DNA probes for differentiating isolates of the pinewood nematode species complex

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SUMMARY

The development of two species specific probes (pBx6 = *Bursaphalenchus xylophilus* and pBm4 = *B. mucronatus*), based on restriction fragment analysis following hybridization with heterologous probes, proved to differentiate isolates of *Bursaphelenchus* spp. within the pinewood nematode species complex (PWNSC). These probes give a " yes "/" no " result when used in a dot blot system to distinguish between *B. xylophilus* and *B. mucronatus*. Southern blot hybridization and double digestion of extracts from sixteen isolates confirms the existence of a *B. xylophilus* group and a *B. mucronatus* group within the PWNSC, and the *B. mucronatus* group comprises at least two subgroups. The technique demonstrates genotypic differences between isolates and clearly separates infraspecific groups of European, Asian, and North American origin.

RÉSUMÉ

Sondes d'ADN permettant de différencier des isolats appartenant au complexe d'espèces du nématode du pin

La mise au point de deux sondes spécifiques (pBx6 spécifique de *Bursaphelenchus xylophilus* et pBm4 spécifique de *B. mucronatus*), basée sur l'analyse des fragments de restriction après hybridation à l'aide d'une sonde hétérologue, permet de distinguer des isolats de *Bursaphelenchus* spp. appartenant au complexe d'espèces du nématode du pin. Utilisées en système « dot blot » afin de distinguer *B. xylophilus* de *B. mucronatus*, ces sondes donnent une réponse « positif/négatif ». L'hybridation sur « Southern blot » après une double digestion des extraits provenant de seize isolats confirme l'existence d'un groupe *B. xylophilus* et d'un groupe *B. mucronatus* au sein de ce complexe d'espèces; le groupe *B. mucronatus* comprend au moins deux sous-groupes. Cette technique prouve l'existence de différences génotypiques entre isolats et sépare clairement les groupes infraspécifiques originaires d'Europe, d'Asie et d'Amérique du Nord.

Pine wilt is the most serious disease of native pines in Japan (Mamiya, 1984) and potentially is the most important nematode disease of conifers worldwide. The disease is caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (= *B. lignicolus*), which has been reported from parts of Canada, the United States (Dropkin & Foudin, 1979) and eastern Asia (Mamiya, 1984; Cheng, Maosong & Ruju, 1986). There are reports of similar nematode species occurring in the temperate forests of Europe and Siberia, but there are no reports of *B. xylophilus* in these areas. There is concern by some that pine wilt could become a more widespread disease in North America (Wingfield *et al.*, 1982), Europe (Magnusson, 1986) and Asia (Mamiya, 1987) as well as in pine plantations of the southern hemisphere (Rutherford & Webster, 1987). The European and Mediterranean Plant Protection Organization (EPPO) has placed *B. xylophilus* on the A1 list of quarantine pests (Anon., 1986) because : 1) it is a perceived threat in view of the devastation this nematode causes in Japan and, 2) of its presumed absence from Europe. This action has profound implications for the marketing of softwoods and softwood products.

Difficulties have arisen with respect to the precise identity of some isolates of *B. xylophilus* and *B. mucronatus* Mamiya & Enda. In particular, the variability and overlap in range of several of the definitive, taxonomic characters of these and similar species is such that their accurate identification is difficult. In Japan, the presence of a mucro on the tail of female *B. mucronatus*, is a major character used in separating this nematode from *B. xylophilus* (Mamiya & Enda, 1979). As well, *B. mucronatus* is more widely distributed in Japan (Mamiya & Enda, 1979) and is reportedly less pathogenic than *B. xylophilus* under field conditions (Mamiya & Enda, 1979; Cheng, Maosong & Ruju, 1986; Tamura & Enda,

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1986). Nematodes in Europe that possess a mucro are not associated with pine mortality in the field and are considered by some to be representatives of *B. mucronatus*, rather than *B. xylophilus* (de Guiran *et al.*, 1985; de Guiran & Boulbria, 1986; Anon., 1986). However, specimens of *B. xylophilus* from North America show a wide variation in female tail shape, from mucro-type to round-type (Wingfield, Blanchette & Kondo, 1983; Fukushige & Futai, 1985). Hybrid crosses of Japanese *B. xylophilus* and *B. mucronatus* reportedly do not produce fertile offspring (Mamiya, 1986). However, fertile offspring were produced from crosses of a French *B. mucronatus* with both Japanese and North American *B. xylophilus* and also with the Japanese *B. mucronatus* (de Guiran & Boulbria, 1986). On the basis of this information, de Guiran and Boulbria (1986) hypothesized that *B. xylophilus* and *B. mucronatus* are members of a " super species "; In view of the capability of exchanging genetic material either directly or indirectly, such as through the French population, it would seem appropriate to regard these two species as members of a " super species ", or species complex until there are more reliable and precise diagnostic characters. We refer to this group of similar nematodes associated with conifers as " the pinewood nematode species complex " or " PWNSC " (Rutherford, Mamiya & Webster, *in press*).

A phoretic relationship with wood boring beetles, primarily *Monochamus* spp., accounts for rapid dispersal of the PWNSC from tree to tree. *Monochamus* species occur, with overlapping distributions, throughout the forested areas of North America (Linsley & Chemsak, 1985), Europe and Asia (Hellrigl, 1971). Such overlapping distribution of the vectors throughout the range of PWNSC provides the opportunity for genetic exchange.

Pathogenic and non-pathogenic isolates of PWNSC exist (Kiyohara & Bolla, 1988), but the pathogenicity tests and taxonomic characters used to differentiate them are impractical or unreliable. De Guiran *et al.*, (1985) overcame this problem of species differentiation among certain *Bursaphelenchus* spp. using enzyme electrophoresis. However, enzymes, like morphological characters, tend to be highly conserved between closely related taxa and their expression is modified by environmental and ontogenic factors, and the conclusions may, therefore, be rather limited. Recombinant DNA technology enables a more reliable characterization of the nematode than does enzyme electrophoresis and so provides an improved method for the differentiation of specific and intraspecific groups (Curran, Baillie & Webster, 1985; Williams, de Salle & Strobeck, 1985; Kalinski & Huettel, 1988).

We here describe the initial stage of development of a rapid, reliable diagnostic technique, based on recombinant DNA technology, for the identification of *B. xylophilus* and *B. mucronatus*. This paper describes 1) the

Table 1

List of *Bursaphelenchus* isolates used in developing and testing DNA probes.

Name	*Code*	*Origin*	*Host tree*
Bursaphelenchus mucronatus group			
RB	Bm	Japan	*Pinus densiflora*
Chiba	Ch	Japan	*P. densiflora*
Norway	Nor	Norway	*P. sylvestris*
French	Fr	France	*P. pinaster*
Bursaphelenchus xylophilus group			
MSP-4	MSP_4	USA (Missouri)	*P. sylvestris*
Ibaraki	Ib	Japan	*P. densiflora*
Fukushima	Fuku	Japan	*P. densiflora*
BxUJA	JA	USA (Alabama)	*P. banksiana*
British Columbia	BC	Canada (B. C.)	*Pinus* sp.
Alberta	Alb	Canada (Alberta)	*P. banksiana*
mm	mm	Canada (B. C.)	(insect vector : *Monochamus maculosa*)
Fids	Fids	Canada	
Q1426	Q14	Canada (Quebec)	chips-mixed conifers
Q52A	Q5	Canada (Quebec)	*Pinus* sp.
St. William	SW	Canada (Ontario)	?
St. John	SJ	Canada (N. B.)	?
China	Chin	China (Nanjing)	*Pinus* sp.
Georgia	Georg	USA (Georgia)	?

development of a " yes/no " dot blot assay to separate populations of *B. xylophilus* and *B. mucronatus* groups within the PWNSC and 2) tentatively the genetic relationships of a range of PWNSC isolates to *B. xylophilus* and *B. mucronatus.*

Materials and methods

Culture conditions

Some seventeen isolates of *Bursaphelenchus* of the PWNSC were obtained from Europe, Asia and North America and maintained in culture. The origin of each isolate is listed in Table 1. Those isolates obtained directly from a field location were extracted in modified Baermann funnels from 500 ml samples of wood chips taken at 30 cm depth from bulk storage piles of chips at mills or the railhead. The isolates were maintained in parafilm-sealed plates of *Botrytis cinerea* grown on potato dextrose agar (PDA) at 27° C. Routine, 2-weekly subcultures were done (PDA plates being inoculated with *B. cinera* 2 weeks before adding the nematodes), the cultures were monitored and those with bacterial and fungal contaminants were discarded. When monoxenic cultures of the nematodes were needed for DNA extraction, the nematodes were surface sterilized in 0.1 % of merthiolate for 20 min prior to inoculation on the culture plate. All laboratory procedures involving culturing of the nematodes were done under standard aseptic conditions, and waste culture plates and nematodes were autoclaved prior to disposal.

DNA isolation

Nematodes that had migrated to the lid of the Petri dish were rinsed off with 0.05 M NaCl, and concentrated by centrifugation at 2 000 rpm for 2 min at room temp. The NaCl solution was discarded and the nematodes were resuspended in seven volumes of Proteinase K buffer (0.1 M Tris pH 8.0, 0.05 M EDTA, 0.2. M NaCl, and 1 % SDS) containing 1.0 mg/ml proteinase K. The solution containing the nematodes was frozen in liquid nitrogen, transferred to a mortar and ground into a fine powder. After thawing the solution was transferred to a 50 ml Falcon tube and was extracted three times with TE (10 mM Tris pH 8.0 and 1.0 mM EDTA) saturated phenol pH 8.0 and twice with 24:1 $CHCl_3$:iso-amyl alcohol. The clean DNA was precipitated by adding two volumes of 95 % ethanol, pelleted, dried and redissolved in TE plus 10 µg/ml RNase A (Maniatis, Fritsch & Sambrook, 1982). The concentration of DNA was determined by running a small sample on an agarose gel with known standards. Plasmid DNA was isolated using the alkaline lysis method (Maniatis, Fritsch & Sambrook, 1982). Phage DNA was isolated using the method of Davis, Botstein and Roth (1980).

Cloning strategies and isolation of species specific probes

Phage libraries were constructed using the lambda cloning vector λ gtWES (Bethesda Research Lab or BRL). Genomic DNA from *B. xylophilus* MSP-4 and *B. mucronatus* RB were digested with EcoRI and ligated into the EcoRI site in λ gtWES following the protocol provided with the vector. The DNA ligation was packaged using Gigapack Gold from Stratagene. The phage were plated on *Escherichia coli* strain C600 on LB plates (Davis, Botstein & Roth, 1980) at 37° C.

The phage libraries were screened using a nick translated, ribosomal clone from *Caenorhabditis elegans* (pCes 370). A phage clone which gave a positive signal to the probe was isolated. DNA was then extracted from the phage digested with Eco RI (BRL) and ligated into pUC19 as described by Snutch (1984). The ligation reaction was transformed into *E. coli* strain JM83 that had been made competent (Morrison, 1979). The transformed cells were plated onto LB plates containing 100 µg/ml ampicillin, 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 160 µg/ml isopropyl-β-D-thiogalactopyranoside. White colonies were selected and DNA was isolated. A subclone from both *B. xylophilus* and *B. mucronatus* was digested with BamHI, EcoRI, EcoRV, KpnI, HindIII, PstI, SalI, XbaI, and XhoI, size fractionated on a 0.7 % agarose gel (Davis, Botstein & Roth, 1980) and transferred to nitrocellulose using the bidirectional transfer method of Smith & Summers (1980). The 5′ and 3′ ends of the 18s and 28s genes from *C. elegans* (provided by Dr. B. Honda) were nick translated (Davis, Botstein & Roth, 1980) using dATP-^{32}P (Amersham). These nick translated fragments were hybridized to the nitrocellulose filters. Analysis of the resulting autoradiographs allowed us to construct a restriction and gene map of the ribosomal cistron for each of the two species.

The large EcoRI/HindIII fragment, containing the nontranscribed spacer region (NTS), the 5′ end of the 18s and the 3′ end of the 28s, was subcloned into pUC19 to facilitate the isolation of the NTS region. To isolate the NTS region from *B. xylophilus* a HaeIII fragment was subcloned into the SmaI site of pUC 19. This clone contained about 250 basepairs of the 5′ end of the 18s coding regions. The coding sequences were removed using the ExoIII method described by Henikoff (1987). The resulting 1.7 kb clone was named pBx6.

A 1.35 kb Sau3A fragment from *B. mucronatus* was identified as containing the NTS region by using the Smith and Bernstiel (1976) restriction site mapping method. This Sau3A fragment was ligated into the BamHI site of pVZI (Henikoff & Eghtedarzadeh, 1987) and the resulting clone, which contained only NTS region, was named pBm4.

This dot blot method was designed to identify two species of nematode from among many isolates of morphologically similar nematodes. A positive control

was made to demonstrate that a negative with both pBx6 and pBm4 was due to the absence of *B. xylophilus* or *B. mucronatus* rather than to lack of DNA on the filter. The positive control was made by ligating 1.1 kb of the 5' region of the 18s gene from *B. mucronatus* into pVZ1. This clone was named pBm3. To confirm that this clone would function as required, the DNA sequence was determined using the Sanger (1977) sequencing method. The sequence from pBm3 was compared with the sequence of the *C. elegans* 18s gene. The overall sequence homology is only 56 %. However, there are runs of homology that are 68 % similar and should, therefore, work well for nematodes that are more closely related to *B. mucronatus* than to *C. elegans*.

Dot blots and hybridizations

Dot blots routinely used 60 ng of DNA in 20 µl of H_2O to which 4 µl of 2 M NaOH was added to denature the DNA, after 10 min 6 µl of 4 M NH_4OAc was added and 10 µl of each sample was spotted in triplicate onto a piece of nitrocellulose in a dot blot apparatus (BRL) while under suction. After all the samples had been spotted onto the nitrocellulose the wells of the dot blot apparatus were rinsed with 1 M NH_4OAc and the nitrocellulose air dried and then baked at 80° C for 2 h.

Hybridizations to the dot blot, using the species specific probes were done at 70° C with 5 × SSPE (1 × SSPE = 0.18 M NaCl, 10 mM $Na_{1.5}PO_4$, 1 mM NaEDTA pH 7.0) and 0.3 % SDS. These were then washed at 68° C using 0.2 × SSPE and 0.2 % SDS. All other filters were hybridized at 65° C and washed at 62° C in 2 × SSPE and 0.2 % SDS and then exposed to X-ray film.

Blind trial for the probes

Once the NTS region had been cloned into a plasmid vector its accuracy was verified by performing a blind trial. Five isolates were obtained from Dr. R. V. Anderson and DNA was extracted and spotted in triplicate onto a nitrocellulose filter. A positive control was included for each of the probes by spotting DNA from MSP-4 and RB onto the filter. The filter was cut into three strips; one tested with the probe for *B. xylophilus* named pBx6, one tested with the probe for *B. mucronatus* named pBm4, and the third a control to test for the quantity of DNA present in each sample, using the pCes370 probe from *C. elegans*.

Sensitivity spectrum of the probes

The sensitivity of the probes was tested by doing a dilution series. The concentration of DNA isolated from Msp4 and RB was determined, and a series of dilutions was made containing 27 ng, 6.9 ng and 1.7 ng of each of the two isolates. An adult *C. elegans* hermaphrodite contains about 1 000 picograms of DNA based on the DNA content of L1 larvae (Sulston & Brenner, 1974). Assuming that *B. xylophilus* and *B. mucronatus* have a similar DNA content, the amount of DNA in each spot is approximately equal to 27, 7 and 2 nematodes respectively.

The probes were tested against a spectrum of popu-

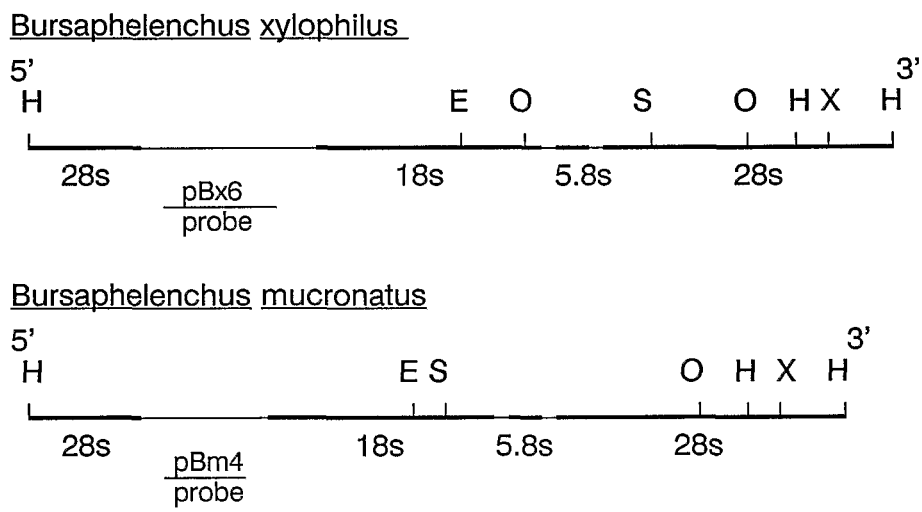

Fig. 1. Restriction map of the DNA containing part of the ribosomal repeat in *Bursaphelenchus xylophilus* MSP-4 and *B. mucronatus* RB; H - Hind III; E = EcoRI; O = Xho I; S = Sal I; X = Xba I. There were no BamHI, EcoRV, KpnI, or PstI sites found in the repeat. The lines under the maps labelled pBx6 probe and pBm4 probe represent the parts of the ribosomal repeat that were cloned to give us the species specific probes.

lations by using sixteen isolates from around the world. These isolates included RB (Japan), Ibaraki (Japan), Fukushima (Japan), Chiba (Japan), French, Norway, MSP-4 (USA), BxUJA (USA), St. John (Canada), St. William (Canada), Q1426 (Canada), Q52A (Canada), mm (Canada), Fids (Canada), British Columbia (Canada), and Alberta (Canada).

Eleven of the isolates, namely Bm, MSP-4, Ch, JA, Nor, Fr, SW, SJ, Q14, Alb and BC (see Tab. 1), were analyzed at the restriction site level. The DNA from these isolates were digested with Xho I, an enzyme that cuts in different places between the two species. The DNA was then run on an agarose gel, transferred to nitrocellulose and probed with pCes370.

DNA from four isolates, namely, Ch, Fr, Nor and Bm (see Tab. 1), of the *B. mucronatus* group were restricted with Sal I and Hind III enzymes, size fractionated and then transferred to nitrocellulose and probed with the total ribosomal coding area.

Results

Restriction and gene maps of the DNA containing a ribosomal repeat were produced for both *B. xylophilus* and *B. mucronatus* (Fig. 1). This map enabled the NTS region, which lies between the 18s and 28s coding regions, to be defined. As the map shows, the restrictions sites were not very conveniently located, and this made isolation of NTS probes difficult. Hence, more sophisticated methods, *i.e.* the ExoIII deletion technique (Henicoff, 1984) and the Smith and Bernstiel (1976) mapping method had to be used to identify the restriction fragment containing the NTS region.

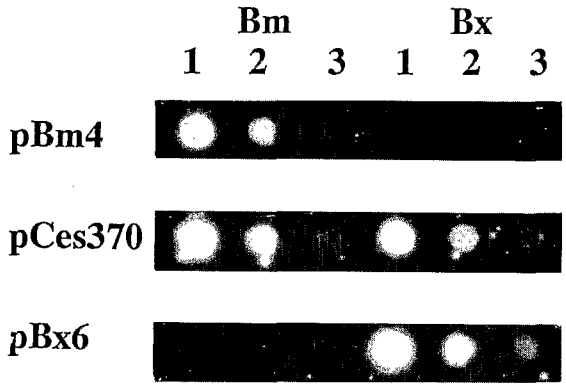

Fig. 2. Two probes, namely pBm4 *(Bursaphelenchus mucronatus)* and pBx6 *(B. xylophilus),* tested together with a control probe (pCes370) against three concentrations of DNA nematode equivalents. DNA was extracted from a large number of infective stage nematodes from both *B. xylophilus* (B.x.) and *B. mucronatus* (B.m.) and diluted so as to have the following concentration of DNA per dot : 1 = 27 ng = 27 nematodes; 2 = 6.9 ng = 7 nematodes; 3 = 1.7 ng = 2 nematodes.

Results of the dilution series test (Fig. 2) show that DNA from as few as two nematodes could be used to reliably identify nematodes. The pBx6 probe reacted positively to *B. xylophilus* (MSP-4) and negatively to *B. mucronatus* (RB), pBm4 was positive with RB and negative with MSP-4. The *C. elegans* ribosomal clone, pCes370, was used as the positive control for presence of DNA as it reacts positively with both species in this test. All five unknowns in the blind trial, namely 1 = Fukushima, 2 = St. John, 3 = Georgia, 4 = China, and 7 = Q1426, gave a positive result with pBx6 indicating that they belonged to the *B. xylophilus* group (Fig. 3). Based on morphological characteristics these isolates had been previously identified as *B. xylophilus.* The results presented here show that our probes could effectively differentiate between unknown *Bursaphelenchus* species.

The two probes clearly segregated the sixteen isolates into two groups (Fig. 4), those positive with pBx6 which included Ib, Fuku, MSP-4, SJ, SW, Q14, mm, Fids, B.C., and Alb, and those positive with pBm4 which were Bm, Ch, Nor and Fr. Close examination of the intensity of each isolate on the dot blot in comparison with the control showed that not all the isolates were equally close to the identifying probe, *i.e.* mm gave more intense spots with the pBm3 control than it did with pBx6. Likewise Nor and Fr each gave less intense spots with pBm4 than with pBm3 (Fig. 4).

Since there were differences in hybridization intensity that were due to sequence divergence rather than to deletions of repeat sequences in the NTS region (data not shown) we used some restriction analysis to clarify

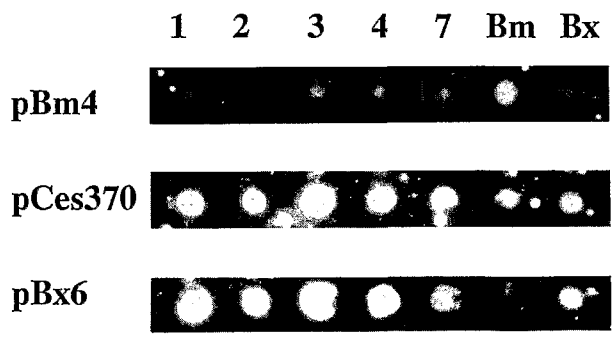

Fig. 3. Results of a blind trial in which a total of 40 adult and juvenile nematodes were picked from each of the plates of " unknowns " and DNA extracted. Each of the probes was labelled with P32 and hybridized to the filters with DNA from the " unknowns " and the standards (*Bursaphelenchus xylophilus,* Bx and *B. mucronatus,* B.m.). All the " unknowns " showed a positive with the pBx6 probe and the control (pCes370) probe and negative with pBm4. All isolates checked out correctly, according to previous identification using morphological characters. Unknowns 1 = Fukushima, 2 = St. John, 3 = Georgia, 4 = China and 7 = Q1426 (cultures of unknowns 5 and 6 were lost).

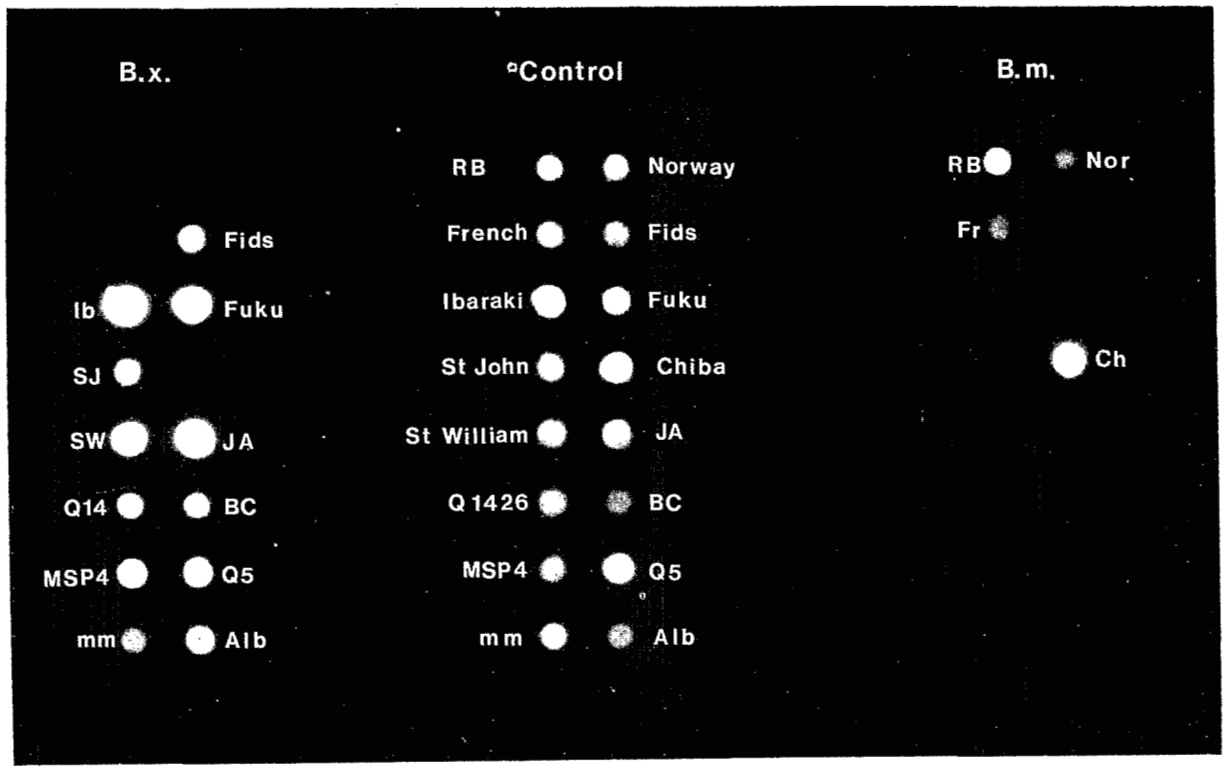

Fig. 4. DNA from sixteen isolates was spotted in triplicate onto nitrocellulose filter so that they could be tested against the probe (pBx6) for *Bursaphelenchus xylophilus* (MSP-4), a probe (pBm4) for *B. mucronatus* (RB), and a control probe (pBm3). The isolates are : RB (Japan); French; Ibaraki (Japan); St. John (Canada); St. William (Canada); Q1426 (Canada); MSP-4 (USA); mm (Canada); Norway; Fids (Canada); Fukushima (Japan); Chiba (Japan); BxUJA (USA); British Columbia (Canada); Q52A (Canada); Alb (Canada). The hybridization conditions were 70° C and wash at 68° C 0.2 × SSPE.

these results. Isolates of both species were digested with the restriction enzyme XhoI (Fig. 5) which is present once in the *B. mucronatus* cistron and twice in the *B. xylophilus* cistron (see Fig. 1). As predicted by the restriction map *B. mucronatus* subgroup isolates gave one major band of 7.3 kb and those of the *B. xylophilus* subgroup gave two bands, one at 5.9 kb and the other at 1.8 kb.

In the *B. mucronatus* isolates differences could be observed in minor secondary bands. To determine if there were differences present within the main repeat of these isolates the isolates were digested with Sal I and Hind III. The results (Fig. 6) show that the isolates from Japan (RB and Chiba) have restriction site differences from the isolates from Europe (Norway and French).

Discussion

In this first stage of developing a reliable and sensitive diagnostic technique for differentiating isolates of *Bursaphelenchus* spp. within the PWNSC, restriction fragment analysis has lead to the development of two species specific probes (pBx6 = *B. xylophilus* and pBm4 = *B. mucronatus*) following hybridization with heterologous probes. These two probes enabled the unequivocal segregation of sixteen isolates into either the *B. xylophilus* group or the *B. mucronatus* group by reciprocal dot-blot tests. Southern blot hybridization and double digestion has confirmed the existence of these two major groups and also indicates that the *B. mucronatus* group comprises at least two subgroups.

The results of this study confirm those of the electrophoretic study of de Guiran *et al.* (1985) in showing a clear taxonomic difference between *B. xylophilus* isolates and *B. mucronatus* isolates. The position of the French specimens is clarified and is shown to have a much closer affinity to *B. mucronatus* than to *B. xylophilus*. As well, the French specimens appear to have a closer affinity with those from Norway than they do with the Japanese isolates (Chiba and RB).

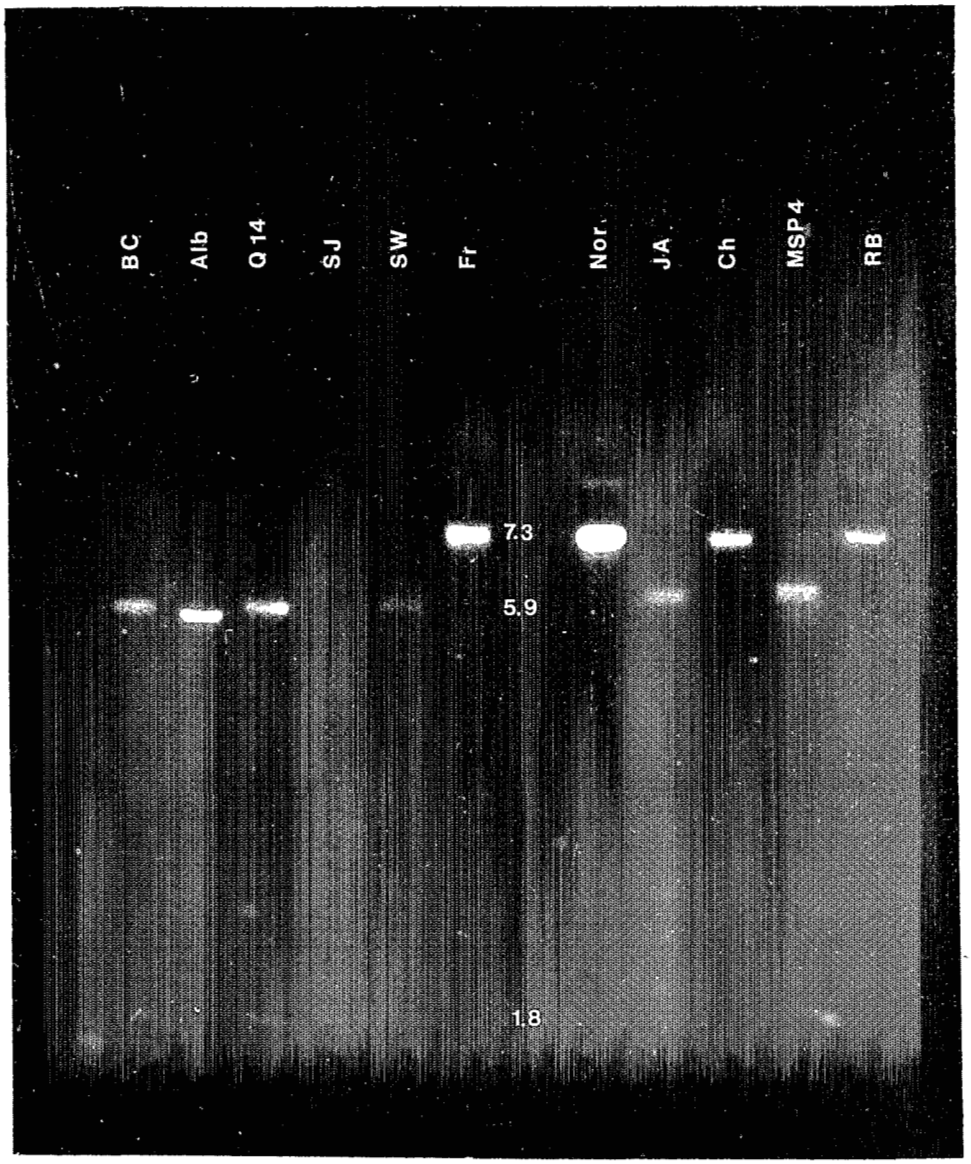

Fig. 5. DNA from eleven isolates was restricted with Xho I, size fractionated on agarose gel, transferred to nitrocellulose and probed with the total ribosomal coding area. In the *Bursaphelenchus xylophilus* subgroup of isolates two bands show (5.9 kb and, faintly, 1.8 kb) and in the *B. mucronatus* subgroup a band occurs at 7.3 kb.

The other major group of isolates within the PWNSC have an affinity for *B. xylophilus*, but the affinity may differ somewhat between isolates. The degree of sensitivity of the dot-blot technique as used here is insufficient to provide definitive answers as to subspecific relationships of a wide range of isolates and so alternative techniques are being examined.

Taxonomic differentiation of *B. xylophilus* and *B. mucronatus* based solely on morphological features is unclear in some circumstances. The most frequently used morphological character that differentiates the two species is the shape of the female tail tip. *B. xylophilus* has a rounded tail, or sometimes one with a short mucro. Whereas, *B. mucronatus* has a long thick mucro (digitate mucro). However, a female form with a digitate mucro was found in France and several intermediate forms with short, filamentous mucros have been reported in North America. This has lead to the terms " r-form ", for round-tailed females, and " m-form ", for mucronate-tailed females of *B. xylophilus*, being used for some

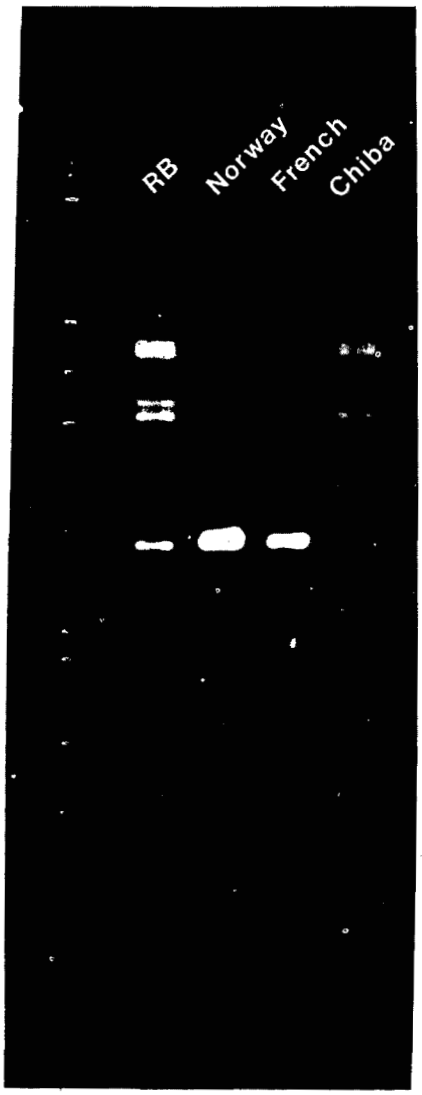

Fig. 6. DNA from four isolates of the *Bursaphelenchus mucronatus* group restricted with Sal I and Hind III, size fractionated, transferred to nitrocellulose and probed with the total ribosomal coding area.

North American specimens. The spicule shape (Yin, Fang & Tarjan, 1988) of males has been used to differentiate *Bursaphelenchus* species though these authors acknowledge the occurrence of intraspecific variation. In addition to the disc-like expansion of male spicule tips de Guiran *et al.* (1985) recognized the distinct vulval flap of females as important characters in common for these two species. These authors clearly separated *B. xylophilus* and a French strain of *B. mucronatus* using enzyme electrophoresis and wisely acknowledged that several strains should be examined so as to clarify whether or not intraspecific variation masked the apparent specific differences that they recorded.

Both the *B. xylophilus* and *B. mucronatus* species are pathogenic to pines but *B. xylophilus* is usually much more virulent. The French strain of *B. mucronatus* spread from infected trees very slowly, and appears to be more virulent than *B. mucronatus* from Japan (de Guiran & Boulbria, 1986). Climatic factors in France would tend to slow the spread of this nematode (Rutherford & Webster, 1987). It has been established that the French strain of the PWNSC interbreeds with both North American and Japanese strains of the PWNSC (de Guiran & Boulbria, 1986; Riga, pers. comm.). It appears that native isolates of the PWNSC may cause pathogenicity on their respective continents but that natural tree distributions and climate ensure pine wilt epidemics do not occur (Rutherford, Mamiya & Webster, *in press*).

The development of infraspecific probes should prove to be helpful in differentiating these economically important, morphologically similar, interbreeding populations of PWNSC. Moreover, in view of the close genetic affinities and differences shown within the *B. mucronatus* group the term " species complex " continues to be appropriate.

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