

MULTIFACETED APPROACH FOR DIFFERENTIATING
ISOLATES OF *BURSAPHELENCHUS XYLOPHILUS*
AND *B. MUCRONATUS* (NEMATODA),
PARASITES OF PINE TREES

by

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Degree: **Doctor of Philosophy**

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**MULTIFACETED APPROACH FOR DIFFERENTIATING ISOLATES OF
BURSAPHELENCHUS XYLOPHILUS AND *B. MUCRONATUS* (NEMATODA),
PARASITES OF PINE TREES**

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Title of Thesis/Project/Extended Essay

Multifaceted approach for differentiating
isolates of Bursaphelenchus
xylophilus and B. mucronatus
(Nematoda), parasites of pine trees

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Abstract

Bursaphelenchus xylophilus (Steiner and Buhner) and *B. mucronatus* (Mamiya and Enda), two closely related nematode parasites of conifers, were studied for the molecular, reproductive, behavioural and pathogenic characteristics which could be used in specific and subspecific identification. Intraspecific cross-hybridization among isolates of *B. xylophilus* produced fertile offspring. Cross-hybridization between *B. mucronatus* from France (female) and Japan (male) produced fertile progeny but the reciprocal cross died out. Most interspecific hybrids between male *B. xylophilus* and female *B. mucronatus* died out, but the reciprocal interspecific crosses were successful.

Mortality of *Pinus sylvestris* seedlings was high after inoculation with *B. xylophilus* populations or their intraspecific hybrids, moderate after inoculation with the French *B. mucronatus*, low with the Japanese *B. mucronatus*, and moderate with the *B. mucronatus* Japanese-French intraspecific hybrid. *Bursaphelenchus mucronatus* and *B. xylophilus* interspecific hybrids were as pathogenic as the *B. xylophilus* parents and more pathogenic than the *B. mucronatus* parents. No correlation was found between the female tail shape of *B. xylophilus* populations and the ability to cause pathogenicity.

Genomic DNA from *B. xylophilus*, *B. mucronatus* and their hybrids was probed with a homologous ribosomal gene clone which produced band pattern differences between the populations of *B. xylophilus* and of *B. mucronatus* and between the French and Japanese populations of *B. mucronatus*.

Males of *B. xylophilus* and *B. mucronatus* were attracted to pheromones released by their homospecific females. However, males or females of *B. mucronatus* were not attracted to *B. xylophilus* females or males. Neither members of *B. xylophilus* nor *B. mucronatus* were attracted to members of *B. fraudulentus* or *Aphelenchoides rhyntium*. Partial characterization of the pheromone of *B. xylophilus* revealed it to be a highly polar, hydrophilic compound.

The results support the hypothesis that *B. xylophilus* and *B. mucronatus* are distinct species and provide additional evidence for the splitting of *B. mucronatus* into two species, one indigenous to France and the other to Japan. In addition, this study provides biological evidence that would lead to the re-evaluation of the embargo against the importation of North America softwood products into several European countries.

Dedication

I am dedicating this work to my mother for being a source of inspiration and challenges in my life.

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Throughout this work, advice, and encouragement has been received from many people. It is with pleasure that I express my gratitude to the following persons:

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General Introduction

In the phylum Nematoda, morphology is highly conserved, probably due to the absence of strong selective pressures. Although, morphological characters are useful taxonomic tools at the higher levels of nematode taxonomy, they are relatively unreliable and difficult to use at the specific and subspecific levels. In recent years, several new methods such as host range tests, protein electrophoresis, immunological techniques and DNA analysis have been developed to differentiate nematodes at the lower taxonomic level. However, taken individually, these techniques do not allow for definitive classification within the same nematode genera. In this study, I have used a multifaceted approach that utilizes a range of biological and phenotypic characters, including the species specificity of nematode pheromones, along with DNA analysis, to supplement the more classical morphological taxonomic characters. To do this I have chosen to compare two closely related species, the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (= *B. lignicolus* Mamiya and Kiyohara) and *B. mucronatus* (Mamiya and Enda); both nematodes are economically important pathogens of coniferous trees.

Bursaphelenchus xylophilus, the causative agent of pine wilt disease, has been associated with heavy losses of native Japanese *Pinus thunbergii* and *P. densiflora*, especially under high temperature (Mamiya, 1983) and dry conditions (Suzuki and Kiyohara, 1978). Takeshita, *et al.* (1975) reported that low summer precipitation (less than 30 mm during 40 days of the summer season) and average temperatures above 25°C throughout the season are responsible for the intensity and spread of pine wilt. In 1979, 650,000 ha, 25% of the total pine forest area in Japan, were affected by the pine wood nematode, and losses of 2.4 million m³ of timber were reported (Mamiya, 1984). *Bursaphelenchus mucronatus* also occurs in Japanese forests and is a close relative of *B. xylophilus*. However, under the climatic conditions in southern Japan it is considered to be less pathogenic than *B. xylophilus* (Mamiya and Enda, 1979; Cheng *et al.*, 1986).

Bursaphelenchus xylophilus has been found in eastern Asia (Mamiya, 1984; Cheng, *et al.*, 1986), and is distributed throughout most of the forested areas of the United States (Dropkin and Foudin, 1979; Robbins, 1982) and Canada (Knowles *et al.*, 1983; Anonymous, 1986a). PWN was first reported in the United States in 1931 but at that time it was not considered a tree pathogen (Nickle *et al.*, 1981). *Pinus sylvestris* mortality occurred in several North American locations following summers with drought conditions and average temperatures higher than the normal seasonal temperatures (Malek and Appleby, 1984), i.e. higher than the 20°C July mean air temperature isotherm (Rutherford and Webster, 1987). Rutherford and Webster (1987) also reported that *P. nigra* in North America dies of pine wilt in locations warmer than the 20°C July isotherm. Similarly, Dwinell (1987) reported that with a few exceptions there was no evidence that pine wilt disease causes a problem in United States southern pines. Therefore, it was speculated that exotic pines like *P. sylvestris* and *P. nigra* could grow free of pine wilt in North America if they are grown in areas cooler than the 20°C July isotherm (Rutherford and Webster, 1987).

Nematodes in all species of *Bursaphelenchus* have a phoretic relationship with insects, mainly with bark beetles and wood boring beetles (Massey, 1974). *Bursaphelenchus xylophilus* and *B. mucronatus* are vectored principally by cerambycid (longhorn) beetles in the genus *Monochamus* (Linit, 1987). In Taiwan, China and Japan, *M. alternatus* Hope is the main vector of *B. xylophilus* (Mamiya and Enda, 1972; Mamiya, 1987). In the United States *B. xylophilus* has been recovered from *M. carolinensis* Oliver, *M. scutellatus* Say, *M. titillator* F., *M. mutator* LeConte, *M. scutellatus oregonensis* (LeConte) and *M. notatus* Drury (Nickle, *et al.*, 1981; Linit *et al.*, 1983; and Wingfield and Blanchette, 1983). In Canada, of 14 potential PWN insect vectors identified by Knowles *et al.* (1983), six are longhorn beetles, namely *M. carolinensis*, *M. marmorator*, *M. mutator*, *M. obtusus* Casey, *M. scutellatus*, and *M. titillator*.

In Japan, *B. xylophilus* has two phases in its life cycle, a dispersal and a propagative phase. Four juvenile stages and female and male adults occur in the live tree host; this phytophagous or propagative stage can be completed in 4-5 days at 25°C (Ishibashi and

Kondo, 1977). During this phase, all developmental stages of the PWN most likely feed on the epithelial cells of the ray canals of the healthy host tree (Mamiya and Kiyohara, 1972). The dispersal or mycophagous stage consists of the first two juvenile stages, followed by a J_3 and a dispersal J_4 , both of which have thicker cuticles than the equivalent propagative juvenile stages, and lipid reserves to withstand adverse conditions. The J_3 nematodes are attracted to the insect pupal chamber where they moult to the J_4 stage, and then enter the tracheae of the young adult beetle just before the beetle emerges from the tree (Ishibashi and Kondo, 1977; Kondo and Ishibashi, 1978). During the mycophagous phase, the nematodes feed on fungi colonizing the dead pine tree.

The J_4 stage of the PWN is transported to a new host tree on the body surface and within the tracheal system of the beetle (Kobayashi *et al.*, 1984). The average number of pine wood nematodes carried by *M. alternatus* in Japan is 15,000 (Mamiya, 1972). The beetle vector emerges from the dead pine tree and starts its maturation feeding early in the pine growing season, between bud break and candle maturation. The feeding is restricted mainly to primary and secondary phloem, cortex and cambial tissues that are produced in elongating candles and 1-year-old twigs of pines (Myers, 1988). During this dispersal stage (Thong and Webster, 1991), the J_4 nematodes exit from the vector beetle (Mamiya and Enda, 1972) and enter the wounded tissue of the pine tree at the insect feeding site (Mamiya, 1983); this is considered primary transmission (Mamiya, 1984). The nematode moults to the adult stage once exposed to the xylem tissues and soon becomes randomly distributed throughout the pine tree (Kiyohara, and Suzuki, 1987). Mature cerambycid beetles oviposit in dead or dying pine trees and undergo development, which is closely coordinated with the life cycle of the PWN. A secondary transmission can occur during oviposition by *Monochamus* in this stage of the PWN life cycle. As the nematode develops it feeds on the fungal hyphae in the dying tree and builds up a population that serves as a reservoir of nematodes to be dispersed by the developed beetles (Wingfield, 1983).

Death of *P. thunbergii* and *P. densiflora* in the warm areas of Japan occurs approximately 3 months postinfection (Mamiya, 1976)

Initially, adult beetles infect healthy pines during maturation feeding from May to July (Mamiya, 1988). Infected beetles feed continually and infect pine trees throughout their lives. From July to August the infected trees begin to show the symptoms of the pine wilt disease, and over 90% of these trees die by October (Mamiya, 1983). In cool areas of Japan, with a mean annual temperature of 10-12°C, pine wilt development is different from that in warm regions. Approximately half of the trees in these areas do not die within the first year of infection, but rather they die from early spring to early summer of the following year (Kishi, 1980; see Mamiya, 1988). In the northern cooler regions of Japan death of only a few branches, rather than the whole tree, infected by PWN has been reported (see Mamiya, 1988). Similar results have been reported in the United States, where trees died within two years following branch death (Malek and Appleby, 1984). The effect of temperature on pine wilt development has been demonstrated by infecting pines with the PWN at 25-30°C and then controlling the progress of the disease by reducing the temperature to 18-20°C (see Mamiya, 1988). In Japan, the life cycle of the *M. alternatus* vector is affected by the cooler temperatures. Most of the beetle populations in cooler areas emerge about one month later than the beetles in warm areas. In addition, many beetle populations in the cooler areas require two years to complete their life cycle (Kobayashi, *et al.*, 1984). By the time the *M. alternatus* populations complete their life cycle in cool areas, the nematode population is low. This results in fewer nematodes carried by the beetles to the next pine host (see Rutherford *et al.*, 1990).

The initial, detectable symptom of pine wilt disease is a decline of variable chlorophyll *a* fluorescence and needle water potential 24-36 h after inoculation of pine seedlings with *B. xylophilus* (Melakeberhan, *et al.*, 1991). Pine wilt disease was initially believed to be caused by the mechanical destruction of vascular tissue and disruption of the normal physiological activities of pines by the PWN (Ikeda and Suzuki, 1984; Kuroda *et al.*, 1988). Other reports suggested that tree death is caused by toxic compounds that are synthesized and released during the nematode-conifer interaction (Dropkin, *et al.*, 1981; Bolla, *et al.*, 1984; Shaheen, *et al.*, 1984). Oku (1988) reported that crude extract from

diseased pines is toxic to pine seedlings, while an extract from healthy pines is not toxic. Several compounds with phytotoxic properties, and low molecular weight were isolated from pines that were infected with *B. xylophilus*, i.e. benzoic acid, catechol, dihydroconiferyl alcohol, 8-hydroxycarvotanacetone and 10-hydroxyverbenone (Oku, 1988). In addition, Oku (1988) reported that the toxicity of the previous abnormal pine metabolites correlates positively to the susceptibility of pines to *B. xylophilus*. Myers (1986) reported that damage to the cambium by *B. xylophilus* caused death of pines through blockage of tracheids by oleoresin and gas or metabolites from dying ray tissues. It was hypothesized that these symptoms are typical of a hypersensitive reaction that might occur in pine trees following PWN migration and that the spreading of the hypersensitive reaction resulted in pine death (Myers, 1988). Kuroda (1989) investigated in detail, the processes that lead to the pine wilt disease. The moving and feeding of *B. xylophilus* causes the production of terpenoid compounds in parenchyma cells of *P. thunbergii*. The degradation of the plasma membrane of parenchyma cells exudes terpenoids into the adjacent tracheids which start to cavitate, i.e. they fill up with gas instead of water. Volatile monoterpenes fill one tracheid and then cavitate to adjacent tracheids. Eventually, the cavitated areas reach the cambium and the tree dies due to water deficit. This is caused in part by the physical blockage of the vascular tissue by the terpenoid compounds and their hydrophobic effect that prevents the cavitated tracheids from refilling with water, and in part by decreased water uptake.

Although the PWN is a serious pest of ornamental pines, it is not a serious forest pest in North America and it causes serious economic problems for the North American forest industry and for forest management practices (Bergdahl, 1988). In 1984, the Finnish Plant Quarantine Service found PWN in shipments of coniferous chips from North America. As a result, Finland enforced an embargo against all raw, softwood products from North America and Japan (Anonymous, 1984). Subsequently, the European and Mediterranean Plant Protection Organization (EPPO) placed *B. xylophilus* on the A1 list of quarantine pests (Anonymous, 1986b). This effectively extends the use of the embargo to most European countries.

Bursaphelenchus xylophilus has not been reported from Europe but a nematode similar to *B. mucronatus* in Japan has been found in European forests (Schauer-Blume, 1987; McNamara and Stoen, 1988).

Bursaphelenchus mucronatus, initially was reported from dead pine trees in Japan but it is less pathogenic to *P. densiflora* and *P. thunbergii* than is *B. xylophilus*. In addition, *B. mucronatus* has a larger area of distribution in Japan than does *B. xylophilus*. *Bursaphelenchus mucronatus* is distinguished morphologically from *B. xylophilus* by the presence of a mucronate tail in both juvenile and adult females (Mamiya and Enda, 1979). The morphological and biometrical characters of *B. xylophilus* and *B. mucronatus* however are variable. This results in overlapping ranges of the morphological characters used that give rise to problems with respect to their use for species identification. For example, a population from *Abies balsamea* in Minnesota is morphologically similar to *B. mucronatus* but it is genetically compatible with only *B. xylophilus* and not *B. mucronatus* (Wingfield *et al.*, 1983). Additionally, populations of *B. xylophilus* and *B. mucronatus* from different geographic locations differ in their morphology (Webster *et al.*, 1990; Kiyohara and Bolla, 1990). In addition, within *B. xylophilus* there exist apparently pathogenic and non-pathogenic isolates (Bolla, *et al.*, 1986). In Japan, Mamiya and Enda (1979) used the presence of a mucro on the tail of *B. mucronatus* females as a major character to distinguish it from *B. xylophilus*. Isolates of *B. xylophilus* from North America however have a female tail shape that ranges from mucro-type to round-type (Wingfield, *et al.*, 1983). These have been referred to as "M" and "R" forms, respectively. De Guiran and Bruguier (1989) noted the range of tail shape and reported that populations of the mucronate tail forms and round tail forms showed some correlation with pathogenicity, i.e. the "R" form is the pathogenic form. Panesar and Sutherland (1989) reported that mortality on *P. sylvestris* and *P. contorta* seedlings occurred more rapidly following inoculation with the "R" form than with the "M" form of the PWN. However, different species of pines showed different degrees of susceptibility to the "M" and "R" forms.

It became apparent that the use of the mucronate tail, even in combination with other morphological characters, was an inadequate

taxonomic tool for separating populations of *B. xylophilus* and *B. mucronatus*, and as a result different experimental approaches were used. Cross-breeding studies on *Bursaphelenchus* spp. provided some answers to the relative distinctiveness of some populations and species but also raised other questions on the relationships between the same species and populations. Cross-breeding experiments between individuals of populations of *B. xylophilus* and *B. mucronatus* showed that F₁ progenies were produced but their numbers were low and some of them were aberrant (Mamiya, 1986). Another approach was the use of enzyme electrophoresis to detect differences between *B. xylophilus* and *B. mucronatus* populations (de Guiran, *et al.*, 1985). However, Kiyohara and Bolla (1990) reported that isozyme patterns of *Bursaphelenchus* spp. cannot separate virulent populations from avirulent populations. The enzyme electrophoresis approach was not definitive, because proteins of closely related species are highly conserved, and their expression is modified by environmental factors. The uncertainty in the taxonomic separation of *B. xylophilus* and *B. mucronatus* populations resulted in 'super species' (de Guiran and Boulbria, 1986), and 'supraspecies' (de Guiran and Bruguier, 1989) descriptors. For supraspecies it was hypothesized that the Japanese *B. mucronatus* and the American *B. xylophilus* populations originated from a Western European population and that the American *B. xylophilus* population was recently introduced to Japan (de Guiran and Bruguier, 1989). The previous hypothesis supports that proposed by Mamiya (1983). In using another descriptor it was hypothesized by Rutherford *et al.* (1990) that the 'pinewood nematode species complex' may be exchanging genetic material either directly between member species or through intermediate forms.

Recombinant DNA technology has been used more recently for the taxonomic separation of *B. xylophilus* and *B. mucronatus* populations. The taxonomic status of some populations was partly clarified using restriction enzyme analysis and hybridization with total genomic DNA (Bolla, *et al.*, 1988), homologous ribosomal probes (Webster *et al.*, 1990; Tares *et al.*, 1992), and the heterologous probe of *unc-22* DNA from *Caenorhabditis elegans* (Abad *et al.*, 1991). In addition, polymerase chain reaction and direct sequencing techniques

have been used (Beckenbach *et al.*, 1992). Results from these cross hybridization studies and molecular studies do not fully concur. For example, de Guiran and Bruguier (1989) reported that the French populations of *B. mucronatus* interbred with both *B. xylophilus* and *B. mucronatus* from Japan even though these two species are reproductively isolated.

It became clear, that no one single technique could satisfactorily differentiate populations of *Bursaphelenchus* species or demonstrate taxonomic relationships within this genus. Kiyohara and Bolla (1990) studied various populations of *Bursaphelenchus* spp. from conifers in the United States and Japan using different ecological, biochemical and genetic markers in order to understand their population biology, genetics and speciation. However, their work is not complete in that it does not include populations of *Bursaphelenchus* spp. from Canada and Europe.

In order to clarify the taxonomic relationships of *B. xylophilus* and *B. mucronatus* it is necessary to evaluate and relate certain biological and genetic characteristics of a range of geographic isolates. The overall purpose of my study is to use cross-hybridization, pathogenicity, DNA analysis and chemical communication studies to explain the biological and taxonomic relationships between *B. xylophilus* and *B. mucronatus* at the subspecific level.

General Materials and Methods

Five geographic populations of *Bursaphelenchus xylophilus*, three Canadian, namely St. John (SJ), St. William (SW), and Q1426 (Q14), one Japanese, Ibaraki (Ib), and one from the USA, MSP4 (MSP4) and two populations of *B. mucronatus*, a Japanese, Chiba (Ch) and a French (Fr) were reared in Parafilm-sealed Petri dishes of *Botrytis cinerea* grown in the dark on 1% potato dextrose agar (PDA) plates at 27°C. The origin of each population is shown in Table 1. Isolates of these populations were subcultured once per week onto

PDA Petri dishes that had been inoculated with *B. cinerea* one week previously. Nematodes were viewed using a dissecting microscope. All laboratory procedures involving nematode cultures were done with standard aseptic conditions. To meet quarantine regulations, objects that came in contact with the PWN, i.e. waste culture Petri dishes, pipettes, infected plant material, gloves, coverslips and the PWN itself were autoclaved prior to disposal.

Nematodes used in experiments were rinsed off the lid of the Petri dish with sterile distilled water. The nematode suspension was filtered through a coarse nylon screen to remove the dead nematodes, and then concentrated by gravity in 100 ml graduated cylinders for 2 hours.

Isolates and hybrids of *Bursaphelenchus* spp. that were not used regularly in the laboratory, were cryopreserved at -180°C in liquid N_2 (Riga and Webster, 1991). Concentrated aqueous suspensions of the nematodes washed off the Petri dish lids were placed into 2.0 ml screw-top tubes (Simport plastics, T500-2) with 1 ml of 15% glycerol (Caledon, code 5350-1), 50% M9 buffer, and 35% S buffer per tube (as described by Brenner, 1974) and frozen according to the method of Brenner (1974) modified by D. L. Baillie (pers. comm., Simon Fraser University) as follows: The nematodes were stored in tubes for 24 h at -70°C and then submerged in a 35 VHG liquid nitrogen tank (Union Carbide) at -180°C . With this method, sufficient numbers of nematodes of any of the isolates and hybrids were available within a few days of thawing to initiate new, vigorously reproducing populations. Nematodes that had been cryopreserved, were used to infect 3-year-old *P. sylvestris* seedlings to ascertain if these nematodes maintained their ability to infect and be pathogenic to pine seedlings.

In the cross-hybridization study, the data were analyzed using a single factor analysis of variance by Ranks (Mann-Whitney-Test). In the pathogenicity study, the data were analysed using Chi-square analysis, single factor analysis of variance and linear regression analysis. In the pheromone study, results were analysed using single factor analysis of variance and a least significance test of F-value for multiple comparisons. For all statistical analysis, significance was stated at the 95% confidence level (Devore, 1987).

Table 1

Description of *Bursaphelenchus xylophilus* and *B. mucronatus* populations

Population	Habitat	Origin	Source
<i>B. xylophilus</i>			
Q1426 (Q14)	<i>Abies balsamea</i>	Canada (Quebec)	I
St. John (SJ)	woodships	Canada (NB)	I
St. William (SW)	nursery	Canada (Ontario)	I
MSP4 (MSP4)	unknown	USA (Missouri)	II
Ibaraki (Ib)	<i>Pinus thunbergii</i>	Japan	IV
<i>B. mucronatus</i>			
French	unknown	France	III
Chiba	<i>P. thunbergii</i>	Japan	IV
Source abbreviations: I = R. V. Anderson, II = R. I. Bolla, III = G. de Guiran and IV = Y. Mamiya			

SECTION I

CLASSICAL AND CONTEMPORARY APPROACHES TO DIFFERENTIATING ISOLATES OF *BURSAPHELENCHUS* *XYLOPHILUS* AND *B. MUCRONATUS*

INTRODUCTION

A species, according to Mayr (1970), is a protected gene pool of actually or potentially interbreeding individuals, and it has its own isolating mechanisms against harmful gene flow from other gene pools. These isolating mechanisms can either be pre-mating or postmating. However, not all isolating mechanisms are perfect at all times and this results in interbreeding or hybridization.

Hence, hybridization of individuals of putative species is a reliable method of assessing the degree of biological compatibility of the organisms. Mamiya (1986) reported that *B. xylophilus* and *B. mucronatus* in Japan are distinct species because the F₁ generation of their interspecific hybrid is not viable, thus fulfilling an essential criterion of a biological species. However, de Guiran and Boulbria (1986) found that fertile offspring were produced from crosses of a French population, identified as *B. mucronatus*, with a Japanese population of *B. mucronatus* and with a Japanese population of *B. xylophilus*. The French population also cross-hybridized with a North American population of *B. xylophilus* from Minnesota (U.S.A.), but only when the parental generation was left with the F₁ offspring. They concluded that *B. xylophilus* and *B. mucronatus* are members of a "super species". De Guiran and Bruguier (1989) proposed the "circle of species" hypothesis in which the original stock of *B. mucronatus* existed before the separation of the North American and the Eurasian plates. This resulted in the existence of the intersterile species, i.e. *B. mucronatus* and *B. xylophilus*, in Japan and North America, respectively. Although these species are intersterile they have both retained breeding compatibility with the original stock of Western Europe. The authors proposed that

populations of *B. mucronatus* and *B. xylophilus* belong to the supraspecies category. Recently, Rutherford *et al.* (1990) referred to *B. xylophilus* and *B. mucronatus* as being members of the pine wood nematode species complex (PWNSC) and cited biological evidence to support this claim. The results of cross-hybridization experiments differ from one laboratory to another, probably due in part to differences in the prevailing environmental conditions (Dobzhansky, *et al.*, 1977) or to the populations used.

Bolla *et al.* (1988) hybridized total genomic DNA from North American *B. xylophilus* population and Japanese *B. mucronatus* with genomic DNA from one of the populations. The hybridization patterns showed differences between *B. xylophilus* and *B. mucronatus* populations. In addition, it was reported that *B. xylophilus* populations from different hosts were genotypically different. These populations, except the French *B. mucronatus*, were used in another study by Kiyohara and Bolla (1990) in which the total cellular DNA of *Bursaphelenchus* spp. populations was hybridized to the total cellular DNA of one of the populations. Restriction fragment length polymorphism analysis showed that *B. xylophilus* populations could be differentiated from *B. mucronatus* populations, and that some geographic populations of *B. xylophilus* could be differentiated from each other (Kiyohara and Bolla, 1990). In other attempts to compare species and populations of *Bursaphelenchus*, Webster *et al.* (1990) used the non-transcribed spacer region from the ribosomal cistron from one of the *Bursaphelenchus* populations to provide evidence for the separation of *Bursaphelenchus* spp. isolates from North America, Europe, Japan and China into a *B. xylophilus* group and *B. mucronatus* group. In addition, the populations of *B. mucronatus* from Japan showed restriction site differences from the European populations. Hence, it was suggested that the *B. mucronatus* group consists of two subgroups (Webster *et al.*, 1990). Abad *et al.* (1991) used a heterologous probe of *unc-22* gene of *Caenorhabditis elegans* to separate populations of *Bursaphelenchus* spp. from Japan, United States and Europe into a *B. xylophilus* group and into a *B. mucronatus* group. Similarly, Tares *et al.* (1992) reported that homologous DNA probes separated geographical populations of *Bursaphelenchus* spp.

into three geographical subgroups i.e. United States, Canada and Japan that belong in the *B. xylophilus* group and a *B. mucronatus* group from Japan and Europe. Abad *et al.* (1991) and Tares *et al.* (1992) concluded, contrary to Webster *et al.*, (1990), that the European and the Japanese populations of *B. mucronatus* are the same species. Beckenbach *et al.* (1992) reported a detailed study, using polymerase chain reaction and direct DNA sequencing, on several populations of *Bursaphelenchus* spp. from North America, Japan and Europe. Their results indicated that the *Bursaphelenchus* spp. populations could be separated into three distinct groups, a *B. xylophilus* group from North America and Japan, a *B. mucronatus* from Japan and, contrary to Abad *et al.* (1991), a "*B. mucronatus*" group from Europe. The evidence of Beckenbach *et al.* (1992) strongly supports the concept of separate species status for the European and Japanese "*B. mucronatus*" populations but these molecular results must be supported by biological and morphological data before species status can be confirmed.

Geographic populations from North America have a female tail shape that ranges from mucro-type to round-type (Wingfield *et al.*, 1983). These have been referred to as "M" and "R" forms, respectively. De Guiran and Bruguier (1989) reported that the mucronate tail forms and round tail forms showed some correlation with pathogenicity. Panesar and Sutherland (1989) reported that mortality on *P. sylvestris* and *P. contorta* seedlings occurred more rapidly following inoculation with the "R" form than with the "M" form of the PWN. Other species of pines however showed different degrees of susceptibility to the "M" and "R" forms. Panesar and Sutherland (1989) reported that Scots pine seedlings were highly susceptible to PWN populations from Alberta, "R" form, and British Columbia, "M" form, and moderately susceptible to two "M" form populations from Quebec, while the susceptibility of Scots pine to the "R" form and the "M" form from Missouri and Quebec, respectively, were similar. In addition, they reported that lodgepole pine was highly susceptible to the "R" form and moderately susceptible to the "M" form of these populations. Regardless of the nature of the host response, large numbers of "M" and "R" form nematodes were found in the tissues of both Scots pine and lodgepole pine.

Although previous authors developed a helpful analysis and working hypothesis, the pathogenicity of *Bursaphelenchus* spp. on pines is known to be influenced by environmental factors, such as temperature, topography, vector distribution and distribution of susceptible tree species. In the susceptible pines of Japan, pine wilt symptoms are more severe under conditions of high temperature and low rainfall. Severe pine death due to PWN has been correlated with drought-stressed pines and average air temperatures above 25°C for 55 days (Takeshita *et al.*, 1975). The Japanese pines, *P. thunbergii* and *P. densiflora* are found both in locations where the PWN is pathogenic and where it is not. The most important difference between the two locations is the temperature. In other words pines do not develop pine wilt symptoms in areas above 700 m elevation (Mamiya, 1984), because the average daily temperature at that altitude is approximately 3.5°C lower than that at sea level (see Rutherford and Webster, 1987). Pine wilt disease in Japan is widespread in southern coastal areas and in inland at low elevation, where average August air temperatures are greater than 25°C (Mamiya, 1984). Rutherford and Webster (1987) hypothesized that the pine wilt disease has influenced the natural distribution of some conifer species in North America and Europe. A prevailing doubt that arises is the degree of pathogenicity of different *Bursaphelenchus* spp. populations on trees of different ages and species under different environmental conditions.

Previous reports have been on individual aspects of the biology and the taxonomic relationships of populations of *B. xylophilus* and *B. mucronatus*, and the data are sometimes contradictory. In order to obtain a clear picture of the relationships of *B. xylophilus* and *B. mucronatus*, various populations from various geographic locations, i.e. North America, Japan and Europe, must be cross-hybridized in order to assess the degree of their biological compatibility. Once there is an indication of a biological compatibility between *B. xylophilus* and *B. mucronatus*, then their isolates must be tested for their ability to cause disease in a susceptible host, e.g. *P. sylvestris* seedlings. Subsequently, molecular analysis of genomic DNA should reinforce the data from similar experiments done previously. Some of the previous molecular studies were done using probes other than ribosomal DNA

(rDNA), whereas I used rDNA as a probe. One of the reasons that rDNA is useful for phylogenetic analysis is that its different regions evolve at different rates, so regions of rDNA can be selected to address almost any systematic and phylogenetic question (Hillis and Dixon, 1991). Experiments similar to the ones that I proposed to do have been done previously but with different geographic populations, under different experimental conditions in different laboratories. By measuring and comparing a select range of phenotypic and genotypic characters of representative nematode populations under controlled conditions, resolution of the taxonomic debate should be possible.

My objective was to provide new information derived from intra- and interspecific cross-hybridizations, from genomic DNA analysis of the parental populations and their intra- and interspecific hybrids using rDNA probes, and from pathogenicity studies to clarify the biological and taxonomic relationships between *B. xylophilus* and *B. mucronatus* and between French and Japanese populations of *B. mucronatus*. A supplementary objective was to determine whether or not there is a correlation between pathogenicity and female tail shape of *B. xylophilus* populations.

MATERIALS AND METHODS

Cross-hybridization

Fourth stage juveniles (J_4) of each sex from each of the seven *Bursaphelenchus* spp. populations (Table 1) were hand picked with a fine glass rod. One J_4 male and one J_4 female were put together in a 6.0 cm diameter Petri dish that was covered with *B. cinerea* and maintained at 29°C. In order to be able to see the nematodes, *B. cinerea* was grown on 15 g agar and 0.64 g potato dextrose broth (i.e. a dilute medium that allows the fungus to grow on a thin layer) for 5 days at 25°C. Intra- and interspecific hybridizations and reciprocal crosses of populations of *B. xylophilus* and *B. mucronatus* were performed with all seven populations on *B. cinerea* Petri dishes. Successful cross-hybridizations (i.e. viable and fertile offspring were produced that gave

rise to subsequent fertile generations) were repeated 10 times, while unsuccessful crosses (i.e. the F_1 or the subsequent generations died out) were replicated 20 times. Intra-population cross-hybridizations also were replicated 10 times. They served as a control and standard for evaluating fecundity of the parental stocks. The parents of *B. xylophilus* and *B. mucronatus* were removed from the Petri dish 4 days after the first day of egg-laying in order to avoid back-crossing with their F_1 progeny. Subsequent inbred generations of each cross-hybridization were reared under the same conditions and monitored. For logistical reasons, and to ensure that there was consistency for all matings, the number of progeny per mating was measured as the number of offspring produced 24 hours following the first oviposition. All observations in this study were performed under a dissecting microscope. The data on the number of progeny of the F_1 generation were analysed using the non-parametric Mann-Whitney-Test (Devore, 1987).

Pathogenicity

All isolates from *B. xylophilus* and *B. xylophilus* populations and some of their intra- and interspecific hybrids that were successfully produced during the cross-hybridization experiment, (f=female, m=male) lb m x SW f, MSP4 m x SW f, SJ m x SW f, Q14 m x SW f, Fr m x SW f, Ch m x SW f, Ch m x SJ f, and Ch m x Fr f, were used to infect 3-year-old Scots pine, *Pinus sylvestris* L., seedlings. Each of 23 Scots seedlings in a batch was inoculated with 2,500 nematodes of one of the parental isolates or their intra- and interspecific hybrids. The nematodes were inoculated into the seedlings according to Panesar and Sutherland (1989) by removing a 20 x 3-5 mm section of the seedling bark on the upper third of the main stem to expose the xylem. A sterile, absorbent cotton ball was placed on the wound and wrapped with parafilm to form a water-tight environment. The nematodes were pipetted onto the cotton. After 72 h the cotton and parafilm were removed, and the wound was wrapped again in parafilm. For controls, 10 seedlings received sterile, distilled water, and 15 seedlings received

B. cinerea filtrate. The seedlings were maintained in a greenhouse conditions according to Panesar and Sutherland (1989), with 18 h light at 28°C and 6 h dark at 23°C. They were watered as needed and observed daily for disease development.

The following observations were recorded: (i) time of appearance of the first symptom of pine wilt disease, i.e. browning of the needles and wilting of all new growth, (ii) days to seedling death, (iii) number of seedlings killed per parental isolate of *B. xylophilus* and *B. mucronatus* and their intra- and interspecific hybrids, and (v) number of nematodes extracted from all seedlings that showed irreversible wilt symptoms and from randomly selected seedlings that did not show symptoms. Seedlings, i.e. roots, stems, branches and needles, were weighed. They then were cut into 2 cm long segments, and the nematodes extracted for 24 h in Baermann funnels. Nematodes in 1 mL of aliquot were counted and expressed as a mean number of three aliquots as nematodes per gram of fresh weight of tissue, from a mean of 3 aliquots per sample.

Chi-square tests (Devore, 1987) were used to analyse the data on numbers of Scots pine seedlings killed by *Bursaphelenchus* nematodes and to compare the pathogenicity of *B. xylophilus* and *B. mucronatus* isolates and their inter- and intraspecific hybrids. Analysis of variance and a least significance test of F-value for multiple comparisons (Devore, 1987) were used to determine the significance of data on the mean number of days to seedling death. Linear regression analysis (Devore, 1987) was used to determine if a relationship existed between (i) the appearance of the first symptoms of pine wilt versus days to seedling death and (ii) number of nematodes present in a seedling versus the day of its death and versus seedling tissue weight (Devore, 1987).

DNA Analysis

Genomic DNA was extracted and purified (as described by Webster *et al.*, 1990) from several plates of nematodes of each of *B. xylophilus* and *B. mucronatus* parental isolates and of each of the

following hybrids which produced viable offspring in the cross-hybridization study and all of which had been tested for their ability to infect 3-year-old *P. sylvestris* seedlings (pathogenicity experiment), lb m x SW f, MSP4 m x SW f, SJ m x SW f, Q14 m x SW f, Fr m x SW f, Ch m x SW f, Ch m x SJ f, and Ch m x Fr f.

Approximately 2 µg of DNA was digested with the restriction endonucleases HindIII and Sall (Boehringer, Mannheim). The enzymes were used as suggested by the manufacturer. The digested genomic DNA was size fractionated on 0.7% agarose electrophoretic gels (Davis *et al.*, 1980), transferred to nylon membranes (Amersham Hybond-N) by the bidirectional transfer method of Smith and Summers (1980) and hybridized to the ³²P labelled ribosomal gene probe pBx2 (Webster *et al.*, 1990) from the Japanese population Ibaraki of *B. xylophilus*. The hybridization conditions were 62°C in 5x SSPE [1x SSPE = 180 mM NaCl, 10 mM (Na_{1.5})PO₄, 1 mM Na₂EDTA pH(7.0)], 0.3% SDS, and 5x Denhardtts [1x Denhardtts = 0.02% w/v of bovine serum albumin, Ficoll 400, and polyvinyl pyrrolidone 40] (Davis *et al.*, 1980). After the overnight hybridization the filters were washed at 45°C in 2x SSPE + 0.2% SDS, air dried, and exposed to X-ray film (Kodak X-Omat K) with intensifying screen (Dupont Lightning Plus) at -80°C for 24 hours.

The DNA band pattern of the hybridizing genomic DNA fragments of *B. xylophilus* and *B. mucronatus* and of their hybrids were compared visually. This DNA analysis was repeated three times with different DNA samples.

RESULTS

Cross-hybridization:

Bidirectional male/female crosses of all isolates of *B. xylophilus* populations produced fertile offspring (F₁). The average number of F₁ offspring, newly hatched juveniles, produced after one day of egg laying ranged from 2.9 to 17.8 (Table 2). The F₁ gave rise to subsequent F_n generations which established populations of several million individuals. There were no significant differences between the mean number of

offspring produced on the first day of egg laying resulting from *B. xylophilus* SJ, SW, lb and Q14 parents and the intraspecific crosses of the parent isolates. However, there was a significant difference between the mean number of offspring produced on the first day of egg laying by the MSP4 parents as compared with those from the MSP4 m x SJ f, and MSP4 m x SW f intraspecific hybrid crosses ($P < 0.05$), but not between the MSP4 parents and the other intraspecific crosses. The cross-hybridization of the male Ch to the female Fr, both *B. mucronatus* isolates, produced subsequent F_n generations. There was no significant difference between the mean number of offspring produced on the first day of egg laying resulting from the intraspecific cross of the parental Ch isolate and the Ch m x Fr f hybrid. However, the reciprocal cross, Fr m x Ch f produced an F_1 generation that died out (Table 2).

Males of *B. mucronatus* Ch isolate cross-hybridized successfully with females of each *B. xylophilus* isolate, producing several fertile generations. There were no significant differences between the mean number of offspring produced on the first day of egg laying resulting from Ch parents in comparison with those from the Ch m x lb f, Ch m x Q14 f and Ch m x MSP4 f intraspecific hybrids. There were significant differences between Ch parents compared with the Ch m x SJ f, and Ch m x SW f intraspecific hybrids ($P < 0.05$). However, the reciprocal crosses between a female *B. mucronatus* Ch isolate and a male of each of the *B. xylophilus* isolates failed to become established and the F_1 died out. However, the SW m x Ch f cross died out after 10 generations (Table 2). There were a significant differences between the mean number of offspring produced on the first day of egg laying by the SJ, lb, Q14 and MSP4 parents as compared with their interspecific crosses with the Ch parent ($P < 0.05$), with the exception of the number of progeny derived from the SW parent in comparison with those from the SW m x Ch f interspecific cross (Table 2). Single females of *B. mucronatus* Fr isolate when mated with single males of each of the three Canadian *B. xylophilus* SW, SJ, Q14 isolates failed to produce viable F_1 offspring, but interbred successfully with *B. xylophilus* MSP4 and lb males from the United States and Japan respectively (Table 2). Significantly less nematodes were produced on the first day of egg laying resulting from the crosses between *B. xylophilus* SJ, SW, Q14

Table 3

Results of inoculating 3-year-old *Pinus sylvestris* seedlings with *Bursaphelenchus xylophilus* and *B. mucronatus* isolates and their intra- and interspecific hybrids

Isolates and hybrids of <i>B. xylophilus</i> and <i>B. mucronatus</i>	Numbers out of 23 seedlings killed	Days to first symptoms (mean \pm SE)	Days to seedling death (mean \pm SE)	Number of nematodes per gram wet tissue (mean \pm SE)
Ibaraki (ib)	23	25.2 \pm 1.9	54.0 \pm 3.0	885.0 \pm 176.8
St. William (SW)	22	22.3 \pm 1.7	55.0 \pm 2.6	621.7 \pm 119.5
MSP4 (MSP4)	22	20.2 \pm 2.1	56.5 \pm 3.8	791.9 \pm 197.0
St. John (SJ)	21	25.2 \pm 1.9	56.5 \pm 2.2	616.0 \pm 124.1
Q1426 (Q14)	20	22.7 \pm 2.6	60.6 \pm 3.0	471.4 \pm 93.0
French (Fr)	12	30.8 \pm 0.4	68.7 \pm 2.0	346.0 \pm 110.5
Chiba (Ch)	6	18.5 \pm 6.2	64.2 \pm 8.5	120.6 \pm 63.9
Ib m x SW f	21	21.7 \pm 2.4	57.0 \pm 2.6	728.0 \pm 148.6
MSP4 m x SW f	23	23.7 \pm 2.5	57.6 \pm 2.4	447.1 \pm 78.8
SJ m x SW f	21	23.3 \pm 1.6	60.3 \pm 1.9	520.1 \pm 86.7
Q14 m x SW f	20	27.5 \pm 3.0	63.5 \pm 2.4	491.0 \pm 96.8
Fr m x SW f	20	22.1 \pm 2.7	63.2 \pm 3.6	573.3 \pm 109.3
Ch m x SW f	15	32.6 \pm 3.5	63.5 \pm 3.1	374.7 \pm 105.3
Ch m x SJ f	22	23.4 \pm 2.1	56.6 \pm 2.2	414.8 \pm 6.3
Ch m x Fr f	16	19.4 \pm 4.2	70.8 \pm 3.4	577.2 \pm 244.1
H ₂ O control*	0			
<i>B. cinerea</i> filtrate **	0			

* based on 10 inoculated seedlings, *** based on 15 inoculated seedlings

and MSP4 parents and each of their interspecific crosses with the *B. mucronatus* Fr parent ($P < 0.05$) (Table 2). However, the MSP4 m x Fr f hybrid produced viable F_1 progeny, while the others did not. There was no significant difference between the mean number of nematodes produced on the first day of egg laying resulting from the *B. xylophilus* lb parents in comparison with that from the lb m x Fr f interspecific hybrids (Table 2).

Pathogenicity:

Numbers of seedlings killed by isolates of *B. xylophilus* SJ, SW, lb, MSP4 and Q14, and *B. mucronatus* Ch and Fr, and their intraspecific and interspecific hybrids lb m x SW f, MSP4 m x SW f, SJ m x SW f, Q14 m x SW f, Fr m x SW f, Ch m x SW f, Ch m x SJ f, and Ch m x Fr f, are shown in Fig. 1 and Table 3. The parental isolates of *B. xylophilus* lb, SW, MSP4, SJ and Q14, and the intraspecific hybrids lb m x SW f, MSP4 m x SW f, SJ m x SW f, Q14 m x SW f killed 86.9%-100% of the infected pine seedlings. Both *B. xylophilus* parental isolates and their hybrids were equally pathogenic to the seedlings (Fig. 1, Table 3). All isolates of *B. xylophilus* populations killed significantly more ($P < 0.05$) seedlings than did the *B. mucronatus* Ch and Fr isolates (Fig. 1, Table 3). The French isolate of *B. mucronatus* Fr killed significantly more ($P < 0.05$) seedlings than did the Japanese isolate of *B. mucronatus* Ch (Fig. 1, Table 3). The intraspecific hybrid Ch m x Fr f, killed significantly more ($P < 0.05$) seedlings than did the Ch parent but did not kill significantly more than the Fr parental isolate (Fig. 1, Table 3). Interspecific hybrids of *B. xylophilus* and *B. mucronatus* Fr m x SW f, Ch m x SW f, Ch m x SJ f, killed significantly more ($P < 0.05$) seedlings than did the *B. mucronatus* Fr and Ch parental isolates but not significantly more than did the *B. xylophilus* parental isolates SW and SJ (Fig.1, Table 3).

There was a significant difference ($P < 0.05$) between the mean number of days to seedling death caused by isolates of *B. xylophilus* SJ, SW, lb, MSP4 and Q14 and *B. mucronatus* Ch and Fr (Table 3). Isolates of *Bursaphelenchus xylophilus* populations killed pine

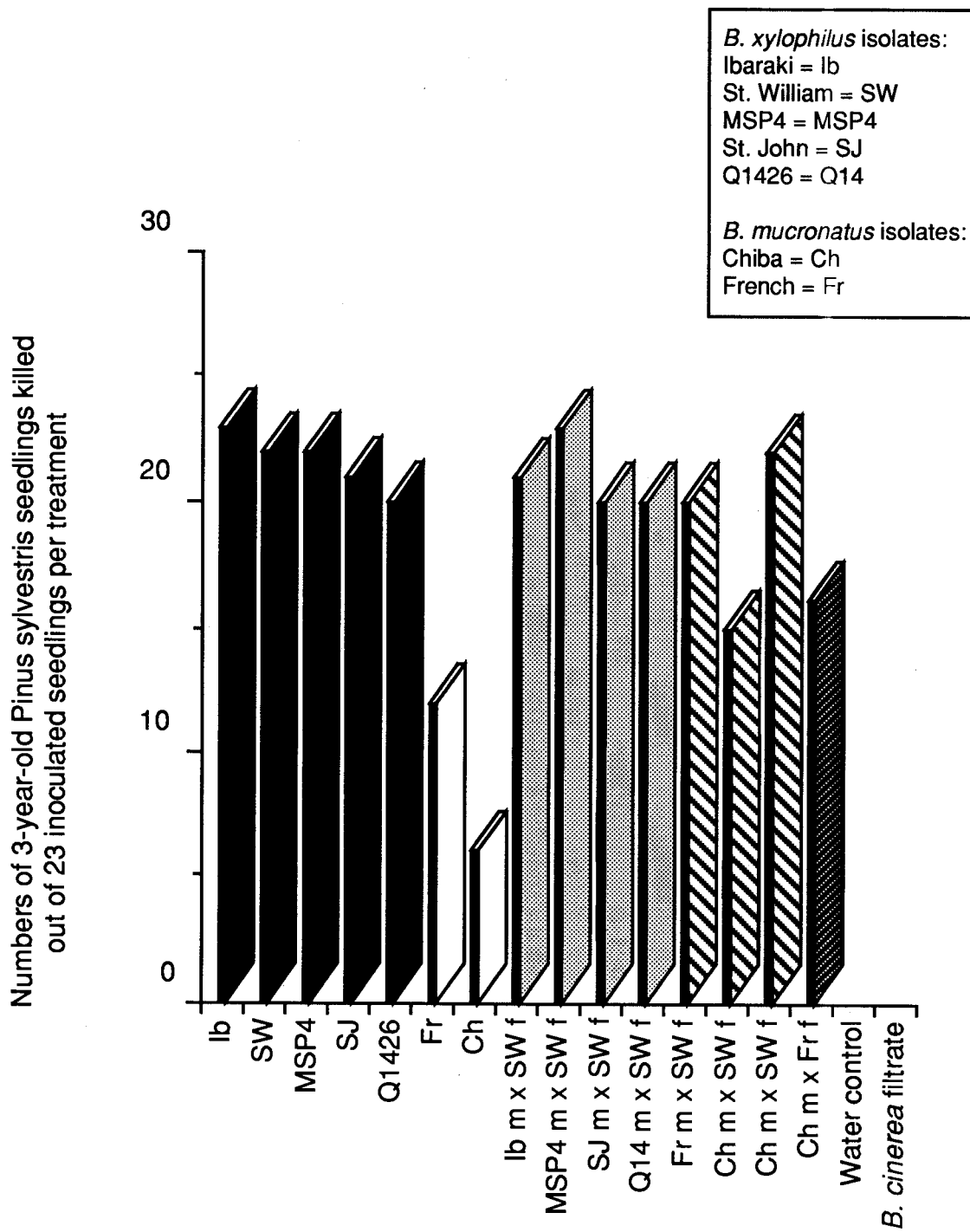
Table 3

Results of inoculating 3-year-old *Pinus sylvestris* seedlings with *Bursaphelenchus xylophilus* and *B. mucronatus* isolates and their intra- and interspecific hybrids

Isolates and hybrids of <i>B. xylophilus</i> and <i>B. mucronatus</i>	Numbers out of 23 seedlings killed	Days to first symptoms (mean \pm SE)	Days to seedling death (mean \pm SE)	Number of nematodes per gram wet tissue (mean \pm SE)
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H ₂ O control*	0			
<i>B. cinerea</i> filtrate **	0			

* based on 10 inoculated seedlings, *** based on 15 inoculated seedlings

Figure 1. Numbers of 3-year-old *Pinus sylvestris* seedlings killed by isolates and intra- and interspecific hybrids of *Bursaphelenchus xylophilus* and *B. mucronatus*; 23, 10 and 15 seedlings were infected with nematodes, water or *Botrytis cinerea* filtrate, respectively (f = female, m = male).



Isolates and intra- and interspecific hybrids of
Bursaphelenchus xylophilus and *B. mucronatus*

seedlings significantly faster than did isolates of *B. mucronatus* populations or the intraspecific *B. mucronatus* Ch m x Fr f hybrid. Linear regression analysis showed a dependent relationship, r^2 values ranging from 0.13 to 0.55 (Appendix I) between the initial appearance of pine wilt symptoms, i.e. foliage browning and wilting, and days to seedling death for *B. xylophilus* isolates, their intraspecific hybrids and most of the interspecific hybrids. However, the Ch m x SW f interspecific hybrid and *B. mucronatus* Ch m x Fr f intraspecific hybrid showed no such relationship (Appendix). No relationship was found between number of nematodes in seedling tissues and the day of seedling death except for *B. xylophilus* Q14 m x SW f intraspecific hybrid, $r^2=0.31$ (Appendix II) and the intraspecific hybrid of *B. mucronatus* Ch m x Fr f, $r^2=0.21$ (Appendix II). There was a dependent relationship, r^2 values ranging from 0.48 to 0.68 (Appendix III), between the number of nematodes of *B. xylophilus* Q14, SW, SJ isolates, their intraspecific hybrids lb m x SW f, SJ m x SW f, *B. mucronatus* Ch isolate, and the interspecific hybrid Ch m x SJ f, present in the seedlings versus seedling tissue weight.

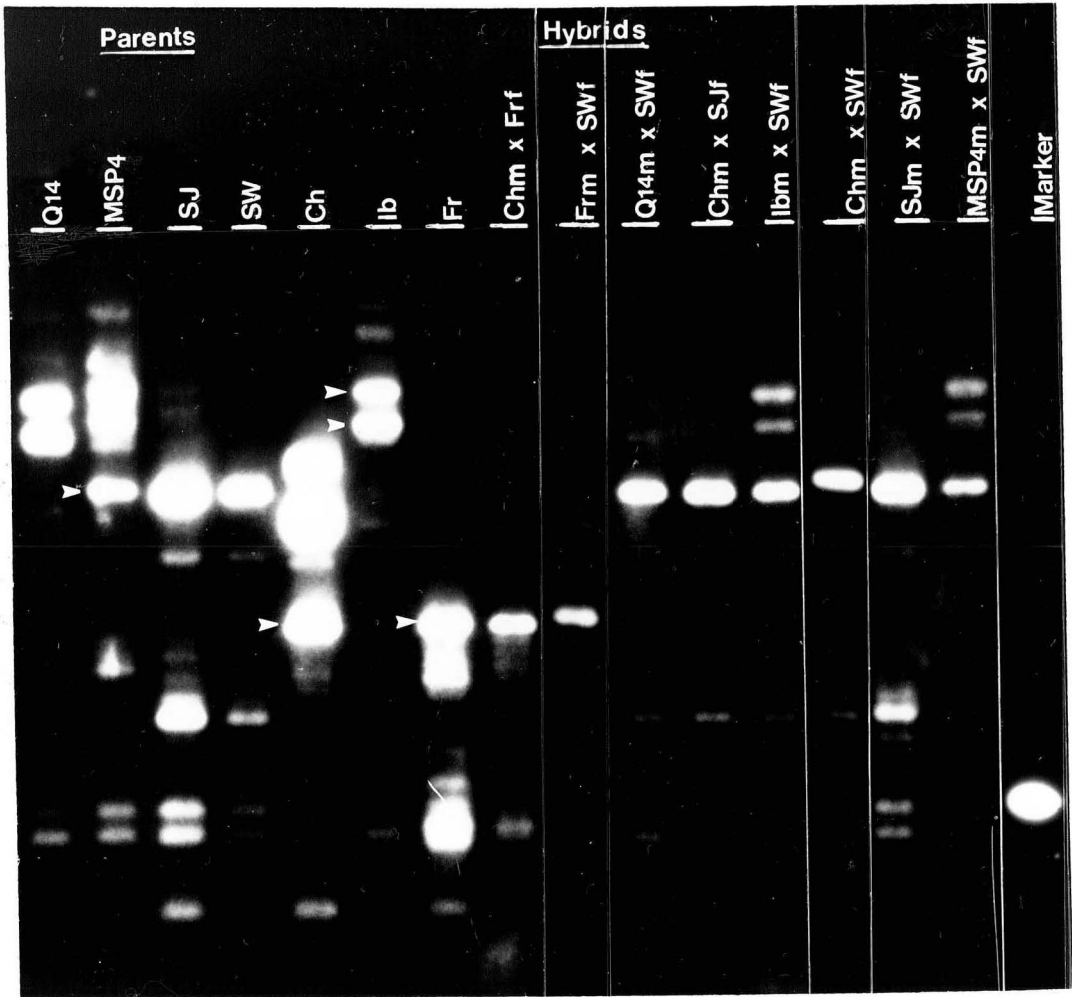
DNA Analysis:

The homologous ribosomal gene probe detected restriction fragment length polymorphism (RFLP) between isolates of *B. xylophilus* Q14, MSP4, SJ, SW and lb populations, and between isolates of *B. mucronatus* Ch and Fr populations. These RFLP's were used to determine whether the components of the genome from both parental populations were present in the inbred hybrid progeny (F_n) (Fig. 2).

Genomic DNA from *B. xylophilus* Q14 m x SW f, lb m x SW f, SJ m x SW f, and MSP4 m x SW f intraspecific hybrids showed RFLP patterns similar to that of both of the respective parental populations. The DNA from *B. mucronatus* Ch m x Fr f intraspecific hybrid showed mostly the Fr RFLP pattern, but one band is shared between the two isolates (Fig. 3). The DNA from the interspecific hybrids Ch m x SJ f and Ch m x SW f (*B. mucronatus* x *B. xylophilus*) showed only the RFLP pattern from the *B. xylophilus* SW parent and the interspecific

hybrid Fr m x SW f showed the RFLP pattern from both *B. xylophilus* SW (very faintly) and *B. mucronatus* Fr parental isolates (Fig. 2).

Figure 2. Composite of total genomic DNA from *Bursaphelenchus xylophilus* (Q14, MSP4, SJ, SW and Ib), and *B. mucronatus* (Ch and Fr) isolates and their hybrids digested with HindIII and Sall, then probed with the homologous total ribosomal gene clone pBx2. The first seven lanes from the left are the parental isolates and the remaining lanes are the intra- and interspecific hybrids. The arrows indicate bands that can be used to identify parental genome types (f = female, m = male).



SECTION II

THE PINEWOOD NEMATODE PHEROMONE AS A TAXONOMIC TOOL FOR SPECIES DIFFERENTIATION

INTRODUCTION

Pheromones are chemicals that are divided, on the basis of an organism's response, into aggregation pheromones and sex pheromones. Aggregation pheromones function among individuals of both sexes in the same species and among juveniles, i.e. dauer-inducing pheromones which serve as a measure of the population density (Riddle, 1988). Sex pheromones function between males and females of the same species (Bone, 1982; Huettel, 1986).

Chance encounter mating of obligatory bisexual nematodes would be inefficient. Greet (1964) was the first to report sexual attraction in nematodes, specifically in the free-living nematode *Panagrolaimus rigidus*. This was soon followed by other reports on nematode chemotaxis that suggested sex attractants may be involved in mate location in both animal and plant parasitic nematodes, i.e. *Ancylostoma caninum* (Beaver, *et al.*, 1964), *Trichinella spiralis* (Bonner and Etges, 1967), *Heterodera rostochiensis* (Green, 1966). To date, more than 30 nematode species have been studied for chemical communication. The free-living *Panagrellus silusiae* and *P. redivivus*, the rat hookworm *Nippostrongylus brasiliensis* and the phytoparasitic *Heterodera* species are the nematodes for which we have the most information.

Chemical communication in nematodes can be demonstrated in an aqueous environment in a small arena. The natural environment of the nematode has been used occasionally as a chemical communication arena; for example the chemical communication of *Ancylostoma caninum* (Roche, 1966) was tested using the intestine of a dog and that of *Ditylenchus dipsaci* was shown in leaf tissue (Windrich, 1973). These natural arenas do not ensure however that it is only the pheromone that influences nematode behaviour, because other stimuli are present. Simpler chemical communication arenas have been

devised, using either salt solutions to simulate the intestinal fluid of the nematode's host or water-agar gels to simulate soil water films (Green, 1980).

Nematodes of several different ages have been tested for secretion of sex pheromones. Females of *N. brasiliensis* (Bone and Shorey, 1977), *P. redivivus* (Duggal, 1978) and *Rhabditis pellio* (Sommers *et al.*, 1977) attracted males only after their final moult. Mated females of *N. brasiliensis* (Bone and Shorey, 1977), and *Heligmosomoides polygyrus* (Riga and MacKinnon, 1988) are attractive to chemicals released by their respective males. Cheng and Samoiloff (1971) reported that males of *P. silusiae* were attracted to either virgin or mated females, but there was a large degree of variability in the male response to mated females. Members of *R. pellio*, a free-living nematode, emit sex pheromones throughout their adult lives (Sommers *et al.*, 1977). Homosexual attraction among males and among females has been reported for *H. polygyrus* (Riga and MacKinnon, 1987), this type of attraction is considered an aggregation attraction. However, homosexual chemical communication experiments among males and among females of *N. brasiliensis* showed neither attraction nor repulsion (Bone and Shorey, 1977).

There have been very few attempts to isolate nematode pheromones. Jones (1966) reported that the sex pheromones released by females of *Pelodera teres* was water soluble. Stringfellow (1974) proposed that pulsed-OH ions produced by female *Pelodera strongyloides* attracted their males. Two fractions of the male exudates of *P. redivivus* which did not fractionate from water into ether, were isolated on a Sephadex G.25 column (Balakanich and Samoiloff, 1974). Similarly, sex pheromones of *H. schachtii* and *G. rostochiensis* did not fractionate from water into ether except when the aqueous layer was made either acidic or alkaline. Greet *et al.* (1968) reported that pheromones of *Heterodera rostochiensis* and *H. schachtii* were partly volatile and partly non-volatile, while Cheng and Samoiloff (1971) reported that the female sex pheromone of *P. silusiae* was non-volatile. Bone *et al.* (1979) separated the pheromones of *N. brasiliensis* by gel filtration. They demonstrated three chromatographic fractions with pheromonal activity. One fraction was attractive to both males and

females and it was found in aqueous extract of both males and females and had a molecular weight of more than 500 Daltons. The second fraction with a molecular weight of less than 400 Daltons, was produced by females and was attractive to only males. The third fraction had a molecular weight less than 140 Daltons, i.e. ionic components, and was attractive to only females.

Recently, a single compound was isolated from aqueous extracts of *H. glycines* females that was attractive to males. The extract was purified by a four-step, reverse-phase, high pressure liquid chromatography procedure. The attractive compound that was isolated was identified as vanillic acid by a combination of ultraviolet spectroscopy and chromatography (Jaffe *et al.*, 1989).

Few studies have been done on species specificity of nematode pheromones. Females of *Cylindrocorpus longistoma* and *C. curzii* released pheromones that attracted only their own males (Chin and Taylor, 1969). Males or females of *N. brasiliensis* were attracted to males or females of *H. polygyrous* (= *N. dubius*) or females of *T. spiralis*. However, the pairing was transient and the pattern of sexual attraction typical for *N. brasiliensis* did not take place (Roberts and Thorson, 1977). Females of *G. rostochiensis* attracted more males of their own species than of the closely related *G. pallida* (Green and Miller, 1969). Chemical communication studies among three genetically different strains of the free-living nematode *Panagrellus redivivus* - *Panagrellus silusia* species complex showed that the male response to sex pheromone was strain specific (Balakanich and Samoiloff, 1974). The attractiveness of pheromone secretions of ten *Heterodera* spp. females to males of the same or different species indicated the existence of at least three sub-generic groups (Green and Plumb, 1970). In view of the relative species specificity of nematode sex pheromones, experiments using this phenomenon could assist in the taxonomic separation of closely related species. However, various studies have been done on the species specificity of insect pheromones. For example, Borden *et al.* (1980) reported that the pheromones of the Ambrosia beetles, *Gnathotrichus retusus* and *G. sultatus* are species specific and that the species specificity is maintained by the enantiomeric composition of their pheromone.

Bursaphelenchus xylophilus is amphimictic and the female of copulates throughout her adult life (Mamiya, 1975). Kiyohara (1982) reported that virgin females of *B. xylophilus* attracted males of *B. xylophilus* (S6-1 isolate); however, mated or gravid females of this species are not attractive to males.

My objective was to investigate chemical communication between the sexes, isolates, and species of *Bursaphelenchus*. The species specificity of *Bursaphelenchus* spp. pheromones would be established and the chemical communication behaviour of these nematodes compared. In addition, I propose to investigate the chemical nature of the *B. xylophilus* pheromone.

MATERIALS AND METHODS

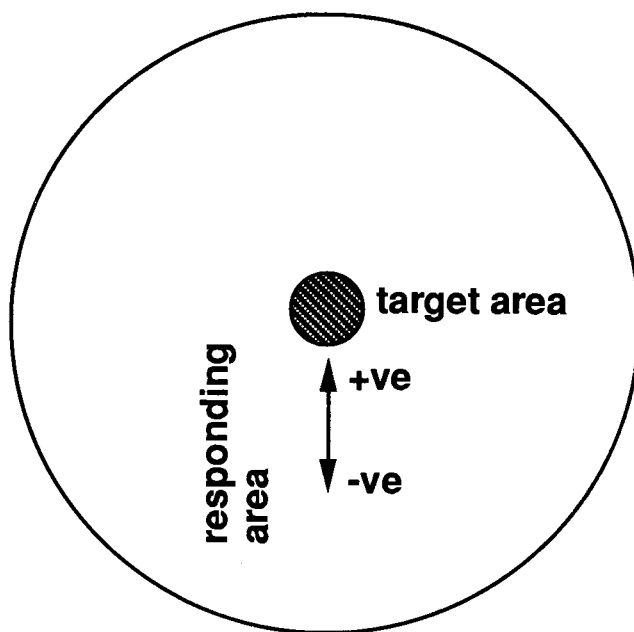
Chemical Communication

Experiments were performed in 6 cm diameter Petri dishes containing a 0.5 cm layer of freshly poured 1.5% agar (Fig.3). This circular arena had a central target area containing a 1 cm diameter plastic filter into which the target nematodes were placed. Test nematodes were released at the origin, a point 1.0 cm from the edge of the Petri dish and 1.5 cm from the target area. The species, isolate, sex and number of target nematodes and test nematodes were varied with each experiment. Female nematodes were not virgin.

Various numbers of the target nematodes were incubated for 1.5 h in the target zone; one test worm was placed at the origin. Its track was recorded one hour later by placing the Petri dish on Ilford (#5 high contrast) black and white photographic paper and exposing it to the light of a photographic enlarger (75 Watt, f22, 10 sec) (Balakanich and Samoiloff, 1974). The distance travelled, i.e. net radial movement (r) toward the target nematodes, was calculated as the difference between the radius of the origin (2.0 cm) and that of the final position. An $r > 0$ was considered a positive response and $r < 0$ was considered a negative response. For controls, no nematodes were placed in the target zone and one male or female was the test nematode.

Figure 3. Diagram of chemical communication arena consisting of a central target area and a responding area. The target area contains a 1 cm diameter plastic filter into which the target nematodes are placed. Test nematodes are released at the origin of the responding area, a point 1.0 cm from the edge of the Petri dish and 1.5 cm from the target zone. The direction of the positive and negative responses are shown.

Petri dish



Chemical communication arena

Four experiments were designed as outlined below. Each treatment and control in each experiment was repeated 20 times using different target and test individuals. Significance was stated at $P < 0.05$ in all cases:

Intra-isolate chemical communication

Target of 0 (control), 1, 5, 10 and 20 *B. xylophilus* SW isolate nematodes, were used with test nematodes of SW. The response of males to females, females to males, males to males and females to females was measured.

Male nematodes of *B. mucronatus* Ch isolate, were tested against 0 (control) or 10 target *B. mucronatus* Ch female.

Intraspecific chemical communication

Intraspecific attraction was investigated among the isolates of *B. xylophilus* using a target of 10 SW female nematodes against males of each of SJ, Q14, Ib or MSP4, as the test nematodes. The reciprocal trials were performed using SW males as the test nematodes and 10 individuals of each of the four previously named isolates as target females. Similarly, intraspecific attraction was investigated among the isolates of *B. mucronatus* using Ch male test nematodes and 10 Fr females as the target nematodes and the reciprocal of Fr male test nematodes and 10 Ch female targets.

Interspecific chemical communication

The response of male *B. mucronatus* Ch or Fr was tested against a target of 10 female *B. xylophilus* nematodes of either Ib, SW or SJ. The reciprocal experiment was performed using *B. xylophilus* males as test worms and 10 *B. mucronatus* females as target nematodes. Also, males of *B. xylophilus* SW or *B. mucronatus* Ch were used as test nematodes with 10 *Aphelenchoides rhyntium* (Aph) or 10 *B. fraudulentus* (Fraud) as the targets.

Chemical nature of the pheromone

Nematodes, 10,000 of *B. xylophilus* SW isolate, were incubated in 1 mL of sterile distilled H₂O for 12 h at 24°C in order to extract any water-soluble, released chemicals. The nematodes then were

separated from the water extract and 2 μ L of this extract, i.e. 10 nematode equivalents, were added in the target area of the bioassay arena (as above) and its biological activity, as measured by its attractiveness to SW males, was assayed. In the control experiment 10 *B. xylophilus* SW females were the target nematodes and one SW male was the tested individual. Different batches of the water extract were stored at -20°C, 4°C, 24°C for 4 days and at 50°C for 24 h and tested for biological activity each day up to 4 days. Newly-prepared water extract was used as a control for each day.

The approximate molecular weight of the *B. xylophilus* SW water extract was estimated using a centricon-3 microconcentrator (Amicon Canada Ltd.) which separates compounds to less than and more than 3,000 Daltons. One mL of water extract was centrifuged for 45 min at 24°C at 5,000 rpm. Two μ L of each of the following fractions were bioassayed for SW male attractiveness: the fraction less than 3,000 Daltons, the fraction more than 3,000 Daltons and both fractions pooled together. The untreated water extract was used as a control.

One mL of the SW water extract was lyophilized, i.e. the water was removed by centrifugation at 4000 rpm at 4°C, and low drying rate in a SpeedVac SVC100 (Savant). The lyophilized sample was reconstituted to the initial volume with distilled water and tested for biological activity, using the SW male attractiveness bioassay. Untreated, non-lyophilized, water extract was used as a control. In addition, the water extract was bioassayed using *B. mucronatus* Ch and Fr males and *B. xylophilus* SJ, and Q14 males as test nematodes. SW males were used as control.

The lyophilized water soluble extract was partitioned with ether into two fractions. The resulting lyophilized ether-soluble fraction, the lyophilized ether-insoluble fraction and both fractions pooled, were tested separately for biological activity using the SW male attractiveness bioassay. An ether blank and lyophilized water-soluble extract served as controls.

Each trial in this experiment was replicated 20 times and each experiment was repeated 3 times.

RESULTS

Chemical communication:

Intra-isolate chemical communication

The net movement in one hour of *B. xylophilus* SW males toward 0 (control), 1, 5, 10 and 20 SW females is shown in Fig. 4a. The greatest net movement of SW males, 0.6 cm, was recorded toward 5 females. Movement of males toward five and 20 target females was significantly different from that in the control. On the other hand, a significant net movement of *B. xylophilus* SW females toward SW males, 0.33 cm, or SW males toward SW males, 0.4 cm, required a target of 20 males (Fig. 4c, 4b). SW females were significantly attracted toward homospecific females at the lowest and highest number of females. Net movement of females was 0.32 cm toward one female and 0.66 cm toward 20 females (Fig. 4d). The net movement of *B. mucronatus* Ch males, 0.25 cm, toward 10 homospecific females was also significant (Fig. 5).

Intraspecific chemical communication

The net movement in one hour of *B. xylophilus* SJ, Q14, lb or MSP4 test males, toward 10 *B. xylophilus* SW target females, was significantly different than the random movement of the same test nematodes toward 0 females (Fig. 6). In the reciprocal experiment, the net movement of *B. xylophilus* SW test males toward 10 *B. xylophilus* SJ, Q14, lb or MSP4 target females was significantly different than toward 0 females (Fig. 7). Similarly, for *B. mucronatus* the net movement of Ch test males toward a dosage of 10 *B. mucronatus* Fr target females was significantly different than toward 0 females (Fig. 6). Also, in the reciprocal intraspecific experiment, the net movement of *B. mucronatus* Fr test males toward a dosage of 10 target *B. mucronatus* Ch females was significantly different than the random movement toward 0 females (Fig. 7).

Interspecific chemical communication

The net movement in one hour of *B. mucronatus* Ch test males toward a dosage of 10 *B. xylophilus* lb or SJ target females was

significantly different than the random movement toward 0 nematode target (Fig. 8a). There was no significant difference between the net movement of *B. mucronatus* Ch or Fr test males toward 10 target *B. xylophilus* SW females and the Ch or Fr controls. Similarly, in the reciprocal trials, the net movement of *B. xylophilus* lb, SW or SJ test males toward a dosage of 10 *B. mucronatus* Ch or Fr target females was not significantly different from that of the corresponding controls (Fig. 8b).

Species specificity of the sex-pheromone

The net movement in one hour of *B. mucronatus* Ch test males either toward a dosage of 10 target *A. rhyntium* (Aph) females or toward 10 *B. fraudulentus* (Fraud) target females was not significantly different than toward 0 females (Fig. 9a). The net movement of SW test males toward 10 Aph target females was significant. However, the net movement of SW males toward 10 Fraud females was not significant (Fig. 9a). In the reciprocal experiment, the net movement in one hour of Aph males or Fraud males toward *B. mucronatus* Ch target females or *B. xylophilus* SW target females was not significant (Fig. 9b).

Figure 4. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW toward 0 (control), 1, 5, 10 and 20 *B. xylophilus* SW nematodes, (SW = St. William isolate); (a) male toward female(s), (b) male toward male(s), (c) female toward male(s) and (d) female toward female(s).

* significantly different from the control ($P < 0.05$).

Error bars represent \pm SE.

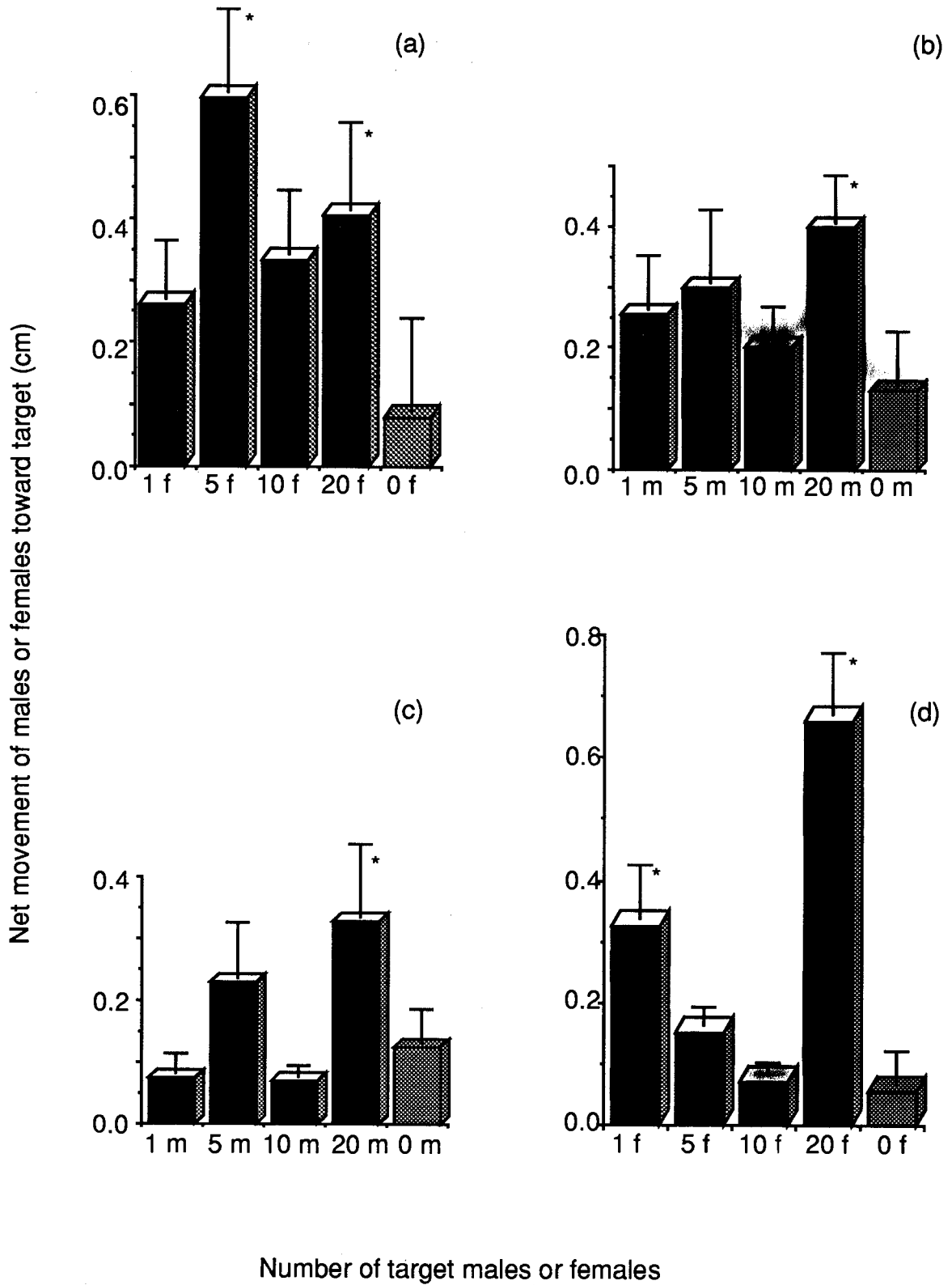
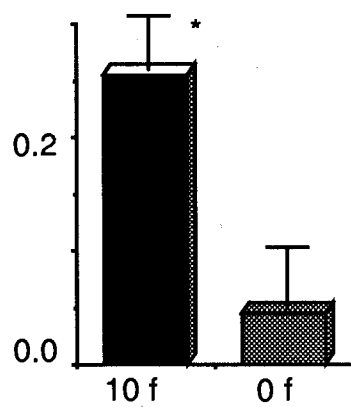


Figure 5. Net movement (cm) in one hour by *Bursaphelenchus mucronatus* Ch male toward 0 (control) and 10 *B. mucronatus* Ch females (Ch = Chiba isolate).

* significantly different from the control ($P < 0.05$).

Error bars represent \pm SE.

Net movement of males toward target (cm)



Number of target females

Figure 6. Net movement (cm) in one hour by *Bursaphelenchus* spp. males toward 0 (control) and 10 *Bursaphelenchus* spp. females (intraspecific chemical communication).

* significantly different from the control ($P < 0.05$).

Error bars represent \pm SE.

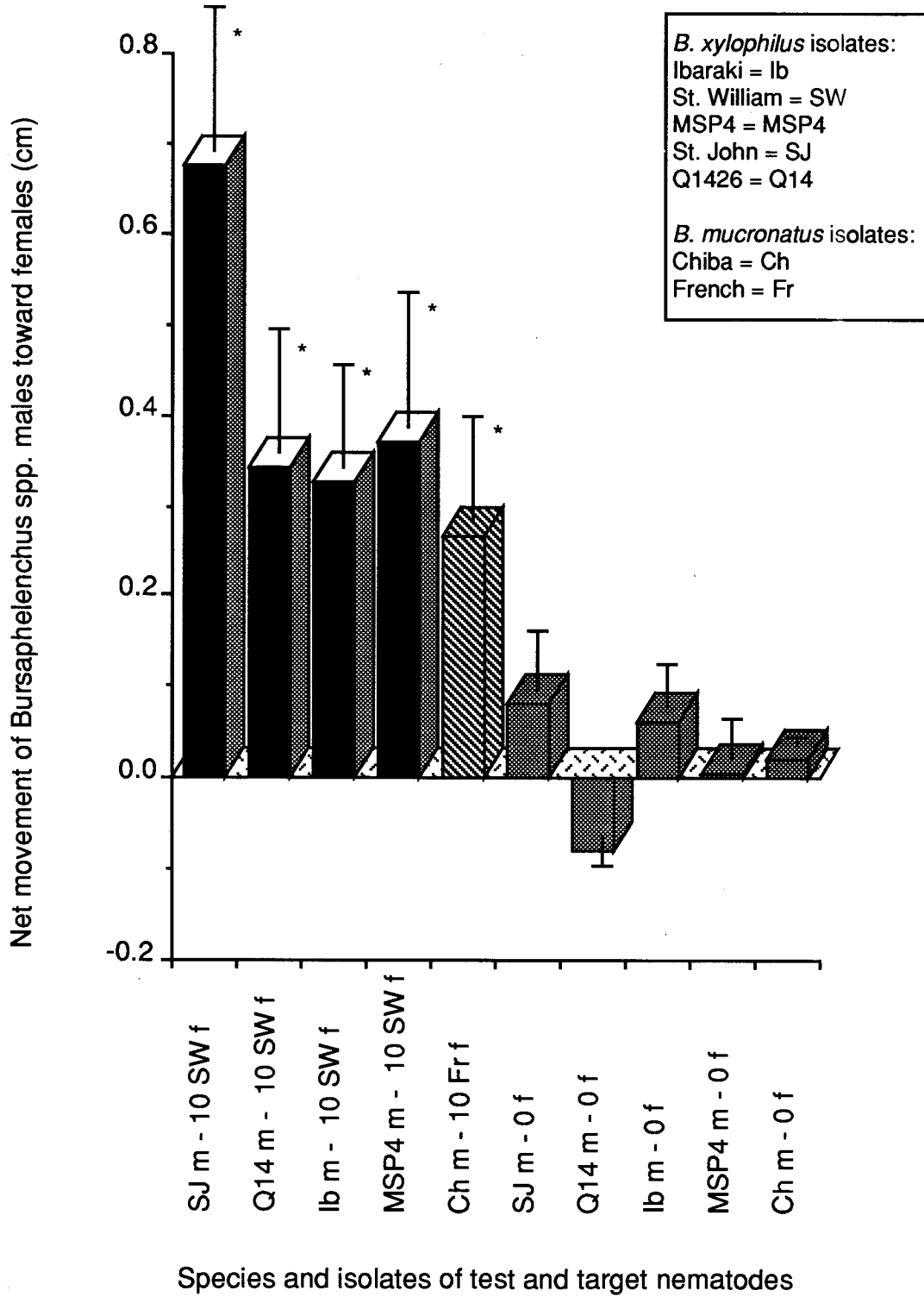
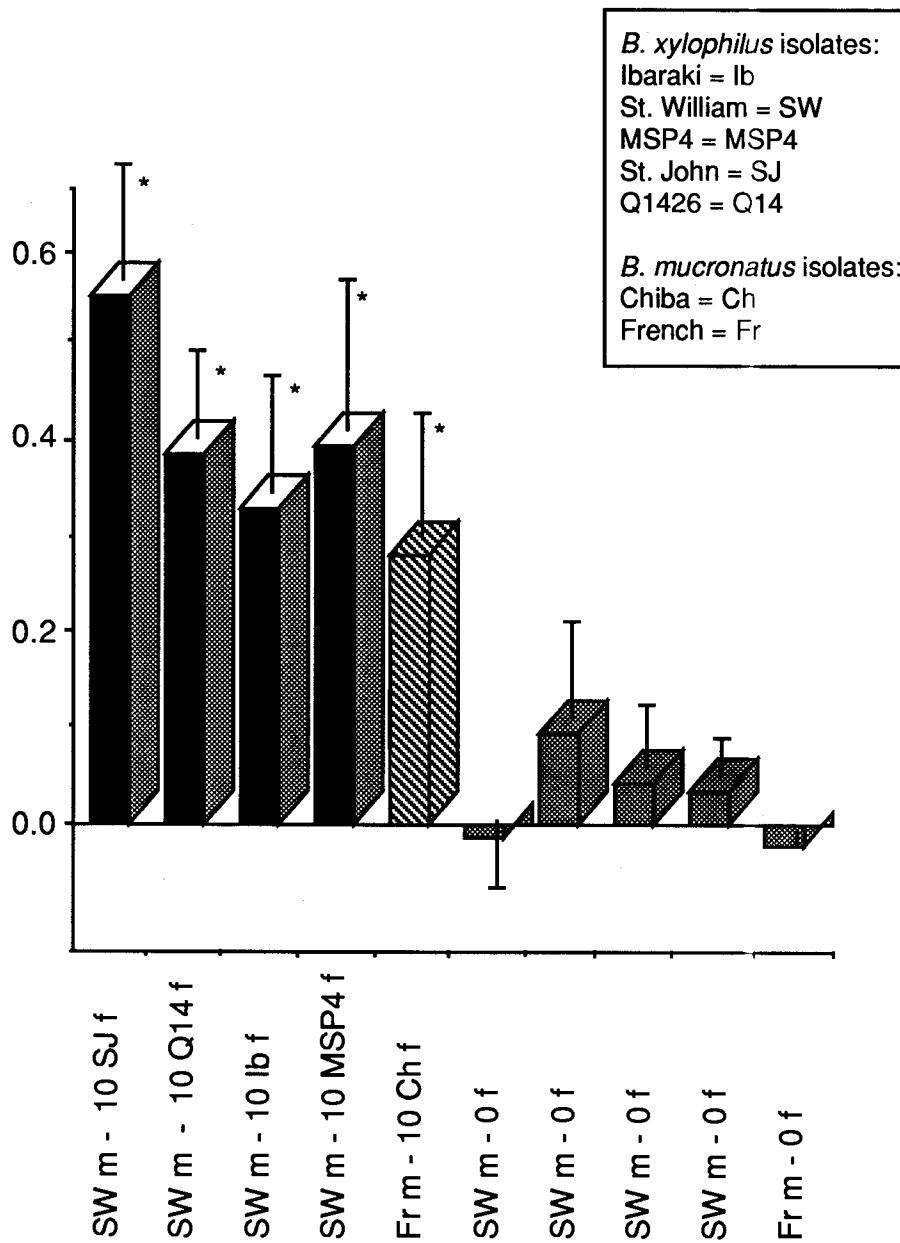


Figure 7. Net movement (cm) in one hour by *Bursaphelenchus* spp. males toward 0 (control) and 10 *Bursaphelenchus* spp. females (reciprocal intraspecific chemical communication of Fig. 5).

* significantly different from the control ($P < 0.05$).

Error bars represent \pm SE.

Net movement of *Bursaphelenchus* spp. males toward females (cm)



Species and isolates of test and target nematodes

Figure 8. Net movement (cm) in one hour in (a) interspecific chemical communication by *Bursaphelenchus* spp. males toward 0 (control) and 10 *Bursaphelenchus* spp. females and in (b) reciprocal interspecific chemical communication.

* significantly different from the control ($P < 0.05$).

Error bars represent \pm SE.

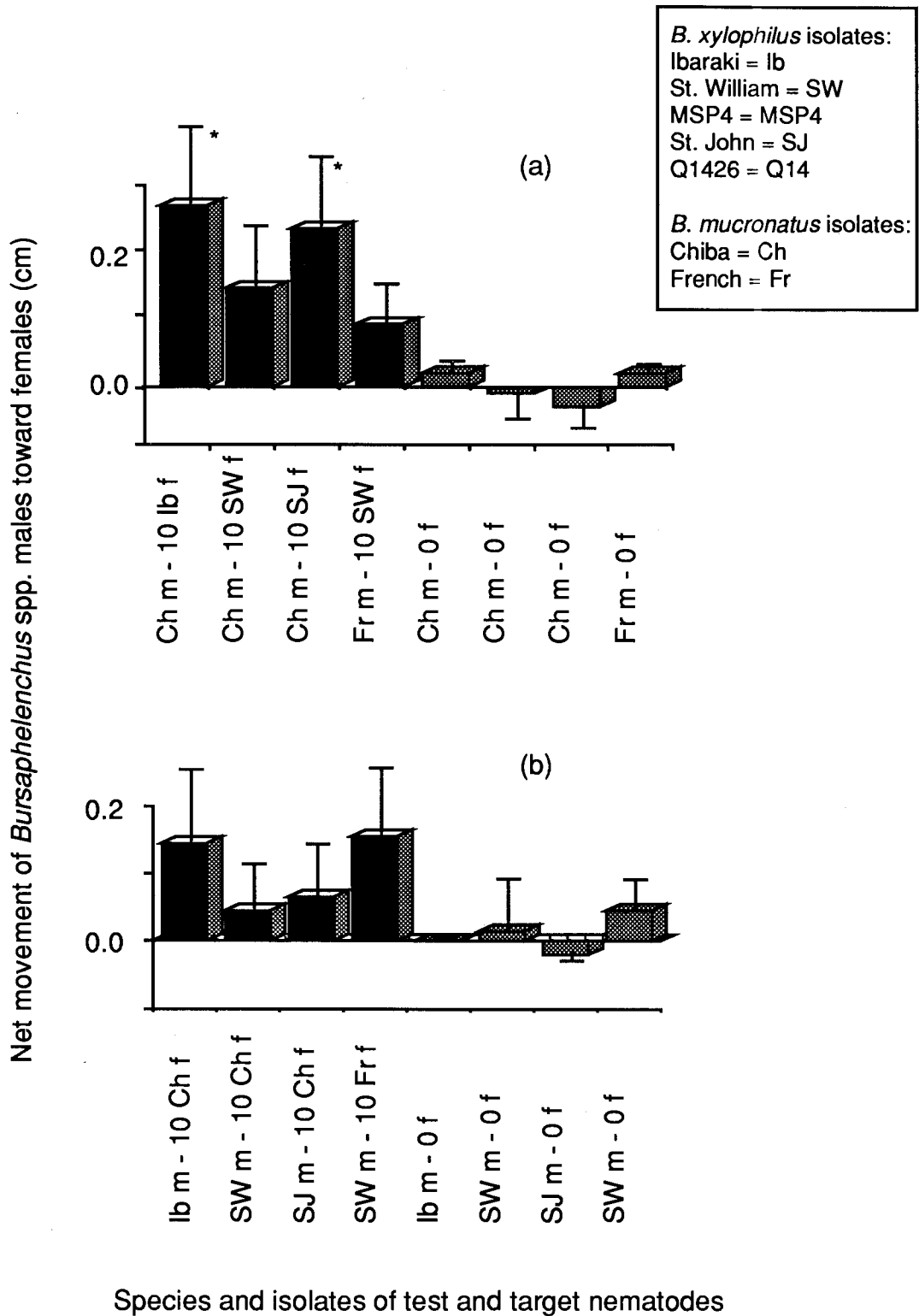
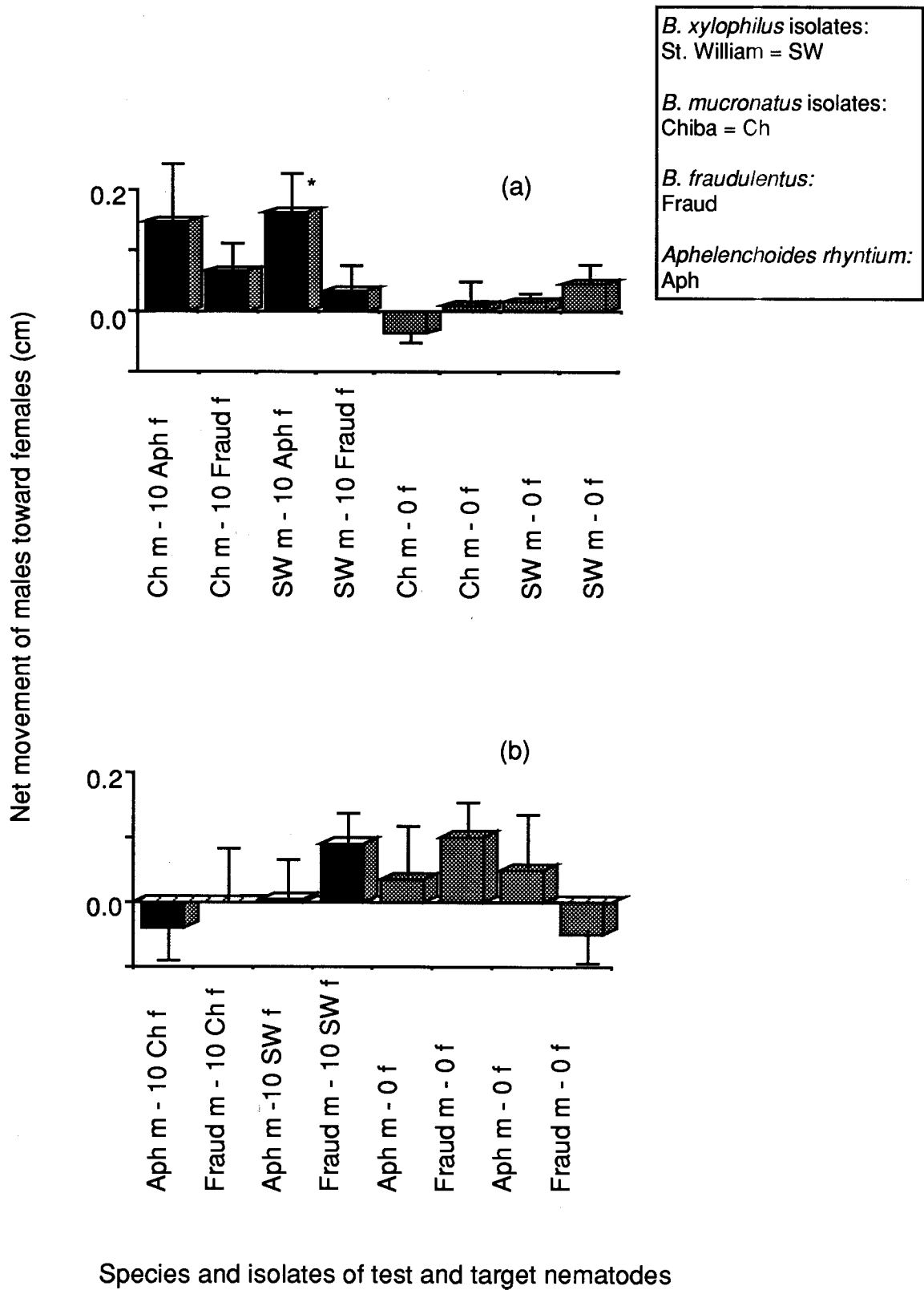


Figure 9. Net movement (cm) in one hour in (a) pheromone species specificity test by *Bursaphelenchus xylophilus* SW (SW = St. William isolate) and *B. mucronatus* Ch males (Ch = Chiba isolate) toward 0 (control), 10 Fraud (Fraud = *B. fraudulentus*) and 10 Aph females (Aph = *Aphelenchoides rhyntium*) and in (b) reciprocal pheromone species specificity test.

* significantly different from its control ($P < 0.05$).

Error bars represent \pm SE.



Chemical nature of the pheromone:

There was no significant difference between the net movement in one hour, 0.48 cm, of *B. xylophilus* SW males toward 10 SW target homospecific female nematodes and toward 2 μ L of the SW water soluble extract, 0.43 cm (Fig. 10). However, the net movement in one hour of *B. xylophilus* SW males toward the SW target homospecific female nematodes and toward the SW water soluble extract, was significantly different from the control (Fig. 10). Storing SW water soluble extract up to 3 days at -20°C or 24°C or 4°C had no significant effect on its attractiveness to the SW males when SW males were used as test nematodes toward the different treatments of the SW water soluble extract in comparison with the controls, i.e. SW water soluble extract (untreated) (Figs 11-13). On the 4th day the biological activity of the SW water soluble extract was significantly lower at -20°C and 24°C but not at 4°C. There was no detectable biological activity in the SW water soluble extract after it was stored one day at 50°C.

There was a significant difference between net movement of *B. xylophilus* SW males toward the <3,000 Daltons fraction than that toward >3,000 Daltons fraction and toward both fractions pooled together, i.e. >3,000 + <3,000 Daltons. There was no significant difference between the net movement of SW males toward the <3,000 Daltons fraction and the freshly prepared SW water soluble extract (Fig. 14).

The net movement of *B. xylophilus* SW test males toward the lyophilized SW water soluble extract was not significantly different from that toward the freshly prepared, untreated SW water soluble extract, i.e. the lyophilization treatment had no deleterious effect on the activity of the pheromone. However, there was a significant difference between the net movement in one hour of *B. xylophilus* SW males toward both of the lyophilized SW water soluble extracts as compared to the blank control (Fig. 15).

The net movement in one hour of *B. xylophilus* SW test males toward the lyophilized SW water soluble extract was significantly different from the net movement of *B. mucronatus* Ch, and Fr test males toward the lyophilized SW water soluble extract (Fig. 16). There

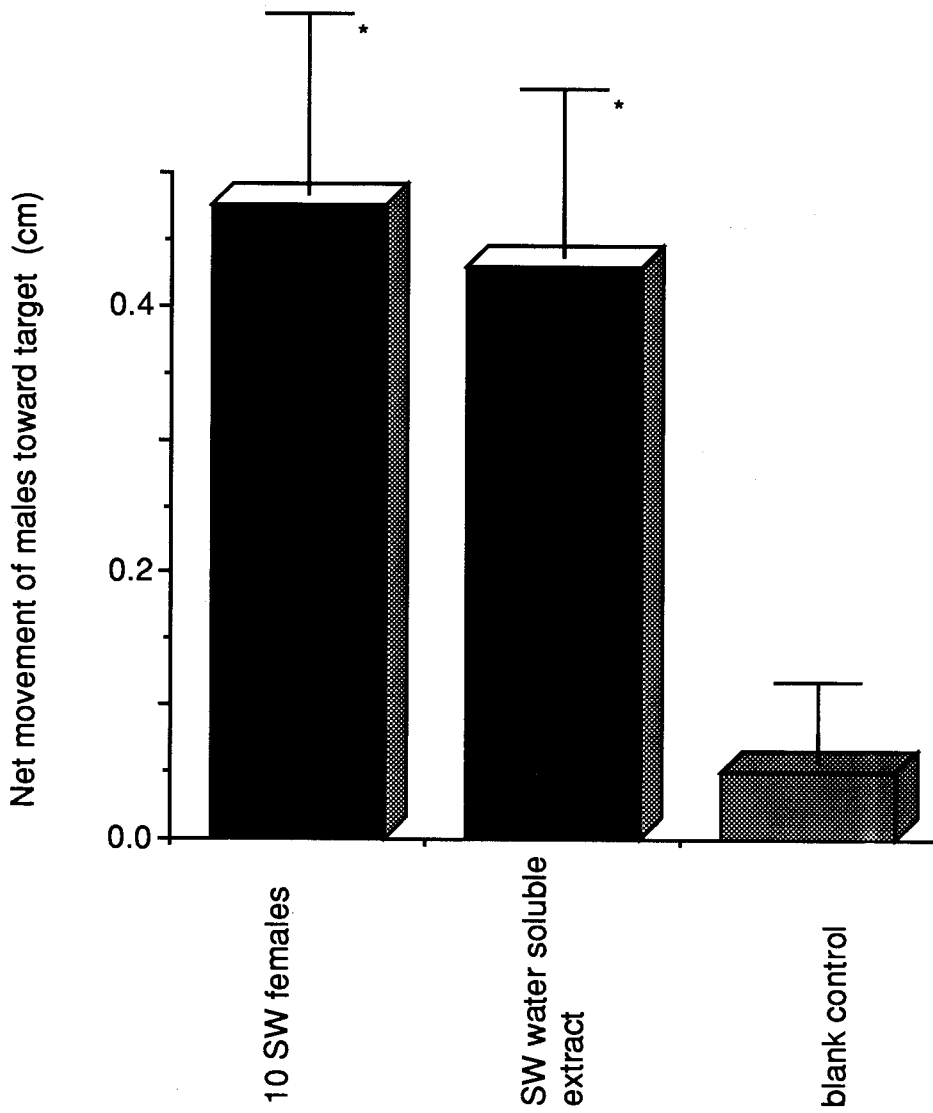
was no significant difference between the net movement of *B. xylophilus* SW test males toward the target lyophilized extract of SW, and the net movement of *B. xylophilus* SJ and Q14 test males toward the target lyophilized extract of SW (Fig. 16).

The net movement in one hour of *B. xylophilus* SW test males was greater toward the target lyophilized SW ether insoluble fraction, the ether insoluble and soluble fractions pooled together, and the lyophilized SW water soluble extract. In addition, there was no significant difference between the previous responses (Fig. 17). There was a significant difference between the net movement in one hour of *B. xylophilus*, SW test males toward the ether insoluble fraction and that toward either the ether soluble fraction or the ether blank control. There was a significant difference between the net movement in one hour of *B. xylophilus*, SW test males toward the target of ether insoluble and soluble fractions pooled together and that toward the ether soluble fraction and the ether blank (Fig. 17).

Figure 10. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW males toward 10 SW females and toward SW water soluble extract (SW = St. William isolate).

* significantly different from the control (blank control) ($P < 0.05$).

Error bars represent \pm SE.

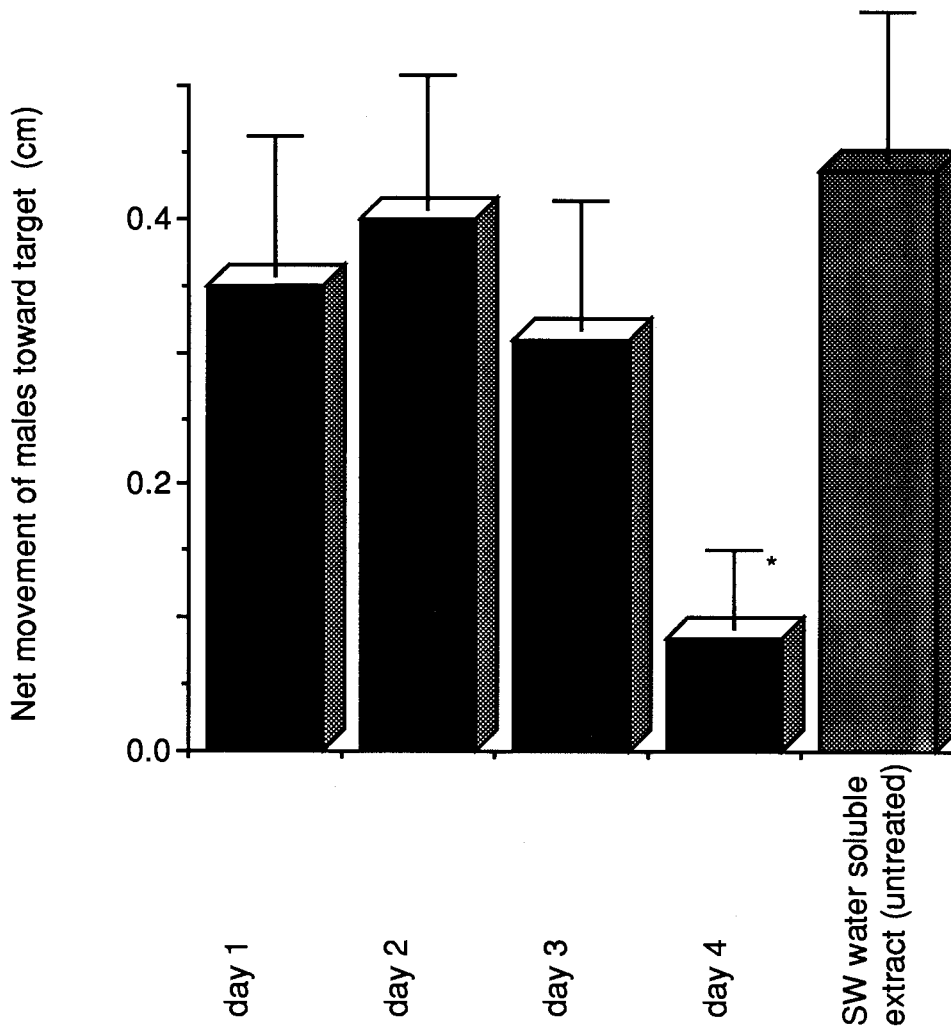


Target of SW nematodes and SW water soluble extract

Figure 11. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW males toward SW water soluble extract that was stored at -20°C for 4 days (SW = St. William isolate).

* significantly different from the control (SW water soluble extract) ($P < 0.05$).

Error bars represent \pm SE.

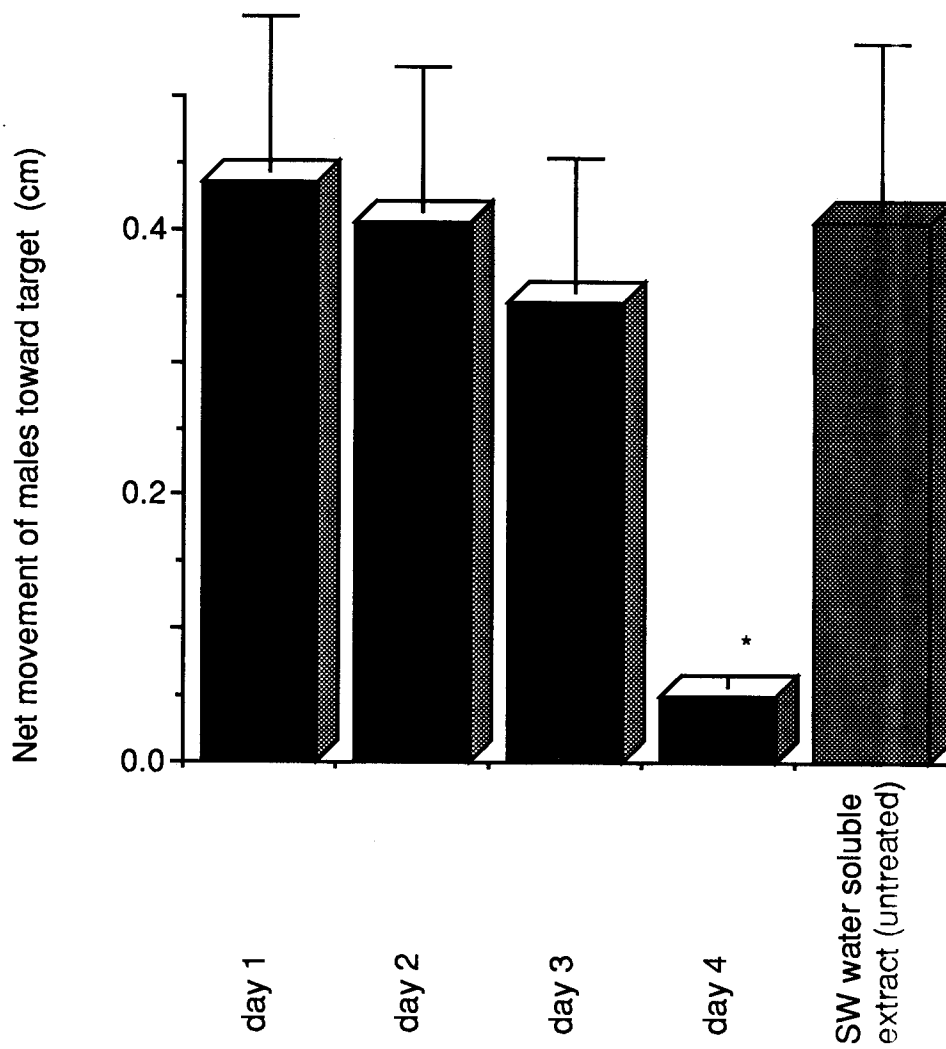


Storage of SW water soluble extract at -20°C at different time periods (days)

Figure 12. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW males toward SW water soluble extract that was stored at 24°C for 4 days (SW = St. William isolate).

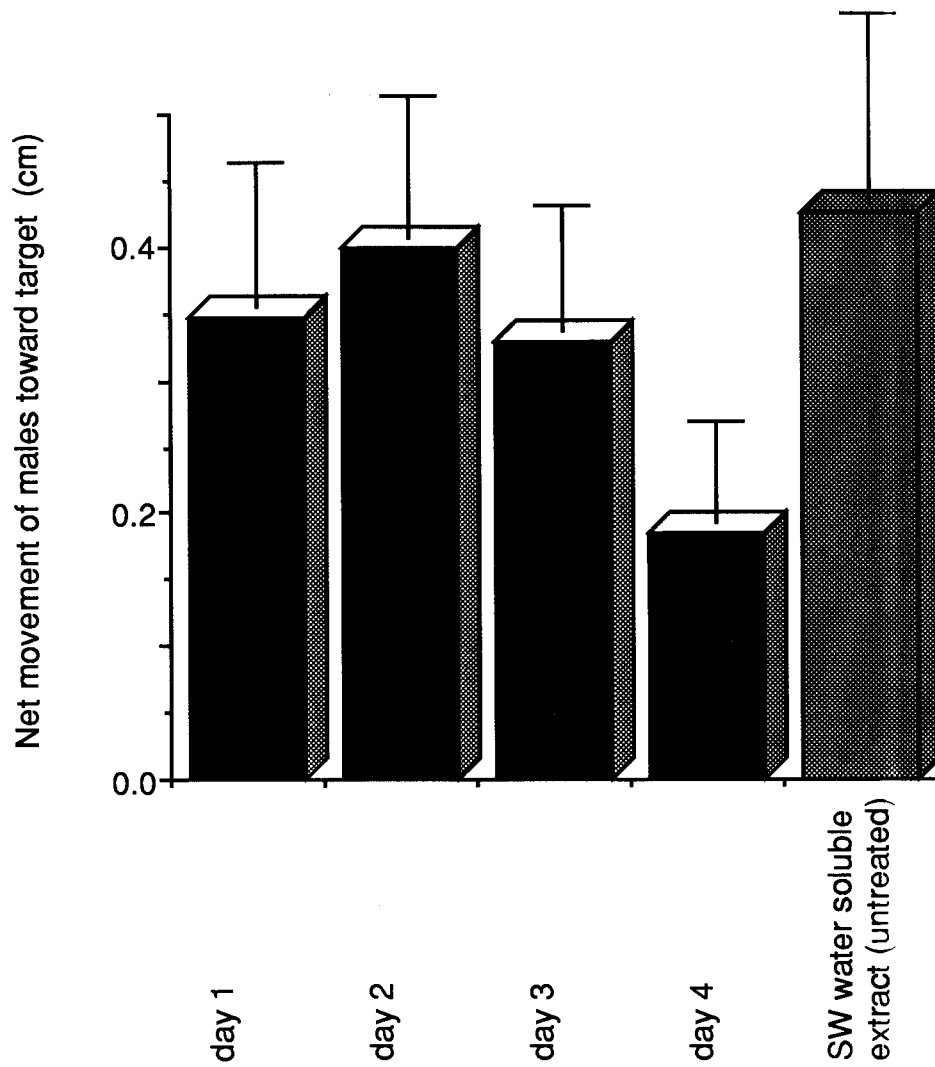
* significantly different from the control (SW water soluble extract) ($P < 0.05$)

Error bars represent \pm SE.



Storage of SW water soluble extract at 24⁰ C at different time periods (days)

Figure 13. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW males toward SW water soluble extract that was stored at 4°C for 4 days (SW = St. William isolate). Error bars represent \pm SE.



Storage of SW water soluble extract at 4⁰ C at different time periods (days)

Figure 14. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW males toward fractions of different molecular weight of the SW water soluble extract (SW = St. William isolate).

* significantly different from the fraction that is more than 3,000 Da and from both fractions pooled together ($P < 0.05$).

Error bars represent \pm SE.

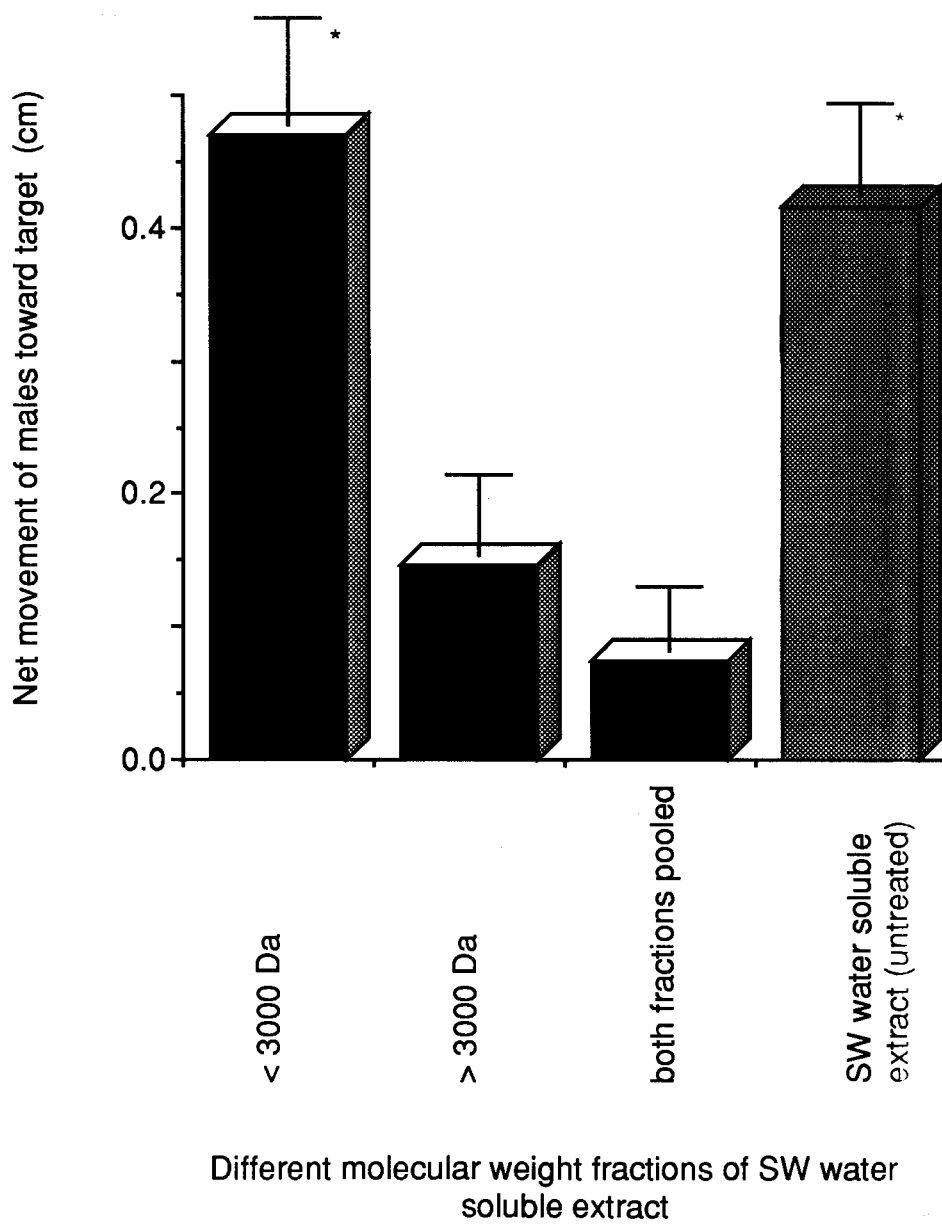
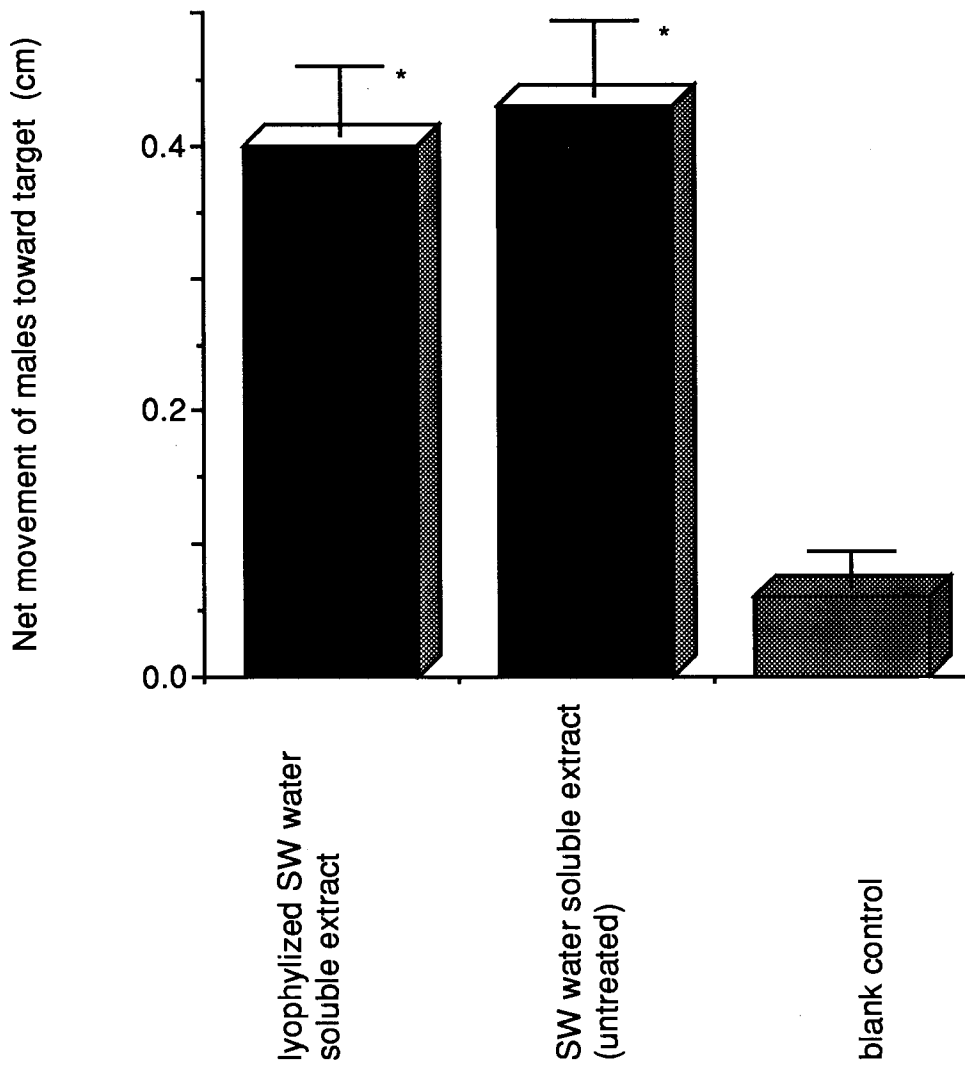


Figure 15. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW males toward SW lyophilized water soluble extract and untreated SW water soluble extract (SW = St. William isolate).

* significantly different from the control (blank control) ($P < 0.05$).

Error bars represent \pm SE.



Lyophilized and SW water soluble extract

Figure 16. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SJ (SJ = St. John isolate) and SW (SW = St. William isolate) males and *B. mucronatus* Ch (Ch = Chiba isolate) and Fr (Fr = French isolate) males toward SW lyophilized water soluble extract (SW = St. William isolate).

* significantly different from the control (SW) ($P < 0.05$).

Error bars represent \pm SE.

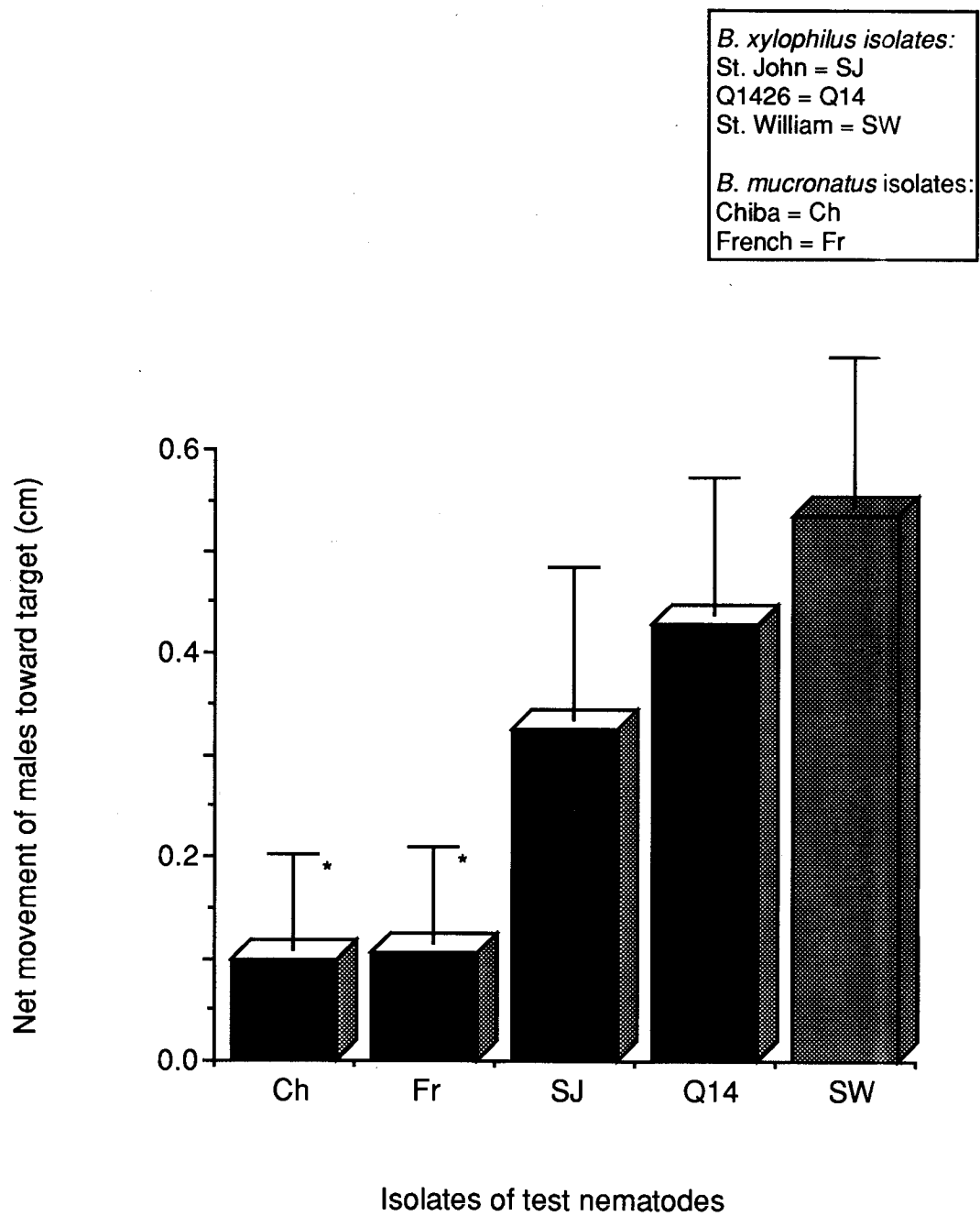
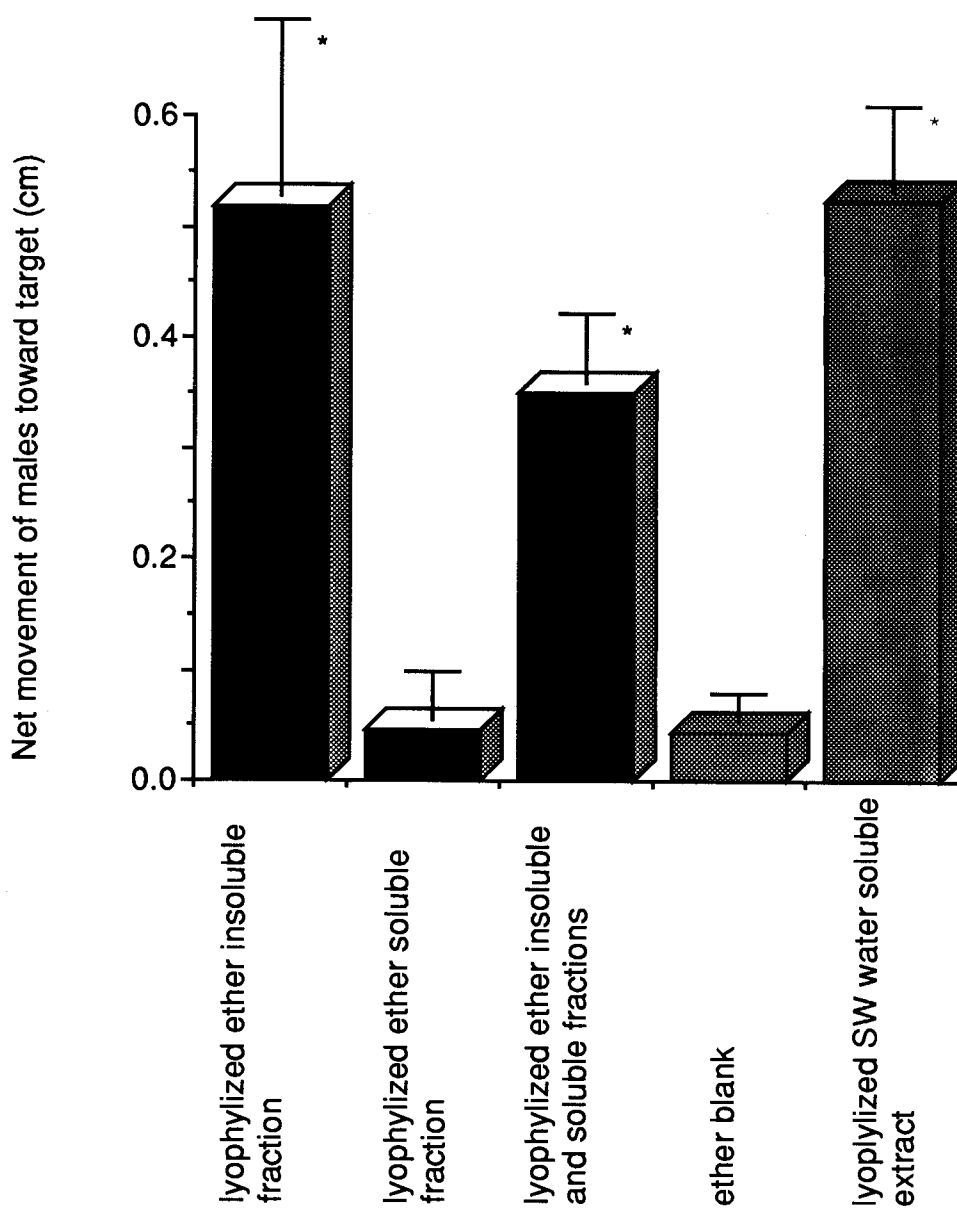


Figure 17. Net movement (cm) in one hour by *Bursaphelenchus xylophilus* SW males toward SW ether extracted fractions (SW = St. William isolate).

* significantly different from the ether soluble fraction and the ether blank control ($P < 0.05$).

Error bars represent \pm SE.



Ether fractions of SW water soluble extract

Discussion

There are contradictory reports on the biological and taxonomic status of the two closely related coniferous parasites, *Bursaphelenchus xylophilus* and *B. mucronatus*. Maggenti (1983) stated that the species is accepted as an objective, naturally occurring unit which becomes an abstraction and subjective when one tries to define it. An understanding of species plasticity, its biological expression, its morphological and cytological characteristics, should lead ideally to a clear, unequivocal classification. In this thesis, I use a multifaceted approach to clarify the taxonomic and biological relationships of *B. xylophilus* and *B. mucronatus*. This approach is based on the cumulative evidence derived from four studies: cross-hybridization, pathogenicity, DNA analysis and chemical communication.

As an initial approach toward the understanding of the taxonomic relationships of *B. xylophilus* and *B. mucronatus*, populations of both species were cross-hybridized. According to Mayr (1970), each species is a genetic system selected through many generations to fit into a definite niche in its environment, and cross-hybridization with other groups usually results in the breakdown of this system and the production of disharmonious types. Therefore, cross-hybridization studies can indicate how closely organisms are related. Intraspecific and reciprocal cross hybridizations of *B. xylophilus* Q14, SJ, SW, MSP4 and Ib geographic populations from Canada, United States and Japan produced fertile progeny that reproduced vigorously over several generations (Table 2). This result concurs with those of similar experiments, with different geographic populations (Mamiya, 1986; de Guiran and Bruguier, 1989). Isolates of the geographic populations of *B. mucronatus* from France and Japan, Fr and Ch respectively, produced fertile offspring only when the male was from the Ch populations and the female was from the Fr populations. According to Mayr (1969), geographic populations have only limited or no gene exchange with other populations of the species, and frequently they have sufficient differences that these populations are ranked at the subspecies level. Prakash (1972) reported that when females of an isolate of *Drosophila pseudoobscura* from the highlands of the Andes near Bogota, Colombia

was cross-hybridized with males of the main body of distribution of *D. pseudoobscura* they yielded fertile females but the male hybrids were sterile. However, the reciprocal crosses produced both fertile male and female hybrids. Based on the initial observation that sterile males were produced, more studies were conducted and as a result the Bogota population of *D. pseudoobscura* was described as a new subspecies, *D. pseudoobscura bogotana* (see Lakovaara and Saura, 1982). The geographical populations of *B. xylophilus* under our laboratory conditions are interfertile. However, the *B. mucronatus* isolates under our laboratory conditions are not always interfertile. I assumed based on the cross-hybridization studies that the populations of *B. mucronatus* from France and Japan are either different subspecific forms or different species.

Although interspecific hybridization between males of the *B. mucronatus* Japanese Ch populations and females of all the *B. xylophilus* populations was successful, all the reciprocal crosses died out (Table 2). The interspecific hybridization between males of the French *B. mucronatus* Fr with females of *B. xylophilus* was successful. However, some of the reciprocal crosses died out (Table 2). Similarly, Mamiya (1986) reported that F_1 hybrids of *B. xylophilus* males with *B. mucronatus* females failed to produce offspring. On the contrary, de Guiran and Bruguier (1989) reported that the French *B. mucronatus* populations gave fertile hybrids from reciprocal crosses with *B. xylophilus*, while in our experiments, reciprocal crosses of French females of *B. mucronatus* populations with males of *B. xylophilus* populations failed in most cases to produce fertile hybrids. When males of *D. montana* were cross-hybridized to females of *D. virilis* the F_1 generation was fertile. However, both males and females of the F_1 generation of the reciprocal cross were sterile (see Throckmorton, 1982). I assume based on my study that matings in nature between *B. xylophilus* and *B. mucronatus* and between the French and Japanese populations of *B. mucronatus* may be rare. However, if hybrid production occurs, these hybrids of *B. xylophilus* and *B. mucronatus* and the Japanese and French hybrids of *B. mucronatus* will probably have more unstable morphological and genetic characters than the ones in the laboratory which may also influence their pathogenic expression. In

addition, I conclude that *B. xylophilus* and *B. mucronatus* populations come from a common ancestor since they can interbreed and produce viable offspring in the laboratory, in some but not all sex combinations of the populations. From my cross-hybridization study, it became clear that more experiments need to be done to understand why the F₁ generation dies out, i.e. is either the male or the female or both sexes sterile, and what are the reasons.

The fact that some of the interspecific crosses between *B. xylophilus* and *B. mucronatus* are non-reciprocal can be used to derive two theories. First, that may be three very closely related but distinct species in which gene flow in the wild may not exist, and which have not yet developed complete reproductive isolation. This could allow partly successful crosses to be generated under laboratory conditions. Alternatively, these results could be generated by a hybrid-definition dysgenesis-like phenomenon, i.e. a syndrome of correlated genetic traits that is spontaneously induced in hybrids between certain mutually interacting strains usually in one direction only (Bregliano and Kidwell, 1983). In this case the maternal cytoplasm of the female of *B. mucronatus* would contain or fail to contain an unknown component which causes abnormal development in heterogenous crosses. For the *B. mucronatus* Fr populations this effect would not be as stable or as predictable as that in the *B. mucronatus* Ch populations. It is also possible that the unknown component is different between *B. mucronatus* Fr and Ch, because when the Ch female was crossed with either the Fr male or *B. xylophilus* males no progeny were produced, while when the Fr female was crossed with either Ch male or *B. xylophilus* males, progeny were produced in some cases. These hypotheses are not mutually exclusive, and it is possible that the hybrid-dysgenesis-like phenomenon is a step toward reproductive isolation and the development of new species.

In the second approach *Bursaphelenchus* spp. nematodes were introduced into *P. sylvestris* seedlings to determine if they induce the same or different levels of pathology. *Bursaphelenchus xylophilus* populations SJ, SW, Ib, and MSP4 are more pathogenic to 3-year-old *P. sylvestris* seedlings than are *B. mucronatus* Ch and Fr populations (Fig. 1, Table 3). These results are in agreement with those of Panesar

and Sutherland (1989) who found that *P. sylvestris* was highly susceptible to *B. xylophilus* populations from Alberta and British Columbia. In addition, my data showed contrary to the results of Panesar and Sutherland (1989) that Q14, which is the same populations as one of those tested by Panesar and Sutherland (1989), is as pathogenic under the conditions specified above as the rest of the tested *B. xylophilus* populations (Fig. 1, Table 3). This study has also shown that *B. xylophilus* Q14, SJ and MSP4 populations, which are "M" forms (R.V. Anderson, pers. comm., Agriculture Canada, Ottawa) were as pathogenic as the Ib and SW populations which are "R" forms. This contrasts with earlier data (de Guiran and Bruguier, 1989) which showed "R forms" to be pathogenic and the "M forms" nonpathogenic. The difference between my data and those of de Guiran and Bruguier (1989) could be attributed to factors such as different experimental conditions or seed sources of seedlings or different geographical populations or loss of the population's pathogenicity with time in culture. The French populations of *B. mucronatus* Fr is more pathogenic than the Japanese populations of *B. mucronatus* Ch (Fig. 1, Table 3). In addition, *B. mucronatus* intraspecific hybrid, Chx Fr, is more pathogenic than the Japanese parental isolate Ch and as pathogenic as the French isolate (Fig. 1, Table 3). Host range testing is used frequently in plant pathology and especially with nematodes that are morphologically similar and possess very few characters for successful species discrimination. For example, the reproduction and effect of *Ditylenchus destructor* from groundnut in South Africa on seven commercial cultivars of potato has been examined. All cultivars tested were poor hosts for the South African race i.e. the nematode caused no damage to the potato tubers (de Waele *et al.*, 1991), while *D. destructor* populations in the northern hemisphere and New Zealand infected most local potato cultivars. Based on these host range tests, de Waele *et al.* (1991) concluded that the South African population of *D. destructor* is a new race. Similar host range tests have been done to differentiate the pathotypes of *Globodera rostochiensis* and *G. pallida* using several cultivars of potatoes (Kort *et al.*, 1977).

Bursaphelenchus xylophilus and *B. mucronatus* interspecific hybrids were more pathogenic than the *B. mucronatus* parental isolates

and as pathogenic as the *B. xylophilus* parental isolates (Fig. 2, Table 2). These interspecific hybrids appear to have inherited their virulence from the *B. xylophilus* parental isolates, possibly in the area of their genomic DNA where the ribosomal gene cluster probe from the *B. xylophilus* Ib isolate binds (Fig. 1, Fig. 2). Appearance of symptoms, i.e. needle wilting and browning, on *P. sylvestris* seedlings inoculated with *B. xylophilus* isolates or with the intra- or interspecific hybrids was always a prelude to seedling death, but this was not so with the *B. mucronatus* isolates, especially the Japanese Ch isolate (Fig. 1, Table 3). This may be because *B. xylophilus* isolates increase their reproductive rates more rapidly with increasing population densities at high temperatures than do the *B. mucronatus* isolates (Rutherford, *et al.*, 1992). No relationship was observed between number of nematodes present in a seedling and the time of seedling death for most isolates and intra- and interspecific hybrids (Table 3). In addition, not all isolates and some of their hybrids show a dependent relationship between the number of nematodes per seedling and seedling weight at the time of death. This is probably due to the fact that pine seedling death is due to the speed with which the water deficit and permanent wilt occurs, and this is affected mostly by the nature and degree of nematode-induced injury or/and host cell response (Kuroda, 1989; Melakeberhan, *et al.*, 1991), and temperature (Rutherford, *et al.*, 1992).

This study on the pathogenicity to 3-year-old *P. sylvestris* seedlings of the pinewood nematode populations and their hybrid crosses has provided evidence on the relative pathogenicity of *B. xylophilus* and *B. mucronatus* populations and their hybrids from a wide geographic range, and on the taxonomic relatedness of *B. xylophilus* and *B. mucronatus*. In particular, data from the pathogenicity study along with data from the cross-hybridization study suggest that the Japanese population of *B. mucronatus* Ch is not the same species as the French population of *B. mucronatus*. The consistent biological differences between *B. mucronatus* Ch and Fr that have been found in my study point out that Ch and Fr from Japan and France, respectively are more than geographic populations or subspecies. In addition, this pathogenicity study does not support the hypothesis that the shape of the female tail i.e. either rounded "R" or mucronated "M", can be used

to separate populations of *B. xylophilus* into pathogenic and non-pathogenic forms.

The third approach in the present study was based on contemporary molecular techniques. DNA analysis is a useful tool for species identification because it is a primary determinant of an organism's genetic composition, it is less subject to environmental effects, and the results can be interpreted objectively. In addition, DNA analysis can reinforce the taxonomic evidence derived from other approaches. Molecular techniques have been applied to taxonomic problems in various phyla in order to understand the relative taxonomic status of the species founded solely on morphological data. Hubby and Lewontin (1966) and Lewontin and Hubby (1966) were the first to apply molecular techniques to taxonomic problems of animal species. From their classic study with different populations of *Drosophila* they reported that whenever a locus is polymorphic in one population, it is also polymorphic in all other populations. In addition, they reported that there was no evidence of geographic races at the level of protein polymorphisms. In the present molecular phylogenetic study of two aphelenchid nematodes, I chose to use the total rDNA as a probe. This particular DNA sequences have been found to be useful for a wide range of phylogenetic studies from mice (Cory and Adams, 1977) and frogs (Gouse and Gerbi, 1980) to dozens of plant species (Learn and Schaal, 1987). According to Hillis and Dixon (1991), the reasons for the systematic versatility of rDNA are due to the different rates of evolution among the different regions of rDNA (both among and within genes), the presence of many copies of rDNA sequence per genome and the pattern of concerted evolution that occurs among repeated copies. The total rDNA probe that I used in my study was able to separate all *B. xylophilus* populations from each other. Similarly, Castaynone-Sereno *et al.* (1991) reported that DNA from several *Meloidogyne incognita* geographic populations was digested and hybridized to a homologous probe. The resulting restriction length polymorphism enables the differentiation of all tested geographic populations of *M. incognita*.

The parental populations of *B. xylophilus* and *B. mucronatus* are readily distinguished as two groups by the RFLP's labelled by the homologous total ribosomal gene probe (Fig. 2). Results from my study

concur with those reported by Bolla, *et al.* (1988), Webster *et al.* (1990), Abad *et al.* (1991) and Tares *et al.* (1992). Recently, molecular techniques have been used on a variety of nematode species ranging from free-living to animal and plant parasitic species. For example, Curran and Webster (1987) used the 5s rDNA nontranscribed spacer region as a homologous probe to identify two strains of *C. elegans* and to separate the *C. elegans* strains from two strains of *C. briggsae*. In another study, restriction endonuclease digestion of genomic DNA of various species of *Trichinella*, *Caenorhabditis*, *Romanomermis*, *Steinernema* and *Meloidogyne* revealed restriction fragment length differences between the species within the genera (Curran *et al.*, 1985). In more recent work, total genomic DNA of 34 *Heterorhabditis* populations, parasitic nematodes of insects, from various geographic locations was digested with restriction enzymes and revealed three groups with different DNA banding patterns: *H. bacteriophora* and *H. heliothidis* group, North-West European group and an Irish group (Smits, *et al.*, 1991).

My study shows that the *B. xylophilus* intraspecific hybrids, in the area of the genome where the probe hybridizes, inherited their DNA from both of the parental populations (Fig. 2). For example, the *B. xylophilus* hybrid Ib m x SW f, shows banding from both the Ib and the SW parents (Fig. 2). Using the *B. xylophilus* rDNA probe I was able to show restriction fragment differences between the Japanese and French populations of *B. mucronatus*. In addition, the DNA analysis of the *B. mucronatus* Ch m x Fr f hybrid shows that the hybrid cross inherited its DNA mainly from the Fr parent but may have obtained some from the Ch parent in view of one shared band (Fig. 2), indicating that there is genetic exchange, in this part of the genome where the probe binds. The data suggest additionally, that the geographic populations of *B. xylophilus* have not developed isolating mechanisms toward each other since they can cross-hybridize successfully and exchange genetic materials in this part of the genome where the probe binds. Although the geographic populations of *B. xylophilus* do not show any exchange of genetic material with the Japanese population of *B. mucronatus* there is a molecular indication that they can exchange genetic material with the French population.

Data from the present DNA study of the *Bursaphelenchus* spp. populations contradicts that of Abad *et al.* (1991), who reported that the European and Japanese populations of *B. mucronatus* are the same species. They based their conclusion on data derived from probing DNA of *Bursaphelenchus* spp. with a heterologous *unc-22* gene from *C. elegans* and in conjunction with a six-base cutter restriction enzyme. This difference of opinion between Abad *et al.* (1991) and the present data could be attributed to the failure of the intergenic region of the *unc-22* gene probe used by Abad *et al.* (1991), to bind to the heterologous species, therefore missing possible variations. Recently, Tares *et al.* (1992) reported that homologous DNA probes could be used to show that the restriction pattern length polymorphism of the Japanese population of *B. mucronatus* is different from the RFLP of the French and Norway populations. Although, Tares *et al.* (1992) found differences between RFLP's of the European and Japanese *B. mucronatus* populations, they concluded that these differences were not sufficient to warrant classification of these populations as separate species. Conclusions from my study in conjunction with conclusions from the cross-hybridization and pathogenicity studies however contradict those of Tares *et al.* (1992), and indicate that the French and the Japanese populations of *B. mucronatus* indeed may be separate species.

A more recent and sensitive technique, the polymerase chain reaction (PCR), has been used as a taxonomic tool. PCR has been used to amplify 1.8 kb sequence of mitochondrial DNA of *M. javanica*, *M. hapla*, *M. incognita* and *M. arenaria* (Harris, *et al.*, 1990). Restriction digestion of the amplified product revealed fragment differences between the tested nematodes with the exception of *M. javanica* and *M. hapla* (Harris, *et al.*, 1990). Recently, Beckenbach *et al.* (1992), using polymerase chain reaction and direct sequencing techniques, reported sequence divergences of 18.9% between *B. xylophilus* and the Japanese *B. mucronatus*, 14.6% between *B. xylophilus* and the French *B. mucronatus*, and 23.5% between the French and Japanese *B. mucronatus*. My studies agree with the results reported by Beckenbach *et al.* (1992).

The DNA analysis of the interspecific hybrids shows the banding pattern from the *B. xylophilus* parent, for example in the Ch m x SW f hybrid (Fig. 3). These DNA banding patterns suggest that under our laboratory conditions, the *B. xylophilus* genome is favoured over the *B. mucronatus*, Ch genome. Similarly, the *B. mucronatus* Fr genome seems to be favoured over the Ch genome. However, the interspecific hybrid of *B. mucronatus* Fr and *B. xylophilus* SW appears to have its genome inherited from both parents. In addition, the *B. xylophilus* and the Fr genome appear to be segregating randomly, i.e. laboratory conditions in my study appear to favour the *B. xylophilus* populations and the Fr population of *B. mucronatus*, while this may not be so for the Ch population of *B. mucronatus*. These cross-hybridization data together with data from the pathogenicity studies support the taxonomic separation of *B. xylophilus* and *B. mucronatus*. In addition the previous data provide evidence toward the separation of the Japanese and French populations of *B. mucronatus* to two different allopatric species.

Pheromones can be valuable taxonomic tools due to their species specific nature. In this final and unique approach to taxonomy, individuals of *Bursaphelenchus* spp. were tested under defined experimental conditions, and they exhibited behavioural responses of sex and aggregation attraction. *Bursaphelenchus xylophilus* SW males were attracted significantly toward a cluster of homospecific females of the same population, and the response of males to females was significant regardless of the number of females present (Fig. 4a). This study shows that the female of the species, in this instance the SW population of *B. xylophilus*, produces chemicals that attract males. This type of attraction is considered a sex attraction. Similarly, *B. mucronatus* Ch males are attracted to homospecific females. This attraction suggests that females of *B. mucronatus* release chemicals that attract homospecific males. In most nematode species examined to date, the male is the responding sex and the female is the main attractor. For example, in *H. polygyrus*, a nematode parasite of mice, the response of the male is dosage dependent (Riga and MacKinnon, 1987), and in *Nippostrongylus brasiliensis* and *T. spiralis* (see Bone, 1982) males exhibit an increasingly positive response to an increased number of target females.

Response by females to male-produced chemicals was reported in *H. polygyrus* (Riga and MacKinnon, 1987) and *N. brasiliensis* (Bone and Shorey, 1977), and it is a weaker response than the male to female attraction. This kind of response may be an aggregation type of attraction. The attractiveness of *B. xylophilus* SW females toward 5 target SW males was not significant in comparison to that of male to female attraction (Fig. 4c). This kind of attraction among the sexes of *B. xylophilus* could be considered either a sex or an aggregation attraction which is not clear without further studies.

Males of *H. polygyrus* were attracted toward target males (Riga and MacKinnon, 1987). Similarly, in the present study a significant, homosexual attraction of SW males was recorded toward the highest number of homospecific males but not toward the low number (Fig. 4b). *Bursaphelenchus xylophilus* SW females showed a significant attraction toward 1 and 20 females, the least and the greatest female number, respectively (Fig. 4d). Females of *H. polygyrus* are attracted to female-produced chemicals. Thus it was suggested by Riga and MacKinnon (1987) that by increasing the local density of females, the concentration of female sex-pheromone increases which in turn increases the probability of mating. Similar attraction has been reported among females of other animal parasitic nematodes *Syphasia obvelata* and of *Aspiculuris tetraptera* (Garcia-Rejon *et al.*, 1982). Therefore, attraction between males and females of *B. xylophilus* may be considered functionally as sex attraction while the attraction among the same sexes of *B. xylophilus* may be considered as aggregation attraction, even though the chemical involved may prove to be identical.

A few studies have compared the response of males to mated and virgin nematodes. Sexual attraction in *B. xylophilus* was reported by Kiyohara (1982), who showed that males of the S6-1 population are attracted to virgin but not to mated or gravid S6-1 females. In addition, Kiyohara (1982) recorded female to male attraction but no homosexual attraction of either males or females. The apparent differences in the behavioural response to chemicals between Kiyohara's study and the one reported here could be attributed to the different type of bioassay arena and/or to the different developmental stage of the female nematodes. All females in my study were mated females. Males of *P.*

silusiae were attracted equally well to either virgin or mated females (Cheng and Samoiloff, 1971), while the attraction of *H. polygyrus* males to females was significantly stronger toward mated than toward virgin females (Riga and MacKinnon, 1988).

The intraspecific chemical communication study between the geographic populations of *B. xylophilus* supports the results of the cross-hybridization study. All populations of *B. xylophilus* tested in this study were attracted to stimuli released from each other and they all mated and cross-hybridized successfully. For example, SJ males were attracted toward SW females and cross-hybridized successfully. Similar results were obtained from the reciprocal experiment. Furthermore, the intraspecific chemical communication between the populations of *B. mucronatus* supports the results of the cross-hybridization studies. For example, Ch males were attracted to Fr females and also they cross-hybridized successfully. Fr males were attracted to Ch females and an F₁ hybrid generation which died out was produced. This result suggests that the two geographic populations of *B. mucronatus* from Europe and Japan are able to recognize the pheromone signals released between each other. However, since one of the hybrid crosses died out, one can assume that these two geographic populations of *B. mucronatus* are in the process of acquiring complete reproductive isolation and becoming two distinct allopatric species.

In the study of interspecific chemical communication between the sexes of *B. xylophilus* lb, SW or SJ and *B. mucronatus* Ch or Fr populations, attraction was not significant in most of the cases. A chemical communication study of two strains of *P. redivivus*, B and N, and C strain of *P. silusiae* (both are members of the *P. redivivus* species complex) showed strain specificity. The B and N strain formed one group and the C strain formed a second group (Balakanich and Samoiloff, 1974). Similarly, female sex attractants of 10 *Heterodera* spp. provided information that led to the division of the genus into 3 sub-generic groups (Green and Plumb, 1970). Intraspecific and interspecific heterosexual and homosexual chemical communication studies of *Cylindrocorpus longistoma* and *C. curzii*, a free-living nematode, showed that the chemical attractants were sex and species specific (Chin and Taylor, 1969). Borden, *et al.* (1981) reported that the

enantiomer-based specificity in pheromone communication between *Gnathotrichus* spp. may have been partly responsible for their speciation

The study of the interspecific chemical communication between populations of *B. xylophilus* and *B. mucronatus* do not always parallel results of the cross-hybridization study. For example, *B. mucronatus* Ch males are attracted toward *B. xylophilus* lb females and in addition they cross-hybridize successfully. In the reciprocal chemical communication study, on the other hand, lb males are not attracted toward *B. mucronatus* Ch females although the cross-hybridization between lb males and Ch females takes place and the F₁ hybrid dies out. According to Ehrman and Powell (1982) when mating of two different species is forced in the laboratory, two isolating mechanisms are enforced to protect the integrity of the species, hybrid sterility and hybrid inviability. It is possible that the isolates of *B. xylophilus* and *B. mucronatus* are in the process of developing isolating mechanisms against each other, but these mechanisms appear to be incomplete. For example, *B. xylophilus* SW males are not attracted to *B. mucronatus* Ch females, but under my laboratory conditions, cross-hybridization takes place. This results in an unsuccessful F₁ hybrid. In the reciprocal experiment, *B. mucronatus* Ch males are not attracted to *B. xylophilus* SW females; however, the F₁ hybrid was successful. All the present chemical communication studies have been performed *in vitro* and the nematodes were not given any choices, i.e. one *B. xylophilus* female and one *B. mucronatus* male were contained within the same Petri dish so an accidental encounter may result in mating. Therefore, it is possible that in nature these animals behave differently, i.e. an isolate of *B. mucronatus*, given a choice, may not cross-hybridize with an isolate of *B. xylophilus*. For example, *D. pseudoobscura* cross-hybridizes more readily with *D. persimilis* in the laboratory than in nature (Lakovaara and Saura, 1982). Similarly, *D. lummei* and *D. virilis* can be crossed in mass culture but in "choice" experiments no cross-hybridization occurs (see, Throckmorton, 1982). The present studies have helped to disclose the biological relationships of these closely related species. However, in order to understand the development of the isolating mechanisms, experiments must be performed *in vivo* on

Bursaphelenchus spp. populations, and chemical communication of the hybrids in comparison with each other and the parental isolates must be investigated .

Male *B. xylophilus* SW and male *B. mucronatus* Ch, in most cases, were not significantly attracted to chemicals released by females of a different genus and different species, i.e. *A. rhyntium* and *B. fraudulentus*. The absence of a significant and consistent attraction of *B. xylophilus* SW male and *B. mucronatus* Ch male toward the females of *A. rhyntium* and *B. fraudulentus*, under laboratory conditions, is further evidence that the pheromones produced by *B. xylophilus* and *B. mucronatus* are species-specific chemicals. In addition, the attraction of male *B. xylophilus* and *B. mucronatus* to only females of their respective isolates, supports the taxonomic distinctiveness of *B. xylophilus* and *B. mucronatus*. According to Dobzhansky (1951) sexual or ethological isolation leads to a weak or lack of mutual attraction between males and females of different species. This ensures that the gene exchange between species, especially sympatric species, is restricted or suppressed so sexual isolation eliminates or reduces the wastage of gametes. *Bursaphelenchus xylophilus* and *B. mucronatus* have developed such sexual isolation toward *A. rhyntium* and *B. fraudulentus*.

Ethological or sexual isolation is the initial and the most effective of all the isolating mechanisms. However, throughout this study it was observed that *B. xylophilus* and *B. mucronatus* populations had not apparently developed a complete sexual isolation. Hybrid inviability or possible hybrid sterility is observed between *B. xylophilus* and *B. mucronatus* populations and between the French and Japanese populations of *B. mucronatus*. When mating is forced in the laboratory, hybrid sterility and hybrid inviability occur (Ehrman and Powell, 1982). It is assumed that *B. xylophilus* and *B. mucronatus* populations are incapable of cross-hybridizing in nature. Therefore, an accidental introduction of *B. xylophilus* to a geographical location i.e. Europe or Australia that does not contain *B. xylophilus* but only *B. mucronatus* will probably not result in the production of interspecific hybrids. However, this hypothesis should be tested by additional *in vivo* experiments. In addition, due to the embargo status of these nematodes in wood

products, pathogenicity studies have been performed in a greenhouse using 3-year-old seedlings as hosts. This greenhouse study has provided useful data concerning the differential pathogenicity of *B. xylophilus* and *B. mucronatus* populations. However, a seedling host probably under represents the actual situation in a pine forest, and greenhouse studies probably provide the extreme case scenario. If *B. xylophilus* were introduced to Europe, the pine forest may not be in danger under the existing environmental conditions. *Bursaphelenchus xylophilus* would probably be as dangerous to the European forests as the French population of *B. mucronatus*. Therefore, the European embargo against importation of North American softwood products should be re-evaluated.

Males of *B. xylophilus*, SW population, had the same net movement in one hour toward the crude pheromone extract released by the SW isolate and the crude pheromone released by the SW females (Fig. 9). Moreover, the SW female isolate water soluble extract maintains its biological active at -20°C and 24°C for up to 3 days and at 4°C for at least 4 days (Fig. 10, Fig. 11, Fig. 12). The active portion of the water soluble extract of the SW isolate has a molecular weight less than 3,000 Da. It maintains its biological activity after lyophilization and reconstitution. Two active components of the pheromonal secretions of *G. rostockiensis* were separated by paper chromatography (Clarke, *et al.*, 1976); one of them has a molecular weight of approximately 400 Da (Greet, 1970). Sex attractants of *H. schachtii* and *G. rostockiensis* were stable up to 90°C, and maintained their biological activity after drying, and became slowly inactivated by U.V. radiation (Green, 1967). In my study, the water soluble extract from lyophilized SW population appears to maintain its species specificity property since males of *B. xylophilus* SJ and Q14 population were attracted towards it but males of *B. mucronatus*, Ch and Fr population were not. The ether insoluble fraction of the water soluble extract from lyophilized SW isolate shows biological activity, leading one to hypothesize that the pheromone of *B. xylophilus* is a highly polar, hydrophilic compound.

My work has provided molecular, biological and behavioural evidence that supports the separate species status of *B. xylophilus* and *B. mucronatus*. Also, I provided evidence that supports the de Guiran

and Bruguier (1989) hypothesis that these species come from a common ancestor. However, substantial evidence has emerged that points to separate species status for the French and Japanese isolates of *B. mucronatus*, i.e. allopatric species. In addition, this study supports the use of nematode pheromones as taxonomic tools.

Conclusion

This thesis represents the first comprehensive attempt to establish nematode pheromones as a taxonomic tool. The species-specific nature of the PWN pheromone was shown to be a useful attribute for nematode taxonomic identification. Pheromone experiments in conjunction with cross-hybridization, pathogenicity, and DNA analysis were shown to be powerful tools for nematode identification at the specific and the subspecific level.

The multifaceted approach provides evidence of the taxonomic distinctiveness of the two closely related coniferous pathogens, *Bursaphelenchus xylophilus* and *B. mucronatus* and for the taxonomic separation of *B. mucronatus* into two species, one endogenous to France and the other to Japan. In addition, my study has raised several questions. For example, in order to ascertain the extent to which individuals of *B. mucronatus* and *B. xylophilus* are attracted to each other and produce offspring, more studies must be undertaken *in vivo*, in mature trees or seedlings. Laboratory conditions mimic the natural environment of the nematodes to limited extent. Consequently, *in vitro* experiments frequently lead us in the right direction but rarely provide all the answers. In addition, the isolating mechanisms that lead to hybrid death need to be investigated in detail as does the behaviour of hybrids. In order to support my statement “we are dealing with three very closely related but distinct species in which gene flow in the wild may not exist and which have not yet developed complete reproductive isolation” additional experiments will have to be performed in pine forests under North American and European conditions. Results from such a study may help re-evaluate the quarantine status of *B. xylophilus*.

This research sets the stage for a new chapter in nematode phylogeny by advocating the collation of data from experiments on the biology and molecular taxonomy of nematodes, with that from the more conventional morphological taxonomy, to identify species. The collective use of these multifaceted techniques will lead to a better understanding of the phylum Nematoda.

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APPENDICES

APPENDIX I

Linear regression analysis of the appearance of the first symptom of pine wilt on Scots pine seedlings caused by *Bursaphelenchus* spp. isolates and hybrids versus the number of days to seedling death

Isolates	r ²	regression equation	correlation*
<i>B. xylophilus</i>			
Ib	0.26	death = 32.6 + 0.75 symptom	0.64
SW	0.14	death = 38.6 + 0.55 symptom	0.34
MSP4	0.14	death = 39.5 + 0.69 symptom	0.38
SJ	0.12	death = 44.3 + 0.38 symptom	0.35
Q14	0.05	death = 46.8 + 0.42 symptom	0.21
Ib m x SW f	0.41	death = 33.3 + 0.83 symptom	0.64
MSP4 m x SW f	0.21	death = 39.9 + 0.64 symptom	0.46
SJ m x SW f	0.23	death = 40.5 + 0.67 symptom	0.48
Q14 m x SW f	0.33	death = 44.9 + 0.52 symptom	0.39
<i>B. mucronatus</i>			
Ch	0.18	death = 44.0 + 0.87 symptom	0.43
Fr	0.41	death = 30.5 + 1.10 symptom	0.64
Ch m x Fr f	0.13	death = 73.9 - 0.35 symptom	-0.36
<i>B. xylophilus</i> x <i>B. mucronatus</i>			
Fr m x SW f	0.02	death = 66.6 + -0.29 symptom	-0.15
Ch m x SW f	0.05	death = 45.6 + 0.39 symptom	0.23
Ch m x SJ f	0.55	death = 34.9 + 0.79 symptom	0.74

* correlation of the appearance of the first symptom of pine wilt and the number of days to seedling death

APPENDIX II

Linear regression analysis of the numbers of *Bursaphelenchus* nematodes in the Scots pine seedlings versus the numbers of days to seedling death

Isolates	r ²	regression equation	correlation*
<i>B. xylophilus</i>			
Ib	0.0	nematode = 99000 + 1 death	0.0
SW	0.04	nematode = 90800 - 280 death	-0.06
MSP4	0.01	nematode = 45100 + 650 death	0.11
SJ	0.02	nematode = 82200 + 250 death	-0.04
Q14	0.27	nematode = 103000 - 720 death	-0.16
Ib m x SW f	0.0.0	nematode = 76400 + 52 death	0.01
MSP4 m x SW f	0.01	nematode = 61500 - 270 death	-0.10
SJ m x SW f	0.12	nematode = 146000 - 1500 death	-0.35
Q14 m x SW f	0.31	nematode = -104500 + 2600 death	0.56
<i>B. mucronatus</i>			
Ch	0.27	nematode = 157000 - 1800 death	-0.52
Fr	0.01	nematode = 80300 - 530 death	-0.10
Ch m x Fr f	0.21	nematode = -315100 + 5800 death	0.45
<i>B. xylophilus</i> x <i>B. mucronatus</i>			
Fr m x SW f	0.05	nematode = 62000 - 310 death	-0.10
Ch m x SW f	0.0	nematode = 62000 - 315 death	-0.09
Ch m x SJ f	0.0	nematode = 61000 - 180 death	-0.07

* correlation of the appearance of the number of nematodes in the seedlings and the number of days to seedling death

APPENDIX III

Linear regression analysis of the numbers of *Bursaphelenchus* nematodes in the Scots pine seedlings versus the weight, (shoot + roots) of the seedlings

Isolates	r ²	regression equation	correlation*
<i>B. xylophilus</i>			
Ib	0.40	nematode = 2800 + 200 weight	0.31
SW	0.23	nematode = 3400 + 430 weight	0.50
MSP4	0.31	nematode = 29300 + 410 weight	0.20
SJ	0.25	nematode = -26000 + 800 weight	0.50
Q14	0.25	nematode = 3400 + 400 weight	0.50
Ib m x SW f	0.68	nematode = -234000 + 3000 weight	0.82
MSP4 m x SW f	0.01	nematode = 533000 - 107 weight	-0.13
SJ m x SW f	0.22	nematode = 10400 + 402 weight	0.47
Q14 m x SW f	0.05	nematode = 30400 + 180 weight	0.22
<i>B. mucronatus</i>			
Ch	0.10	nematode = -21000 + 390 weight	0.94
Fr	0.04	nematode = -1200 + 300 weight	0.19
Ch m x Fr f	0.01	nematode = 60200 - 160 weight	-0.10
<i>B. xylophilus</i> x <i>B. mucronatus</i>			
Fr m x SW f	0.14	nematode = 8600 + 500 weight	0.37
Ch m x SW f	0.31	nematode = 16300 + 150 weight	0.22
Ch m x SJ f	0.48	nematode = 5000 + 220 weight	0.56

* correlation of the number of nematodes in seedlings versus the weight of the seedling