stages in the life cycle. This arrest is a form of dormancy, not diapause, is readily reversible,

and ends as soon as favourable environmental conditions return. Anhydrobiosis, the ability to survive almost complete dehydration, is a form of dormancy known to occur in many soil and plant nematodes. The importance of the rate of water loss for subsequent survival following rehydration has been known for many years (Ellenby 1968; Crowe and Madin 1975). In general, nematodes have a better chance of recovery if they are dried slowly. Many nematodes show adaptations such as coiling and clumping to reduce the rate of water loss. Barrett (1991) suggested that rapid dehydration could lead to mechanical distortion and physical damage to a nematode, which would kill it when it re-hydrated. In addition, rapid drying may not allow time for the biochemical changes needed for successful induction of anhydrobiosis. These changes include, for example, accumulation of trehalose (Womersley 1987), and thickening of the outer lipid layers on the cuticle (Bird and Buttrose 1974).

The experiment described here is an attempt to gain a better understanding of the biology of the nematodes isolated in this work and their ability to survive desiccation. Four nematode species were tested, and exposed to either water or sucrose pre-treatments before being desiccated. The sucrose pre-treatment caused some water loss in the nematodes, and would have induced some biochemical and physiological adaptation before they were placed at 0% RH.

7.2 Materials and Methods

7.2.1 Nematode preparation

Four nematode species (*Laimaphelenchus preissii*, Aphelenchid K1, Aphelenchid K2 and Aphelenchid H1) were washed from culture plates with water. The nematode suspension was then concentrated by using a 45 μm sieve to reduce the volume. The numbers of nematodes were counted, and the volume was adjusted to make the suspensions about 100 nematodes per 1 ml. All procedures were conducted at room temperature.

7.2.2 Treatments

The methods used for the desiccation experiments were taken from Charwat (1994). She showed that the aphelenchid nematode *Aphelenchus avenae* survived desiccation best when preconditioned in sucrose solutions with concentrations between 0.8 and 1.2 M. Given these data, 1 M sucrose solutions were used here for pre-treatment of the aphelenchid nematodes used in desiccation experiments.

For each of the four species tested, three treatments were applied. Control nematodes were incubated in tap water throughout the experiment, to check for survival. In the second treatment, nematodes were kept in tap water for 2 and 12 h respectively, before being desiccated for 48 h or 1 week. In the third treatment, nematodes were kept in 1 M sucrose for 2 and 12 h respectively, before being desiccated for 48 h or 1 week. After desiccation, nematodes were returned to tap water, and checked at 30 min intervals for determination of survival (based on movement).

For all treatments, the procedure was as follows. One-millilitre aliquots of nematode suspension were collected, and added to 1 ml water or 1 ml of 2 M sucrose solution in glass blocks. Thus, the nematodes were pre-treated in either water or a 1 M sucrose solution for 2 or 12 h. After the respective pre-treatments, nematodes were transferred to 25 mm Millipore filter discs (0.8 μm pore size) placed on top of a scintered glass funnel attached to an Erlenmeyer flask. Excess water was rapidly removed from the nematodes by using suction. For desiccation, filter discs with nematodes were then placed in a glass desiccator containing silica gel, and left for either 48 h or 1 week. For the control, filter discs with nematodes were returned to water. All treatments were conducted at room temperature and replicated twice.

After 48 h or 1 week, filter discs with nematodes were removed from the desiccator and returned to tap water in glass cavity blocks. After 30 min, they were agitated within the water to wash the nematodes from the filter papers, and checked for movement, i.e., recovery from desiccation. This was taken as an indication of

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survival. They were subsequently checked for movement at 30 min intervals for up to 4.5 h, and then occasionally until the nematodes had been in water for 24 h after re-hydration. The survival rates of nematodes in the controls were only checked twice; after 48 h and one week respectively.

7.2.3 Data analysis

Analyses of variance (ANOVA) were performed using GenStat Release 8.2 (PC/Windows 2000). Means of the numbers of nematodes recovering, treatment means and mean times for recovery were compared using analysis of variance. Where significant differences were detected between the means, the Least Significant Difference (LSD) method was used.

7.3 Results

7.3.1 *Laimaphelenchus preissii*

Recovery of *Laimaphelenchus preissii* after two days and one week of desiccation is shown in Fig. 7.1 and Fig. 7.2. After two days desiccation, all surviving nematodes had recovered within 2 h of rehydration. Of nematodes pre-treated for 2 h in water before desiccation, 77% recovered; and 79% of those pre-treated for 2 h in 1 M sucrose recovered. Of nematodes pre-treated for 12 h in water and sucrose before desiccation, 80 and 86% recovered respectively, while the rate of survival in the water control was 95%. In comparison, after one week of desiccation, all nematodes surviving had recovered within 4 h of return to water. The survival rates of nematodes pre-treated for 2 h in water and sucrose were 88 and 80% respectively; and for those pre-treated in 12 h in water and sucrose it was 89%. The rate of survival of nematodes in the water control was 90%.

The recovery rate was high for all developmental stages of *L. preissii*. Recovery rates after both pre-treatments (water and sucrose) were lower than survival in the water control after 48 h and one week of desiccation. ANOVA indicated that these

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differences were significant (LSD, P<0.05). However, the recovery rates after desiccation following pre-treatments of 2 h (77%) and 12 h in water (80%) were not significant (LSD, p>0.05); nor after the pre-treatments of 2 h for (79%) and 12 h (86%) in sucrose (LSD, p>0.05).

The nematode recovery rate in the first 30 min after return to water was significantly different after 48 h and one week of desiccation. After 48 h, the nematode recovery rates within 30 min of being put back into water ranged from 23 to 43% for the 4 pre-treatments (2 h water, 2 h sucrose, 12 h water and 12 h sucrose). However, after one week of desiccation, the recovery rate within 30 min of returning to water ranged from 2 to 18% for the 4 pre-treatments. There was no significant difference in the recovery rates between the water and sucrose treatments.

7.3.2 Aphelenchid K1

Recovery rates of Aphelenchid K1 are shown in Figs. 7.3 and 7.4. After desiccation for 48 h, only 6.7% of the nematodes pretreated with 2 h in water recovered when returned to water, compared to 55% of those pretreated with 2 h in sucrose. Of Aphlenchid K1 nematodes pretreated with 12 h in water before desiccation, 33% recovered; and of those pretreated with sucrose, 82% recovered. After one week of desiccation, only 2.8% of nematodes pretreated in water for 2 h recovered; 25% of those pretreated for 2 h in sucrose; and 6% and 59% following pre-treatments of 12 h in water and sucrose, respectively.

After 48 h or one week of desiccation, Aphelenchid K1 recovery rate was significantly different between each treatment (P<0.001, LSD 5%=15.8% recovery). Referring to Figs 7.3 and 7.4, it is clear that pre-treating Aphelenchid K1 with 1 M sucrose solution before desiccation significantly improved their recovery rates. After a week of desiccation, the recovery rates of Aphelenchid K1 pretreated by 2 h in water (2.8%) or 12 h in water (6.3%) did not differ. Survival of all desiccated K1 nematodes, with the exception of those pretreated with 1 M sucrose for 12 h, was
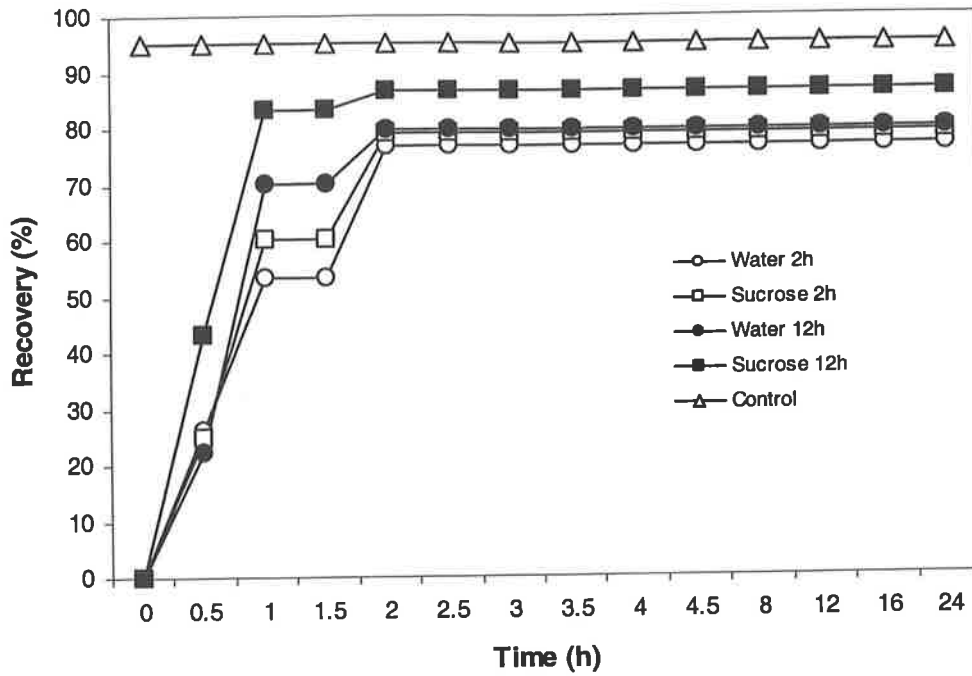
Fig. 7.1. Recovery of *L. preissii* pre-treated in water and 1 M sucrose solutions for 2 or 12 h respectively, and desiccated for 2 days. Two replicates for water and sucrose treatment each.
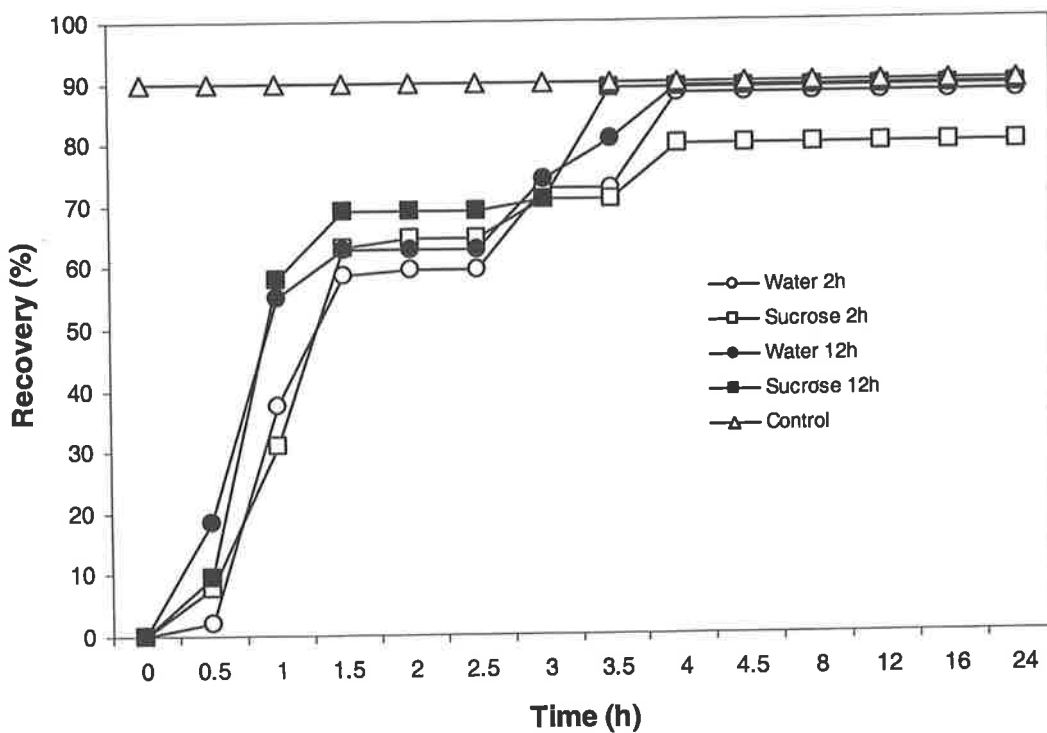
Fig. 7.2. Recovery of *L. preissii* pre-treated in water and 1 M sucrose solutions for 2 or 12 h respectively, and desiccated for 7 days. Two replicates for water and sucrose treatment each.
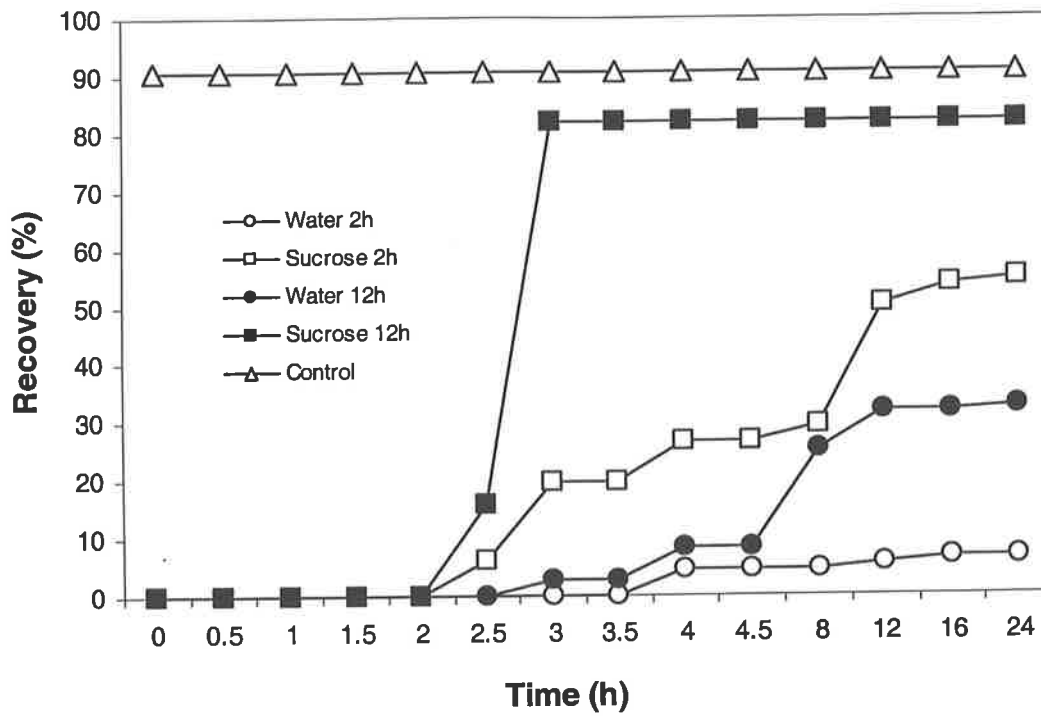
Fig. 7.3. Recovery of K1 pre-treated in water and 1 M sucrose solutions for 2 or 12 h respectively, and desiccated for 2 days. Two replicates for water and sucrose treatment each.
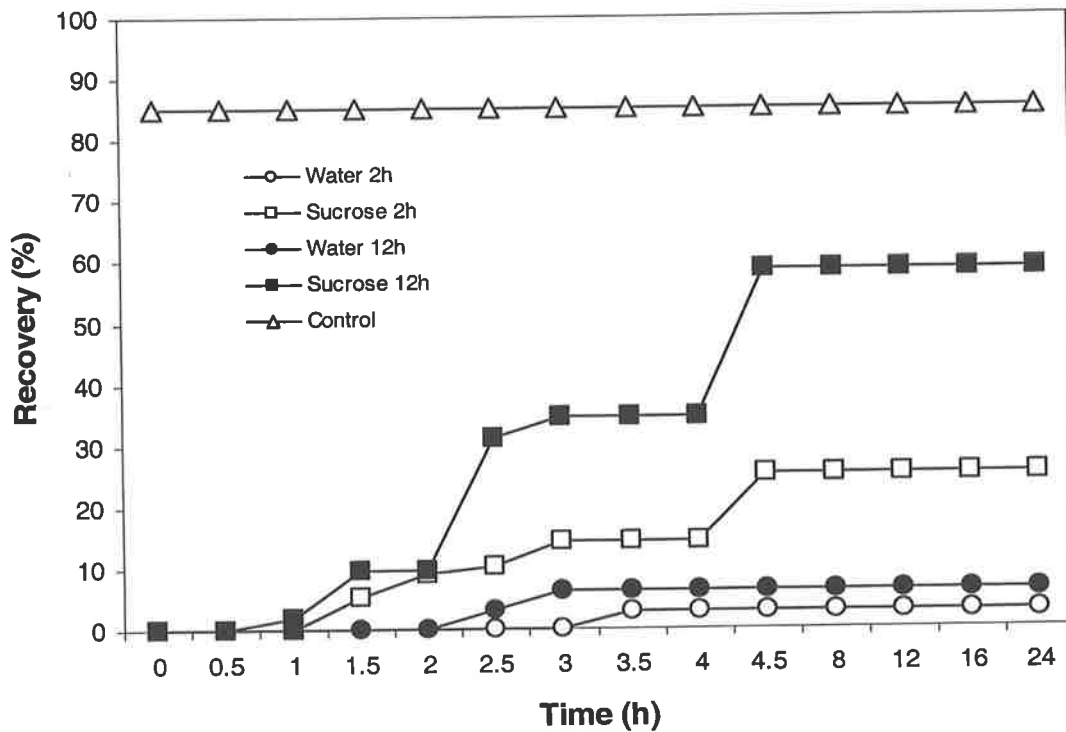
Fig. 7.4. Recovery of K1 pre-treated in water and 1 M sucrose solutions for 2 or 12 h respectively, and desiccated for 7 days. Two replicates for water and sucrose treatment each.
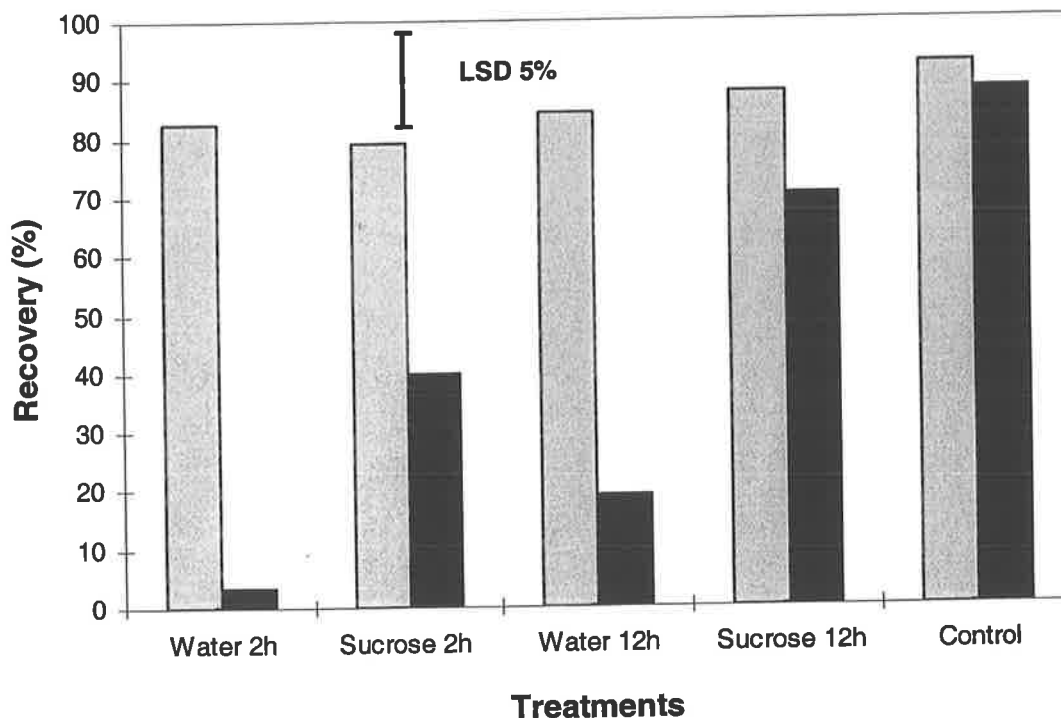
Fig.7 5. Nematode percentage recovery following various treatments. (■) *Laimaphelenchus preissii*; (■) Aphelenchid K1; LSD 5%=15.8. Four replicates for each water and sucrose treatment, two replicates for control.

highly significantly reduced compared with the water control (90.6% after 48 h, and 84.9% after one week) (P<0.001, LSD 5%=19.39% recovery).

There were significant differences in the times for Aphelenchid K1 recovery (P=0.045, LSD 5%=4.99% recovery). When nematodes were returned to water after 48 h desiccation, the average time for recovery of Aphelenchid K1 was 11.3 h. After one week of desiccation, the average time for K1 recovery was 6.06 h. The time at which recovery of Aphelenchid K1 was seen was shorter following pre-treatment with sucrose solution (2-2.5 h), compared to water pre-treatment (3.5-4.5 h). Similarly, after a week of desiccation, nematode recovery following the 2 and 12 h pre-treatment with sucrose solution occurred at about 1-1.5 h, but at 4-4.5 and 2-2.5 h following pre-treatment with water for 2 and 12 h, respectively.

The percentage survival of *Laimaphelenchus preissii* and Aphelenchid K1 in different treatments is shown in Fig. 7.5. It is clear that survival of Aphelenchid K1 was affected by each treatment significantly, however, it was not significantly affected for *L. preissii*. Response of the two species to the treatments is significantly different.

7.3.4 Aphelenchid K2 and Aphelenchid H1

Ability of Aphelenchid K2 and Aphelenchid H1 to tolerate desiccation was tested. However, no nematodes recovered after 48 h or 1 week of desiccation for either species. In contrast, 80% of Aphelenchid K2 in the water control was alive after 48 h and 67% after one week. Only 40% of Aphelenchid H1 was alive after 48 h in the water control, and only 0.8% survived after 7 days.

7.4 Discussion and Conclusions

When subjected to extreme, and rapid, dehydration conditions (direct transfer from water to 0% relative humidity (RH)), different species of nematodes show different abilities to survive. Charwat (1994) studied water loss of 5 species (*Anguina funesta* (J2), *Ditylenchus dipsaci* (J4), *Rhabditis strongyloides* (J2 to J4), *Aphelenchus avenae* (J2 to adult), and *Heterorhabditis bacteriophora* (J3)) during storage after 72 h at 0% RH. Only juveniles of *A. funesta* and *D. dipsaci* were able to survive three days exposure to 0% RH without preconditioning. Ellenby (1969) reported that *Anguina* survived for 7 d at 0% RH in a desiccator, while *Ditylenchus* survived for 3 days.

In contrast, of the species tested here, many *L. preissii* could survive 7 d of desiccation while Aphelenchid H1 and Aphelenchid K2 could not recover after only 48 h of desiccation. As mentioned in Section 5.2, the nematode *L. preissii* was isolated from native *Callitris* growing on the roadside at Burdett, near Murray Bridge in South Australia. This area is very hot and dry in summer, with an annual average rainfall of about 375.8 ml, average maximum temperature of about 22.4°C, and the highest temperature of 46.7°C see (Chapter 3). In addition, *Callitris* has a

thin bark compared to that of *P. radiata,* which may possibly dry out faster. The nematode's strong ability to survive desiccation may be an adaptation of this species to the harsh summer environmental conditions. Conversely, nematodes Aphelenchid K1, Aphelenchid K2 and Aphelenchid H1 were isolated from diseased *P. radiata* at Knoxfield and Heidelberg in Melbourne. Melbourne has a wetter, cooler climate than that at Burdett. The trees were large with thick bark, which may hold more moisture over the summer. In addition, they were collected from wood. The differing responses of the nematodes to the desiccation experiment suggest that nematodes from bark have a strong capability to adapt to their environment.

Charwat (1994) studied the effects of various desiccation regimes on different nematodes, and found that the water content of nematodes reflected time of exposure and pre-treatment (sucrose concentration). *Aphelenchus avenae* (J2 to adult) did not survive in dry air unless they had been pre-treated in sucrose for 24 h. The dehydration of *A. avenae* causes changes in the biochemistry of the nematode, first observed by Madin and Crowe(1975). Chemical analysis showed a degradation of glycogen and lipids and the synthesis of trehalose and free glycerol. At least four days of slow drying at 97% RH was needed for nematodes to shift their metabolic process from lipid-glycogen to glycerol-trehalose storage. Similar observations were also made for *A. tritici* (Womersley *et al.* 1982). *Anguina tritici* accumulated trehalose and inositol but no glycerol from the onset of dehydration. In the experiment here, the recovery of Aphelenchid K1 pre-treated with a sucrose solution before desiccation was significantly higher than for those pre-treated with water. In general, this nematode survived better if pre-treated with sucrose solution than if kept in water before desiccation. This implies that Aphelenchid K1, which lose their body water gradually, and presumably have time to adapt physiologically, can increase their ability to survive desiccation. However, recovery after the water treatment and sucrose treatment was not significantly different for *L. preissii*. The reason for this could be that *L. preissii* intrinsically has a high ability to become anhydrobiotic.

The nematode coiling response to desiccation has been described in many nematode species. Charwat (1994) observed different nematode species coiling after exposure to rapid water loss in dry air. Ellenby (1969) observed that *D. dipsaci* in the centre

of an aggregate of dried nematodes survived dehydration better than those on the surface. Similarly, Huang and Huang (1974) reported that *Aphelenchoides besseyi* survived better in larger aggregates than in smaller ones when dehydrated. The reason for this is that dehydration of the nematodes on the surface of the aggregate slows down the dehydration of those in the centre (Ellenby 1968). Here, nematodes were observed coiling in both the desiccation experiment and in the laboratory cultures. In the culture plates, some nematodes moved on to the lid of the petri dish, and aggregated together to form many clusters of different sizes. Such clusters were observed in all the cultures in the laboratory.

Here, two nematode species, Aphelenchid H1 and Aphelenchid K2, had no survivors after 48 h or 7 d of desiccation. This result revealed that on the one hand, both Aphelenchid H1 and Aphelenchid K2 have a weak ability to survive desiccation compared to the nematode *L. preissii* and Aphelenchid K1. Given the short life cycle of Aphelenchid H1 (Section 6.3.1), its lack of survival for a week was expected. On the other hand, in this experiment, the nematodes were exposed to extreme conditions that would occur rarely in nature. Bird and Buttrose (1974) reported that the cuticle of *A. tritici* was sticky and concluded that epicuticles of different individuals may fuse and form a barrier to water loss (Bird and Buttrose 1974). Charwat (1994) also pointed out that sticking and coiling might help to reduce the rate of water loss of individual nematodes, thus allowing more time for biochemical adaptations during dehydration. Thus, the structure of the cuticle of a nematode may be related to its ability to dehydrate, and it would be interesting to examine sections of fresh and dried aphelenchid nematodes for differences in the thickness of the epicuticle.

Chapter 8: Pathogenicity Studies

8.1 Introduction

Since the pinewood nematode, *Bursaphelenchus xylophilus* was first identified as the pathogen causing pine wilt disease in Japan (Mamiya and Enda 1972; Mamiya and Kiyohara 1972), the mechanism of the disease has not been clearly elucidated. Some reports demonstrated that *B. xylophilus* is the only pathogen responsible for pine wilt disease (Mamiya 1975; Mamiya 1983; Myers 1988; Yang 2002), but others have shown that surface sterilisation of the nematode can cause it to lose pathogenicity (Cao 1997). In addition, it was also reported that bacteria were associated with the pinewood nematode (Oku *et al.* 1980; Higgins *et al.* 1999) and pine wilt disease was induced by both the pinewood nematode and the bacteria it carries (Zhao *et al.* 2003). It also has been reported that *B sexdentati* is highly virulent, causing mortality to inoculated seedlings, and that *B. leoni* causes inoculated seedlings to die (Melakeberhan and Webster 1992). Isolates of *B. mucronatus* from Norway and Germany have also been reported to be pathogenic to conifers (Panesar and Sutherland 1989; Mamiya 1999). Therefore, it is possible that nematodes other than *B. xylophilus* can cause sudden death of pine trees, and the cause of such deaths needs to be carefully examined.

From diseased *Pinus radiata* trees at Knoxfield and Heidelberg, Victoria, five nematode morphospecies were extracted and four were successfully cultured (Chapter 4). From the morphological and molecular analyses presented in Chapters 4 and 5, it is clear that none of these extracted nematodes belong to the genus *Bursaphelenchus*. However, the possibility that they are pathogenic to pine trees cannot be ruled out. In order to examine whether they are pathogens, inoculation studies using aphelenchid morphospecies Aphelenchid K1, Aphelenchid K2, Aphelenchid H1, *Laimaphelenchus* Heidelberg and *Laimaphelenchus preissii* were performed using young *Pinus radiata* in a shadehouse

8.2 Materials and Methods

8.2.1 Nematode subculturing

Nematodes were reared on cultures of *Botrytis cinerea* in 90 mm Petri dishes. The growth medium was potato dextrose agar (PDA, Difco Laboratories Detroit, USA). The nematodes were subcultured from their respective stock cultures, stored at 5°C. From the stock culture (as described in Chapter 3), 10 pieces (10 x 10 mm) of agar with *B. cinerea* and nematodes were removed and two pieces were transferred to newly growing *B. cinerea* on a fresh PDA plate. Based on the optimal temperatures for the respective nematodes, five subcultures of *Aphelenchus avenae*, Aphelenchid K1, Aphelenchid K2, Aphelenchid H1 and *Laimaphelenchus* Heidelberg were incubated at 25°C, and 15 subcultures of *L. preissii* were incubated at 20°C, until harvest.

8.2.2 Nematode harvest

To obtain sufficient numbers, the nematodes were harvested after different periods of incubation reflecting the different multiplication rates (Chapter 6). The nematodes of *Aphelenchus avenae*, Aphelenchid K1 and *Laimaphelenchus* Heidelberg were harvested after 10 d, Aphelenchid H1 and Aphelenchid K2 after 8 d, and *L. preissii* after 45 d. The nematodes were harvested from the Petri dishes by washing with distilled water on the day of tree inoculation. The respective nematodes were collected in a 50 ml tube and allowed to settle. As much supernatant as possible was removed by suction, then 1% streptomycin was added to the tube (the final concentration of streptomycin was about 0.8%, and the purpose of this was to kill bacteria associated with the nematodes). After 10 min incubation, the nematodes were centrifuged at 1000 rpm at 5°C, and then washed by shaking in sterile water. This was repeated twice. The first two supernatants, which contained fungus and agar debris, were discarded. The last supernatant was collected for control inoculation. After washing, the nematodes were suspended in sterile water

and the density was determined by counting in a dish under a dissecting microscope. The final density was adjusted to 15,000 nematodes per ml for inoculation.

8.2.3 Plant inoculation

Two hundred three-year-old *P. radiata* trees were transplanted into 150 x 150 mm plastic pots with UC mix (Appendix C) in a shadehouse six months prior to the inoculation. The plants were irrigated daily during the week and once on the weekend. Inoculations were performed as described by Skarmoutsos and Michalopoulos (2000), with slight modification. Briefly, the inoculation point on the plants was immediately below the base of the new season's growth. Pine needles around the point were removed with a scalpel; and a 15 mm long slit was made vertically in the bark on the stem. A wad of thin sterile cotton wool was inserted slightly under the bark (Fig. 8.1). A piece of Parafilm (American National Can™, Chicago, USA) was then loosely wrapped around the stem at the inoculation site. Using a pipette, the nematode suspension was added slowly to the cotton wool (volume of 100μl for lower number treatment and 1000μl for high number treatment). Finally, the top of the cotton was closed with Parafilm. In the control plants, the inoculation procedure was carried out as above, except that the nematode suspension was replaced with their corresponding supernatant. Two nematode concentrations were applied (1000-2000 and 10,000-20,000 nematodes per tree). There were five replicates for each treatment, giving 15 trees for each of the six nematodes tested. A laboratory culture of the soil nematode *Aphelenchus avenae* was used as a negative control. In total, 90 young pine trees were inoculated (Fig. 8.2). The plants were observed on a weekly basis for the development of any symptoms. The experiments were performed twice, first in the winter and second in the summer.

8.2.4 Extraction of nematodes from young pine trees

The survival of inoculated nematodes in the young pine trees was evaluated six months after inoculation by collecting about 50 mm of stem above and below the point of inoculation. The removed stems were cut equally in three parts (about 30

mm each) and were marked as bottom, middle (inoculation section) and top, respectively. The three parts were further chopped into small pieces and placed in a misting cabinet (Section 3.3.1) to extract live nematodes. After 48 h extraction, 10 ml tubes were used to collect water from the clamped tube attached to the extraction funnel. The water with nematodes was kept on the bench for about 1 h to allow the nematodes to settle, after which the volume of water was reduced by suction to about 2 ml. The nematodes were counted in a counting dish under a dissecting microscope to calculate the recovery rates.

8.3 Results

In both summer and winter experiments, no symptoms of disease were seen in any of the inoculated trees. No nematodes were found to have migrated to the stem sections above or below the inoculation section. No nematodes were recovered from young pine trees, inoculated by supernatant only. However, nematodes were extracted from many of the inoculated sections of the young pine trees (Table 8.1). They were morphologically identical to those applied.

The recovery rates for the inoculation in the winter were: from 10 of 10 trees for Aphelenchid K1 and *Laimaphelenchus* Heidelberg, 9 of 10 trees for *Aphelenchus avenae*, 4 of 10 trees for Aphelenchid K2, but only 2 of 10 trees for the Aphelenchid H1. The average numbers of nematodes recovered from inoculated trees are given in Table 8.1. It is clear that Aphelenchid K1 and *Laimaphelenchus* Heidelberg had the highest recovery rates, followed by *A. avenae*; Aphelenchid K2 and Aphelenchid H1 had lower recovery rates.

In the experiment conducted in summer, similar recovery rates were obtained for five species. Nematodes of *A. avenae*, Aphelenchid K 1 and *Laimaphelenchus* Heidelberg were found in all the 10 inoculated trees, *L. preissii* was recovered from 8 out of 10 trees, Aphelenchid H1 was recovered from 4 out of 10 trees, but Aphelenchid K2 was not found in any inoculated trees. In trees from which nematodes were recovered, the average numbers of nematodes collected are given in Table 8.1. The results showed that the recovery rates of different nematodes

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Fig. 8.1. Inoculation of young pine trees with nematodes.

Fig. 8.2. Inoculated pine trees in the shadehouse.

Table 8.1 Mean recovery of nematodes from winter and summer inoculated pine trees (n = 10).

Nematode	Adult	Juvenile	Total [log(X +1)]	Total*
Winter				
Aphelenchus avenae	20.4	12.1	1.36	21.9
Aphelenchid K1	7.7	6.8	1.11	11.9
Aphelenchid K2	2.9	4.0	0.49	2.1
Aphelenchid H1	1.1	0.5	0.19	0.6
Laimaphelenchus Heidelberg	38.3	22.2	1.49	29.9
LSD 5%			0.412	
Summer				
Aphelenchus avenae	19.0	7.2	1.39	23.6
Aphelenchid K1	24.6	22.3	1.39	23.6
Aphelenchid K2	0.0	0.0	0.00	0.0
Aphelenchid H1	1.4	0.1	0.25	0.8
Laimaphelenchus Heidelberg	66.8	39.3	1.88	74.9
Laimaphelenchus preissii	2.9	3.0	0.64	3.4
LSD 5%			0.395	

* Total(backtransformed).

varied; with *Laimaphelenchus* Heidelberg having the highest number recovered from the inoculated trees, but Aphelenchid K2 was not recovered at all.

Comparing the winter and summer experiments, the average number of the respective nematodes recovered was similar. This indicated that season played only a small role and had little effect on the survival of these nematodes. This result

showed that the four species of nematodes extracted from the diseased trees in Melbourne were not able to infect the young pine trees over winter or summer periods, under the shadehouse conditions.

Statistical analyses showed that there was no significant effect of the initial inoculation density. However, there were significant differences in the number of nematodes recovered for the different species (Table 8.1). So the means in this table combine both initial inoculation densities.

8.4 Discussion and Conclusions

Relatively small numbers of the nematode species applied were recovered from both the summer and winter inoculation experiments. In most cases some juveniles were recovered. The recovery of juveniles is significant as it indicates that the nematodes had reproduced despite the population declining well below the inoculation density. It could be argued that these juveniles had survived in an anhydrobiotic state from the time of inoculation. However, this is unlikely because juveniles of Aphelenchids K2 and Aphelenchid H1 were found and these species were not able to survive desiccation well (see Chapter 7). In addition the trees were watered from above, and moisture should not have been limiting. Therefore, it is most likely that over six months the nematode population continued to cycle but the population declined to an equilibrium density that could be supported by the available food resources, or it was still declining. This is significant, because had the nematode been virulent in this host, there was opportunity for them to cause disease and increase in population density. It is not unreasonable to conclude that *P. radiata* is not susceptible to these nematodes and that a suitable fungus was not present for the nematode to feed on.

In considering their desiccation ability (Chapter 7), relatively high recovery was obtained with the nematodes with better ability to survive desiccation (Aphelenchid K1) except *L. preissii*. Trudgill and Philips (2006) described the various reproductive strategies of nematodes. Some grow large and have long life cycles with low rates of population increase (K strategists), others are relatively small, have short life cycles and potentially higher reproductive rates (r strategists). It is a characteristic of K strategists that they do best in stable environments where

populations are usually close to the equilibrium density (the population density that can be sustained). In contrast, r strategists increase rapidly where the environment is favourable, often overshooting the equilibrium density. Here, all 6 nematode isolates had a low rate of recovery compared with the original number inoculated. Statistically there was no significant difference in survival of the nematodes in the summer and winter inoculations, suggesting that temperature was not important to these nematodes. However, the recovery numbers differed between species, with more *A. avenae*, Aphelenchid K1 and *Laimaphelenchus* Heidelberg recovered than *L. preissii*, Aphelenchid K2 and Aphelenchid H1. This result, on the one hand, is consistent with the desiccation study in Chapter 7. Aphelenchid K1 is better able to withstand drying than are Aphelenchid K2 and Aphelenchid H1. On the other hand, it is contradictory to the desiccation study result with *L. preissii*. *Laimaphelenchus preissii* is an exception in this experiment; an unfavourable host change to *P. radiata* from *C. preissii* and the hot temperature of summer could have caused its low recovery rate. It has a low optimal temperature for multiplication and long times for population doubling (Chapter 6).

Since Kiyohara and Tokushige (1971) first demonstrated, by inoculation tests, that *Bursaphelenchus xylophilus* was the causal organism of pine wilt disease, intensive studies have been carried out on the nematode's pathology, biology and ecology (Mamiya 1983; 1988; Braasch 2001). In recent years, new evidence suggested that the *B. xylophilus* is not the only pathogen to cause pine wilt disease, and other nematodes such as *B. sexdentati* and *B. leoni* are now considered pathogenic to pines (Michalopoulous-Skarmoutsos *et al.* 2003). In this experiment, no symptoms appeared in any inoculated pine trees. It may be concluded that the nematodes inoculated are not pathogens of *P. radiata* under the experimental conditions tested. However, two critical questions remain from this experiment. Firstly, *B. xylophilus* could not be used as a positive control for the experiment due to quarantine restrictions; secondly, the nematodes from Knoxfield and Heidelberg were originally extracted from the wood of diseased trees. Here, however, no nematodes were extracted from the stem of inoculated trees above or below the inoculation point, and the nematode number had declined dramatically by 6 months after inoculation. This indicated that the nematodes did not enter the wood of the tree, and it seems that in the shadehouse they did not behave as they did in the diseased

trees in Melbourne. Therefore, it is unclear if any of these morphospecies extracted from diseased trees was the pathogen responsible for the death of the trees in Melbourne.

There are several possibilities:

1) The nematodes extracted are not pathogens of pine trees. There are many aphelenchid nematodes associated with pine trees reported from around the world (Massey 1960; Kaya 1984; Nickle 1992; Hunt 1993). However, there are no reports that any aphelenchid nematodes other than several species of *Bursaphelenchus* can cause severe disease in pine trees. Based on both morphology and molecular studies (Chapter 4 and 5), none of the isolates from the diseased trees in Melbourne was *Bursaphelenchus* spp. Therefore, they do not have a high probability of being pathogens.

2) While the nematodes extracted are not pathogens of pine trees, they may have a role in speeding up the development of symptoms, and possibly of tree death. The fungus *Diplodia pinea* was isolated from wood samples of diseased trees in Melbourne (I. Smith, pers. com. 2005). This fungus is the most damaging to exotic and native pine species in the USA and has been reported to cause pine mortality (Brookhouser and Peterson 1971; Phillips 1999). Diplodia canker (*D. pinea*) is known to cause leader dieback, crown wilt and whorl canker. It is also a major cause of blue stain of timber (van de Hoef and Hill 2003). However, the fungus causes a slow death process, dissimilar to that of pine trees infested by pinewood nematode. Therefore, if the fungus *D. pinea* was the factor causing the pine trees die in Melbourne, the nematodes may have had a role in symptom development.

3) The nematodes from the diseased trees may be pathogens but they may lose their pathogenicity after rearing *in vitro* and several washes of the nematodes during the nematode preparation before inoculation. Zhao *et al.* (2003) reported that pinewood nematode (PWN) always carried bacteria in their natural environment, and axenic PWN alone could not cause pine wilt disease, unless associated with phytotoxic bacteria GcM5-1A (*Pseudomonas fluorescens*). Comparing the bacterial isolates from Japan and China, three bacteria (*Bacillus* spp.) were isolated from PWN in

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Japan, which could produce substances toxic to callus and black pine seedlings (Kawazu and Kaneko 1997). Twenty-four strains of bacteria were isolated from nematodes in samples in China, 17 of which produced phytotoxins (Zhao *et al.* 2003). *Bacillus* species were found in China. This suggests that the bacteria carried by PWN may differ between regions. As to the Melbourne isolates, no attempt was made to isolate bacteria. Notably, 16S sequences of the bacteria *P. fluorescens* were obtained several times during the amplification of the ITS region of ribosomal DNA of the nematodes, Aphelenchid K1 and Aphelenchid H1 (Chapter 5). This outcome is coincidental to the Chinese report that *P. fluorescens* is responsible for pine wilt disease. There could have been two reasons for the finding the 16S sequences: firstly, contamination may have occurred during the amplification of the ribosomal RNA because of the abundance of *P. fluorescens* in the natural environment and the sensitivity of the PCR; secondly, the bacteria could be directly associated with the nematodes, consistent with the Chinese finding (Zhao *et al.* 2003). The nematodes tested here were unlikely to have been carrying any bacteria on the cuticle, as these should have been washed off, and the wash water inoculations also gave no evidence of pathogenicity. Therefore, it seems unlikely that bacteria were involved in the inoculation experiment described here. However, bacteria associated with the nematodes from Heidelberg and Knoxfield, Victoria need to be further investigated.

4) There may be other nematode species that are pathogens but which were not recovered from the diseased trees. Since 2000, more than thirty cases of pine deaths in Victoria were investigated. From these, a putative species, *B. hunanensis,* was collected from most of the samples (D. Smith, pers. com. 2005) but was not successfully cultured. Although the pathogenicity of *B. hunanensis* is not known, the high frequency of nematodes of this appearance in the dying trees should be further investigated.

5) The conditions in the shadehouse might not have been suitable for the nematodes to cause pine disease. Futai and Akema (Futai and Akema 2003) reported that drought could exacerbate pine wilt damage and mycorrhizal associations could mitigate drought stress and reduce pine wilt damage. Different topography could also cause differences in pine damage. In this experiment, the young pine trees

were well cared for in the shadehouse. They were fertilised and watered regularly and did not face harsh conditions like those of the diseased trees in Melbourne. Therefore, the tested trees may have had strong natural defence responses to foreign objects such as nematodes.

Chapter 9: General Discussion

Pine wilt disease is a problem of international significance. In 1999, the pinewood nematode was found in Portugal, which makes Europe the third continent with *Bursaphelenchus xylophilus*. Less than 200 years since the first exotic pine plantation was planted in Australia, today there are more than 1×10^6 ha of pine plantations (Kelly *et al.* 2005). The scale of Australian softwood forestry has made it important, not only economically, but also ecologically and environmentally, and this is likely to continue into the future (Section 2.2.2.4). Pine wilt nematode has not been identified in Australia, but its insect vector *Monochamus* has been found in isolated instances. The 2000-2002 pine disease incidents in Melbourne acted as a serious alert to the Australian softwood industry. They indicated that Australian pine plantations are threatened by some exotic diseases. *Bursaphelenchus xylophilus* was seen as a potential causal agent for the incidents in Australia. Given the pathogenicity of *B. xylophilus* to *P. radiata* (the most commonly grown plantation species), it is essential to protect Australia from entry of this potential devastating nematode.

An extensive survey was carried out to determine the distribution and biodiversity of above-ground nematodes in pine forests of south-eastern Australia. Understanding of the above ground nematode fauna of *Pinus* and related conifers in Australia was a basic aim of this study. Since the project started in 2003, sites from areas representing about 60% of Australian pine plantations and three states in Australia were surveyed. Samples were collected across more than 1000 km from South Australia (SA), through Victoria (Vic.) to New South Wales (NSW). Five nematode trophic groups were identified from the survey. Of these, aphelenchids were the predominant group in all *Pinus* plantations. Twelve commonly collected nematodes were described, including 3 new species and 8 morphospecies. No nematodes were extracted from the wood of healthy *Pinus* spp. and *Callitris* spp. While the 39 surveyed sites were reasonably representative of the pine forests in southeast Australia, more sampling will be needed to get a full picture of the distributions of above-ground nematodes in pine forests in Australia. Several problems need still to be addressed. Firstly, the limited number of samples. Although over 1200 samples were taken in this study, it is still a restricted number

to represent such a large area of forest, particularly given the patchy nature of nematode distribution. Secondly, the surface area and volume of the wood samples taken was small, and nematodes with a patchy distribution were probably missed. However, the absence of nematodes from the healthy wood samples is consistent with findings in Greece (Michalopolulos *et al.* 2003), so nematodes may be naturally absent from the wood of healthy pine trees. As part of the work conducted for this thesis, a biological control (*Sirex* woodwasp) site was selected for survey in the South-East region of SA, and it was expected that some tylenchids (*Deladenus siricidicola*) would be extracted from the wood samples. However, no tylenchids were extracted, suggesting that the sampling cores taken might have been too small for detection of nematodes with a patchy, restricted distribution. Thirdly, in theory the south east side of tree trunks in Australia, being shaded, should contain more moisture compared to the other sides of the tree, and this side may be better for nematodes to avoid the hot summer. This idea was the rationale for selection of samples here, but it has not been experimentally investigated. It would be better to collect more samples from different positions on the trunk of the tree, and to compare the nematode populations from different positions. In addition, as mentioned in Chapter 3, in pine trees from which the putative *Macrolaimus* was isolated, these nematodes may be interacting with other bacterial feeding nematodes. As a saprophagous nematode, *Macrolaimus* may compete with them, and could suppress or be suppressed by their populations. Because the actual numbers of nematodes were not counted here, it is not possible to comment on possible changes in population composition.

Counting the actual numbers of all trophic groups present in the survey could also suggest interactions between trophic groups. Aikawa *et al.* (2006) showed that the population structure of *B. xylophilus* within a single tree varied both with the virulence level of the nematode populations transmitted to the tree and their transmission order. In the survey reported in this thesis, most samples contained one numerically dominant nematode species. This is probably related to the environmental conditions of individual trees including food resources and populations of competitors. For example, when nematodes were first extracted from samples of diseased wood from Melbourne, the dominant nematode from trees at Knoxfield was Aphelenchid K1 and from trees at Heidelberg was Aphelenchid

H1. Six months later, in a second extraction from the same samples (stored at 16°C), the dominant nematode extracted from Knoxfield was Aphelenchid K2 and from Heidelberg was *Laimaphelenchus* Heidelberg. Although Aphelenchid K1 and Aphelenchid H2 were still present in the respective samples, they appeared to be in small proportions. For the Melbourne disease incidents, it is therefore possible that when the sampling for this work was carried out, the composition of the nematode populations were already changed from the initial sampling (not tested). When the dead pine trees were first examined in Melbourne, a nematode tentatively identified as *Bursaphelenchus hunanensis* was isolated from diseased wood (Smith, pers.com.). Changes in population structure over time could explain why the putative *B. hunanesis* was not extracted from the later samples of diseased wood. The survey reported in this thesis has provided baseline information about above-ground nematodes in pine plantations in southeast Australia.

Nematode morphological taxonomy was one of the main tasks of this study. Twleve morphospecies of nematodes were studied in detail. Of these, 2 new species of *Laimaphelenchus* have been described, 6 morphospecies of *Laimaphelenchus* were found, 3 morphospecies of *Aphelenchoides* and one of a putative *Acugutturus*. *Laimaphelenchus* had previously been recorded from every continent except Australia (Hunt 1993, Swart 1997, Peneva & Chipev 1999). *Acugutturus* was described from the West Indies (Hunt, 1993). Thus, findings of both *Laimaphelenchus* and *Acugutturus* are first records for Australia, and contribute to our knowledge of the world distributions of these genera.

However, the considerable problems of nematode taxonomy, particularly of aphelenchids, make it difficult to determine new species (Chapters 2 and 4). The small size and conservation (similarity) of morphological characters of nematodes makes it difficult to distinguish species. In this study, only 3 species have been fully described, and the remainder were identified and described only as morphospecies. For example, the genus *Laimaphelenchus* is a small group in the Aphelenchida, and contains only 12 species, including the 2 new species added from this study. In this genus, the nematode tail structures are the most distinctive feature, but in several forms can only be seen using scanning electron microscopy (SEM). If reference is only made to the descriptions given in Hunt (1993), it is

difficult to judge to which genus some new collections of aphelenchids belong. In contrast, the genus *Aphelenchoides* is a large group in the Aphelenchida, consisting of over 180 species (Nickle 1992; Liu *et al.* 1999). Because of the large numbers of described species and large numbers of invalid species, it is difficult to compare new with existing species. Thus, whether the group is small or large, it can be difficult to describe a new species. SEM imaging may be essential for studying nematode morphology and molecular techniques are also needed for precise data for nematode taxonomy. Diagnosis of the genus *Laimaphelenchus* should be expanded to include the morphological features observed in the forms described in this study (Chapter 4), particularly of the tail. The genus *Aphelenchoides* needs revision (Hunt 1993).

Clearly, molecular taxonomy was a key point in this study. The internal transcribed spacer restriction fragment length polymorphism (ITS-RFLP), D2D3 and 18S of ribosomal DNA sequences, and cytochrome oxidase subunit I (COI) sequences are widely used in nematode taxonomy and phylogeny studies worldwide. These techniques can easily and precisely discriminate multiple species. For this thesis, ITS-RFLP and D2D3 fragments of six nematode species (Aphelenchid K1, Aphelenchid K2, Aphelenchid H1, *Laimaphelenchus* Heidelberg, *L. australis* and *L. preissii*) were studied. In addition, 18S of ribosomal DNA sequences were studied in five species, and COI sequences were made for three of the six species. Comparisons of three different loci of ribosomal DNA sequences of the nematodes strongly supported the morphology taxonomy (Chapter 4). For example, males of *L. preissii* have a bursa, and using Hunt's key (1993), *Bursaphelenchus* rather than *Laimaphelenchus* is indicated. Genetically it is closer to *Laimaphelenchus* than *Bursaphelenchus*, and in addition it has a knob-like tail structure. Thus, it has been described as a new species of *Laimaphelenchus*. Similarly, in *Laimaphelenchus* Heidelberg, the tail structure is different from that described for *Laimaphelenchus* by Hunt (1993). However, genetically it is close to *Laimaphelenchus*, and has also been described as a new species of *Laimaphelenchus*. Phylogenetic trees clearly showed that, of the 6 species from Australia, none fell into the *Bursaphelenchus* clades (Chapter 5). However, a disadvantage for molecular taxonomy of nematodes at the moment is the limited information available in GenBank. Not enough DNA sequence information is available to be used for comparisons. The lack of gene

sequence information, and the limited sequence comparisons possible may give misleading conclusions. Therefore, in the future, more DNA sequences of aphelenchids are needed. It would be interesting to look at the genetic relationships of nematodes collected from native conifers and exotic pines, as part of work to determine which nematodes are endemic to Australia and which are introduced. Sequences from the same species of nematode collected from different locations could be made to check for genetic drift.

In this study, we found that the nematodes from the survey could not rapidly be distinguished. Thus, the normal nematode populations in the pine plantations could confuse surveillance efforts. Given the vulnerability of the Australian softwood industry to pine wilt disease caused by *B. xylophilus*, and potentially other *Bursaphelenchus* spp. such as *B. sexdentati* and *B. leoni*, and the speed at which molecular tools can be used to identify nematodes, the Australian forestry industry and the quarantine services should obtain and/or develop specific probes for the detection of these *Bursaphelenchus* spp. This should be coupled with a service able to handle samples of meaningful size and with the capacity to rapidly process large numbers, similar to the DNA-based testing service provide for soil borne pathogens (Herdina *et al*. 2003; 2004).

Biology studies of Aphelenchid K1, Aphelenchid K2, Aphelenchid H1, *Laimaphelenchus* Heidelberg and *L. preissii*, have contributed to the knowledge of aphelenchid nematodes. Much previous research had been done on the development of nematodes on differing food sources and under differing conditions (Chapter 6). For example, Giblin *et al*. (1984) reported that host, temperature and media had additive effects on the growth of *Bursaphelenchus seani*. Trudgill and Philips (2006) discussed various reproductive strategies of nematodes. Some large nematodes with long life cycles have low rates of population increase; other relatively small nematodes with short life cycles have potentially higher reproductive rates. In this study, *L. preissii,* Aphelenchid K1 and Aphelenchid H1 differ in body length (Chapter 4). Their multiplication rates under different temperature were tested. Body size in the aphelenchids tested appears correlated with their multiplication rate. Nematodes with long body size had a longer mean doubling time, and vice versa. This suggests that some of the aphelenchids

collected are 'r' strategists and others 'K' strategists, and has implications for possible interactions between them.

Temperature and rainfall would be expected to play an important role in the nematode multiplication rate in the field. Even in wetter regions, climate factors affect the distribution of nematodes (Cao 1997). The most important environmental factor influencing the occurrence and spread of pine wilt disease is temperature. In Japan, pinewood nematode can commonly occur and damage pine forest seriously in areas where the mean annual temperature is above 14 °C, and can occur in but not seriously damage pine forest in areas where the mean annual temperature is 10-14°C (Yang 2003). In Australia, the mean annual temperature in South Australia, Victoria and New South Wales is above 14°C. Thus, temperature in these areas is suitable for *B. xylophilus*. Therefore, understanding of the biological characters of nematodes found in Australian pine plantations is essential, particularly for those collected from diseased trees. As discussed in Chapter 6 and 7, it would be expected that *L. preissii*, from a semi-arid area, has evolved a higher optimal temperature for multiplication and more tolerance of dry conditions. However, the optimal temperature for multiplication of *L. preissii* is lower (20°C) than that for Aphelenchid K1 (25°C) and Aphelenchid H1 (28°C) collected from a wetter, cooler climate. This could be because Burdett is dry in the summer, and *L. preissii* probably only reproduces in the winter months when it is wet. The nematodes isolated from Melbourne may be able to develop throughout the year. Moreover, the biological characters of Aphelenchid H1 and Aphelenchid K1 are similar in terms of temperature sensitivity and reproduction activities (Chapter 6 and 7). They were present in the same samples (Chapter 3), and could compete with each other, suggested by the differences in their relative numbers in individual samples. In contrast, biologically, *L. preissii* is quite different from H1 and K1. The environmental conditions to which they are adapted may play a major role in these differences. In addition, they were collected from different species of hosts, and probably fed on different fungi or lichens.

Studies of nematode feeding habits are essential to understand their biology and role in an ecosystem (Ikonen 2001). Fungal or hyphal-feeding (Yeates 1998) nematodes

feed on many different species of fungi, including saprophytic, pathogenic and mycorrhizal fungi (Freckman and Caswell 1985; Giannakis and Sanders 1989; Ruess and Dighton 1996). Giblin-Davis and Kaya (1984) tested *B. seani* on eighteen fungi, and Ikonen (2001) tested *Aphelenchoides bicaudatus* and *Aphelenchus avenae* on six different fungi. Kondo *et al.* (1982) tested *B. xylophilus* on three fungi (*Ceratocystis ips*, *Diplodia pini* and *Trichoderma* sp.). These studies confirmed that different nematodes show different population development on different food sources. In this study, Aphelenchid K1 was tested on three different fungi. Its population mean doubling time and population multiplication rate indicated that *Botrytis cinerea* was the best of the food sources tested. However, the fungus *Diplodia pini* was isolated from the diseased pine trees in Melbourne (Ian Smith, pers. comm. 2005), and may have acted as a food source for nematodes. All nematode isolates should be tested with this fungus in the future.

Different species of nematodes show different abilities to survive desiccation. For this study, five nematode species were exposed to desiccation and their recovery rates were checked. *Laimaphelenchus preissii* had the best ability to survive desiccation compared with 3 species from Melbourne. This makes sense because *L. preissii* was isolated from native *Callitris*, from a hot area that is dry in summer and has a limited annual average rainfall. In addition, *Callitris* has a thin bark compared to that of *P. radiata,* and it may dry out faster. Conversely, nematodes Aphelenchid K1, Aphelenchid K2 and Aphelenchid H1 were isolated from diseased *P. radiata* at Knoxfield and Heidelberg in Melbourne, which has a wetter, cooler climate than that at Burdett. The *Pinus* trees were large with thick bark, which may hold more moisture over the summer. In addition, the nematodes were collected from wood. The differing responses of the nematodes to the desiccation experiment suggest that nematodes from bark have a strong capability to adapt to their environment.

Finally, the pathogenicity of nematodes isolated from diseased trees in Melbourne was assesed. *Bursphelenchus xylophilus* is known to cause pine wilt disease, but nematodes such as *B. sexdentati* and *B. leoni* are also pathogenic to pines (Michalopoulous-Skarmoutsos *et al.* 2003; Zhao *et al.* 2003). Therefore, even though none of the nematodes collected from diseased trees in Melbourne is *Bursaphelenchus* spp. (Chapter 4 and 5), they cannot be ruled out as possible

pathogens. In the experiment described in Chapter 8, Aphelenchid K1, Aphelenchid K2, Aphelenchid H1 and *Laimaphelenchus* Heidelberg from diseased pine trees in Melbourne were tested, but no symptoms appeared in any inoculated pine trees. It may be concluded that the nematodes are not pathogens of *P. radiata* under the experimental conditions tested. However, questions still remain from this experiment. Firstly, no positive control could not be included in the experiment due to the understandable quarantine restrictions that prevent importation of known pathogens into Australia. Secondly, the nematodes from diseased trees in Melbourne did not behave as they did in dying trees, and no nematodes were extracted from the stem above or below the inoculation point, indicating that the nematodes did not enter the wood of the young and/or healthy pine trees. Therefore, it is still unclear if any of these morphospecies extracted from diseased trees was the pathogen responsible for the death of the trees in Melbourne. However, it is most likely that they were secondary colonisers, not primary disease causing agents.

In conclusion, from the nematode survey, taxonomy studies, and biology and pathogenicity tests, a first picture of the numbers and composition of above-ground nematodes in pine plantations in Australia has emerged (Chapter 3). However, more work needs to be done in the future to clarify several issues. As mentioned in Chapter 4, more species of nematodes were present in the samples than have been described. For this study, only morphospecies commonly observed in the extracts from bark and wood were collected and mounted for examinations. Rare nematodes could have included potential pathogens, but this was not considered likely. Moreover, it was difficult to determine to what genus the aphelenchid nematodes found in the pine trees in Australia belong. To identify new nematodes to genus or species, SEM pictures and considerable nematode taxonomic expertise are needed. Therefore, methods for dealing with large wood or bark samples and for detecting *B. xylophilus* and other pathogens of *Bursaphelenchus* spp. are needed. In order to protect Australian pine plantations successfully from pine wilt disease, it is important to enhance quarantine surveillance against *B. xylophilus* entry from overseas; and to gain more knowledge of nematodes of pine plantations in Australia.

Appendices

Appendix A: **Zhao, Z. Q., K. A. Davies, I. T. Riley and J. M. Nobbs (2006).** *Laimaphelenchus preissii* **sp. nov. (Nematoda: Aphelenchina) from native** *Callitris preissii* **in South Australia.** *Transactions of the Royal Society of South Australia* **130: 10-16.**

Appendices

Appendix A: Zhao, Z. Q., K. A. Davies, I. T. Riley and J. M. Nobbs (2006).
Laimaphelenchus preissii **sp. nov. (Nematoda: Aphelenchina) from native**
Callitris preissii **in South Australia.** *Transactions of the Royal Society of South*
Australia 130: 10-16.

NOTE: This publication is included in the print copy of the thesis
held in the University of Adelaide Library.

Appendix B: Zhao, Z. Q., K. A. Davies, I. T. Riley and J. M. Nobbs (2006).
Laimaphelenchus australis **sp. nov. (Nematoda: Aphelenchina) from exotic pines,**
Pinus radiata **and** *P. pinaster***, in Australia.** *Zootaxa* **1248: pp. 35-44.**

Appendix C: Specification for University of California soil mix (Waite Precinct version)

400 L of coarse washed sand sterilised at 100°C for 30 minutes

300 L of peatmoss added and mixed (at 80°C).

Mixture is cooled and the following fertilisers are added:

Calcium hydroxide 700 g

Calcium carbonate 480 g

Nitrophoska (15:4:12) 600 g

      Total nitrogen 15% (5% NH_4, 4% NO_3, 1% NH_2, 5% IBDU)

      Total phosphorus 3.9%

      Potassium sulphate 12.4%

      Magnesium carbonate 1.25%

      Dicalcium phosphate 3.4%

      Sulphate 5.3%

      Iron oxide 0.3%

      Copper oxide 0.0002%

      Zinc oxide 0.007%

      Calcium borate 0.01%

      Molybdenum oxide 0.0003%

Appendix D: Sequences of ITS rRNA

1. *Laimaphelenchus preissii*

LOCUS   *Laimaphelenchus preissii*   969 bp.   1-184 partial 18S; 184-476 ITS1; 477-598 5.8S; 599-920 partial ITS2.

DEFINITION   *Laimaphelenchus preissii*

```
  1 acttgcattg attacgtccc tgccctttgt acacaccgcc cgtcgctccc cgggactgag
 61 ttgtttcgag aaagccacgg accggtgata cttcatgctt cggtatgatt gtcgctggaa
121 agtggtttaa tcgcaacggc ttgaaccggg gaaaagtcgt aacaaggtca cggtaggtga
181 acctgccgtg agatcaggta aacgactata attgtaacat tggtgctctt gcatacaaca
241 ctcggcagaa gccgtgagtg tgcttgagtt aaccgctttg attgaatcta acggcttgtc
301 tgggcctcta agttagtcgg agcagatgtg ttaaacgtcc gtgtctgcaa cgacggctaa
361 cgatagcgat cgacttcggt cggacgtgag agttgatgac ccggtcgggc acccagaacc
421 atccacacat ttccactatt tactgtcatt taataaagtc aagttatgtc ggcgaatcac
481 ttggctcgtg ggtcgatgaa gaacgcagtg aattgcgtta ataagcacga attacagata
541 ttttgagtgc cttgtttcg attgcatatt gcgccgttgg gttttgccct tcggcataca
601 ctactcaggg tgttttcatg acaaaggaaa gccaacttga ttgttgcttg cttaccttag
661 ccaagtgcac gcttccccaa gggacagctt gtgtacgttg aactggtgaa actgcaaagt
721 ttccgttgtt gagttgtgct tacactgtac ttgccaaaca atcctaagcg taaggcccta
781 tcgagtggcg gtcgttagct cagtaaagta gtaacatact ttttgattat gctgactatc
841 gactgttgct tgactatggg ttggattggc agtgtattgt ctagttcctc gggattgagg
901 ctgacagcac cgaccaatag ttaactattg gtttaattcc accctgagtt gtgtatgact
961 accctgcga
```

2. Aphelenchid H1

LOCUS   aphelenchid   805 bp.   1-304 ITS1; 305-405 5.8S; 406-805 ITS2

DEFINITION   Aphelenchid H1

```
  1 aaaagacact gtttttgtgt cccacgaagg ggggtgtaaa aaaaaacaaa gccctgtcat
 61 tggtcagccc ctgattcgtc ggtgggttgg gttgatgatg gaagattgag aacgaaacgg
121 cttggctagg attctaggtt tcggaggagc agttgtgtta cacgtcccgg ggctgcgttg
181 attccgattc ggtagcggtt ccaattccat tggggctgta gaattaatga ccccgagagg
241 gcgcctagca aattctttta tttttcattt ttttcaaaac aaaagcttaa ctaagttgta
301 tcgatggatc acttggttcg tgggtcgatg aagatcgcag tgaattgcga taataaatat
361 gaattacaga tatttagaat acagtgttat cgaatgcata ttgcgttgtt gagtttttgc
421 tcttcaacct gtacgattca gggtgttttc tctcaaaaca cttcttattg attctcaaag
481 tgtggttttg gacgaggatt gttttgcaat cccttaatcc gggccgtttg cctagtcatt
541 taagtatgcg ggcgaatggg ctaacaaaac taggctccgt aaatttattc tcaaacaagc
601 gtttaagcac cgttcggttc ttatcggctc cctcttgttg ccccgttttg gaaattattg
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661 tttctgatga tgggtctcga tgggttggga cggtaaagga tgctccgtac ggtgtcagct
721 cgcatcttgt gtgcgtgagc gcactcttgg cggagaggcc taatttgatt aaaccacctg
781 aattgtgcaa gatcacccgc ggaac
```

3. Aphelenchid K1

LOCUS   aphelenchid   721 bp. 1-38 partial of 18S; 39-329 ITS1; 330-483 5.8
             S; 484-621 ITS2; 622-721 partial 28 S.

DEFINITION Aphelenchid K1

```
  1 cgtaacaagg tagctgtagg tgaacctgct gatggatcac taacgtccac aaacgactac
 61 ttgttacttg aaagtaagaa aatcattgag atttgaactt ccaagtgcaa caaagctgac
121 tgtctaggtc taaaaagcct caacagtttc agcatttaac ggcttgcttg ggtatctatg
181 gttggttgag cagttgtgtc acacgtccgt ggctgctaag acactgaccg gtagagtcgc
241 tttgatggct gactttagat tcgatgatcc gttgtggcgc ccaagaatca ttattcattt
301 aaatactttt atgaactaaa tagcaattta gctatatcgg tggatcactt ggctcgtaga
361 tcgatgaaga acgcagtgaa ttgcgttaag aatcacgaat tacagatatt atgagtgata
421 tgttttcgat tgcatattgc accgttgggc atttgctcat cggtataccc aattcagggt
481 gtgtatccca atcaaattga tacaatgcac ttgttggttg tatcaaaatt cataagttgt
541 tgttttgtgt agaaaggcca ttgtgtgttt taaaatgaaa gttataagac atgcatggac
601 gtcaaagcaa tgatttaacc gaatccacct gaattggata tgatcacccg ctgaacttaa
661 gcatatcagt aagcggagga aaagaaacca acagggattt ccttagtaac ggcgagtgaa
721 a
```

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Appendix E: Partial sequences of LSU rRNA

1. Partial LSU rRNA gene of Aphelenchid K1 743bp

LOCUS   466 aphelelenchid   743 bp

DEFINITION 466 Aphelenchid K1 D2D3

```
  1 gaagttgaaa gcacctttga aaagagagtg caagagaacg tgaaaccgtt atagtggaag
 61 cggatggagc cgacgtatcc ggtgcgtatt cagctgttag ttagtgcatt ccatttgttt
121 tgggttctgc aaagtcctga gcaggtgagg atgtattaac gagcggtgca tttgcgtaca
181 gagtgcgccg aatcggtcga gcacgctgtt ggaaggtcga aaagaggact agcttgctag
241 aaacctggtt ggccggaagg cggttgngct tggctgtttt aaagggattg gtgaattcgt
301 gatcagttgc gggcaataga gtggttttag tgcgatgcat gcatttcgct ttaatggctt
361 tgttgtccgt ggttggttgc tctgtccccc attcggtgtc tagtcggtca cccatttgac
421 ccgtcttgaa acacggacca aggagtttaa ggtgtgcgca agtcaatagg ctcagcatag
481 acctataggc gtaatgaaag tgggctactt agtggctgtg gtgtgatcgg ttgttttttc
541 gaaggcagcc gagcaacatc gacccatgac tggaacttgt tctgttgtgg agtctgagcg
601 tatgctttga gacccgaaag atggtgaact atgcctgaac aggacgaagt cagaggaaac
661 tctgatggaa gttccgaagc ggttctgacg tgcaaatcga ttcgttctga tttgggtata
721 ggggcgcaag acaatcgaac ctc
```

2. Partial LSU rRNA gene of Aphelenchid H1 682 bp

LOCUS   468 aphelelenchid   682 bp

DEFINITION 468 Aphelenchid H1 D2D3

```
  1 ggaagttgaa aagcactttg aaaagagagt gcaagagaac gtgaaatcga tgcaatggaa
 61 gcggatgaag tcgccgtatc tagtgcgtat tcagttgttg tggtcgggga tcgctggtgt
121 gtcattccgc aaggttggct agccggtgtt gtcttcggtt gcggcaatgc atttgcgtac
181 agagtgcaac gggtcgttga ggaccgtgga acggagcgca gtttagagga ccagcctcgg
241 ttggaaacct ggattgtgcc gagagccatt ggtcttcttc ggttttgtat ggttcatggc
301 ttacgactcg gtcgggtctg ttttatgggg tttgaggtga acgcatgcgt tcgcttctta
361 tgctgtggaa tgggctgggt cggtgtcact tgaatcgtct cgttgtattg gcgatcactt
421 atccgacccg tcttgaaaca cggaccaagg agtttatatt acacgcgatt tattgagtgg
481 tgaaaactca aaagagcaat gaaagtgaac aactgtcttc ggacagcaac gtgattgttg
541 gctcattcgt gggccagcac gcaacgttgc cccattccag aggcttgcct cggggtggag
601 gttgagcgtg tgaaatgaga cccgaaagat ggtgaactat gcctgaacag gatgaagcca
661 gaggaaactt ctggtggaag tc
```

3. Partial LSU rRNA gene of *Laimaphelenchus preissii* 730 bp

LOCUS   467 *Laimaphelenchus*   730 bp

DEFINITION 467 *Laimaphelenchus preissii* D2D3

```
  1 ggaaagttga aaagcacttt gaaaagagag tgcaagagaa cgtgaaaccg acataatgga
 61 agcggatgga gccgacgtat cgtgcatgta ttcaattgca cagcgttgca ggctgctggt
121 ttggcattcc gtaaggttgc cgatcggttg ttcttgttgc gtttgtgtaa tgcatttgca
181 tgcagagtgc gccgagtggg ttggtttcgc cgttggaagc tgctatagag gactagcctt
241 cgggttggaa ccctgttgca gtggatgata gatgaggctg gcttgtcaac tgcaggtgaa
301 ttcgcgattt gcgtcttcgg tctggttaac ttgcgagtag cacatgtgtt gcttgcttga
361 ttgctaggct gttggcgttg attgcccttg catttaactc ggtgttcagt cggtcaccca
421 ttcgacccgt cttgaaacac ggaccaagga gtttatggtg tacgcgagtc attgggcttt
481 taaacccaaa ggcgtaatga aagtgagaca tttagtgtct gtgatgtgaa cggttttcgt
541 ttcgacggtt gccgtgcaac atcgccctat gcctgggact tgtcctggcg tggaggttga
601 gcgtacgcta tgagacccga aagatggtga actatgcctg aacaggatga agccagagga
661 aactctggtg gaagtccgaa gcggttctga cgtgcaaatc gatcgtctga tttgggtata
721 ggggcgcaag
```

4. Partial LSU rRNA gene of *Laimaphelenchus australis* 718 bp

LOCUS   753 *Laimaphelenchus*   718 bp

DEFINITION 753 *Laimaphelenchus australis* D2D3

```
  1 tttgaaagag agtgcaagag aacgtgaaac cgacgtaatg gaagcggatg gagccgacgt
 61 atctgatacg tattcaatca cgtctcgttg cgatctgcga cgtggtattc cgcaaggttg
121 cctagttgta gttgttgttg cgatacgtga tgcatttgcg tacggagtgc gccgagtggg
181 ttgatattgc tgttggatgt caacaaagag gacctcattt cggtgtggaa ccctgagttg
241 gcggacggtg gataatgttg actcgtatat gaatactggt gaattcgcga ttagtctcta
301 tagtgaaaca agtcttggca tggcacatgt gttatgtcta aatttggtgt gctatgtatg
361 tttgttgccc atgtattcga ctcggtgtat agtcggtcac ccatttgacc cgtcttgaaa
421 cacggaccaa ggagtttaag gtgtacgcaa gtcattgggc ttataaaact caaaggcgta
481 atgaaagtga gatacttggt gtctgtgatg tgagtagttg ttgtttcgac gtcaactatg
541 caacatcacc ccatggctag ggcttgctct gtcgtggagg ttgagcgtac gcgttgagac
601 ccgaaagatg gtgaactatg cctgaacagg ataaagtcag aggaaactct gatggaagtc
661 cgaagcggtt ctgacgtgca aatccgatcg tggagatttg ggtatagggg cgcaagac
```

References

Abad, P., S. Tares, N. Brugieur and G. De Guiran (1991). Characterization of the relationship in the pinewood nematode species complex (*Bursaphelenchus* spp.) using a heterologous unc-22 DNA probe from *Caenorhabditis elegans*. *Parasitology* **102**: 303-308.

Adams, B. A. (1998). Species Concepts and the Evolutonary Paradigm in Modern Nematology. *Journal of Nematology* **30**: 1-21.

Aikawa, T., T. Kikuchi and H. Kosaka (2006). Population structure of *Bursaphelenchus xylophilus* within single *Pinus thunbergii* trees inoculated with two nematode isolates. *Forest Pathology* **36**: 1-13.

Al-Banna, L., L. Williamson and S. L. Gardner (1997). Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S r DNA. *Molecular Phylogenetic Evolution* **7**: 94-102.

Aleshin, V. V., O. S. Kedrova, I. A. Milyutina, N. S. Vladychenskaya and N. B. Petrov (1998). Relationships among nematodes based on the analysis of 18S rRNA gene sequences: molecular evidence for monophyly of Chromadorian and Secernentian nematodes. *Russian Journal of Nematology* **6**: 175-184.

Andrássy, I. (1976). Evolution as a Basis for the Systematization of Nematodes. London, UK, Pitman Publishing Ltd.

Andrássy, I. (1984). Klasse Nematoda. Stuttgart, Gustav Fischer Verlag.

Arakawa, Y. and K. Togashi (2002). Newly discovered transmission pathway of *Bursaphelenchus xylophilus* from males of the beetle *Monochamus alternatus* to *Pinus densiflora* trees via oviposition wounds. *Journal of Nematology* **34**: 396-404.

Bajaj, H. K. and K. K. Walia (2000). A new species of *Laimaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchina) from Kalesar forest, Haryana, India. *Indian Journal of Nematology* **30**: 86-110.

Barrett, J. (1991). Anhydrobiotic nematodes. *Agricultural Zoology Reviews* **4**: 161-167.

Basham, H. G. (1970). Pathogenicity and development of wilt in loblolly pine inoculated with blue-stain fungi in the genus *Ceratocysis*. *Phytopathology* **60**: 50-54.

Bastian, H. C. (1865). Monograph on the Anguillulidae, or free nematoids, marine, land, and freshwater; with descriptions of 100 new species. *Transactions of the Linnean Society of London* **25**: 73-184.

Baujard, P. (1981). Revue taxonomic du genre *Laimaphelenchus* Fuchs, 1937 et description de *Laimaphelenchus pini* n. sp. *Revue de Nématologie* **4**: 75-92.

Bedding, R. A. (1972). Biology of *Deladenus siricidicola* (Neotylenchidae), an entomophagous-mycetophagous nematode parasitic in siricid woodwasps. *Nematologica* **18**: 482-493.

Berryman, A. A. (1974). Dynamics of bark beetle populations: Towards a general productivity model. *Environmental Entomology* **3**: 579-585.

Bird, A. F. and M. S. Buttrose (1974). Ultrastructural changes in the nematode *Anguina tritici* associated with anhydrobiosis. *Journal of Ultrastructure Research* **48**: 177-189.

Blaxter, M. L., P. De Ley, J. R. Garey, L. X. Liu, P. Scheldeman, A. Vierstraete, J. R. Vanfleteren, L. Y. Machey, M. Dorris, L. M. Frisse, J. T. Vida and W. K. Thomas (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**: 71-75.

Bohmer, B. and B. Weil (1978). Methodenverleich zur extraktion von blatt und Knospennemtoden (*Aphelenchoides* spp.) aus Erdbeerpflanzensperossen. *Nachrichtenblatt. Deutschen Pflschutzdienst (Stuttgart)* **30**: 85-88.

Braasch, H. (1978). (Contribution to knowledge of the nematodes of pine forests) Vortragstagung (4) zu aktuellen Problemen der Phytonematologie am 8-6. 178 in Rostock. Manuskriptdruck der Vortrage. *Biologische Gesellschaft*: 66-75.

Braasch, H. (2001). *Bursaphelenchus* species in conifers in Europe: distribution and morphological relationships. *EPPO Bulletin* **31**: 127-142.

Braasch, H. and H. Schmutzenhofer (2000). *Bursaphelenchus abietinus* sp. n. (Nematoda: Parasitaphelenchudae) associated with fir bark beetles (*Pityokteines* spp.) from declining silver fir trees in Austria. *Russian Journal of Nematology* **8**: 1-6.

Brookhouser, L. W. and G. W. Peterson (1971). Infection of Austrian, Scots, and Ponderosa pines by *Diplodia pinea*. *Phytopathology* **61**: 409-414.

Campbell, A. D., R. B. Gasser and N. B. Chilton (1995). Differences in a ribosomal DNA sequence of *Strongylus* species allow identification of single eggs. *International Journal of Parasitology* **25**: 359-365.

Cao, Y. (1997). Studies on toxin of pine wilt disease caused by pine wood nematode. Ph.D. thesis. Nanjing Forest University.

Charwat, S. (1994). Variations in anhydrobiotic survival of nematodes. Ph.D. thesis. Der Christian-Albrechts-Universität zu Kiel, Germany.

Cherry, T., A. L. Szalanski, T. C. Todd and T. O. Powers (1997). The internal transcribed spacer region of *Belonolaimus* (Nemata: Belonolaimidae). *Journal of Nematology* **29**: 23-29.

Chilton, N. B., R. B. Gasser and I. Beveridge (1995). Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the *Hypodontus macropi* complex (Nematoda: Strongyloidea). *International Journal of Parasitology* **25**: 647-651.

Chitwood, B. G. (1937). A revised classification of the Nematoda. Papers on helminthology, 30 year jubileum. K. J. Skrjabin (eds.). Moscow, All-Union Lenin Academy of Agricultural Sciences: pp. 67-79.

Cobb, N. A. (1919). A newly discovered nematode (*Aphelenchus cocophilus* n. sp.) connected with a serious disease of the coconut palm. *West Indian Bulletin* **17**: 203-210.

Cooper, A. F., Fr. and S. D. Van Gundy (1971). Senescence, quiescence and cryptobiosis. In: Plant Parasitic Nematodes. B. M. Zuckerman, W. F. Mai and R. A. Rohde (eds.). New York, Academic Press: pp. 297-318.

Courtright, E. M., D. H. Wall, R. A. Virginia, L. M. Frisse, J. T. Vida and W. K. Thomas (2000). Nuclear and mitochondrial DNA sequence diversity in the Antarctic nematode *Scottnema lindsayae*. *Journal of Nematology* **32**: 143-153.

Cracraft, J. (1983). Species concepts and speciation analysis. *Current Ornithology* **1**: 159-187.

Cracraft, J. (1989). Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and process of differentiation. In: Speciation and its consequences. D. Otte and J. A. Endler (eds.). Sunderland, MA, Sinauer: pp. 28-59.

Crowe, J. H. and A. F. Cooper, Jr. (1971). Cryptobiosis. *Scientific American* **225**: 30-36.

Crowe, J. H. and K. A. C. Madin (1975). Anhydrobiosis in nematodes: Evaporative water loss and survival. *Journal of Experimental Zoology* **193**: 323-333.

Davies, K. A. and R. M. Giblin-Davis (2004). The biology and association of *Fergusobia* (Nematoda) from the *Melaleuca leucadendra*-complex in eastern Australia. *Invertebate Systematics* **18**: 291-319.

De Ley, I. T., P. De Ley, A. Vierstraete, G. Karssen, M. Moens and J. Vanfleteren (2002). Phylogenetic analysis of *Meloidogyne* small subunit rDNA. *Journal of Nematology* **34**: 319-327.

De Ley, P. and M. Blaxter (2002). Systematic Position and Phylogeny. In: The Biology of Nematodes. D. L. Lee (ed.). London, Taylor and Francis: pp. 1-30.

Dorris, M., M. E. Viney and M. L. Blaxter (2002). Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. *International Journal of Parasitology* **32**: 1507-1517.

Doucet, M. E. (1992). A new species of the genus *Laimaphelenchus* Fuchs, 1937 (Nemata: Aphelenchina). *Fundamental and Applied Nematology* **15**: 1-6.

Dowsett, A. P. and M. W. Young (1982). Differing levels of dispersed repetitive DNA among closely related species of *Drosophila*. *Proceedings of the National Academy of Sciences* **79**: 4570-4574.

Dropkin, V. H., A. Foudin, E. Kondo, M. S. Lin, M. Smith and K. Robbins (1981). Pinewood nematode: a threat to U. S. forest? *Plant Disease* **65**: 1022-1027.

Dropkin, V. H. and A. S. Foudin (1979). Report of the occurrence of *Bursaphelenchus lignicolus*-induced pine wilt disease in Missouri. *Plant Disease* **63**: 904-905.

Duncan, L. W., R. N. Inserra, W. K. Thomas, D. Dunn, I. Mustika, L. M. Frisse, M. L. Mendes, K. Morris and D. T. Kaplan (1999). Molecular and morphological analysis of isolates of *Pratylenchus coffeae* and closely related species. *Nematropica* **29**: 61-80.

Dwinell, L. D. (1986). Ecology of the pinewood nematode in southern pine chip piles. Research paper SE-258, USDA Forest Service Southeastern Forest Experiment Station, Asheville, NC.

Edwards, O. R. and M. J. Linit (1992). Transmission of *Bursaphelenchus xylophilus* through oviposition wounds of *Monochamus carolinensis* (Coeloptera: Cerambycidae). *Journal of Nematology* **24**: 133-139.

Ellenby, C. (1968). Desiccation survival of the infective larva of *Haemonchus contortus. Journal of Experimental Biology* **24**: 84-85.

Ellenby, C. (1969). Dormancy and survival in nematodes. *Experimental Biology* **23**: 572-576.

Elmiligy, I. and E. Geraert (1971). *Laimaphelenchus deconincki* n. sp. (Nematoda: Tylenchida). *Biologisch Jaarboek* **39**: 145-149.

Emmons, S. W., M. R. Klass and D. Hirsh (1979). Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans. Proceedings of the National Academy of Sciences* **76**: 133-137.

Enda, N. (1989). Current status of pine wilt disease in Korea. *Forest Pest* **38**: 148-152.

Evans, H. F., D. G. Mcnamara, H. Braasch, J. Chadoeuf and C. Magnusson (1996). Pest risk analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monchamus. EPPO Bulletin* **26**: 199-249.

Fallas, G. A., M. L. Hahn, M. Fargette, P. R. Burrows and J. L. Sara (1996). Molecular and biochemical diversity among isolates of *Radopholus* spp. from different areas of world. *Journal of Nematology* **28**: 422-430.

Felsenstein, J. (1985). Confidence limits on phylogenies, an approach using the bootstrap. *Evolution* **39**: 783-791.

Felsenstein, J. (1993). PHYLIP (Phylogeny inference package), Version 3.5. Seattle, WA, University of Washington.

Ferris, V. R., J. M. Ferris and J. Faghihi (1993). Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* **16**: 177-184.

Ferris, V. R., J. M. Ferris, J. Faghihi and A. Ireholm (1994). Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein pattern and ribosomal DNA. *Journal of Nematology* **26**: 144-151.

Ferris, V. R., L. I. Miller, J. Faghihi and J. M. Ferris (1995). Ribosomal DNA comparisons of *Globodera* from two continents. *Journal of Nematology* **27**: 273-283.

Filipjev, I. N. (1934). The classification of the free-living nematodes and their relation to the parasitic nematodes. *Smithsonian Miscellaneous Collections* **89**: 1-63.

Fitch, D. H. A., B. Bugaj-Gaweda and S. W. Emmons (1995). 18S ribosomal RNA gene phylogeny for some Rhabditidae related to *Caenorhabditis*. *Molecular Biology and Evolution* **12**: 346-358.

Floyd, R., E. Abebe, A. Papert and M. L. Blaxter (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology* **11**: 839-850.

Folmer, O., M. Black, W. Hoeh, R. Lutz and R. Vrijenhoek (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294-299.

Freckman, D. W. and E. P. Caswell (1985). The ecology of nematodes in agroecosystems. *Annual Review of Phytopathology* **23**: 275-296.

Futai, K. and T. Akema (2003). Some environmental conditions that influence the spead of pine wilt. In: The pinewood nematode, *Bursaphelenchus xylophilus*. M. M. Mota and P. Vieira (eds.). Leiden-Boston, Brill: pp.167-176.

Gasser, R. B. and H. Hoste (1995). Genetic markers for closely related parasitic nematodes. *Molecular and cellular Probes* **9**: 315-319.

Giannakis, N. and F. E. Sanders (1989). Interactions between mycophagous nematodes, mycorrhizal and other soil fungi. *Agriculture, Ecosystems and Environment* **29**: 163-167.

Gibb, K. S. and J. M. Fisher (1986). Co-ordination of the life cycle of *Contortylenchus grandicolli* (Nematoda: Allantonematidae) with that of its host *Ips grandicollis* (Scolytidae). *Nematologica* **32**: 222-233.

Giblin, R. M. (1985). Association of *Bursaphelenchus* sp. (Nematoda: Aphelenchoididae) with nitidulid beetles (Coleoptera: Nitidulidae). *Revue de Nématologie* **8**: 369-375.

Giblin, R. M. and H. K. Kaya (1984). Host, temperature and media additive effects on the growth of *Bursaphelenchus seani*. *Revue de Nématologie* **7**: 13-17.

Giblin-Davis, R. M., K. A. Davies, G. S. Taylor and W. Kelley Thomas (2003). Entomophilic Nematode Models for Studying Biodiversity and Cospeciation. In: Nematology, Advances and Perspectives. Z. X. Chen, S. Y. Chen and D. W. Dickson (eds.). New York, Tsing-hua University Press: pp. 492-538.

Giblin-Davis, R. M. (1993). Interaction of nematodes with insects. In: Nematode interactions. M. W. Khan (ed.). London, UK, Chapman and Hall: pp. 302-344.

Goodey, J. B. (1963). Soil and Freshwater Nematodes. Methuen and Co., London. 2nd Edition.

Hall, N., R. D. Johnston and G. M. Chippendale (1970). Forest Trees of Australia. Canberra, Australian Government Publishing Service.

Hasagawa, M., H. Kishino and T. A. Yano (1985). Dating of the human ape splitting by a molecular clock of mitochondrial-DNA. *Journal of Molecular Evolution* **22**: 160-174.

Heegaard, O., O. A. Jensen and J. U. Prause (1986). Hexamethyldislazane in preparation of retinal tissue for scanning electron microscopy. *Ophthalmic Research* **18**: 203-208.

Herdina, S. Neate, S. Jabaji-Hare and K. Ophel-Keller (2003). Persistence of DNA of *Gaeumannomyces graminis* var. *tritici* in soil as measured by a DNA-based assay. *FEMS Microbiology Ecology* **1598**: 1-10.

Herdina, K., Ophel-Keller, S. Wiebkin, I. Dumitrescu, R. Burns and A. Mckay (2004). The detection of dead soil-borne pathogens measured by DNA-based assay. 3rd Australiasian Soilborne Disease Symposium, South Australia. pp. 130.

Higgins, D. F., M. A. Harmy and D. L. Jones (1999). Pathogenicity related gene expression in *Bursaphelenchus xylophilus*. In: Sustainability of Pine Forests in relation to Pine Wilt and Decline. Proceedings of International Symposium, Tokyo, Japan October 1998. K. Futai, K. Togashi and T. Ikeda (eds.). Kyoto, Japan, Shokado: pp. 23-28.

Hillis, D. M. and J. J. Bull (1993). An empirical test of bootstrapping as a method for assessing confidence on phylogenetic analysis. *Systematic Biology* **42**: 182-192.

Hillis, D. M., C. Morita and B. K. Mable (1996). Molecular systematics. Sunderland, MA: Sinauer Associates.

Hodda, M. (2000). Nematoda, http://www.ento.csiro.au/science/nematode.html. Date accessed, 18/03/2003.

Hoste, H., N. B. Chilton and I. Beveridge (1995). Differences in the second internal transcribed spacer (ribosmal DNA) between five species of *Trichostrongylus* (Nematoda: Trichostrongylidae). *International Journal of Parasitology* **25**: 75-80.

Huang, C. S. and S. P. Huang (1974). Dehydration and the survival of rice white tip nematode, *Aphelenchoides besseyi. Nematologica* **20**: 9-18.

Huelsenbeck, J. P., J. J. Bull and C. W. Cunningham (1996). Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* **11**: 152-158.

Huelsenbeck, J. P. and F. Ronquist (2001). MR BAYES: Bayesian inference of phylogenetic tress. *Bioinformatics* **17**: 1754-1755.

Hunt, D. J. (1993). Aphelenchida, Longidoridae and Trichodoridae: Their Systematics and Bionomics. Cambridge, University Press.

Hunt, D. J. and N. G. M. Hague (1974). A redescription of *Parasitaphelenchus oldhami* Rühm, 1956 (Nematoda: Aphelenchoididae) a parasite of two elm bark beetles: *Scolytus scolytus* and *S. multistriatus*, together with some notes on its biology. *Nematologica* **20**: 174-180.

Ikonen, E. K. (2001). Population growth of two aphelenchid nematodes with six different fungi as a food source. *Nematology* **3**: 9-11.

Inglis, G. W. (1983). An outline classification of the phylum nematoda. *Australian Journal of Zoology* **31**: 243-255.

Ishibashi, N. and E. Kondo (1977). Occurrence and survival of the dispersal forms of pine wood nematode, *Bursaphelenchus xylophilus*, Mamiya and Kiyohara. *Applied Entomology and Zoology* **12**: 293-302.

Iwahori, H., N. Kanzaki and K. Futai (2000). A simple, polymerase chain reaction-restriction fragment length polymorphism-aided diagnosis method for pine disease. *Forest Pathology* **30**: 157-164.

Iwahori, H., K. Tsuda, N. Kanzaki, K. Izui and K. Futai (1998). PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundamental and Applied Nematology* **21**: 655-666.

Judith, H. H. and A. H. Matthew (1993). Detection and identification of *Bursaphelenchus* species with DNA Fingerprinting and Polymerase Chain Reaction 1. *Journal of Nematology* **25**: 406-415.

Kaisa, T. R., A. L. Harman and D. M. Harman (1995). Two new species of Aphelenchoididae (Nemata) from declining red pines in Maryland. *Journal of Nematology* **27**: 213-221.

Kampfer, S., C. Sturmbauer and J. Ott (1998). Phylogenetic analysis of rDNA sequences from Adenophorean nematodes and implications for the Adenophorea-Secernentea controversy. *Invertebrate Biology* **117**: 29-36.

Kanzaki, N. and K. Futai (2002). A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus* group. *Nematology* **4**: 35-41.

Kanzaki, N. and K. Futai (2002). Phylogenetic analysis of the phoretic association between *Bursaphelenchus conicaudatus* (Nematoda: Aphelenchoididae) and *Psacothea hilaris* (Coleoptera: Cerambycidae). *Nematology* **4**: 759-771.

Kawazu, K. and N. Kaneko (1997). Asepsis of the pine wood nematode isolate OKD-3 causes it to lose its pathogenicity. *Japanese Journal of Nematology* **27**: 76-80.

Kaya, H. K. (1984). Nematode Parasites of Bark Beetles. In: Plant and Insect Nematodes. W. R. Nickle (ed.) New York, Marcel Dekker: pp. 727-754.

Kelly, M., G. Tredinnick, G. Cutbush and G. Martin (2005). Impediments to investment in long rotation timber plantations. Forest and Wood Products Research and Development Corporation, Melbourne: pp. 1-93.

Kishino, M. and Hasegawa (1989). Evolution of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominodea. *Journal of Molecular Evolution* **29**: 170-179.

Kiyohara, T. (1984). Pine wilt resistance induced by prior inoculation with avirulent isolate of *Bursaphelenchus xylophilus*. Proceedings of the United States-Japan Seminar, Honolulu, Hawaii. pp. 178-187.

Kiyohara, T. and Y. Tokushige (1971). Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. *Journal of the Japanese Forestry Society* **53**: 210-218.

Kondo, E., A. Foudin, M. S. Lin, M. Smith, R. I. Bolla, R. Winter and V. Dropkin (1982). Pine wilt disease - nematological, entomological, biochemical investigations. Agricultural Experiment Station, University of Missouri-Columbia. pp. 56.

Kumar, S., K. Tamura and M. Nei (1993). MEGA: Molecular evolutionary genetic analysis, Version 1.01. University Park, PA, The Pennsylvania State University.

Lambe, R. C., A. P. Elliott, R. L. Wicks, S. A. Alexander, H. J. Heikkenen and P. B. Shultz (1984). Pinewood Nematode Disease of Ornamental Conifers. Virginia, Virginia Polytechnic Institute and State University College of Agriculture and Life Sciences.

Larget, B. and D. L. Simon (1999). Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**: 750-759.

Lewis, N. B., I. S. Ferguson, W. R. J. Sutton, D. G. M. Donald and H. B. Lisboa (1993). Management of Radiata Pine. Melbourne, Inkata Press.

Li, D. (2002). Immune reactions involved in parasitoid - host interaction. Ph.D. thesis. Adelaide University.

Linit, M. J. (1988). Nematode-vector relationships in the pine wilt disease system. *Journal of Nematology* **20**: 227-235.

Liu, J., R. E. Berry and A. F. Moldenke (1997). Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. *Journal of Invertebrate Pathology* **69**: 246-252.

Liu, W. Z., X. Wu and Y. Liu (1999). A new species of genus *Aphelenchoides*, leaf nematode of American ginseng, *Aphelenchoides panaxofolia* n. sp. (Nematoda: Aphelenchoidae). *Acta phytopathological sinica* **29**: 360-363.

Lorenzen, S. (1981). Entwurf eines phylogenetiscgen Systems der freilebenden Nematoden. *Veroeffentlichungen. Institut fuer Meeresforschung in Bremerharen. Supplement* **7**: 1-472.

Lorenzen, S. (1994). The phylogenetic systematic of freeliving nematodes. London, The Ray Society.

Lownsbery, J. W. and B. F. Lownsbery (1985). Plant-parasitic nematodes associated with forest trees in California. *Hilgardia* **53**: 1-16.

Luc, M., A. R. Maggenti, R. Fortuner, D. J. Raski and E. Geraert (1987). A reappraisal of Tylenchina (Nemata) 1. For a new approach to the taxonomy of Tylenchina. *Revue de Nématologie* **10**: 127-134.

Madin, K. A. C. and J. H. Crowe (1975). Anhydrobiosis in nematodes: Carbohydrate and lipid nematolism during dehydration. *Journal of Experimental Zoology* **193**: 335-342.

Maggenti, A. R. (1991). Nemata, Higher classification. In: Manual of Agricultural Nematology. W. R. Nickle (eds.). New York, Marcel Dekker Inc.: pp. 147-187.

Maggenti, A. R., M. Luc, D. J. Raski, R. Fortuner and E. Geraert (1987). A reappraisal of Tylenchina (Nemata) 2. Classification of the suborder Tylenchina (Nemata: Diplogasteria). *Revue de Nématologie* **10**: 135-142.

Magnusson, C. (1981). Nematoder som konsumenter på skogsbildonde barrträd. (in Swedish, English summary). *Växtskyddsrapporter Jordbruk* **16**: 61-69.

Magnusson, C., H. Overgaard, H. Nyeggen, K. Thunes, S. Haukeland Salinas and B. Hammeraas (2004). Survey of the pine wood nematode (PWN) *Bursaphelenchus xylophilus* in Norway 2002. *Plante forsk* **8**: 1-13.

Magnusson, C., K. H. Thunes, S. Haukeland Salinas and B. Økland (2000). Survey of the pine wood nematode, *Bursaphelenchus xylophilus*, in Norway in 2000. In: The Pine Wood Nematode, *Bursaphelenchus xylophilus*. M. M. Mota and P. Vieira (eds.). Leiden, Brill: pp. 101-112.

Majiwa, P. A. O. and P. Webster (1987). A repetitive deoxyribonucleic acid sequence distinguishes *Trypanosoma aimiae* from *T. congolense*. *Parasitology* **95**: 137-144.

Mamiya, Y. (1972). Pine Wood Nematode, *Bursaphelenchus lignicolus* Mamiya and Kiyohara, as a causal agent of pine wilting disease. *Review of Plant Protection Research* **5**: 46-60.

Mamiya, Y. (1975). Behaviour of *Bursaphelenchus lignicolus* in the wood of pine seedings and pathological responses of pine to nematode infection. *Transaction of the 86th Meeting of the Japanese Forestry Society.* **86**: 285-286.

Mamiya, Y. (1983). Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus. Annual Review of Phytopathology.* **21**: 201-215.

Mamiya, Y. (1984). The Pine Wood Nematode. In: Plant and Insect Nematodes. W. R. Nickle (ed.). New York, Marcel Dekker Inc.: pp. 589-626.

Mamiya, Y. (1987). Origin of the pine wood nematode and its distribution outside the United States. In: Pathogenicity of the pine wood nematode. M. J. Wingfield (ed.). St. Paul, American Phytopathological Society Press: pp. 50-65.

Mamiya, Y. (1988). History of pine wilt disease in Japan. *Journal of Nematology* **20**: 219-226.

Mamiya, Y. (1999). Review on the pathogenicity of *Bursaphelenchus mucronatus.* In: Futai, K, Togashi, K and Ikeda, T (eds) Sustainability of pine forests in relation to pine wilt and decline. Proceeding of International symposium, Tokyo, Japan, 27-28 October 1998 Shokado, Tokyo. pp. 57-64.

Mamiya, Y. and N. Enda (1972). Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematologica* **18**: 159-162.

Mamiya, Y. and N. Enda (1979). *Bursaphelenchus mucronatus n. sp.* (Nematoda: Aphelenchoididae) from pine wood and its biology and pathogenicity to pine trees. *Nematologica* **25**: 353-361.

Mamiya, Y. and T. Kiyohara (1972). Description of *Bursaphelenchus lignicolus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and histopathology of nematode-infested trees. *Nematologica* **18**: 120-124.

Marx, D. H. and C. B. Davey (1969). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. IV. Resistance of

naturally occurring mycorrhizae to infections by *Phytophthora cinnamomi*. *Phytopathology* **59**: 559-565.

Massey, C. L. (1960). Nematode parasites and associates of the California five-spined engraver, *Ips confusus* (Lec.). *Proceedings of the Helminthological Society of Washington* **27**: 14-22.

Massey, C. L. (1966). The nematode parasites and associates of *Dendroctonus adjunctus* (Coleoptera: Scolytidae) in New Mexico. *Annals of the Entomological Society of America* **59**: 424-440.

Massey, C. L. (1971). Nematode associates of several species of *Pissodes* (Coleoptera: Curculionidae) in the United States. *Annals of the Entomological Society of America* **64**: 162-169.

Massey, C. L. (1974). Biology and taxonomy of nematode parasites and associates of bark beetles in the United States. Agriculture Handbook (ed.). Washington, Forest Service: 233 pp.

Mayr, E. (1942). Systematics and the Origin of Species from the Viewpoint of a Zoologist (ed.). New York, Columbia University Press.

Mayr, E. (1963). Animal Species and Evolution. Cambridge, MA, Harvard University Press.

McLeod, R., F. Reay and J. Smyth (1994). Plant Nematodes of Australia listed by Plant and by Genus (eds.). Sydney, NSW Department of Agriculture.

Melakeberhan, H. and J. M. Webster (1992). The insignificance of the energy requirements of *Bursaphelenchus xylophilus* as a causal factor in *Pinus xylophilus* seedling mortality. *Fundamental and Applied Nematology* **15**: 179-182.

Michalopoulous-Skarmoutsos, H., G. Skarmoutsos, M. Kalapanida and A. Harageorgos (2003). Surveying and recording of nematodes of the genus

Bursaphelenchus in Conifer forests in Greece and pathogenicity of the most important species. In: The pinewood nematode, *Bursaphelenchus xylophilus*. M. M. Mota and P. Vieira (eds.). Leiden-Boston, Brill: pp. 113-126.

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

Myers, G. S. (1952). The nature of systematic biology and of a species description. *Systematic Zoology* **1**: 106-111.

Myers, R. F. (1988). Pathogenesis in pine wilt caused by pinewood nematode, *Bursaphelenchus xylophilus*. *Journal of Nematology* **20**: 236-244.

Nadler, S. A. (1992). Phylogeny of some Ascaridoid nematodes, inferred from comparison of 18S and 28S sequences. *Molecular Biology and Evolution* **9**: 932-944.

Nadler, S. A. (2002). Species delimitation and nematode biodiversity: phylogenies rule. *Nematology* **4**: 615-625.

Nadler, S. A. and D. S. S. Hudspeth (1998). Ribosomal DNA and phylogeny of the Ascaridoidea (Nemata, Secernentea): Implications for morphological evolution and classification. *Molecular Phylogenetic Evolution* **10**: 221-236.

Nelson, G. J. and N. I. Platnick (1981). Systematics and Biogeography: Cladistics and Vicariance. New York, Columbia University Press.

Nickle, W. R. (1970). A taxonomic review of the genera of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). *Journal of Nematology* **2**: 375-392.

Nickle, W. R., A. M. Golden, Y. Mamiya and W. P. Wergin (1981). On the taxonomy and morphology of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Burhrer 1934) Nickle 1970. *Journal of Nematology* **13**: 385-392.

Nickle, W. R. and D. J. Hooper (1991). The Aphelenchina: bud, leaf and insect nematodes. In: Manual of Agricultural Nematology. W. R. Nickle (ed.). New York, Marcel Decker: pp. 456-507.

Nixon, K. C. and Q. D. Wheeler (1990). An amplification of the phylogenetic species concept. *Cladistics* **6**: 211-223.

Nunn, G. B. (1992). Nematode Molecular Evolution. Ph.D. thesis. University of Nottingham, U.K.

Ogura, N. and H. Kosaka (1991). Biology of a tylenchid nematode parasitic on the Japanese pine sawyer, *Monchamus alternatus*. *Nematologica* **37**: 455-469.

Oku, H., T. Shirashi and S. Kurozumi (1979). Participation of toxin in wilting of Japanese pines caused by a nematode. *Naturwissenschaften* **66**: 210.

Oku, H., T. Shirashi, S. Ouchi, S. Kurozumi and H. Ohta (1980). Pine wilt toxin, the metabolite of a bacterium associated with a nematode. *Naturwissenschaften* **67**: 198-199.

Oliver, I. and A. J. Beattie (1996). Invertebrate morphospecies as surrogates for species: a case study. *Conservation Biology* **10**: 99-109.

Panesar, T. S. and J. R. Sutherland (1989). Pathogenicity of Canadian isolates of the *Bursaphelenchus xylophilus* (pinewood nematode) to provenances of *Pinus sylvestris* and *Pinus contorta* as grown in Finland: a green house study. *Scandinavian Journal of Forage Research* **4**: 549-557.

Peneva, V. and N. Chipev (1999). *Laimaphelenchus helicosoma* (Maslen,1979) n. comb. (Nematoda: Aphelenchida) from the Livingston Island (the Antarctic). *Bulgarian Antarctic Research Life Sciences* **2**: 57-61.

Phillips, C. (1999). Monterey Pine Aphid - a new pest of pine trees. *Australian Forest Grower* **22**: 1-12.

Posada, D. and K. A. Crandall (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

Powers, T. O. (2004). Nematode molecular diagnostics: From bands to barcodes. *Annual Review of Phytopathology* **42**: 367-383.

Powers, T. O., T. C. Todd, A. M. Burnell, P. C. B. Murray, C. C. Fleming, A. L. Szalanski, B. A. Adams and T. S. Harris (1997). The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. *Journal of Nematology* **29**: 441-450.

Prager, E. M. and A. C. Wilson (1988). Ancient origin of lactalbumin from lysozyme analysis of DNA and amino acid sequence. *Journal of Molecular Evolution* **27**: 326-335.

Ridley, G., J. Bain and M. Dick (2001). Exotic nematode found in pine trees in Melbourne, Victoria. *New Zealand Journal of Forestry* **46**: 41-42.

Riga, E., K. Beckenbach and J. M. Webster (1992). Taxonomic relationship of *Bursaphelenchus xylophilus* and *B. mucronatus* based on interspecific and intraspecific cross-hybridization and DNA analysis. *Fundamental and Applied Nematology* **15**: 391-396.

Ritzema Bos, J. (1890). De bloemkoolziekte der aardbeien, veroorzaakt door *Aphelenchus fragariae* nov. spec. (Voorloopipe mededeeling). *Maandblad Natuurwetensch* **19**: 107-117.

Robbins, K. (1982). Distribution of the pine wood nematode in the United States. In: Proceedings of the National Pine Wilt Disease Workshop, Rosemont IL. J. E. Appelby and R. B. Malek (eds.). University of Illinois, Urbana, USA: pp3-6.

Rosen, D. E. (1978). Vicariant patterns and historical explanation in biogeography. *Systematic Zoology* **27**: 159-188.

Rosen, D. E. (1979). Fishes from the uplands and intermontane basin of Guatemala: revisionary studies and comparative geography. *Bulletin of the American Museum of National History* **162**: 267-376.

Ruehle, J. L. (1972). Nematodes of Forest Trees. London-New York, Academic Press.

Ruess, L. and J. Dighton (1996). Cultural studies on soil nematodes and their fungal hosts. *Nematologica* **42**: 330-346.

Rühm, W. (1956). Die Nematoden der Ipiden. *Parasitologische Schriftenreihe* **6**: 1-435.

Sabrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular Cloning: A Laboratory Manual. New York, Cold Spring Harbor Press.

Saitou, N. and M. Nei (1987). The neighbor-joining method, a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Sanderson, M. J. (1989). Confidence limits on phylogenies: the bootstrap revisited. *Cladistics* **5**: 113-129.

Sanderson, M. J. (1995). Objections to bootstrapping phylogenies: a critique. *Systematic Biology* **44**: 299-320.

Sasaki, S., K. Odani, Y. Nishiyama and Y. Hayashi (1984). Development and recovery of pine wilt disease studied by tracing ascending sap flow marked by water soluble stains. *Journal of the Japanese Forestry Society* **66**: 141-148.

Schwartz, M. (1911). Die Aphelenchen der Veilchengallen und Blattflecken an Farnen und Chrysanthemum. *Arbeiten der kaiserlichen biologischen Anstalt fuer Land und Forstwirtschaft* **8**: 303-334.

Seinhorst, J. W. (1966). Killing nematodes for taxonomic study with hot f.a. 4:1. *Nematologica* **12**: 175.

Siddiqi, M. R. (1980). The origin and phylogeny of the nematode orders Tylenchia Thorne, 1949 and Aphelenchia n.ord. *Helminthological Abstracts Series B* **49**: 143-170.

Simpson, G. G. (1961). Principles of Animal Taxonomy. New York, Columbia University Press.

Skarmoutsos, G. and H. Michalopoulos (2000). Pathogenicity of *Bursaphelenchus sexdentati*, *Busaphelenchus leoni* and *Bursaphelenchus hellennicus* on European pine seedlings. *Forest Pathology* **30**: 149-156.

Sousa, E., M. A. Bravo, J. Pires, P. Naves, A. C. Penas, L. Bonifacio and M. M. Mota (2001). *Bursaphelenchus xylophilus* (Nematoda; Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal. *Nematology* **3**: 89-91.

Spencer, D. (2001). Conifers in the Dry Country: A report for the RIRDC/L&W Australia/FWPRDC Joint Venture Agroforestry Program.

Stamps, W. T. and M. J. Linit (2001). Interaction of intrinsic and extrinsic chemical cues in the behaviour of *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae) in relation to its beetle vectors. *Nematology* **3**: 295-301.

Steiner, G. (1914). Freilebende nematoden aus der Schweiz. 1. Teil einer vorlaüfigen Miteilung. *Archiv für hydrobiologie und Planktonkunde* **9**: 259-276.

Steiner, G. and E. Buhrer (1934). *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. *Journal of Agricultural Research* **48**: 949-951.

Sutherland, J. R. and J. M. Webster (1993). Nematode Pests of Forest Trees. In: Plant Parasitic Nematodes In Temperate Agriculture. K. Evans, D. L. Trudgill and J. M. Webster (eds.). Wallingford, CAB International: 351-380 pp.

Swart, A. (1997). Description of *Laimaphelenchus patulus* sp. n. (Nematoda: Aphelenchoididae) from *Pinus pinaster* Ait. in South Africa. *African Plant Protection* **3**: 23-28.

Swofford, D. L. (2002). PAUP*, Phylogenetic analysis using parsimony (* and other methods). Version. 4. Sunderland, Massachusetts, Sinauer Associates.

Templeton, A. R. (1983). Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and apes. *Evolution* **37**: 221-224.

Thong, C. H. S. and Webster, J. M. (1991). Dauerlarvae: Is there one in *Bursaphelenchus xylophilus*? *Revue de Nématologie* **14**: 635-636.

Todd, E. H. and J. G. Atkins (1958). White tip disease of rice. I. Symptoms, laboratory culture of nematode and pathogenicity tests. *Phytopathology* **48**: 632-637.

Togashi, K. and Y. Arakawa (2003). Horizontal transmission of *Bursaphelenchus xylophilus* between sexes of *Monochamus alternatus*. *Journal of Nematology* **35**: 7-16.

Trudgill, D. L. and M. S. Phillips (2006). Nematode population dynamics, threshold levels and estimation of crop losses, http://www.fao.org/docrep/V9978E/v9978e07.htm. Date accessed, 05/04/2006.

van de Hoef, L. and B. Hill (2003). Radiata pine for farm forestry. Department of Primary Industries. http://www.dpi.vic.gov.au/dpi/nreninf.nsf/LinkView. Date accessed, 19/02/2006.

Van Gundy, S. D. (1965). Factors in survival of nematodes. *Annual Review of Phytopathology* **3**: 43-68.

Webster, J. M., R. V. Anderson, D. Z. Baillie, K. Beckenbach, J. Curran and T. A. Rutherford (1990). DNA probes for differentiating isolates of the pine wood nematode species complex. *Revue de Nématologie* **13**: 255-263.

Whitehead, A. G. (1998). Plant Nematode Control. London, UK: CAB International.

Wiley, E. O. (1978). The evolutionary species concept reconsidered. *Systematic Zoology* **27**: 17-26.

Williams, B. D., B. Schrank, C. Huynh, R. Shownkeen and R. H. Waterston (1992). A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics* **131**: 609-624.

Womersley, C., S. N. Thompson and L. Smith (1982). Anhydrobiosis in nematodes II. Carbohydrate and lipid analysis in undesiccated and desiccated nematodes. *Journal of Nematology* **14**: 145-153.

Womersley, C. Z. (1987). A re-evaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. In: Vistas on Nematology. J. Veech and D. W. Dickson (eds.), Hyattsville, Society of Nematologists: pp.165-173.

Yang, B. J. (2002). Advance in research of pathogenetic mechanism of pine wood nematode. *Forest Pest and Disease* **1**: 27-31.

Yang, B. J. (2003). The history, dispersal and potential threat of pine wood nematode in China. In: The Pine Wood Nematode, *Bursaphelenchus xylophilus*. M. M. Mota and P. Vieira (eds.). Leiden, Koninklijke Brill: pp. 21-24.

Yeates, G. W. (1998). Feeding in free-living soil nematodes: a functional approach. In: The physiology and biochemistry of free-living and plant parasitic nematodes. R. N. Perry and D. J. Wright (eds.). Wallingford, UK, CAB International Publishing: pp. 245-269.

Yin, K., Y. Fang and A. C. Tarjan (1988). A key to species in the genus *Bursaphelenchus* with a description of *Bursaphelenchus hunanensis* sp. n. (Nematoda: Aphelenchoididae) found in pinewood in Hunan Province, China. *Proceedings of the Helminthological Society of Washington* **55**: 1-11.

Zhao, B. G., H. L. Wang, S. F. Han and Z. M. Han (2003). Distribution and pathogenicity of bacteria species carried by *Bursaphelenchus xylophilus* in China. *Nematology* **5**: 899-906.

Zhao, Z. Q., K. A. Davies, I. T. Riley and J. M. Nobbs (2006). *Laimaphelenchus australis* sp. nov. (Nematoda: Aphelenchina) from exotic pines, *Pinus radiata* and *P. pinaster*, in Australia. *Zootaxa* **1248**: 35-44.

Zhao, Z. Q., K. A. Davies, I. T. Riley and J. M. Nobbs (2006). *Laimaphelenchus preissii* sp. nov. (Nematoda: Aphelenchina) from native *Callitris preissii* in South Australia. *Transactions of the Royal Society of South Australia* **130**: 10-16.

Zheng, J. W., S. A. Subbotin, S. S. He, J. F. Gu and M. Moens (2003). Molecular characterisation of some Asian isolates of *Bursaphelenchus xylophilus* and *B-mucronatus* using PCR-RFLPs and sequences of ribosomal DNA. *Russian Journal of Nematology* **11**: 17-22.

Zijlstra, C., A. E. M. Lever, B. C. Uenk and C. H. van Silfhout (1995). Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology* **85**: 1231-1237.

Zijlstra, C., B. J. Uenk and C. H. van Silfhout (1997). A reliable, precise method to differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs. *Fundamental and Applied Nematology* **20**: 59-63.