MOLECULAR APPROACHES TO IDENTIFICATION OF INTRASPECIFIC DIFFERENCES WITHIN TWO *BURSAPHELENCHUS* SPECIES

by

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MOLECULAR APPROACHES TO IDENTIFICATION OF INTRASPECIFIC DIFFERENCES WITHIN TWO *BURSAPHELENCHUS* SPECIES

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ABSTRACT

A DNA probe (pBm45) which identifies unique polymorphic DNA patterns has been isolated. The probe shows consistent differences between the morphologically similar nematode species *Bursaphelenchus mucronatus* and *B. xylophilus* and between their subspecific forms. Such molecular techniques can supplement classical taxonomy based on morphological characters especially in those cases where the morphological characters are inadequate for the classification of these nematodes.

Initially, a Sau3A partial library of *B. xylophilus* St. William genomic DNA and EcoR1 libraries of *B. xylophilus* Ibaraki, *B. xylophilus* Q1426, and *B. mucronatus* RB were constructed to aid this project as well as future studies. Additionally, eight heat shock protein homologies were isolated from the *B. mucronatus* RB library to augment possible molecular approaches to nematode classification.

The probe pBm45 was isolated from the *B. mucronatus* RB ZAP library on the basis of its similarity to the transposable element Tc1 from *Caenorhabditis elegans*. A range of nematode isolates probed with pBm45 exhibited unique band

patterns by genomic Southern analysis. Cross hybridization experiments with Tc1 to *Bursaphelenchus* spp. and pBm45 to *C. elegans* suggested a possible sequence identity. Subcloning and partial sequencing of pBm45 failed to demonstrate any signifigant homologies between pBm45 and Tc1.

Although the probe pBm45 does not show the expected similarity to Tc1, it has proven to be useful in the differentiation of nematode isolates. The use of such tools should lead to a more thorough understanding of the taxonomic affinities of *Bursaphelenchus* spp. and in the precise identification of subspecific forms of *B. xylophilus*. This in conjunction with other biological information, may help in alleviating the economic problems for the forestry industry that are associated with the presence of this nematode.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Bursaphelenchus xylophilus is the causative agent of pine wilt, the most serious disease of native pines in Japan (Mamiya, 1984). Because of the perceived threat to forest areas outside of Japan, the European and Mediterranean Plant Protection Organization (EPPO) considers *B. xylophilus* a quarantine pest and has placed an embargo on importation of lumber and lumber products from those countries that have *B. xylophilus*. *Bursaphelenchus xylophilus* has been reported to occur in parts of Canada including British Columbia (Anon, 1986, 1987), in the United States (Dropkin and Foudin, 1979) and in eastern Asia (Cheng, Maosong and Ruju, 1986; Mamiya, 1984). However, pine wilt does not occur as a disease in Canada despite the presence of the causative organism (Rutherford *et al.*, 1990). Climatic factors and the natural distribution of susceptible trees in British Columbia inhibit the pathogenicity of the nematodes in these forests. *Bursaphelenchus mucronatus*, as well as the pine wilt disease causing organism *B. xylophilus* (Mamiya and Enda, 1979), occurs in Japan. So far, *B. mucronatus* has been observed in Europe and is recorded from areas of France (de Guiran and

Boulbria, 1986), Norway (McNamara and Stoen, 1988), Sweden (Magnusson and Schroeder, 1989), and Finland (Tomminen *et al.*, 1989). However, there have been no reports of pine wilt disease in Europe caused by this pine wood nematode to date (Rutherford *et al.*, 1990).

It is known that the pathogenicity of *Bursaphelenchus* spp. to coniferous trees is influenced by environmental factors such as temperature. Additionally, *B. mucronatus* does not have the same degree of pathogenicity to pines as *B. xylophilus*, and is not regarded worldwide as a pest (Panesar and Sutherland, 1988; Riga *et al.*, 1991). The economic impact of trade embargoes on forest management practices and on export trade as a result of this infection could have serious consequences on the economy (Bergdahl, 1988). *Bursaphelenchus. xylophilus* has a significant impact on the economy of British Columbia due to the embargoes against infested wood chips and other forest export products. Because the potential of tree loss is high and can translate into high dollar values as it has in Japan (Bergdahl, 1988), it is extremely important to be able to characterize precisely the intraspecific identity of the nematode in question.

Bursaphelenchus xylophilus becomes associated with its vector, various species of pine sawyer (*Monochamus* spp.), while the beetle is dormant in pupal chambers in dead wood in the spring (Mamiya, 1984). The most efficient vector of *B. xylophilus* in Japan is the beetle *Monocamus alternatus*. The life cycle of *B. xylophilus* and its close relative *B. mucronatus* are virtually identical though the species of vector differs in different parts of the world. Within the wood of decaying trees the nematodes are in their mycophagous phase. *Bursaphelenchus xylophilus* juveniles are attracted to the beetles over winter and are present in the tracheae of the insects when they emerge in spring. The *Monochamus* spp. beetles seek out only healthy pines for maturation feeding as soon as they exit their pupal chambers. While the *Monochamus* is feeding, the dauerlarvae, dispersal fourth-stage nematodes, exit the insect tracheae and infect the trees through the wound caused by the feeding insect (Mamiya and Enda, 1972). The nematodes migrate into the woody tissues, and molt to the adult stage (Mamiya, 1984). This is the phytophagous phase of the nematode life cycle. Once in the healthy tree the nematodes enter the resin canals where they feed on epithelial cells and begin to multiply and spread

throughout the tree through the resin canal network. Within hours of the initial inoculation, effects on tree physiology can be detected. Nematode-infected trees which seem healthy in early summer can die before autumn under high temperature conditions. The initial symptoms noticed are: reduction of oleoresin exudation, transpiration and photosynthesis reduction, and chlorosis (Mamiya, 1984; Melakeberhan and Webster, 1990). Large populations of the nematode rapidly build up in the dying and dead trees. The dispersal, fourth-stage juveniles or dauerlarvae are adapted to surviving unfavorable conditions and to being carried by the insect vector to a new habitat.

The impetus for this study was the recognition that it is difficult to precisely identify *B. xylophilus* and *B. mucronatus* isolates using morphological characteristics. These species are morphologically very similar and the characters somewhat plastic. Furthermore, different geographic isolates of each of these species appear to have different degrees of pathogenicity on pines (Riga *et al.*, 1991). In order to engage in an effective control and management program, it is necessary to know the species, its geographic origin and its pathogenicity to mature pines. To this end, host

range and pathogenicity studies are an extremely important complement to molecular diagnostic studies. Many nematode pests can be controlled by crop rotation after the disease causing organism is identified. Obviously, crop rotation of pine trees is not a viable means of control. Accurate identification of nematode subspecific forms and a complementary knowledge of isolate pathogenicity would enable meaningful appraisals of the risk to soft wood production in various regions to be made. The knowledge gained should allow quarantine control measures to be more appropriately and intelligently administered.

For the purpose of general, high level taxonomy, morphological characters are usually sufficient for precise identification. However, the differences between intraspecific groups are often more subtle and require the use of more sophisticated techniques. The difficulty in precisely and reliably differentiating between *B. xylophilus* and *B. mucronatus* using classical morphological methods and the economic threat of quarantine embargoes against *B. xylophilus* infected wood products, necessitate the development of a sensitive and reliable technique of identification.

Until the advent of recombinant DNA technology, studies of the taxonomic relationships between nematodes used morphology, differential hosts and cultivar range tests, serological techniques, gel electrophoresis and biochemical methods (Burrows, 1990).

One of the key morphological feature that is used to differentiate between *B. xylophilus* and *B. mucronatus* is the shape of the female tail tip (Wingfield *et al.*, 1983). *Bursaphelenchus xylophilus* has a rounded tail whereas *B. mucronatus* most frequently has a digitate mucro. However, within populations of both species a range of tail shapes occurs. It has been suggested that the term "r-form" for round-tailed females and "m-form" for mucronate-tailed females be used to distinguish populations with different tail shapes within *B. xylophilus* (R.V. Anderson, pers. comm.; de Guiran and Bruguier, 1989). However, as a phenotypic trait, tail shape has the potential to be modified by developmental or environmental factors, and intermediate forms exist. Similarly, in males of *Bursaphelenchus* spp. intraspecific variation occurs in the spicule shape of males which makes this a less useful distinguishing character (Yin *et al.*, 1988). Consequently, geographic populations are

extremely difficult to categorize by morphology alone and a more reliable means of differentiation is required. Nematodes exist in their environment in a number of morphological forms with associated different lifestyles, appearances and exudates. Any useful identification system must be able to take this variable nature of biological expression into account. Some species of plant-parasitic nematodes (eg. *Meloidogyne* spp.) which show no or only small morphological differences have been separated into host races using host plant differential tests which recognize that different isolates have definite differences in food preference (Sturhan, 1971).

Some species of plant-parasitic nematode are commonly diagnosed on the basis of host range and pathogenicity to crop species and cultivars. Conventional screening for resistance to nematodes such as *Heterodera avenae*, a common nematode pest of cereals, relies on the use of seedlings grown in soil as a bioassay (Dropkin, 1988). The obvious problem with such an identification system is that the assay requires a full nematode life cycle until the infected roots can be examined by trained observers. In the case of *Bursaphelenchus* spp. such a biological assay for host specificity would require a great deal of time and expense and the

results may not be definitive. For example, the seedlings must be grown in greenhouses for extended periods before inoculation and then must be quarantined for the duration of the study. Such tests for the host range and pathogenicity of *Bursaphelenchus* spp. are extremely important, but not as a diagnostic tool. Biochemical and molecular methods of identification provide a more reliable method of isolate lineage because they reflect more completely the genetic differences that are only partially reflected in morphological variability (Sturhan, 1971). Single base changes of the DNA sequence can result in silent mutations which may lead to the production of identical protein products in spite of the molecular difference in the DNA code. It is also possible to produce proteins from identical sequences which can be differentially modified by environmental factors. Therefore, protein differences, a phenotypic expression, may not necessarily be indicative of any DNA difference.

Protein serology involves the reaction of antibodies to the surface proteins of the nematode cuticle and to the nematodes proteinaceous secretions and exudates. The results of early attempts (Webster and Hooper, 1968) to use polyclonal antibodies raised against nematode

proteins for nematode identification were unsatisfactory. The broad reactivity of such antibodies, variation in antigen expression and antigen conservation between closely related taxa caused such serological techniques to be viewed as too insensitive for identification purposes. The more recent advances in monoclonal antibody technology may lead to more routine use of serology (Burrows, 1990), but at present it is not the most sensitive and reliable technique available for nematodes.

Gel electrophoresis allows high resolution of protein differences on the basis of size and net charge. Studies by Dickson *et al.* (1971) and Hussey (1979) demonstrated the power of this technique over morphological identification. The procedure in general consists of extracting nematodes in a salt solution which contains reducing agents that inhibit phenol oxidase activity that would cause the denaturation of proteins. The nematodes are then homogenized and centrifuged to yield a clear solution of proteins which are then separated by electrophoresis on acrylamide gel. Proteins can be identified as discernable bands when separated on the basis of amino acid composition and molecule size because the smallest molecules move fastest

through the matrix. The protein bands can be examined after staining with a non-specific protein stain.

The problem with gel electrophoresis is that different proteins may behave similarly on the gel while having entirely different amino acid composition. Different amino acids may hold the same charge and have similar molecular weights and thus would appear similar on a protein gel. Amino acid sequence analysis would be more accurate but is laborious and not economic for nematode study. Moreover, proteins are the phenotypic expression of the genotype and, as such, they are subject to environmental influence that can result in detectable inconsistencies. Variability in results can arise also because different developmental stages of nematodes may have slightly or grossly different protein expression (Hussey and Krusberg, 1971). Similarly, in plant-parasitic nematodes, the host plant can influence the protein banding pattern (Hussey, 1979). Finally, as with other means of identification, different experimenters can and do use slightly different techniques. Variations in the extraction of protein, for example, can lead to variability in the final result (Burrows, 1990).

As the goal of diagnosis and taxonomy is the classification of genotypes of organisms, the examination of protein patterns is valid as proteins are coded for by the nucleic acid which contains all the nematode genetic information. However, the variation in protein may not always reflect the true genotype through the differential expression of many proteins. As well, the small fraction of the nematode's genome which is examined by this methodology, only 15-20%, is an important constraint. Direct examination of the nematode genome avoids problems associated with phenotypic variation in taxonomic characters (Curran, 1991). It seems logical, therefore, that the most accurate means of comparison of individuals is through comparison of DNA sequences. This was done first on parasitic nematodes by Curran *et al.* (1985). Unfortunately, direct comparison of DNA sequence is not presently practical for the rapid identification of nematodes. Advances in DNA cloning and nucleotide sequencing and restriction enzyme analysis allow direct comparisons of existing polymorphisms without the requirement of elucidating the entire genome sequence (Burrows, 1990; Hyman, 1990). For example, base changes and sequence rearrangements are examinable without direct

sequencing of the entire genome. Techniques that enable recognition of these alterations provide a means of identification of the species or host race using genomic data.

For an identification system to be diagnostically useful, it need only display differences between isolated groups. In the case of *Bursaphelenchus*, derivation of the evolutionary relationships is not being examined. Therefore, any segment of DNA which differentiates between groups is useful.

The availability of probes which detect restriction fragment length differences (RFLPs) in the DNA of separate populations has allowed for more precise determination of the relationship of those individuals. RFLP analysis of repeated nuclear DNA and mitochondrial DNA has been employed to differentiate between isolates of several plant pathogenic nematodes (Hyman, 1990; Burrows, 1990). Variation among repeated coding DNA restriction patterns is commonly observed when different species are compared. An RFLP is rarely detected among reiterated sequences derived from intraspecific populations. Although useful for detecting variation at or above the species level, repeated coding DNA is less suitable for demonstrating

intraspecific differences. Several investigations on the evolution of tandem and interspersed repeated DNA families have revealed an unexpectedly higher amount of homogeneity within families than between species. This has been well documented for ribosomal genes (reviewed by Gerbi 1985; Pace *et al.*, 1986; Vahidi, 1988) and has been attributed to a process of unknown mechanisms, called concerted evolution. The fact that there is a process involved in maintaining homogeneity at levels different from the rest of the genome makes such an avenue less suitable for molecular genetic studies. The danger of studying a single gene or region of the genome is that the data may reflect the evolution of the gene rather than the organism, and the organism does not necessarily manifest the evolutionary changes of the genome.

As there could be difficulties in the determining differences between intraspecific groups by RFLP analysis of repetitive coding DNA, it is necessary to consider other regions of the genome for study. One such region with potential for diagnostic use is that of single copy coding DNA. RFLPs in low or single copy DNA sequence can be detected by the Southern blot hybridization. The heat shock protein genes are examples of single copy

type coding DNA fragments, and this type of DNA probe was considered for use in this study. However, as such genes contain more highly conserved sequences they are most useful at higher taxonomic levels eg., genus to phylum. For this reason there is a lower probability of finding differences between intraspecific groups using such probes.

To increase the probability of differentiating between subspecific groups it may be necessary to study more variable sequences. Such sequences are the repetitive non-coding DNA which exist in relatively large amounts in the genome. It is possible to assay the genome of organisms through the study of randomly cloned DNA fragments (Curran, 1991; Curran and Webster, 1987). In this approach, random clones are screened against genomic digests for RFLPs. The randomness of the probes ensures that information derived in this fashion does not limit study to one area of the genome or type of DNA sequence. Screens can be established to test for both species specificity and isolate specificity. It has been estimated that a screen of 30-100 clones would provide discrimination of isolate groups (Curran and Webster, 1987). This method would almost certainly provide the necessary sensitivity required to differentiate nematode subspecific

groups. However, a screen of such a large number of clones would be extremely time consuming. As other approaches were available it was decided that the same goal could be accomplished through the use of a different assay system.

The transposable element Tc1 has been shown to be useful in differentiating closely related species in the genus *Caenorhabditis* as well as in the separation of subspecific groups of *C. elegans*. In genomic DNA hybridization studies of *C. elegans* Bo and *C. elegans* N2 DNA differential band patterns were observed using the Tc1 molecular probe. A transposable element is a genetic unit capable of integrating into many sites in the genome. They have been identified in several eukaryotes including *Drosophila*, maize and *C. elegans*. In these organisms the transposon contains terminal inverted repeats and a long open reading frame. Although transposon size is conserved, the random insertion and location of transposable elements is responsible for the repetitive pattern observable through genomic hybridization studies. Their location on different size fragments appears as a ladder of bands after size fractionation and Southern hybridization. It has been observed that the copy number of Tc1 elements varies between 30 in the

Bristol strain and 300 in the Bergerac strain of *C. elegans* (Emmons *et al.*, 1983; Liao *et al.*, 1983). On the basis of these observations, the isolation and use of a transposable element from *Bursaphelenchus* spp. was regarded as a useful focus of study.

The objective of this study was to develop probes which could be used to characterize the plant-parasitic nematodes *Bursaphelenchus mucronatus* Mamiya and Enda 1979 and *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle 1970 and to differentiate between these species and their intraspecific forms. Specifically, the approach was focused on examining the potential of the heat shock gene and transposable element as probes.

MATERIALS AND METHODS

Restriction enzymes used throughout this research were obtained from Pharmacia and BRL. Klenow and ligase were obtained from Pharmacia. All radioactive nucleotides were from ICN and the reaction conditions outlined by the suppliers were followed.

Strains and Culture Methods:

Isolates of *Bursaphelenchus* were obtained from North America, Asia and Europe. See Table 1 for isolate origins and sources. Field samples of *Bursaphelenchus* were obtained from 30 cm below the surface of the chip storage pile and the nematodes were extracted from wood chips in modified Baermann funnels. Isolates were maintained in Parafilm-sealed plates of *Botrytis cinerea* grown on potato dextrose agar at 27°C. The stock cultures were subcultured at bi-weekly intervals and those plates with bacterial or fungal contaminants discarded. To obtain monoxenic cultures for DNA extraction, the nematodes were surface sterilized in 0.1% merthiolate for 20 min prior to innoculation of the culture plate.

Table 1: Name, origin, and source of isolates of *Bursaphelenchus xylophilus* and *B. mucronatus*

Name	Code	Origin	Host/Habitat	Source
B. mucronatus				
Norway	Nor	Norway	unknown	D.G. MacNamarra
Chiba	Ch	Japan	*Pinus thunbergii*	Y. Mamiya
RB	Bm	Japan	unknown	R.I. Bolla
French-3	F3	France	unknown	G. de Guiran
B. xylophilus				
Ibaraki	Ib	Japan	*Pinus thunbergii*	Y. Mamiya
China	Chin	China (Nanjing)	*Pinus* (spp.)	Y. Mamiya
BC	BC	Canada (BC)	*Pinus* (spp.)	R.V. Anderson
Q1426	Q14	Can. (Quebec)	mixed conifers	R.V. Anderson
St. Williams	SW	Can. (Ontario)	nursery	R.V. Anderson

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D.G. McNamarra: European Plant Protection Organisation, Paris, France
Y. Mamiya: Forestry and Forest Products Research Institute, Ibaraki, 395 Japan

Extraction of Genomic DNA:

The nematode cultures were monitored daily and at about maximum yield the nematodes were washed off the plate covers using approximately 25 ml of 0.1M NaCl. The nematodes were partially cleaned by shaking the suspension and centrifuging at 1000 rpm for 2 min. and the pelleted nematodes were then rinsed in fresh 0.1M NaCl and repelleted. To extract the DNA, a modification of the general technique outlined in Maniatis *et al.* (1982) was used as follows: the nematodes were resuspended in 2-10 volumes of 1X proteinase K buffer [0.1M Tris (pH 8.5), 0.05M EDTA, 0.2M NaCl, 1% sodium dodecyl suphate (SDS)] containing 200 g/ml proteinase K and then instantly frozen in liquid nitrogen. The frozen nematodes were ground in a mortar and pestal and the powder warmed gradually to 65°C. The sample was held at 65°C for 10 min until the solution became viscous and clear and was then transferred from the warm mortar to 50 mL phenol-resistant Falcon tubes and extracted twice with phenol, once with 1:1 phenol:SEVAG [chloroform/isoamyl alcohol 24:1 (v/v)], and once with SEVAG. The DNA was precipitated 1hr to overnight in 2 volumes 95% ethyl alcohol, pelleted, washed in 75% ethyl alcohol, and dried under vacuum. The pellet was resuspended in

1X TE [10 mM Tris, 1 mM EDTA (pH 7.4)], and the resultant DNA was treated with one-twentieth volume boiled RNAse I (1 mg/ml) for 30 min at 37°C and stored at 4°C until required.

Cloning Strategies:

Phage libraries were constructed using either the lambda cloning vector Lambda ZAP (Stratagene) or Charon 40 phage (Dunn and Blattner, 1987).

The Lambda ZAP libraries were constructed using genomic DNA from Bm, Q14, and Ib nematode isolates digested with EcoRI. Vector DNA was treated following the protocol provided by the manufacturer. The DNA ligation was packaged using Gigapack Gold (Stratagene) following the instructions provided by the manufacturer. The recombinant phage were plated on NZYCM medium and grown at 37°C prior to harvest.

The Charon 40 library of the SW isolate was constructed using genomic DNA partially digested with SauIIIA. The DNA was ligated into the EcoRI site of the vector.

Plasmid Minipreparation:

Small scale plasmid DNA was purified by the alkaline lysis procedure (Maniatis *et al.*, 1982) with minor modifications. Luria broth (LB)

containing 50 g/ml ampicillin was innoculated with JM83 or DH5Á cells containg pVZ1 plasmids and grown overnight at 37°C. JM83 bacteria were centrifuged at 4000 rpm for 4 min and supernatant poured off. The pellet was resuspended in 1.4 ml 1X TE [10 mM Tris, 1 mM EDTA (pH 8.0)] and transferred to a microcentrifuge tube. Bacteria were repelleted and resuspended in an ice-cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0) and 5 mg/ml lysozyme. This was incubated at room temperature for 5 min prior to addition of 400 l ice-cold 0.2N NaOH/1% SDS. This was mixed gently by inversion and stored on ice for 5 min. 300 l ice-cold 3M potassium acetate/acetic acid solution [11.5 ml glacial acetic acid and 28.5 ml H_2O added to 60 ml 5M potassium acetate] was then added, mixed gently and stored on ice for 5 min. After centrifugation at 4°C for 10 min the supernatant was transferred to a new microcentrifuge tube and equal volumes of phenol, 1:1 phenol/SEVAG, and SEVAG were used sequentially to extract the sample. Two volumes 95% EtOH and 1/10 volume ammonium acetate at room temperature were added and the mixture allowed to sit on ice for 20 min prior to centrifugation, 70% EtOH wash and vacuum drying. 50 l 1X TE was added with 1 l of 1 mg/ml RNAse and incubated at room

temperature for 30 min. Plasmid minipreps were then stored at 4°C.

Southern Hybridization:

DNA was electrophoresed in agarose gels and transferred bidirectionally (Smith and Summers, 1980) to nitrocellulose for 2 h to overnight. The agarose gel was soaked first in 0.25M HCl for 20 min, 0.5M NaOH/1.5M NaCl solution for 30 min, and a 1M ammonium acetate/0.02M NaOH solution for 1 h. After transfer, filters were soaked for 1 min in 2X SSPE [5X = 0.9 M NaCl, 0.05 M disodium hydrogen phosphate, 5 mM EDTA (pH 7.0)] for 1 min and baked for 1 to 2 h at 80°C under vacuum.

Filters were prehybridized and hybridized at 1.0 ml per 100 cm^3 of filter in 5X SSPE/ 0.3% SDS.

The radioactive probe was denatured prior to hybridization by boiling for 10 min then plunging on ice. Prehybridization temperature for 0.5 hr. Filters were then hybridized overnight at 62°C.

The filters were washed either at low stringency [62°C, 2X SSPE, 0.2% SDS] or moderate stringency [65°C, 2X SSPE, 0.2% SDS]. Filters were exposed to Kodak XAR film at -70°C with intensifying screens.

Agarose Gel Electrophoresis:

DNA samples were electrophoresed in agarose gels (Maniatis *et al*, 1982) of 0.5% to 1.0% concentrations depending on the size of fragments. The agarose was dissolved in 1X Tris/borate/EDTA (TBE) buffer [89 mM Tris, 89 mM boric acid, 2.5 mM EDTA;(pH 8.3)] and contained 0.75 mg/ml ethidium bromide. Loading buffer was added to a 1X final concentration [10X LB = 25% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol in 1X TBE buffer]. The gels were run at 0.7 V/cm and DNA samples visualized by fluorescence with 302 or 365 nm ultraviolet light.

Gel Purification of DNA:

Individual DNA bands were isolated from agarose gels by excision with a spatula (Maniatis *et al*, 1982). Each band was placed in a dialysis bag with 600 uL 10 mM Tris 1 mM EDTA and placed in 1X TBE buffer and run at 1.0 V/cm for 4 hours. The current was reversed for 30 seconds to release the DNA from the side of the dialysis bag and placed in a microcentrifuge tube. The sample was then spun at 40 k for 10 min and precipitated at -20°C in 2 volumes 95% ethyl alcohol 1/10 volume 5M ammonium

acetate. After vacuum drying the DNA fragment is resuspended in 1X TE.

Sequencing of DNA:

To prepare a template for sequencing, 5 ml cultures were mini-prepped using the Miniprep Kit Plus (Pharmacia). The only modification to the described protocol involved running 50 L of sample through the column instead of the recommended 25 L.

Cloned DNA was sequenced by the dideoxy method (Sanger, 1977). Sequencing reactions were carried out according to the Sequenase procedure described, using the forward and reverse primers. All reagents for the sequencing reactions were supplied by Pharmacia. ^{35}S ATP was used in the reactions instead of ^{32}P ATP because of advantages of less exposure to radioactivity, longer half-life, and sharper bands (Williams *et al.*, 1986).

Sequencing reactions were run on 6% acrylamide, 8M urea gels at 50 W for approximately 3.5 hours. Gels were dried under vacuum at 80°C for 30 minutes. The dried gel was placed directly on Kodak XAR film and developed 24 to 48 hours later.

To determine the appropriate subclones for sequencing, each of the subclones was digested out of the vector and Southern blotted onto

nitrocellulose filters. The most strongly hybridizing bands were then chosen for sequencing.

Subcloning strategies:

A 4.5 kb EcoR1 fragment from *B. mucronatus* RB containing homology to Tc1 was isolated by gel electrophoresis and electroelution. The fragment was restricted with TaqI and Sau3A and ligated into the multiple cloning site of the pVZ1 plasmid vector. The TaqI fragments were ligated into the AccI site of the vector and the Sau3A fragments were ligated into the BamH1 site of the vector. This strategy results in overlapping subclones allowing for orientation during sequencing. The subclones were transformed into DH5Á cells (BRL), which give very high efficiency of transformation and are nuclease-free.

RESULTS

In this study three approaches were taken to develop probes for *Bursaphelenchus* spp. isolates. These approaches used probes that identified either: i) random restriction fragment length differences (RFLD), ii) specific genes, namely the gene for the heat shock 70kd protein, or iii) transposable element homologies.

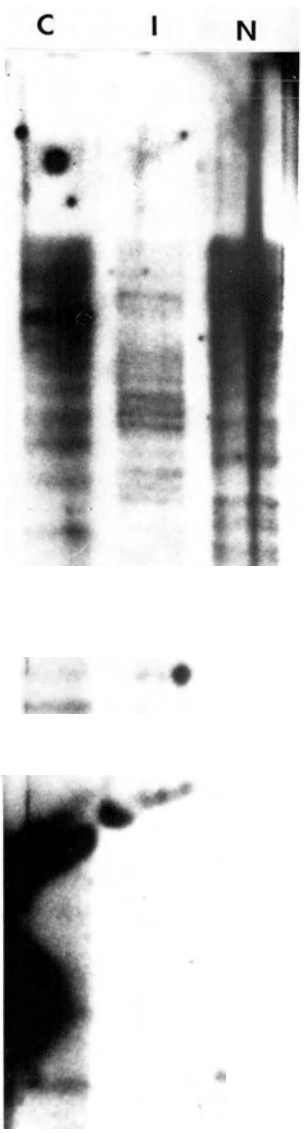
Since no libraries of *Bursaphelenchus* DNA existed prior to this study, which commenced in 1987, the first step of this research was the construction of genomic libraries. Two types of libraries were constructed; insertion libraries using ZAP and a replacement library using Charon 40. The ZAP libraries were made with DNA from three different nematode isolates.

1. Construction of Genomic Libraries:

B. xylophilus St. William isolate DNA was used to construct a Sau3A partial library in the Charon 40 vector. As partial libraries contain only partially digested DNA they are particularly suitable for genomic studies requiring overlapping DNA fragments.

Figure 1: Hybridization of random genomic probe rp1 to *Bursaphelenchus* species genomic DNA

Lanes 1-3 show genomic DNA from *Caenorhabditis elegans* N2 (C), *B. xylophilus* Ibaraki (I), and *B. mucronatus* Norway (N) was digested with EcoR1 and size-fractionated on 0.7% agarose gel. After Southern transfer of the DNA to nitrocellulose membrane the DNA was probed with P32-labelled random genomic probe rp1.


C
I
N

The Lambda ZAP vector was used to create EcoR1 genomic libraries. Three nematode isolates were constructed with the ZAP vector to yield total genomic libraries, namely *B. xylophilus* Ibaraki, *B. xylophilus* Q1426 and *B. mucronatus* RB.

2. Isolation of Random Genomic Clones

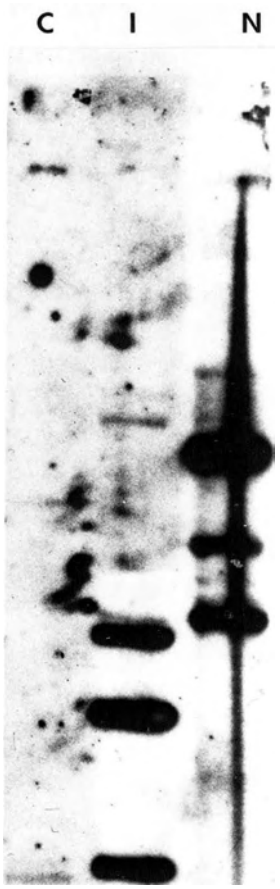
The *Bursaphelenchus mucronatus* RB library was used to isolate 20 genomic EcoR1 clones. Samples of distinctly separate phagemid plaques were obtained and the associated insert excised. The subsequent 20 unique clones were available for use to distinquish inter/intraspecific differences on the basis of hybridization patterns. Initial hybridization studies using a random probe (rp1) demonstrated the potential usefullness of this method. When hybridized to genomic DNA from *B. xylophilus* Ibaraki and *B. mucronatus* Norway numerous bands were observed (Fig. 1).

3. Identification of Heat Shock Protein Homologies:

The *B. mucronatus* RB Lambda ZAP library was screened with the hsp70A gene from *C. elegans* (Heschl, 1988). A total of eight positives were selected from the screen and isolated as plasmid

Figure 2: Hybridization with *Bursaphelenchus*-derived heat shock gene probe hc1

Lanes 1-3 show genomic DNA from *Caenorhabditis elegans* N2 (C), *B. xylophilus* Ibaraki (I), and *B. mucronatus* Norway (N) was digested with EcoR1 and size-fractionated on 0.7% agarose gel. After Southern transfer of the DNA to nitrocellulose membrane the DNA was probed with P32-labelled heat shock gene homologous probe hc1.


C
I
N

clones. One of these clones (hc3) was identified by sequence analysis as being a member of the heat shock protein gene family. Hc3 was used to probe digested genomic DNA from *B. xylophilus* Ibaraki and *B. mucronatus* Norway. In this hybridization (Fig. 2), band differences are visible between the two nematode species.

4. Identification of a repetitive DNA element in *Bursaphelenchus mucronatus* Norway genomic DNA:

A. Genomic Southern Blots

Initially, EcoRI digested genomic DNA from the *B. mucronatus* Norway strain was probed with a Tc1 probe from *C. elegans* pCes100. The probe was a 1.2 kb fragment containing the 5' end of the coding region of the transposable element Tc1. Hybridizations were performed at moderate stringency. *Bursaphelenchus mucronatus* gave 5 bands with this heterologous probe (see Figure 3). Furthermore, genomic DNA digested with restriction enzymes BamHI, SalI and HindIII showed multiple copy band patterns which suggest either hybridization to a multiple gene family or to a transposable element type family.

Figure 3: Identification of a Repetitive DNA Element in *Bursaphelenchus mucronatus* Norway genomic DNA

Figure 3a shows EcoR1-digested *Caenorhabditis elegans* (C) N2 DNA when size-fractionated in 0.7% agarose gel. After unidirectional transfer of the gel onto nitrocellulose membrane, the filter was hybridized with the probe pCes100. The probe contains the 5' end of the coding region of the *C. elegans* transposable element Tc1 and was labelled by the random-priming method. Hybridizations were performed at moderate stringency (65 C, 2X SSPE).

Figure 3b shows a DNA size marker in lane 1. Lanes 2 and 6 show EcoR1-digested *Bursaphelenchus mucronatus* Norway (N) isolate (4ug/lane), Lane 3 shows EcoR1-digested *B. xylophilus* Ibaraki (I) isolate (4ug/lane), Lanes 4 and 5 show HindIII genomic digests of the Norway and Ibaraki isolates respectively. The probe used was pCes100.

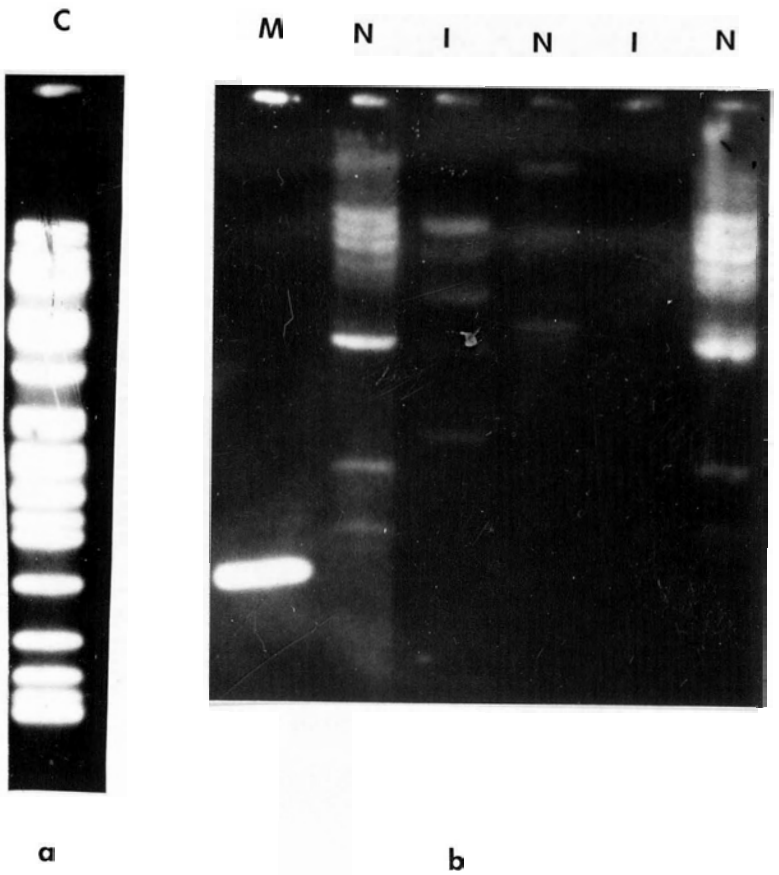
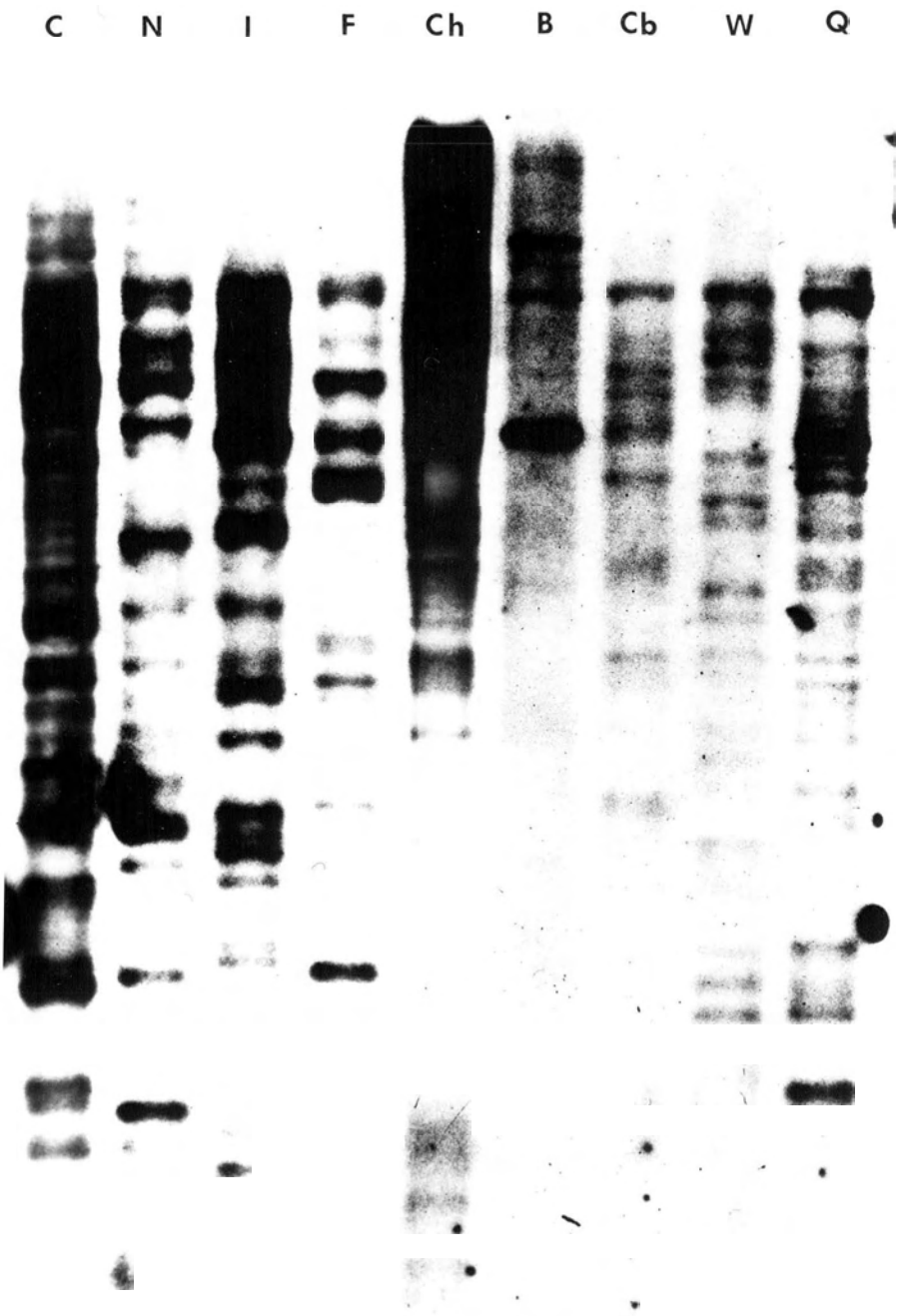

C
M
N
I
N
I
N
a
b

Figure 4: Hybridization of probe pBm45 to *B. mucronatus* and *B. xylophilus* intraspecific groups

Genomic DNA (4 ug/lane) from *C. elegans* (C), *B. mucronatus* isolates Norway (N), Chiba (Cb), and French-3 (F) and from *B. xylophilus* isolates Ibaraki (I), China (Ch), BC (B), Q1426 (Q), and St. William (W) was digested with EcoR1 and size-fractionated on 0.7% agarose gel. Probe pBm45 was labelled with 32P by the random priming method and hybridized to the transferred DNA at low stringecy.


C
N
I
F
Ch
B
Cb
W
Q

B. Screen of *Bursaphelenchus mucronatus* library

As bands were seen to exist in *B. mucronatus* RB an attempt was made to recover clones homologous to the transposable element Tc1. The Lambda ZAP *B. mucronatus* RB strain library was screened with the pCes100 probe at moderate stringency and five positives were identified. Each of the five clones selected contained a 4.5 kb Eco R1 insert and two of the clones also contained an additional 7.0 kb Eco R1 fragment. The 4.5 kb band common to all the positives was chosen for further investigation and is referred to as TE1.

C. Hybridization to *B. mucronatus* and *B. xylophilus* intraspecific groups

The TE1 element was hybridized at low stringency to three isolates of *B. mucronatus* (Norway, Chiba, French-3) and five isolates of *B. xylophilus* (Ibaraki, China, BC, Q1426, St. William). Band patterns of each of these hybridizations of these subspecific groups were completely different. Approximately 4 ug of DNA were loaded in all lanes except for the Q1426 isolate which showed less intense bands than the other isolates. (Fig. 4) This blot also demonstrated that the pBm45 probe hybridizes to *C. elegans* and displays a multiple

band pattern similar to the pattern observed when Tc1 is hybridized to *C. elegans*.

D. Characterization of the TE1 element

i. Sequencing

In order to characterize the TE1 element further the 4.5 kb TE1 fragment and four selected subclones were sequenced. For all subclones only the ends of the fragments were examined. The resultant sequence covers over 700 bp of the original TE1 fragment. The sequence data obtained were compared to the Tc1 sequence from *C. elegans*.

ii. Comparison to Tc1

The computer program FASTA was used to compare the sequence obtained from pBm45 to the published sequence of Tc1 (Harris, 1988). Only partial homology was identified over a region of 25 base pairs.

DISCUSSION

In order to achieve the objectives of this study a number of different methods were developed and tested before an effective and informative method was chosen for this study. Molecular studies of this type require genomic libraries that contain the cloned DNA of isolates involved in the study.

In studies of the molecular nature of an organism the primary tool is the genomic library. The utility of the library is twofold. Firstly, it immortalizes the DNA of the organism in question. In the case of difficult-to-obtain strains or crosses this is extremely important for the subsequent research. Specifically, it takes a great deal of time to make the crosses from which the DNA is isolated. When the cross has been made and the strain establised, it is best to preserve the DNA of that isolate in a recombinant DNA vector so that there is available a supply of the exact same material for later tests. In some cases it may not be possible to duplicate the DNA of the isolate under study. Secondly, the library allows the simple and rapid isolation of specific fragments of DNA for further study in a nondegenerative fashion.

Equally important, one can isolate and amplify sequences for further molecular characterization such as DNA sequence analysis. The libraries constructed in this study appear to be of high quality as they have yielded satisfactory clones which have been used in this and other projects *eg.*(Beckenbach *et al.*, 1992). It is hoped that the libraries will continue to be used and demonstrate their utility on other projects.

With respect to this study of *Bursaphelenchus* spp., it was important to have the cloned DNA from the progeny of single female crosses in order to minimize variation within the library. Different individuals contain different sequences which would confuse the analysis if the studies were of DNA from a population of individuals rather than DNA clones from one individual. Sampling a single individual from the population allows one to assert that patterns observed are genetically related.

There is no single strategy for cloning of genomic DNA. Similarly, there is no vector suitable for cloning all DNA fragments. Libraries of eukaryotic DNA can be prepared by either complete or partial digestion and insertion of the fragments into an appropriate bacteriophage vector. Complete digestion with an enzyme like EcoR1 yields

relatively large fragments of ~2-10 kb in length which can be cloned into an insertion vector like Lambda ZAP (Stratagene). These fragments are small enough to be added to the DNA of the bacteriophage without inhibiting its ability to package and replicate normally. Alternatively, the DNA can be digested partially with an enzyme which cuts more frequently in the genome and inserted into a replacement vector such as Charon 40 (Dunn and Blattner, 1987). This technique results in larger inserts and, as a result, certain parts of the bacteriophage must be removed in order for the phage to grow normally. The latter method has the advantage of allowing one to "walk" along the chromosome from adjacent, overlapping fragments. Walking is possible by using an isolated clone as the hybridization probe for overlapping clones in both directions from the original clone. Partial libraries do not interfere with sequences of interest which may have a restriction site that would otherwise be digested to completion.

In this study, I have used the vector Lambda ZAP and Charon 40. The Lambda ZAP vector was chosen for its simplicity of use in recovering subclones from the library. The Lambda ZAP vector simplicity lies in its ability to directly isolate subclones in

the Bluescript plasmid vector by excision from the lambda bacteriophage (Short *et al.*, 1988). Other vectors require intermediate steps of isolation of lambda clones and their subsequent cloning into a plasmid vector. The Charon 40 phage allowed for the somewhat larger size of fragments generated for constructing an overlapping library (Blattner, 1988).

1. Evaluation of Approaches:

The rationale chosen to effectively differentiate between the three different approaches is described below.

i. Random probes

The technique of using randomly generated DNA probes to identify genomic DNA differences has previously been demonstrated for the free-living nematode *C. elegans* (Rose *et al.*, 1982), the plant-parasitic nematode genus *Meloidogyne* (Hyman, 1990) and the filarial nematodes (Rollinson *et al.*, 1986). Preliminary results obtained in this study demonstrate the potential of the method for use in nematode identification. The high degree of RFLP variability when the random probe rp1 was used to probe genomic DNA of different species of

Bursaphelenchus, indicates that it is highly likely that random probe hybridizations would be an effective method of differentiating the *Bursaphelenchus* spp. isolates. The technique relies on randomly cloned DNA fragments from one isolate to be hybridized to other isolates and the fraction of differences is used to demonstrate relationships. Many hybridizations with differentially restricted DNA are required to develop the required probes with the same end achieved as if previously defined genes had been used. Therefore, as the goal of the project was the elucidation of specific and subspecific differences and other methods were available, it was decided to use another approach that was more economical in its use of DNA and time.

ii. Specific gene (heat shock protein gene)

RFLP analysis among single copy DNA is used to survey sequence divergence with a major fraction of the genome where substantial genetic variation can exist. Single copy RFLPs should provide a more sensitive assay of genotypic divergence between populations than would repeated coding DNA, because there are more different single copy sequences than different repetitive coding DNA sequences (Hyman, 1990). However, as specific gene DNA probes (such

as the heat shock gene) survey sequence of coding DNA there are obviously constraints on the variability which can be observed. Sequence conservation dictates that, in general, such highly conserved sequences (heat shock genes, histone genes, etc.) are most useful at higher taxonomic levels. Indeed, variability in *Bursaphelenchus* spp. was indicated in this study using such probes. It is unlikely that highly conserved sequences would be useful for the study of intraspecific groups. In addition to the requirement of differentiation of species, this study examines isolates which are very closely situated geographically and highly similar morphologically. The determination of intraspecific variation in this case requires a highly rigorous strategy. Therefore, there seemed to be a greater probability of detecting variation through the analysis of DNA sequences that are not under such tight, conservative influence as are coding DNA regions.

iii. Transposable Element

Transposable elements can also be used to determine relationships between isolates. In this thesis, DNA sequences from the free-living nematode, *C. elegans*, hsp70 and Tc1 transposable element were

used to isolate probes which have been used to identify differences between isolates of *B. mucronatus* and *B. xylophilus*. After an initial analysis of the characteristics of the hsp70 gene family in *Bursaphelenchus* spp., the transposable element Tc1 was chosen as the molecular probe for this study.

2. Repetitive DNA patterns

The results presented above demonstrate the presence of a distinctive repeated element within the genome of *Bursaphelenchus* spp. isolates. Apart from the utility of the genomic hybridization pattern in identifying isolates, it is interesting to consider why the repeat DNA is present. Analysis of the molecular data from various organisms suggests that there are three possible explanations for a repetitive pattern. These possibilities will be discussed and evaluated below as they relate to the *Bursaphelenchus* spp. data.

Firstly, as others have reported, repetitive coding DNA has been used for distinguishing patterns of restriction fragment length polymorphisms in a number of different applications (reviewed in Hyman, 1990). In such cases, related coding regions which are duplicated yet contain rare differences in

sequence are identified during genomic hybridization or through visualization on ethidium stained agarose gels.

There are two common types of multigene families: 1) tandem, and 2) dispersed. In higher eukaryotes the ribosomal DNA genes are found in a single DNA unit which is tandemly repeated (Vahidi *et al.*, 1988). As the tandem repeat changes at widely separated intervals, there exists a number of very nearly identical repeats which may be cut at different restriction sites. Genomic hybridization with a probe to such a repeat yields a "ladder" of fragments corresponding to the varying number of repeat units present.

Collagen genes in *C. elegans* are an example of a dispersed multigene family. Collagen genes are present in separate chromatin domains and are separately controlled. As there are different copies in different areas of the genome hybridization with a collagen probe will also yield a number of bands on a genomic Southern blot corresponding to each of the different genes at a different location of the genome. However, in *Bursaphelenchus* there are a large number (>10) of bands visible at moderate hybridization stringency. It is unlikely that such a large number of different

copies is required to code for one of the major structural proteins such as collagen, actin, tubulin, or histone genes that usually make up this type of gene family. Therefore, as a repetitive coding gene does not seem to be a likely candidate for the pattern of repetitive DNA observed in *Bursaphelenchus* spp., other possibilities were investigated.

The second type of repetitive DNA commonly found is that of transposable elements. A transposable element is a genetic unit capable of integrating into many sites in the genome. They have been identified in several eukaryotes including *Drosophila*, maize and *C. elegans*. In these organisms the transposon contains terminal inverted repeats and a long open reading frame. In the cases of the *Drosophila* foldback FB elements and the sea urchin TU elements (Potter *et al.*, 1980; Liebermann, 1983; reviewed in Felsenstein and Emmons, 1987) an inverted repeat alone is the repetitive unit. Although transposon size is conserved the random insertion and location of transposable elements is responsible for the repetitive pattern observable through genomic hybridization studies. Their location on different size fragments appears as a ladder of bands after size fractionation and

Southern hybridization. It has been observed that the copy number of Tc1 elements varies between 30 in the Bristol strain and 300 in the Bergerac strain (Emmons *et al.*, 1983; Liao *et al.*, 1983). Of particular relevance to this study, Tc1 identifies a sequence subsequently isolated from *B. mucronatus*, which is useful for describing relationships between isolates. Sequence analysis suggests that although the detected fragment was isolated with the transposable element Tc1, it is not in fact very similar to the transposable element. It is highly likely that in this case the base composition is responsible for the original clone being extracted. It is known that Tc1 exhibits a relatively high A-T rich base composition similar to that found in non-coding repetitive DNA. This similarity could be responsible for the original similarity during library screening.

The third major class of repetitive DNA commonly observed is that of non-coding repetitive satellite DNA. A large fraction of the DNA of eukaryotic genomes consists of families of short, interspersed repeated sequences a few hundred nucleotides in length (Felsenstein and Emmons, 1987; Jelinek and Schmid, 1982). The human genome contains many dispersed tandem-repetitive "mini-

satellite" regions. These regions are highly polymorphic and thus can provide an individual-specific fingerprint when probed with a tandem repeat of the core sequence (Jeffreys *et al.*, 1985a,b). Similarly, species-specific tandem repeats exist in the nematode *C. elegans* (Felsenstein and Emmons, 1987) and in *Trypanosoma cruzi* (Borst *et al.*, 1981; Gonzalez *et al.*, 1984) where it is used as a probe for parasite detection and identification. In addition a cloned repeat from the filarial nematode *Brugia malayi* has been used to distinguish *B. malayi* from the closely related *B. pahangi* by restriction site polymorphisms and by differences in specific regions of the DNA sequence (McReynolds et al., 1986). These molecular probes are proving to be extremely useful in the collection of data regarding important parasitic diseases.

In conclusion, this work has shown the feasability of molecular approaches to subspecies classification of nematodes. Construction of genomic libraries allowed isolation and characterization of molecular probes useful for identification purposes. In particular, the unique probe pBm45 was used to demonstrate the effectiveness of this molecular approach. The

isolation of other probes, for example the heat shock protein homologous sequences, will allow for a more thorough understanding of *Bursaphelenchus* spp. biology and its associated economic implications.

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APPENDIX

Sequence comparison of subclone s9t3 from *Bursaphelenchus mucronatus* RB genomic library clone pBm45 to the *Caenorhabditis elegans*-derived transposable element Tc1. Comparison was performed using the FASTA sequence software.

sequence a: s9t3 413 nucleotides

sequence b: tc1 1610 nucleotides

74.1% identity in 27 nucleotide overlap

```
     350       360       370
a) AATGCCCCTGGCTTAAGCATGGCGATG
   X::::::     :::::::::X : ::
b) AATGCCCAACCGTTAAGCATGGAGGTG
     870       880       890
```