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Detection and Identification of *Bursaphelenchus* Species with DNA Fingerprinting and Polymerase Chain Reaction[1]

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Abstract: We have evaluated the potential of DNA-based methods to identify and differentiate *Bursaphelenchus* spp. and isolates. The isolation of a DNA probe, designated X14, and development of a DNA fingerprinting method for the identification and differentiation of *Bursaphelenchus* species and strains is described. Polymerase chain reaction (PCR) amplification of DNA isolated from *Bursaphelenchus* species using two primers derived from the sequence of the cloned repetitive DNA fragment X14 resulted in multiple band profiles. A 4-kb fragment thus amplified from *B. xylophilus* DNA was not amplified from *B. mucronatus* or *B. fraudulentus* DNA. In addition to this fragment, several other fragments are amplified from the three species. The banding patterns obtained allowed species identification and may have value in determining taxonomic affinities.

Key words: Bursaphelenchus, differentiation, DNA fingerprinting, identification, nematode, pinewood nematode species complex (PWNSC), polymerase chain reaction, systematics, taxonomy.

Bursaphelenchus xylophilus (23,33) is the causative agent of pine wilt disease (15,18, 21), a serious disease of native pine trees in Japan, and is transmitted to pine trees by insect vectors, primarily *Monochamus alternatus* (19). This nematode also occurs in Canada (16), China (44), and parts of North America (41,42). Another species, *Bursaphelenchus mucronatus,* has been described from Japan (20) and Europe (9,22). A third species, *Bursaphelenchus fraudulentus,* has also been described (27), *Bursaphelenchus fraudulentus* has not been shown to be pathogenic to healthy pine trees, and *B. mucronatus* is pathogenic to only stressed pine trees (20). Because pine wilt disease is regarded as a serious threat to European forests (17), the EC has instituted a ban on the import of coniferous timbers that have not been kiln dried from regions where the nematode is endemic. The protective policy of the EC can be effectively pursued only if a reliable test system exists for the screening of imported timbers for the presence of *B. xylophilus.* With this aim in mind, we investigated DNA-based detection systems that could be applied to timber prior to importation to pine wilt-free areas.

The identification of *B. xylophilus* is difficult (24) because of its morphological similarity to *B. mucronatus* (20) and *B. fraudulentus* (27). Unequivocal identification of these nematodes is further complicated by the possible interfertility of *B. xylophilus* and *B. mucronatus* (26,29). *Bursaphelenchus fraudulentus* is morphologically similar to both *B. mucronatus* and *B. xylophilus* but does not mate with either (30). The morphological characteristics of these nematode species are highly variable (35), in particular tail shape (43), which was used as the definitive morphological trait (20,24) but cannot be relied upon as a basis for accurate identification. For example, a *B. xylophilus* isolate, US10, has a mucronate tail (43), a morphological feature characteristic of *B. mucronatus.* These three different species are thought to be derived from common origins and to constitute a supraspecies (10).

Although protein profiles (14), immunological methods (31,37), and enzyme electrophoresis (9) have aided in the identification of nematode species, the value of these methods is limited by differential gene expression during the life cycle or external influences such as environmental conditions. DNA-based methods, however,

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offer an attractive alternative because DNA is independent of developmental stage and phenotypic variation due to external influences. DNA and restriction fragment length polymorphism (RFLP) profiles have been used with some success (1,4,8,14,25,35); more recently, sequence differences in ribosomal genes and nontranscribed spacer regions have been utilized (38). Recently, a method involving PCR amplification of heat shock protein (Hsp) genes and subsequent sequence analysis of the amplified fragment has been used to identify phylogenetic relationships among *Bursaphelenchus* spp. (2).

DNA amplification fingerprinting, using arbitrary oligonucleotide primers (6, 39,40) or primers targeting repeated DNA sequence elements (28), has been applied to the identification of a wide range of organisms.

The purpose of this study was to develop DNA and PCR-based methods for discriminating among *B. xylophilus, B. mucronatus,* and *B. fraudulentus.* We used repeated DNA sequences from *Bursaphelenchus xylophilus* as DNA fingerprinting probes to distinguish between *B. mucronatus* and *B. xylophilus.* One DNA probe, X14, was specific for *B. xylophilus* and *B. mucronatus* and did not hybridize to DNA from other nematode species or from pine wood (*Pinus sylvestris*) DNA under the conditions employed. In addition to differentiating between *B. xylophilus* and *B. mucronatus,* this probe also differentiated between different isolates of the two species. The PCR amplification, using sequences derived from within the sequence of the X14 probe as primers, discriminated among *B. xylophilus, B. mucronatus,* and *B. fraudulentus.* Our results confirm the division of the PWNSC into three distinct species.

Materials and Methods

Bursaphelenchus isolates were maintained on mats of a sterile *Botrytis cinerea* mutant (strain 389S) as described (38) and were subcultured every 2 to 4 weeks. The isolates used in this study are listed in Table 1.

Nematode DNA extraction: DNA from mixed life stages of nematodes was extracted by a modification of a described method (38). Nematode pellets (ca. 100 µl) were suspended in five volumes of extraction buffer (100 mM Tris · HCl [pH 8.5], 200 mM NaCl, 50 mM ethylenediaminetetraacetic acid [EDTA], 1% sodium dodecyl sulfate (SDS), and 2 mg/ml Proteinase K [Sigma Chemical Co., St. Louis, MO]) and

Table 1. Geographical origins and hosts of the nematode isolates used in this study.

Isolate	Origin	Host
	Bursaphelenchus xylophilus	
US4	United States (Florida)	*Pinus elliotti*
US9	United States (Tucson, Arizona)	*Pinus halepensis*
US10	United States (Cloquet, Minnesota)	*Abies balsamea*
US15	United States (Cook Co., Illinois)	*Pinus sylvestris*
Bc	Canada (British Columbia)	*Pinus spp.*
St. Will	Canada (St. William, Ontario)	*Pinus spp.*
C2	Canada (Quebec)	*Abies balsamea*
J3	Japan (Ueki, Kumamota Pref.)	*Pinus densiflora*
J10	Japan (Nishiaizu, Fukushima Pref.)	*Pinus densiflora*
	Bursaphelenchus mucronatus	
J13	Japan (Yachiyo, Chiba Pref.)	*Pinus thunbergii*
BmN	Norway	*Pinus sylvestris*
BmF	France (Forêt de Campet, Landes)	*Pinus pinaster*
	Bursaphelenchus fraudulentus	
BfHU	Hungary	*Quercus robur*
	Heterorhabditis spp.	
K122	Ireland (Wexford)	*Galleria mellonella*

incubated at 65 C for 45 minutes with occasional mixing. The DNA was extracted twice with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) and twice with an equal volume of chloroform: isoamyl alcohol (24:1). The DNA was recovered by ethanol precipitation.

Wood DNA extraction: A modification of a described method (11) was used to extract DNA from primary wood tissue of *Pinus* spp. The material was ground in a coffee mill together with broken glass Pasteur pipettes. The ground material was incubated in 2 volumes of a solution of 220 mM Tris · HCl (pH 8.0), 140 mM sorbitol, 22 mM EDTA, 0.8% cetyltrimethyl ammonium bromide (CTAB), 1% N-lauroylsarcosine, and 1 mM β-mercaptoethanol at 65 C for 20 minutes with occasional shaking. The DNA was extracted twice with an equal volume of chloroform: isoamyl alcohol (24:1) and precipitated with two volumes of ice cold isopropanol at −20 C. The pelleted DNA was washed three times with 30 mM sodium acetate and 12.5 mM $MgCl_2$ in 70% ethanol. The nucleic acid pellet was resuspended in 4 ml TE buffer (10 mM Tris · HCl [pH 8.0], 1 mM EDTA), and lithium acetate was added to a final concentration of 2.0 M. RNA was pelleted by centrifuging at 1,250*g* for 10 minutes. DNA was precipitated by adding 2 volumes of 100% ethanol and pelleted by centrifuging at 12,000*g* for 10 minutes. The DNA pellet was resuspended in TE buffer, phenol-extracted, and ethanol-precipitated.

Isolation of fungal DNA: Botrytis cinerea was grown in Vogel's minimal medium (36) for 48 hours. Mycelium was collected by filtering the culture through Whatman No. 1 paper. The mycelium was ground in a mortar and pestle in extraction buffer, and the DNA was isolated as for nematodes.

Construction and screening of genomic library: The *B. xylophilus* (J10) genomic DNA was digested with Hind III and ligated into the Hind III site of plasmid vector pKS450 (34). Ligation reactions were used to transform *Escherichia coli* DS941 cells prepared according to a described procedure (13). Transformed cells were grown on LB agar plates containing 50 μg/ml ampicillin. Colonies were lifted onto a Nytran membrane (Schleicher & Schuell, Dassel, Germany) and lysed in 10% (w/v) SDS. The DNA was denatured, neutralized, and fixed according to the manufacturer's instructions. The library was screened with ^{32}P-labeled Sau3A-digested *B. xylophilus* (J10) genomic DNA. DNA was labeled as described (12). Membranes were hybridized at 42 C in a solution of 6× SSC (20× SSC is 2.99 M NaCl and 0.299 M sodium citrate), 10× Denhardt's solution (50× Denhardt's is 1% bovine serum albumin [BSA], Ficoll, and polyvinylpyrollidone [PVP]), and 1% SDS containing 50 μg/ml sheared salmon sperm DNA. The filters were washed three times for 20 minutes each in a solution of 10× SSC and 1% SDS at room temperature, twice in a solution of 1× SSC and 1% SDS at 37 C, and twice in a solution of 0.1× SSC and 1% SDS at 65 C. Filters were air dried and exposed to X-ray film. DNA was isolated from selected clones by alkaline lysis miniprep (3), digested with Hind III to release inserted DNA, fractionated by electrophoresis through a 1% (w/v) agarose gel, and transferred to a Nytran membrane according to the manufacturer's instructions. Filters were hybridized with ^{32}P-labeled Sau3A-digested genomic DNA from *B. mucronatus* (BmF) as described above.

DNA hybridization: Genomic DNA was digested with restriction endonucleases according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). DNA electrophoresis was conducted in agarose gels containing 50 μg/ml ethidium bromide in TBE buffer (133 mM Tris · HCl [pH 8.8], 74 mM boric acid, 2.5 mM EDTA). Optimum resolution was achieved with a Ficoll-based loading buffer (15% [w/v] Ficoll containing bromophenol blue). ^{32}P-Labeled probes were prepared by random primer labeling (12). Fractionated DNA was transferred to nitrocellulose membrane by Southern blotting (32). Nitrocellulose filters were hybridized with

^{32}P-labeled X14 DNA at 42 C in a solution of 25 mM potassium phosphate (pH 7.4), 50% (v/v) formamide, 5× SSC, and 5× Denhardt's solution containing 50 μg/ml sheared salmon sperm DNA. Nitrocellulose filters were washed twice for 30 minutes each in a solution of 2× SSC and 0.1% SDS at room temperature, three times in a solution of 1× SSC and 0.1% SDS at 45 C, and three times in a solution of 0.2× SSC and 0.1% SDS at 60 C. Filters of probe hybridization to Bgl II-digested DNA received an extra wash in a solution of 0.1× SSC and 0.1% SDS at 62 C. Filters were air dried and exposed to X-ray film.

PCR amplification: Approximately 50 ng of genomic DNA was amplified by thermal cycling in 100 μl of a solution of 10 mM Tris · HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1% Triton X-100 containing 50 pmol of each primer (HAR1 and HAR2) and 40 μM dATP, dTTP, dGTP and dCTP, and 0.2 μCi (50 pmol) α-[^{32}P]dGTP (Amersham, UK). PCR primers HAR1 and HAR2 were designed for other purposes but were used here as arbitrary primers for DNA amplification fingerprinting (6,39,40). The sequence of HAR1 5′-3′ is TTGTGAGGGAGCCCTTTC and HAR2 5′-3′ is AGGGGCTTTGTCCCAAATT. Melting temperatures (Tm) were calculated using "melt, 2.0" (J. Nash, National Research Council of Canada). On the basis of the Tm values of 49.3 C and 47.7 C for HAR1 and HAR2, respectively, an annealing temperature of 42 C was selected. The reaction mix was incubated at 97 C for 10 minutes before adding 5 units of Taq polymerase (Cetus, Emeryville, CA) and beginning thermal cycling. Amplification was performed by 25 cycles of 97 C for 20 seconds, 42 C for 30 seconds, and 72 C for 2 minutes with a Perkin-Elmer (Norwalk, CT) Cetus thermal cycler. Amplification products were analyzed by electrophoresis on 1.5% agarose gels. Following electrophoresis, the DNA was fixed by washing the gel in 50 mM sodium acetate in 1% CTAB for 1 hour (7). The gel was dried and exposed to X-ray film for 3 hours.

Results

Screening the genomic library: A genomic library constructed from *B. xylophilus* (J10) was probed with ^{32}P-labeled. Sau3A-digested genomic DNA from the same species. Highly repetitive DNA hybridizes readily, and sequences occurring less frequently hybridize less easily. Therefore, when homologous genomic DNA is hybridized, the length of time taken to develop a strong signal reflects the degree of the repetitiveness and size of the cloned DNA. The length of exposure time required to develop a strong signal was therefore used as the basis for selecting potential clones for further analysis. Figure 1 shows filters hybridized and exposed for 4, 16, and 96 hours. Colonies that appeared after 4 hours should represent clones containing highly repetitive DNA sequences, such as short tandem repeats. Colonies

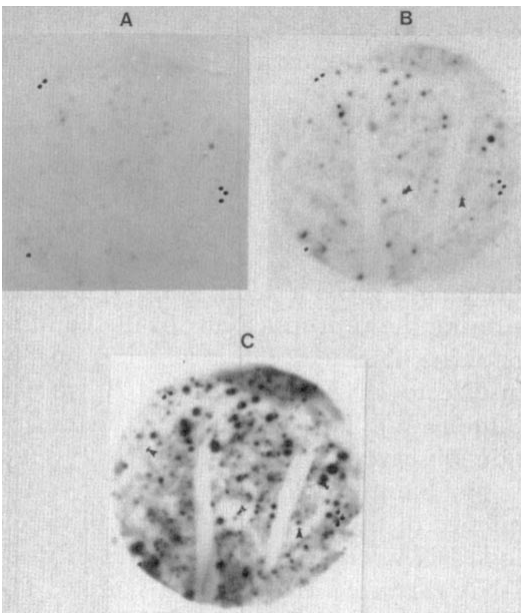

FIG. 1. Screening the *Bursaphelenchus xylophilus* J10 genomic library. A genomic library of Hind III restriction fragments from *B. xylophilus* J10 was plated out and the DNA was transferred to a nylon membrane. Immobilized DNA was hybridized with ^{32}P-labeled Sau3A-digested genomic DNA from *B. xylophilus* J10. After hybridization, the filters were washed, air dried, and exposed to X-ray film for 4 hours (A), 16 hours (B), or 96 hours (C). Colonies selected for further analysis are indicated by arrows.

that appeared after the 16-hour exposure may represent moderately repetitive DNA sequences. Such DNA is an ideal candidate for DNA fingerprinting probes, as it provides multiple possible sites for hybridization. Colonies that appeared after 96 hours of exposure should represent unique or low copy number DNA sequences. Based on this rationale, a number of *B. xylophilus* clones were selected for further analysis. Clones X1 to X15 appeared after a 16-hour exposure time, and clone X16 appeared after 96 hours.

Because the prime objective was to detect and discriminate *B. xylophilus* isolates, we analyzed the selected clones for homology to *B. mucronatus* (BmF) DNA. A 1,191-bp DNA insert, X14, appeared not to hybridize with *B. mucronatus* DNA (data not shown) and was therefore selected as a likely candidate for a *B. xylophilus*-specific probe.

DNA fingerprinting of nematode DNA: Figure 2 depicts the results of hybridizing the X14 insert to Hind III-digested genomic DNA from a number of nematode isolates. The probe hybridized to only one (J13 and BmF) or two bands (BmN) in the *B. mucronatus* isolates but hybridized to multiple bands in the *B. xylophilus* isolates. Samples M and X represent DNA isolated from parallel cultures of BmF and J10, respectively, and provide blind controls demonstrating the reproducibility of the banding patterns obtained. The probe did not hybridize to DNA isolated from *P. sylvestris* (sample W). The lack of probe hybridization observed with J10-infected *P. thunbergii* (Sample IW) is probably a consequence of the very low amount of nematode DNA relative to the amount of wood DNA extracted from such a sample. The banding patterns obtained were species specific, clearly differentiating between *B. mucronatus* and *B. xylophilus* on the basis of the number of hybridizing bands observed.

Although the banding patterns observed were also isolate specific, the number of bands obtained with Hind III-digested *B. xylophilus* DNA was too great and the profile too compressed to allow unambiguous comparison of these banding patterns. We therefore investigated the hybridization profiles obtained by probing genomic DNA digested with alternative restriction enzymes. Figure 3 shows the profiles obtained when X14 was hybridized to genomic DNA digested with Bgl II. By running the gel for long periods (up to 48 hours), many of the smaller fragments ran off the gel, resulting in a fingerprint based on fragments larger than 1 kb, which gave a reduced number of well-spaced bands. The probe clearly distinguished between

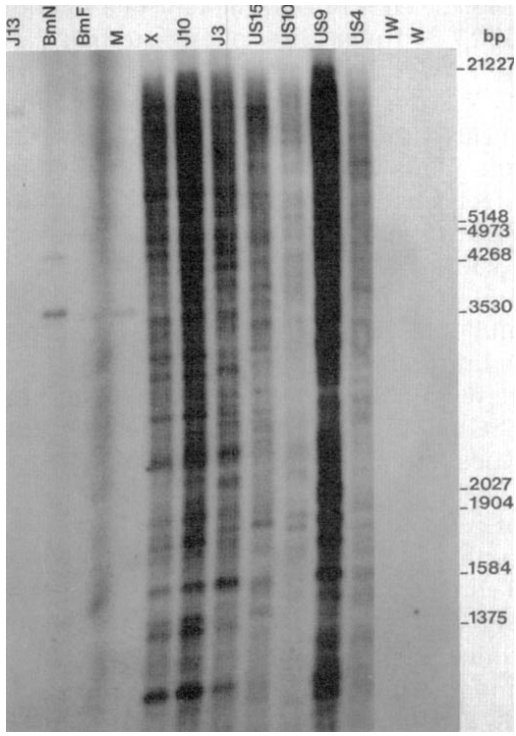

FIG. 2. Hybridization of the X14 probe to Hind III-digested genomic DNA from *Pinus* spp. and *Bursaphelenchus* spp. Total genomic DNA was digested with Hind III and fractionated by electrophoresis through a 0.8% agarose gel. Fractionated DNA was transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled X14 insert DNA. After hybridization, the filter was washed, air dried, and exposed to X-ray film. Isolates (abbreviations as in Table 1) are shown at the top of the figure. W shows DNA isolated from *P. sylvestris* and IW shows DNA isolated from J10-infected *P. thunbergii*. Samples X and M show DNA isolated from parallel cultures of J10 and BmF, respectively, and provide blind controls on the reproducibility of the hybridization pattern.

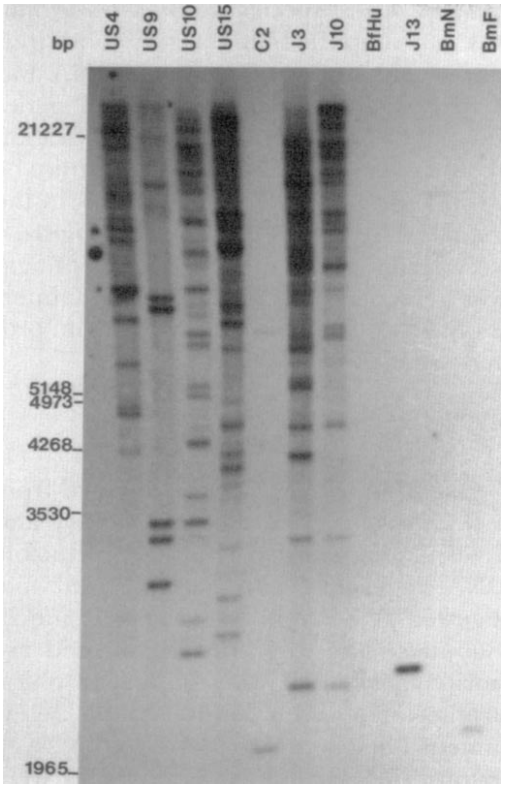

FIG. 3. Hybridization of X14 to Bgl II-digested genomic DNA from *Bursaphelenchus* spp. Total genomic DNA was digested with Bgl II and fractionated by electrophoresis through a 0.7% agarose gel. Fractionated DNA was transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled X14 insert DNA. After hybridization, the filter was washed, air dried, and exposed to X-ray film. Isolates are listed at the top of the figure. Abbreviations as in Table 1. The positions of molecular weight markers are indicated in base pairs (bp).

B. mucronatus and *B. xylophilus* isolates on the basis of the number of hybridizing bands. The probe hybridized to only a single band in *B. mucronatus* isolates BmN and J13 and two bands in BmF, whereas the probe hybridized to multiple bands in the *B. xylophilus* isolates. In addition to clearly differentiating the two species on the basis of band number, the probe gave a characteristic "fingerprint" for each of the isolates. The Japanese *B. xylophilus* isolates J3 and J10 had two common bands of approximately 2.2 kb and 3.3 kb but also contained many unique bands. Although the probe did not hybridize to any band common to all the American *B. xylophilus* isolates (US4, US9, US10, and US15), a number of bands were shared between isolates. US10, which is a mucronate *B. xylophilus* isolate, showed a multiple band profile with the X14 probe, and a band of ca. 3.4 kb was common to both US9 and US10. Although isolate C2 was supplied to us as a mucronate *B. xylophilus,* our X14 probe hybridized to only two bands in DNA from this isolate, suggesting that the C2 isolate is in fact a *B. mucronatus* type. The probe was specific for *B. xylophilus* and *B. mucronatus* but did not hybridize with DNA isolated from a third species *B. fraudulentus* (BfHu) under the conditions employed, nor did it hybridize to DNA from *Heterorhabditis* isolate K122 (data not shown).

DNA amplification fingerprinting: PCR amplification of genomic DNA with primers HAR1 and HAR2 resulted in multiple band profiles for all isolates examined (Fig. 4). Amplification of DNA from *B. mucronatus* isolates BmF and BmN resulted in three common bands (indicated by ●) absent from *B. xylophilus* profiles. There was, however, a suggestion of the top band in *B. xylophilus* isolates J10, J3, and US9. The middle band appeared as a minor component in *B. xylophilus* isolates Bc, J10, J3, and US10. Amplification of *B. mucronatus* J13 resulted in a very different profile, with one band in common with *B. mucronatus* isolate BmN (shown by ○) but absent in *B. mucronatus* BmF. The uppermost band in *B. mucronatus* isolate J13 also occurred in *B. xylophilus* US15. Isolate C2 shared a band (○) with *B. mucronatus* J13 and BmN and three additional bands (●) with *B. mucronatus* BmF and BmN. An additional 365-bp fragment (▶) was common to *B. mucronatus* BmF, BmN, and isolate C2 and *B. xylophilus* isolates.

Amplification of *B. xylophilus* DNA resulted in a ca. 4-kb band (■) common to all *B. xylophilus* isolates studied and a number of minor bands. The 4-kb band was also present as a minor component of *B. mucronatus* BmN. Another band (□ ◁) was common to all *B. xylophilus* isolates except the St. Will isolate and the US10 isolate, a

mucronated *B. xylophilus* morphologically distinct from the other *B. xylophilus* isolates. The Japanese isolates (J10 and J3) shared an additional four bands (□), possibly indicating common origins. A fainter, lower, ca. 365-bp band (▶) was common to all *B. mucronatus* and *B. xylophilus* isolates, except J13 and St. Will. A ca.2.3-kb band (■), common to *B. xylophilus* isolates J3, US15, US10, and US4, was also evident when DNA from *B. fraudulentus* was amplified, possibly indicating a degree of relatedness between these two species. *Bursaphelenchus fraudulentus* did not share any bands in common with the *B. mucronatus* isolates.

Because the nematodes were cultured on *Botrytis cinerea* mats, a control amplification with *B. cinerea* DNA resulted in a band profile with no fragments common to the *Bursaphelenchus* profiles, showing that the observed nematode PCR product profiles were not a result of amplification of DNA from contaminating fungal hyphae. PCR amplification of another nematode species, *Heterorhabditis* isolate K122, similarly produced a completely different band profile (Fig. 4). DNA from *Pinus sylvestris*, a host species for *Bursaphelenchus*, was not amplified under the conditions employed. Because *P. sylvestris* DNA was amplified using primers to rRNA genes (data not shown), the lack of amplification products with HAR1 and HAR2 cannot be attributed to inhibitory compounds in the sample. The results presented in Figure 4 were obtained by autoradiography of agarose gel separations. Similar, albeit fainter, profiles were obtained from ethidium bromide stained gels.

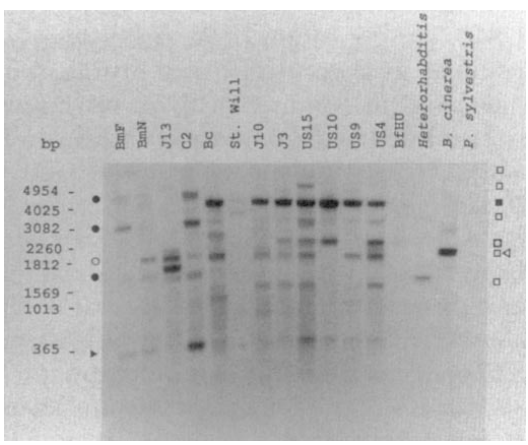

FIG. 4. Amplification products from *Bursaphelenchus mucronatus, B. xylophilus, B. fraudulentus, Botrytis cinerea, Pinus sylvestris,* and *Heterorhabditis* K122. Genomic DNA was amplified by polymerase chain reaction and the amplification products were fractionated by electrophoresis through a 1.5% agarose gel. Following electrophoresis, the gel was dried and exposed to X-ray film. Isolate names as described in Table 1. Symbols indicate diagnostic bands described in text. Molecular weights are indicated in base pairs (bp).

DISCUSSION

Our DNA probe (X14) was isolated from a *B. xylophilus* (J10) genomic library and gives a characteristic and reproducible DNA fingerprint with restriction enzyme-digested DNA from *B. macronatus* and *B. xylophilus*. The fingerprints obtained are isolate specific, allowing the geographical origins of a given sample to be determined. The banding patterns we observed in our fingerprinting experiments were consistent over time, as repeated experiments using DNA isolated from subcultures over a year-long period produced the same pattern for a given *Bursaphelenchus* isolate. In addition, DNA isolated from parallel cultures of the same isolates produced the same band profile in our DNA hybridization experiments. The sample designated "X" was sent from Japan, where it has been cultured for some years. It gave a profile identical to the J10 supplied to us by Georges de Guiran, which has been cultured in his laboratory for a number of years.

Our X14 hybridization results agree with those obtained using a heterologous *unc*-22 DNA probe from *C. elegans* (1), a homologous DNA probe (35), or sequences from the non-transcribed spacer (NTS) region of ribosomal RNA genes (38). These findings clearly demonstrate the existence of a *B. xylophilus* group and *B. mucronatus* group within the pinewood nematode species complex (PWNSC). Under the conditions employed, the X14 probe does not hybridize to DNA from *B.*

fraudulentus, which is reproductively isolated from *B. xylophilus* and *B. mucronatus* (30). Although once thought to form a supraspecies with the other two *Bursaphelenchus* spp. (10), this nematode is no longer considered part of the pinewood nematode species complex (35). Our results from arbitrarily primed PCR are also consistent with the division of the PWNSC into a *B. mucronatus* group and *B. xylophilus* group (38), with *B. fraudulentus* representing a third species (1). On the basis of amplification products of common molecular weight, there may be a distant relationship between *B. fraudulentus* and *B. xylophilus.*

In addition to confirming and extending the results of other workers (1,35,38), we have identified isolate C2 as a *B. mucronatus* on the basis of its hybridization pattern with the X14 probe. In our PCR experiments, isolate C2 was distinct from all other *Bursaphelenchus* isolates studied, but it clearly fell within the *B. mucronatus* group because it shared bands with all *B. mucronatus* isolates. Although this isolate was believed to be *B. xylophilus* at the time of our experiments, since submission of this manuscript, isolate C2 has been classified as *B. mucronatus* on the basis of mating potential and chromosome number (5), thereby illustrating the usefulness of both methods for differentiation of *B. xylophilus* and *B. mucronatus.*

Although all *B. xylophilus* isolates produced related profiles, there were clear differences between the various isolates. The Japanese and American strains of *B. xylophilus* may be derived from common origins (1,10), and our PCR results support this. Because Norwegian and French *B. mucronatus* isolates shared several bands in our PCR experiments, they may be closely related—unlike the Japanese *B. mucronatus,* which was quite distinct. This finding supports the hypothesis of Beckenbach et al. (2) that there exist two distinct *B. mucronatus* groups: one group containing the European isolates and another containing the Japanese isolates. Other workers using DNA hybridization techniques obtained similar results (1,38).

In agreement with other workers' data (1), the X14 probe identified US10, a mucronated *B. xylophilus,* (43) as part of the *B. xylophilus* group. This isolate was clearly related to the other *B. xylophilus* isolates by PCR, but a band common to the other American, Japanese, and Canadian Bc isolates was absent from this particular isolate. Similarly, Abad et al. (1) obtained a different DNA hybridization pattern for this isolate with an *unc-22 C. elegans* probe than the pattern they obtained for other American and Japanese isolates.

The PCR banding profiles were reproducibly obtained in two separate experiments. The use of [^{32}P]dGTP in PCR reaction mixes is unconventional, but the low number of cycles used resulted in very faint ethidium bromide staining. Inclusion of [^{32}P]dGTP resulted in increased sensitivity and enhanced band definition. Although the use of [^{32}P]dGTP could conceivably cause a bias in favor of G-rich sequences, ethidium bromide staining of DNA is also a function of both DNA size and base composition. The total dGTP (labeled and unlabeled) concentration is 40.5 μM, compared to 40.0 μM of the other dNTPs, which is not sufficient to cause a bias in favor of G-rich sequences. ^{32}P-Labeled dNTPs have been used by other workers for visualization of arbitrarily primed PCR products (39). We used amplification products of similar molecular weight as an index of relatedness between species and isolates, but these fragments are not necessarily homologous.

Although the primers HAR1 and HAR2 were designed to sequence the X14 probe, they were found to lie within regions of compressions in the sequence and did not in fact anneal to the X14 probe. In the PCR experiment presented herein, these primers behaved essentially as arbitrary PCR primers.

Although our collection of isolates is not exhaustive, the results presented indicate that the X14 insert can be used to discriminate between both species and isolates of *B. mucronatus* and *B. xylophilus.* Our efforts to identify these nematodes within DNA samples isolated from infected timber using the X14 probe proved unsuccessful.

(As these were laboratory-infected wood samples, they may not accurately reflect the situation in vivo with respect to nematode numbers and distribution.) This apparent failure may limit the usefulness of the X14 probe for routine diagnosis. However, the X14 method provides a clear, well-resolved fingerprint with a low number of bands, facilitating assessment of the degree of band-sharing and representing an improved fingerprint compared to many published profiles. Furthermore, the method can be readily performed with basic equipment, without resorting to sequencing or other technically demanding procedures. One limitation of the X14 fingerprinting method is the requirement for significant quantities of DNA. The PCR-based method overcomes that limitation, as only small amounts of DNA (as little as 10 ng) are needed.

The PCR amplification results concur with those obtained from the X14 fingerprinting, although the profiles obtained by amplification fingerprinting of *B. mucronatus* isolates are less distinct than those obtained by fingerprinting with X14. Nevertheless, the PCR band profiles are clearly different for each species and group of isolates examined. The significance of these differences merits further investigation. The preliminary results presented above are encouraging, and, with refinement, this technique may prove of value in defining the phylogenetic relationships within the PWNSC. With further research, possibly using additional primer pairs, PCR may develop into a rapid technique for identifying a given isolate and tracing taxonomic relationships at the genetic level. Another advantage of PCR over DNA fingerprinting is speed: PCR can be performed in a single day with results within 24 hours, whereas X14 DNA fingerprinting can require a week to yield results.

The value of DNA-based techniques for identification purposes is illustrated by the close agreement between our results and those of other workers using DNA hybridization and PCR-based methods (1,2,4,8, 35,38). Because *P. sylvestris* DNA was not amplified under the conditions employed, the PCR method may provide a suitable means for direct detection of *Bursaphelenchus* in wood samples. This possibility is currently under investigation.

Literature Cited

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

2. Beckenbach, K., M. J. Smith, and J. M. Webster. 1992. Taxonomic affinities and intra- and interspecific variation in *Bursaphelenchus* spp. as determined by polymerase chain reaction. Journal of Nematology 24:140–147.

3. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7:1513–1523.

4. Bolla, R. I., C. Weaver, and R. E. K. Winter. 1988. Genomic differences among pathotypes of *Bursaphelenchus xylophilus*. Journal of Nematology 20: 309–316.

5. Bolla, R. I., and M. Boschert. 1993. Pinewood nematode species complex: Interbreeding potential and chromosome number. Journal of Nematology 25:227–238.

6. Caetano-Anolles, G., B. J. Bassam, and P. M. Gresshoff. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Biotechnology 9:553–557.

7. Cockerill, P. N. 1988. Two methods that facilitate autoradiography of small 32P-labeled fragments following electrophoresis in agarose gels. Analytical Biochemistry 168:450–454.

8. Curran, J., and J. M. Webster. 1987. Identification of nematodes using restriction fragment length differences and species-specific probes. Canadian Journal of Plant Pathology 9:162–166.

9. de Guiran, G., M. J. Lee, A. Dalmasso, and M. Bongiovanni. 1985. Preliminary attempt to differentiate pinewood nematodes (*Bursaphelenchus* spp.) by enzyme electrophoresis. Revue de Nématologie 8:85–92.

10. de Guiran, G., and N. Bruguier. 1989. Hybridization and phylogeny of the pinewood nematode (*Bursaphelenchus* spp.). Nematologica 35:321–330.

11. Doyle J. J., and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–15.

12. Feinberg, A., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 132:6–13.

13. Hanahan, D. 1985. Techniques for transformation of *E. coli*. Pp. 109–135 *in* D. M. Glover, ed. DNA cloning. A practical approach, vol. 1. Oxford: IPC Press.

14. Kiyohara, T., and R. I. Bolla. 1990. Pathogenic

variability among populations of the pinewood nematode. *Bursaphelenchus xylophilus*. Forest Science 36: 1061–1076.

15. Kiyohara, T., and Y. Tokushige. 1971. Inoculation experiments of a nematode, *Bursaphelenchus* sp. onto pine trees. Journal of Japanese Forest Society 53:210–218.

16. Knowles, K., Y. Beaubien, M. J. Wingfield, F. A. Baker, and D. W. French. 1983. The pinewood nematode, new in Canada. Forest Chronicles 59:40.

17. Magnusson, C. 1986. Potential for establishment of *Bursaphelenchus xylophilus* and the pine wilt disease under Nordic conditions. European Plant Protection Organisation Bulletin 16:465–467.

18. Mamiya, Y. 1984. The pine wood nematode. Pp. 589–626 *in* W. R. Nickle, ed. Plant and insect nematodes. New York: Marcel Dekker.

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

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

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

22. McNamara, D. G., and M. Stoen. 1988. A survey for *Bursaphelenchus* spp. in pine forests in Norway. European Plant Protection Organisation Bulletin 18: 353–363.

23. Nickel, W. R. 1970. A taxonomic review of the genera of the Aphelenchoididea (Fuchs, 1937) Thorne, 1949. (Nematoda: Tylenchida). Journal of Nematology 2:375–392.

24. Nickle, W. R., A. M. Golden, Y. Mamiya, and W. P. Wergin. 1981. On the taxonomy and morphology of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer 1934) Nickle 1970. Journal of Nematology 13:385–392.

25. Okimoto, R., H. M., Chamberlin, J. L. Macfarlane, and D. R. Wolstenhulme. 1991. Repeated sequence sets in mitochondrial DNA molecules of root knot nematodes (*Meloidogyne*): Nucleotide sequences, genome location and potential for host-race identification. Nucleic Acids Research 19:1619–1626.

26. Riga, E., J. R. Sutherland, J. M. Webster. 1991. Pathogenicity of pinewood nematode isolates and hybrids to Scots pine seedlings. Nematologica 37:285–292.

27. Ruhm, W. 1956. Die nematoden der ipiden. Parasitologische Schriftenreihe 6:1–487.

28. Rogowsky, P. M., K. W. Shepherd, and P. Langridge. 1992. Polymerase chain reaction based mapping of rye involving repeated DNA sequences. Genome 35:621–626.

29. Rutherford, T. A., E. Riga, and J. M. Webster. 1992. Temperature-mediated behavioral relationships in *Bursaphelenchus xylophilus, B. mucronatus* and their hybrids. Journal of Nematology 24:40–44.

30. Schauer-Blume, M. 1989. Report of the panel on the pine wood nematode. European and Mediterranean Plant Protection Organisation Bulletin 89/ 2095:11–12.

31. Schots, A., F. J. Gommers, J. Bakker, and E. Egberts. 1990. Serological differentiation of plant-parasitic nematode species with polyclonal and monoclonal antibodies. Journal of Nematology 22: 16–23.

32. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98: 503–517.

33. Steiner, G., and E. M. Buhrer. 1934. *Aphelenchoides xylophilus*, n. sp. a nematode associated with bluestain and other fungi in timber. Journal of Agricultural Research 48:949–951.

34. Stirling, C. J., G. Stewart, and D. J. Sherratt. 1988. Multicopy plasmid stability in *Escherichia coli* requires host encoded functions that lead to plasmid site-specific recombination. Molecular and General Genetics 214:80–84.

35. Tares, S., P. Abad, N. Bruguier, and G. de Guiran. 1992. Identification and evidence for relationships among geographical isolates of *Bursaphelenchus* spp. (pinewood nematode) using homologous DNA probes. Heredity 68:157–164.

36. Vogel, H. J. 1964. Distribution of lysine pathways among fungi: Evolutionary implications. American Naturalist 98:435–466.

37. Webster, J. M., and D. J. Hooper. 1968. Serological and morphological studies on the inter- and intra-specific differences of the plant-parasitic nematodes *Heterodera* and *Ditylenchus*. Parasitology 58:879–891.

38. Webster, J. M., R. V. Anderson, D. L. Baillie, K. Beckenbach, J. Curran, and T. A. Rutherford. 1990. DNA probes for differentiating isolates of the pinewood nematode species complex. Revue de Nématologie 13:255–263.

39. Welsh, J., and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18:7213–7218.

40. Williams, J. G. K., K. J. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531–6535.

41. Wingfield, M. J., R. A. Blanchette, T. H. Nicholls, and K. Robbins. 1982. Association of pine wood nematode with stressed tress in Minnesota, Iowa, and Wisconsin. Plant Disease 66:934–937.

42. Wingfield, M. J., R. A. Blanchette, T. H. Nicholls, and K. Robbins. 1982. The pine wood nematode: A comparison of the situation in the United States and Japan. Canadian Journal of Forest Research 12:71–75.

43. Wingfield, M. J., A. Blanchette, and E. Kondo. 1983. Comparison of the pinewood nematode, *Bursaphelenchus xylophilus* from pine and balsam fir. European Journal of Forest Pathology 13:360–372.

44. Yang, B., and Wang, Q. 1989. Distribution of the pinewood nematode in China and susceptibility of some Chinese and exotic pines to the nematode. Canadian Journal of Forest Research 19:1527–1530.