

Genomic Differences among Pathotypes of *Bursaphelenchus xylophilus*¹

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Abstract: Total genomic DNA from *Bursaphelenchus xylophilus* pathotypes MPSy-1 and VPSt-1 and from *B. mucronatus* was digested with restriction endonucleases. DNA fragments were electrophoretically separated, Southern blotted to nitrocellulose, and hybridized to genomic DNA from one of the isolates. The resulting hybridization patterns indicate genomic differences in repetitive DNA sequences among these populations. Greatest differences were seen between *B. xylophilus* and *B. mucronatus*, but genomic differences were also apparent between *B. xylophilus* pathotypes MPSy-1 and VPSt-1 and between a population from *P. nigra* in New Jersey and a population of a mucronate form from *Abies balsamea* in Quebec, Canada.

Key words: *Abies balsamea*, *Bursaphelenchus mucronatus*, *B. xylophilus*, DNA, genetics, pathotype, pinewood nematode, *Pinus nigra*, *P. strobus*, *P. sylvestris*, restriction endonuclease.

Bursaphelenchus xylophilus ((Steiner and Buhner), Nickle), the causal agent of pine wilt, has been reported in the United States in 33 states from 23 pine and 8 nonpine conifers and from both pine and fir in Canada (8,9,15,18). Regional variations have been reported in susceptibility of conifers to infection by *B. xylophilus* and in host expression of disease symptoms (1,2,13,17,22). In Missouri and neighboring states, where heavy infections by *B. xylophilus* are widespread, *Pinus sylvestris*, *P. nigra*, and *P. resinosa* are susceptible hosts and *P. strobus* is resistant (13). In the upper north central states, *P. strobus* is susceptible, whereas *P. sylvestris* appears to be resistant (1,23). Similarly in Vermont, *P. sylvestris* and *P. nigra* are resistant, but *P. strobus* is susceptible (2). *P. thunbergii* and *P. nigra* are susceptible hosts in New Jersey, with other pines showing variable resistance (17). Furthermore, *B. xylophilus* appears to be the primary cause of pine wilt in some regions of the United States, but in other areas this nematode is

a secondary invader, accelerating the decline of trees already stressed by bacterial or fungal pathogens (1,21,23). In Canada and Minnesota, a mucronate form of this nematode has been isolated from *Abies balsamea*, but it is apparently nonpathogenic to pine (22).

Recent evidence suggests that regional variation in the susceptibility of pines and in the expression of pine wilt symptoms may be due to development of *B. xylophilus* pathotypes. Comparison of susceptibility of several pine species to infection by *B. xylophilus* isolated from *P. sylvestris* in Missouri (pathotype MPSy-1) and by *B. xylophilus* isolated from *P. strobus* in Vermont (pathotype VPSt-1) indicates that maximum nematode population size and full development of disease symptoms occur only in those pines from which the pathotypes were isolated (3). Dwinell (10) demonstrated host preference of *B. xylophilus* isolates from *P. virginiana* in South Carolina, Georgia, and Alabama. Wingfield and coworkers (22,23) have reported host specificity of *B. xylophilus* from *P. sylvestris* and from *A. balsamea* to pine and fir, respectively, in the upper north central states.

Restriction endonuclease cleavage of DNA and comparative DNA:DNA hybridization have the potential to detect changes in transcribed and nontranscribed DNA sequences and are particularly suited for evaluating differences in repetitive sequences of DNA (5-7,12,19,20). Studies with a variety of other organisms have

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shown that genomic changes are found in repetitive DNA sequences, but such changes do not greatly affect gene transcription (14).

Curran et al. (5,6) successfully used DNA restriction endonuclease analysis to demonstrate genomic differences among *Trichinella spiralis*, *Caenorhabditis briggsae*, *Romanomermis* sp., and *Steinernema* sp., as well as among species and races of *Meloidogyne*.

The objective of this research was to use restriction fragment length polymorphisms (RFLP) to differentiate between pathotypes MPSy-1 and VPSt-1 and between *B. xylophilus* and *B. mucronatus*. RFLP among other populations of *B. xylophilus* from different geographical regions are also reported.

MATERIALS AND METHODS

Source and culture of isolates: Isolates of *Bursaphelenchus* spp. were obtained from the following pine species, geographical areas, and cooperators: *B. xylophilus* MPSy-1—*P. sylvestris*, Columbia, Missouri, V. H. Dropkin; *B. xylophilus* VPSt-1—*P. strobus*, Vermont, D. R. Bergdahl via R. F. Myers; *B. xylophilus* NJPn-1—*P. nigra*, New Jersey, R. F. Myers; *B. xylophilus* AzPh-1—*P. halepensis*, Arizona, M. A. McClure; *B. xylophilus* COPSy-1—*P. sylvestris*, Ontario, Canada, B. E. Hopper; *Bursaphelenchus* sp. CQAb-1 with a mucronate tail—*A. balsamea*, Quebec, Canada, B. E. Hopper; *B. mucronatus*—*P. thunbergii*, Japan, Y. Mamiya via V. H. Dropkin. All were supplied on cultures of *Botrytis cinerea* except VPSt-1 which was on *Pyrenochaeta* sp. Nematodes were maintained at 26 C on *B. cinerea* growing on potato dextrose agar (PDA). Subcultures were made monthly, and all cultures were routinely assayed for contaminating bacteria and fungi (2). Infectivity of each nematode isolate was assessed monthly by inoculation of 2–3-year-old pine seedlings (3).

DNA isolation: DNA was extracted essentially as described by Emmons et al. (11). Proteinase K, dissolved in 200 mM Tris-HCl (pH 8.0), 10 mM EDTA, 400 mM

NaCl, and 2% SDS (sodium dodecyl sulfate), was added to 5×10^3 nematodes, suspended in 0.5 ml of the same buffer, to a final concentration of 10 mg/ml. After incubation for 1 hour at 65 C, an additional 5 mg/ml Proteinase K was added to the digest and the incubation was continued for 30 minutes. Nucleic acid was extracted from the nematode digest by the addition of two volumes of phenol saturated with 50 mM Tris-HCl containing 1 mM EDTA (pH 8.0). The aqueous phase was recovered and the phenol extraction repeated. One volume of buffer saturated phenol plus one volume of chloroform was then added to the aqueous phase. Residual phenol was removed from the aqueous phase by one extraction with two volumes of chloroform: isoamyl alcohol (19:1, v:v) (16). Total nucleic acid then was precipitated with 2.5 volumes of 100% ethanol. The nucleic acid pellet was washed twice with 70% ethanol and suspended in 0.5 ml of a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 150 mM NaCl. RNA was then digested by incubation of the nucleic acid with 100 µg/ml pancreatic RNase and 50 µg/ml RNase T1 for 30 minutes at 37 C. The RNase had been previously heated at 100 C for 10 minutes and rapidly cooled to 4 C to inactivate any endogenous DNase activity (16). DNA was extracted and precipitated with 100% ethanol. Purified DNA was suspended in sterile distilled water, and the concentration was determined by absorbance at 260 nm (16).

Restriction endonuclease digestion of DNA: Restriction endonuclease digestions were done according to manufacturers' suggestions. Twelve units of restriction endonuclease were added to a 50-µl reaction mixture containing 6 µg nematode DNA, and the mixture was incubated for 2–4 hours at 37 C. When two different restriction enzymes were used sequentially, the DNA was extracted from the first incubation, suspended in an appropriate buffer, and incubated with the second enzyme. Several restriction endonucleases, differing in rec-

ognition sequence, were used singly or in pairs. Reactions were stopped by adding EDTA to a final concentration of 10 mM.

Agarose gel electrophoresis: A 10- μ l aliquot of the digestion mixture, which contained 1.2 μ g DNA, was mixed with 3 μ l loading buffer composed of 5 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5% bromophenol blue, 0.5% xylene cyanol, and 50% glycerol. The samples were then loaded into wells of a 15 \times 25-cm horizontal 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide. Sixteen-well gels were used so that all DNA samples from the different nematode populations could be separated in triplicate on the same gel for comparison. Gels were electrophoresed at 2 V/cm in 1 \times TBE running buffer (13). A 1-kilobase (kb) ladder of DNA fragments was used as a marker. Gels were observed at 310 nm and photographed.

Comparative hybridization: DNA fragments were transferred to nitrocellulose paper (16), and the nitrocellulose papers were cut to give sections containing a single lane each of the nematode DNA. Each section was then hybridized to genomic DNA from either VPSt-1, MPSy-1, or *B. mucronatus*. DNA, used as a hybridization probe, was radioactively labeled by nick translation with [³²P]-dCTP and [³²P]-TTP (13). Nick translation reactions were adjusted to yield radioactive DNA with a specific activity of 1.0–1.5 \times 10⁷ cpm/ μ g DNA, and 3.0 \times 10⁶ cpm were used in each hybridization reaction. Southern blots (16) were prehybridized in 5 \times SSPE composed of 4.4 M NaCl, 0.2 M NaH₂PO₄, 0.03 M EDTA, pH 7.4, 2 \times Denhardt's solution (16), 0.1% sodium dodecyl sulfate (SDS), and 100 μ g/ml salmon sperm DNA for 2 hours at 65 C. Radioactive hybridization probe DNA was denatured for 15 minutes at 100 C, rapidly cooled to 4 C, and added to the reaction. Hybridization was at 60 C for 16 hours. The hybridized filters were washed twice for 15 minutes each wash with 1 \times SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS at room temperature and twice with 0.2 \times SSC containing 0.5%

SDS for 30 minutes at 65 C. Filters were exposed to Kodak XAR-5 X-ray film with Dupont Cronex Lightning-Plus CH intensifying screens for 6 or 24 hours at -70 C (16). Autoradiograms and photographic negatives were analyzed with an E-C 910 Transmission Densitometer, and the densitometric tracings were integrated using a Zeineh Videophoresis II Electrophoresis Reporting Integrator Program (Biomed Instruments, Fullerton, CA) on an Apple IIe computer.

In control experiments, pBR322 DNA was cleaved with Hae III, the fragments were electrophoresed in agarose gels, and the gels were analyzed by densitometry. The test was used to determine the accuracy of the method for detection of fragments differing by 100 or more base pairs.

RESULTS

Restriction endonuclease-generated fragments of DNA from populations of MPSy-1 and VPSt-1 and from *B. mucronatus* hybridized with radioactively labeled genomic DNA from one of the nematode isolates showed distinct differences between species and among pathotypes within species of *Bursaphelenchus* (Fig. 1). The differences were apparent after consecutive cleavages of the genomic DNA with two restriction endonucleases or after cleavage with a single enzyme (Figs. 1, 2). The size range of fragments in which hybridization pattern differences could be discerned depended on the restriction endonucleases and the hybridization probe DNA used (Figs. 1–3). Greatest differences were seen between isolates of *B. xylophilus* and *B. mucronatus* (Figs. 1, 3). DNA from *B. xylophilus* isolates hybridized intensely with itself but only weakly with DNA from *B. mucronatus*, and DNA from *B. mucronatus* showed intense self hybridization but only weak hybridization to *B. xylophilus* DNA (Fig. 1). Hybridization intensity of the restriction endonuclease fragments of DNA from MPSy-1 or VPSt-1 to DNA from these pathotypes was similar (Figs. 1, 2).

MPSy-1 and VPSt-1 genomic DNA,

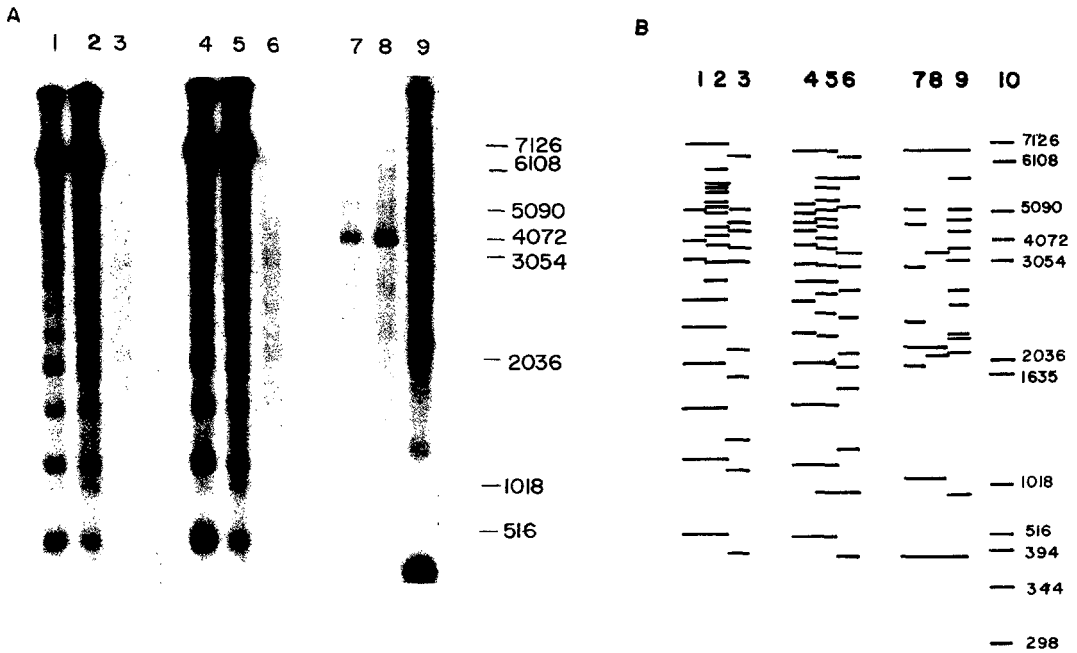


FIG. 1. Analysis of genomic differences between *Bursaphelenchus xylophilus* and *B. mucronatus* and among MPSy-1 and VPSt-1 by comparative hybridization of total genomic DNA to genomic DNA cleaved with *Rsa* plus *Pvu*II. A) Autoradiogram. Lanes 1-3 were hybridized to MPSy-1 genomic DNA, lanes 4-6 to VPSt-1 genomic DNA, and lanes 7-9 to *B. mucronatus* genomic DNA. DNA fragments in each lane are as follows: lanes 1, 4, and 7—VPSt-1; lanes 2, 5, and 8—MPSy-1; and lanes 3, 6, and 9—*B. mucronatus*. Marker DNA fragments are indicated on the left. B) Line drawing from autoradiograms shown in A indicating hybridizing fragments. Lane 10 indicates position marker DNA fragments seen in the gel stained with ethidium bromide.

cleaved with *Rsa* plus *Pvu*II and hybridized to genomic DNA from either MPSy-1 or VPSt-1, showed RFLP in fragments larger than 3,000 base pairs (Fig. 1). Smaller fragments from both pathotypes were nearly identical. RFLP also were seen when *Rsa*-*Pvu*II DNA fragments from MPSy-1 and VPSt-1 were hybridized with *B. mucronatus* DNA, but these differences encompassed a greater range of fragment sizes (Fig. 1).

There were also RFLP between MPSy-1 and VPSt-1 when their DNA was cleaved with *Hind* III or *Hinf* and hybridized to MPSy-1 DNA (Fig. 2). RFLP were generally in fragments larger than 4,000 base pairs and were most apparent when genomic DNA was cleaved with both endonucleases (Fig. 2). In the latter case, RFLP were in fragments larger than 2,500 base pairs. Smaller DNA fragments were similar (Fig. 2). RFLP between MPSy-1 and VPSt-1 and between *B. xylophilus* and *B. mucronatus* DNA were seen consistently,

regardless of the restriction endonuclease used to cleave the DNA or whether one or two enzymes were used (Fig. 3).

RFLP determined by cleavage of genomic DNA with *Dra* I plus *Pvu*II and hybridization of the fragments to MPSy-1 genomic DNA indicated differences among several other *B. xylophilus* isolates (Fig. 4). NJPn-1 and AzPh-1 differed from each other and the other *B. xylophilus* isolates, as well as from *B. mucronatus*. RFLP and hybridization intensity of genomic DNA of COPSy-1 and CQAb-1 to MPSy-1 genomic DNA were similar, suggesting that these isolates differ greatly from *B. mucronatus*. The restriction fragment pattern of COPSy-1 and CQAb-1 suggest some similarity to VPSt-1 and MPSy-1, although differences are present (Fig. 4).

DISCUSSION

The analysis of restriction endonuclease-generated DNA fragments supports the

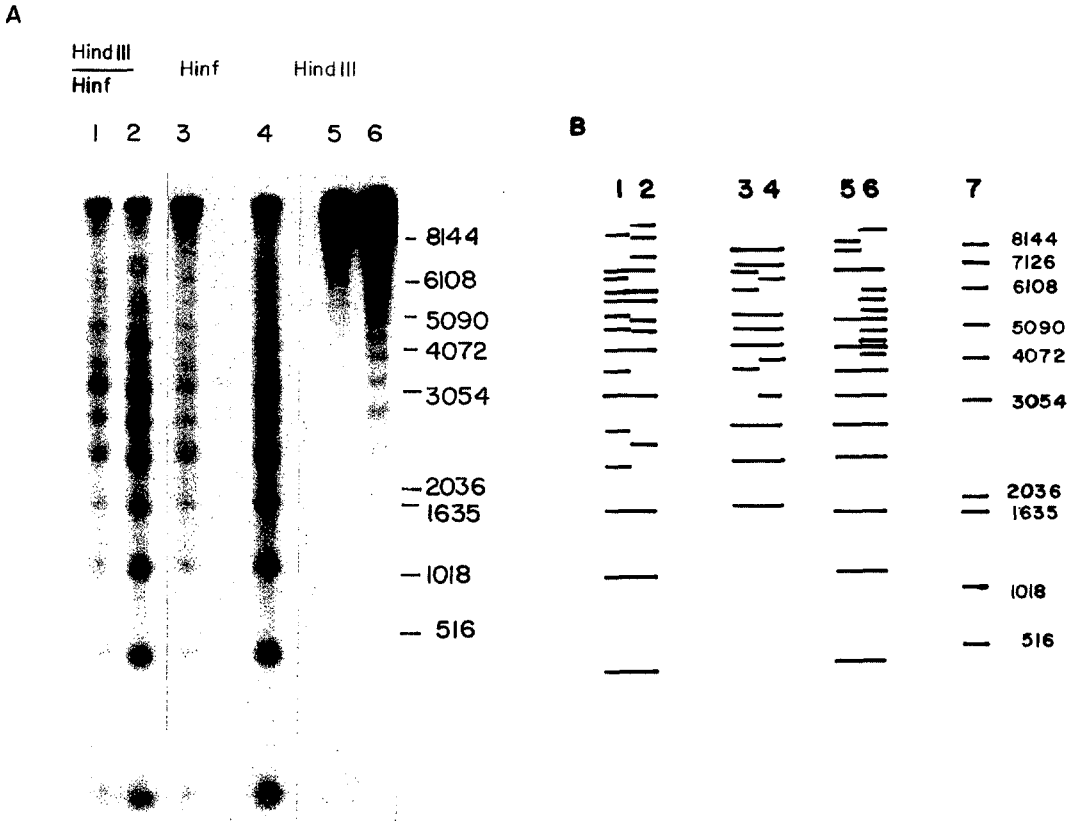


FIG. 2. Analysis by comparative hybridization of DNA from *Bursaphelenchus xylophilus* pathotypes MPSy-1 and VPSt-1. Genomic DNA was cleaved with Hind III, Hinf, and Hind III plus Hinf and hybridized to nick-translated MPSy-1 genomic DNA as described in text. A) Autoradiogram. Lanes 1, 3, and 5 contain VPSt-1 DNA; lanes 2, 4, and 6 contain MPSy-1 DNA. Mobility of marker DNA fragments are indicated on the left. B) Line drawing from autoradiogram shown in A indicating fragments hybridizing to MPSy-1 DNA. Lane 7 indicates position of marker DNA fragments seen in the gel stained with ethidium bromide.

hypothesis that *B. xylophilus* pathotypes MPSy-1 and VPSt-1, which are different in host specificity, differ genotypically. There are several possible explanations for the development of the pathotypes, including 1) feeding specificity of the carrier insect on pines, perhaps brought about by the selective pressure of the major pine species present in a given area; 2) geographical isolation of the nematode related to the dispersal of the insect vector; 3) the species of insect vector present; and 4) intraspecific differences within populations of a given host pine species. The observed differences in restriction endonuclease patterns probably result from mutational events, such as base-pair substitutions or insertion or deletion of small or large DNA se-

quences, and from recombination or transposition (7,14,19). The nature of these changes, however, cannot be determined solely from RFLP analysis. This analysis does reveal inherent differences that distinguish one pathotype or species from another. Genomic differences between VPSt-1 and MPSy-1 apparently do not involve major rearrangements or changes in the coding pattern of the DNA, because the pathotypes retain the ability to mate and produce viable, infective F₁ and F₂ generations (4, Bolla, unpubl.). That MPSy-1 and VPSt-1 are host specific may reflect either these genetic differences or physiological plasticity of this nematode. The mechanism cannot be determined from the results presented.

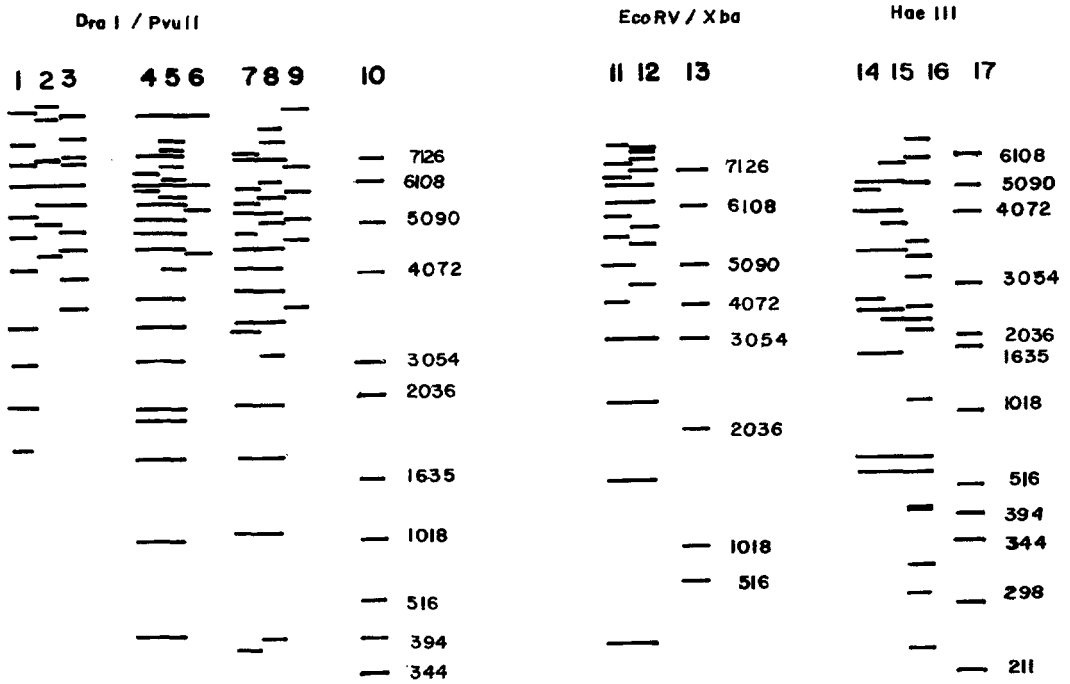


FIG. 3. Composite line drawing from computer analyzed densitometric tracings of autoradiograms of *Bursaphelenchus* spp. genomic DNA after reaction with restriction endonuclease and hybridization to nick translated genomic DNA. Lanes 1, 4, 7, 11, and 14 contain restriction fragments of VPSt-1 genomic DNA; lanes 2, 5, 8, 12, and 15 contain MPSy-1, and lanes 3, 6, 9, and 16 contain *B. mucronatus*. Lanes 1-3 were hybridized to *B. mucronatus* genomic DNA; lanes 4-6, 11, 12, and 14-16 to MPSy-1 genomic DNA; and lanes 7-9 to VPSt-1 genomic DNA. Lanes 10, 13, and 17 indicate position of marker DNA fragments within each gel. All experiments were done in triplicate.

Host specificity has not yet been determined for isolates NJPn-1, AzPh-1, COPSy-1, and CQAb-1. RFLP analysis and comparative DNA:DNA hybridization, however, suggest that NJPn-1 and AzPh-1 differ genotypically from the other *B. xylophilus* isolates and that they are not similar to each other. Although isolate CQAb-1 has a mucronate tail, RFLP and comparative DNA:DNA hybridization suggest that it is more similar to COPSy-1, MPSy-1, and VPSt-1 than it is to *B. mucronatus*. These results suggest that the different isolates may show host specificity.

Definition of a species generally is based on variation in morphology, cytogenetics, and production of viable offspring. Restriction endonuclease mapping of DNA usually supports differentiation of species defined in this manner (5,7,19,20). Molecular techniques further aid in the definition of subspecific variation (5-7,20). Thus these

techniques, coupled with genetic analysis from mating studies and cytogenetics, become powerful tools in describing the taxonomic structure of subpopulations of a variety of organisms, including nematodes, and in describing evolution by determining rate of DNA sequence change (7).

Although restriction endonuclease analysis of genomic DNA to define infraspecific forms of plant-parasitic nematodes must undergo further testing, the observations of genotypic differences among isolates of *Bursaphelenchus* suggest a strategy by which an analytical system can be designed. Such a system will depend on the ability to identify a specific genomic fragment characteristic of a single standard isolate and to use this fragment as a hybridization probe for other populations of the nematode. Results of our study, although not conclusive, suggest a method for analysis of nematode populations within a geographical region

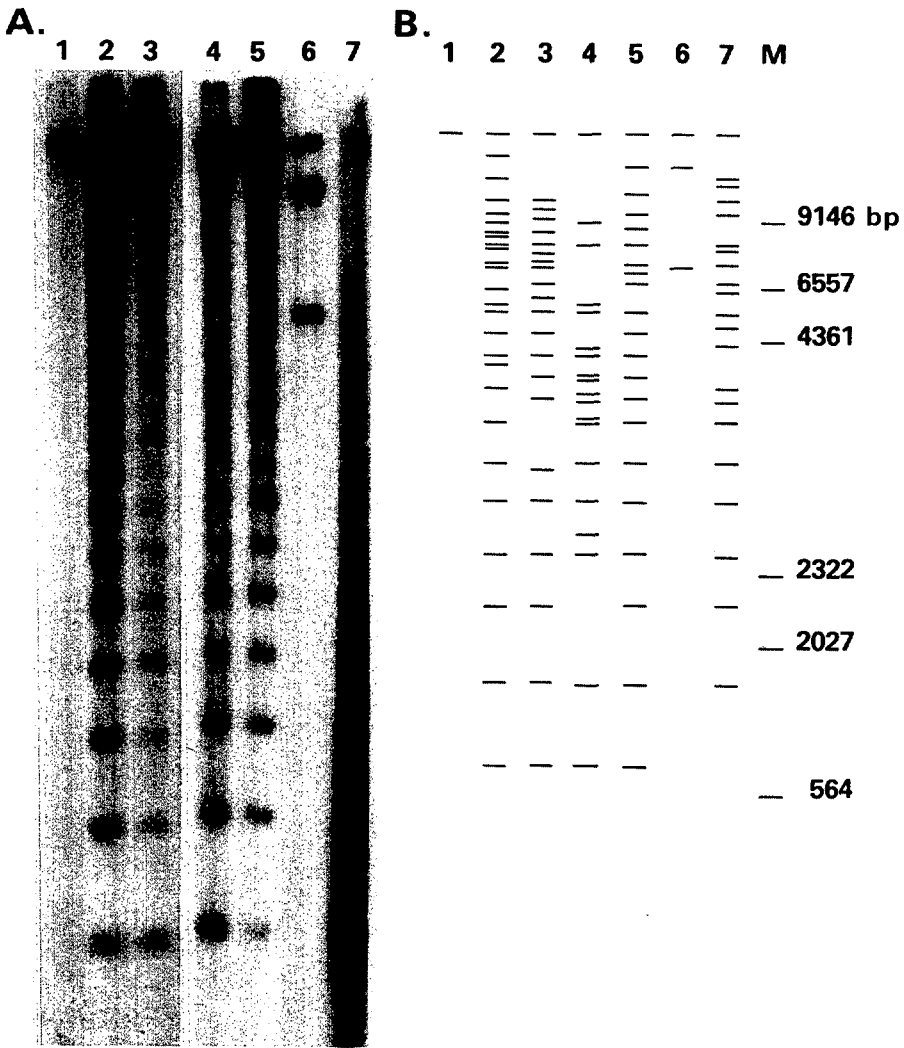


FIG. 4. Analysis of genomic differences between *Bursaphelenchus xylophilus* and *B. mucronatus* and among isolates of *Bursaphelenchus* spp. by comparative hybridization of MPsy-1 genomic DNA to genomic DNA cleaved with Dra I plus PvuII. A) Autoradiogram. Lane 1, NJPn-1; lane 2, MPsy-1; lane 3, VPSt-1; lane 4, AzPh-1; lane 5, CQAb-1; lane 6, *B. mucronatus*; lane 7, COPsy-1. B) Line drawing from autoradiogram shown in A indicating fragments hybridizing to MPsy-1 DNA. Position of marker DNA fragments seen in the gel stained with ethidium bromide is indicated in lane M.

to determine interactions of the populations.

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