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Intraspecific rDNA Restriction Fragment Length Polymorphism in the *Xiphinema americanum* group

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Summary - *Xiphinema americanum sensu lato* is a plant parasitic nematode vector of nepoviruses, which is widely distributed in North America. Considerable variation in the capability to vector nepoviruses and in many morphological characters is found between different populations. The concept of this species has been considerably restricted by some authors while others recognized the variability but hesitated to split the species. In this study we separated populations of the *X. americanum* group using restriction fragment length differences in the 5.8s gene and the internal transcribed spacers (ITS) of ribosomal DNA. Two plasmid clones from *Xiphinema bricolensis* (Xb) 18s and 26s ribosomal genes were isolated from a genomic library using a complete repeat of the ribosomal cistron of *Caenorhabditis elegans* (Ce) as a probe. Conserved sequences between Xb and Ce were identified and two 21-mer oligonucleotides primers were designed to amplify the ITS region using the polymerase chain reaction (PCR). The 1.5 Kb amplified product from the ITS region of each of sixteen populations of the *X. americanum* group was analyzed for restriction length polymorphisms (RFLP's). The RFPL's were recorded, dissimilarity coefficients were calculated, and a cluster analysis was generated arranging the sixteen populations as a dendrogram with five clusters. Two populations of *X. rivesi* were well separated from other *X. americanum* populations. *X. bricolensis* and two populations from Washington State were grouped together, while *X. pacificum* and an undescribed population from California were in another cluster. Mixed populations of *X. rivesi* and *X. americanum* from Pennsylvania and West Virginia could not be resolved. The taxonomic separation of this complex of species is an important step towards determining the vectors of nepoviruses in North America.

Résumé - *Polymorphisme intraspécifique de fragments de restriction de l'ADN ribosomal dans le groupe* Xiphinema americanum - *Xiphinema americanum sensu lato,* parasite de plantes et vecteur de nepovirus est présent dans nombre de régions d'Amérique du Nord. Il existe dans les différentes populations une variabilité importante chez de nombreux caractères morphologiques. La définition de cette espèce a été restreinte d'une manière considérable par certains auteurs tandis que d'autres, tout en reconnaissant la variabilité, ont hésité à diviser l'espèce. Cette étude sépare certaines populations du groupe *X. americanum* en utilisant la différence de longueur des fragments de restriction de séquences du gène 5.8s et des régions intergéniques transcrites de l'ADN ribosomal. Deux plasmides contenant des fragments clones des gènes ribosomaux 18s et 26s de *Xiphinema bricolensis* ont été isolés à partir d'une librairie génomique en utilisant comme sonde l'opéron complet de l'ADN ribosomal de *Caenorhabditis elegans* (Ce). Les séquences homologues entre Xb et Ce ont été utilisées comme amorces d'amplification des ITS utilisant la réaction de polymérase en chaîne. Le produit d'amplification de chacune des seize populations du groupe *X. americanum,* un segment d'environ 1.5 Kb, a été analysé pour la longueur des fragments de restriction (RFLP's). Les polymorphismes ont été notés, des coefficients de dissimilarité entre populations ont été calculés, et une analyse en grappes a permis de grouper les seize populations en cinq groupes. Deux populations de *X. rivesi* sont bien séparées des autres populations du groupe *X. americanum. X. bricolensis* et deux populations de l'état de Washington sont regroupées, tandis que *X. pacificum* et une population de Californie non encore identifiée forment une autre branche. Des populations de Pennsylvanie et de Virginie de l'Ouest, contenant un mélange de *X. americanum* et de *X. rivesi,* n'ont pu être séparées. L'étude taxinomique de ce complexe d'espèces est un pas important vers la détermination des vecteurs de nepovirus d'Amérique du Nord.

Key-words : Restriction fragment length polymorphism, ribosomal DNA, *Xiphinema americanum* group.

Xiphinema americanum Cobb. 1913 is widely distributed in numerous areas of North America. It is the vector of several economically important nepoviruses : tobacco ringspot virus (Hibben & Walker, 1971), tomato ringspot virus (Breece & Hart, 1959), peach rosette mosaic virus (Klos *et al.*, 1967), and cherry rasp leaf virus (Nyland *et al.*, 1969). The efficiency of transmission of nepoviruses seems to vary between populations (Georgi, 1998*b;* Griesbach & Maggenti, 1989). It would be useful to relate morphological differentiation to virus vector characters (Tarjan, 1969; Lima, 1975; Luc & Southey, 1980; Heyns, 1983; Malik & Jairajpuri, 1983; Lamberti & Golden, 1986; Griesbach & Maggenti, 1990; Cho & Robbins, 1991). Unfortunately, it has not been possible to establish links between virus vector ability and morphological differences (Griesbach & Mag-

genti, 1989). Fifteen new species were named by Lamberti and Bleve-Zacheo (1979) from their examination of several hundred populations formerly identified as *X. americanum*. The usefulness or the validity of these relationships have recently been questioned (McKenry, 1987; Georgi, 1998*a;* Griesbach & Maggenti, 1990). Thirteen years after their definition, the taxonomic status of many species within the *X. americanum* group is still debated.

These nematodes are notorious for their susceptibility to changes in their environment, and have frustrated many nematologists in their attempts to maintain populations in laboratory or greenhouse culture. This problem has made it difficult to study their biology and to use ecological or physiological data to separate populations into different species. Males are extremely rare or nonexistent in the populations examined, suggesting that parthenogenesis is a common means of reproduction. Therefore, the taxonomy of this group has been primarily based on morphometrics. However, it is now recognized that many characters used to separate the recently described species often overlap, making diagnostics at best difficult and too often merely tentative (Heyns, 1983; Georgi, 1998*a;* Griesbach & Maggenti, 1990).

Platzer (1981) anticipated that techniques of molecular biology would be applied to help resolve problems of identification of plant parasitic nematodes, and the topic has recently been reviewed (Burrows, 1990; Hyman & Powers, 1991). Restriction fragment length polymorphisms (RFLP's) have been used to separate species and genera of plant parasitic nematodes (Powers & Sandall, 1988). Comparative analysis of ribosomal RNA and DNA (rDNA) sequences has become a popular tool for the construction of phylogenetic trees and in the determination of evolutionary relationships. Qu *et al.* (1986) used sequences from 28s ribosomal RNA to demonstrate phylogeny in Helminths. Williams *et al.* (1985), separated geographic variants of *Drosophila* on the basis of variation in the nontranscribed spacer of rDNA. While these and other direct analytical methods are of proven utility, similar analysis with *Xiphinema* populations were precluded due to difficulties in obtaining sufficient quantities of nucleic acids.

A complete repeat of the ribosomal cistron of *Caenorhabditis elegans* (Ce) has been cloned and sequenced (Ellis *et al.,* 1986). Like the ribosomal cistron of many other organisms it contains three spacers separating the 5.8s, 18s, and 26s genes. In this study we have made use of the polymerase chain reaction (PCR) to amplify a region of the ribosomal gene (rDNA) and developed a sensitive method to examine sequence variability within the 5.8s gene and the internal transcribed spacers (ITS) of rDNA. We have amplified the ITS region of rDNA from sixteen North American populations of the *X. americanum* group. Cluster analysis of the RFLP's allowed us to group these populations, and our results have the potential to clarify the status of some recently described species.

Materials and methods

Nematode preparation

Nematodes from field populations in Pennsylvania, West Virginia, and Iowa State in the USA, and from Quebec, Canada, were extracted, frozen in water when still alive, and shipped in dry ice by J. Halbrendt, J. Kotcon, D. Norton, and G. Bélair respectively. Samples of soil from the states of California, Oregon and Washington in the USA were provided by M. McKenry, R. Ingham, and F. McElroy respectively. Several populations (Dobbins, Firestone, and Murido) were only tentatively identified as *X. americanum*. The *Xiphinema bricolensis* (Xb) and *X. pacificum* populations were from the type localities of these species in British Columbia (Ebsary *et al.,* 1989).

Soils containing *Xiphinema* nematodes were processed by a sieving flotation technique (Graham *et al.,* 1988). The organic matter residues and the nematodes caught on the lower sieve (250 μm diam. pores) were placed on Baermann pans. Nematodes were collected from the pans every other day. Molten 2.5 % low gel temperature agarose at 40 °C was added to an equal volume of the nematode suspension, and the mix quickly poured into a Petri dish. The nematodes moved to the surface of the gel in 2 hours or more, leaving all soil debris behind. They were handpicked, placed in 50 μl of distilled water in a 1.5 ml silanized (treated with dimethyldichlorosilane) microcentrifuge tube and stored at – 80 °C.

All conditions of nucleic acid isolation, digestion, ligation, transformation, and hybridization, were according to laboratory manuals by Sambrook *et al.* (1989).

Nucleic acids isolation

Frozen pellets of nematodes were ground in liquid nitrogen. Total nucleic acids were isolated after digestion of the homogenate with Proteinase K, followed by phenol/chloroform extraction, treatment with RNase, and precipitation with ethanol. Xb DNA for cloning purposes was further purified on cesium chloride/ethidium bromide gradients. Plasmid DNA was isolated by the alkaline lysis method.

Characterization of *X. bricolensis* ribosomal clones

Total Xb DNA was digested to completion with EcoR1. The DNA fragments were ligated into alkaline phosphatase-treated pUC 13 (Pharmacia, Uppsala, Sweden). Competent *Escherichia coli* NM522 or JM109 cells were transformed, and selected on ampicillin agar plates with isopropylthio - B - D galactosidase (IPTG), and 5 - bromo - 4 - chloro - 3 - indolyl - B - D - galactoside (xGal). Transformants were screened by hybridization to a clone of an entire ribosomal cistron of

Table 1. Origin, location and identification of the 16 *Xiphinema* populations examined. The population labels c to r correspond to lanes on the agarose gel pictures and diagrams of Figures 2 to 5.

c	*X. americanum,* Fin 8, West Virginia, USA (Kotcon).
d	*X. americanum,* Dobbins, Woodlands, Washington, USA (transmits TomRSV, McElroy).
e	*X. americanum,* Firestone, raspberry, Vancouver, Washington, USA (TomRSV, McElroy).
f	*X. americanum,* Corvallis, raspberry, Oregon State University, Oregon, USA (TomRSV, Ingham).
g	*X. americanum,* Murido, Murido vineyard, Highway 43, S.E. Edge, California, USA (McKenry).
h	*X. americanum,* Parlier, Kearney Vineyard, S.E. Corner, Parlier, California, USA (McKenry).
i	*X. rivesi,* Frelighsburg, Apple orchard, Quebec, Canada (Belair).
j	*X. rivesi,* Idlewood, black walnut grove, Idlewood County Park, Floyd County, Iowa, USA (Norton).
k	*X. americanum,* Hinds Farm, Corn, Iowa State University, Story County, Iowa, USA (Norton).
l	*X. rivesi* 92 % and *X. americanum* 8 %, Ken peach, Pennsylvania, USA (Halbrendt).
m	*X. americanum* 92 % and *X. rivesi* 8 %, Peach, Pennsylvania, USA (Halbrendt).
n	*X. rivesi,* Adams Peach, Pennsylvania, USA (Halbrendt).
o	*X. americanum* 84 % and *X. rivesi* 16 %, Adams Grass, Pennsylvania, USA (Halbrendt).
p	*X. americanum* 72 % and *X. rivesi* 28 %, Apple, Pennsylvania, USA (Halbrendt).
q	*X. pacificum,* Plutt, Osoyoos, British Columbia, Canada.
r	*X. bricolensis,* Ste Claire, Peachland, British Columbia, Canada.

Fig. 1. Position of *Xiphinema* clones pXb101 and pXb481 flanking the Internal Transcribed Spacer (by homology to the ribosomal cistron of *Caenorhabditis elegans*), with sequences and positions of 18s and 26s primers.

Ce provided by D. L. Baillie, Simon Fraser University, Burnaby, Canada. Both ends of selected ribosomal clones were sequenced by the Sanger dideoxy-mediated chain termination method. The position of the Xb rDNA clones, relative to the gene map of the Ce ribosomal operon, was ascertained by computer searches of the alignment of cloned Xb sequences with Ce ribosomal sequences from the EMBL database, using SEQNCE PC 3.1 software (Delaney Software Ltd., Vancouver, Canada).

Amplification

Two 21 nucleotide sequences from clones pXb101 and pXb481, positioned respectively on the 18s and the 26s genes (Fig. 1), were synthesized as primers in an Applied Biosystems DNA synthesizer (Biotechnology Department, University of British Columbia). The DNA fragment containing the ITS region was amplified by PCR. The reaction mixture contained 10 mM Tris pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.1 % gelatin, 1.25 mM each of dATP, dCTP, dGTP, and dTIP, 0.5 µM of both primers, 1 to 50 ng of *Xiphinema* DNA, 2.5 units of Taq DNA polymerase, and deionized water to a volume of 50 µl. A negative control without DNA added, and a positive control with Ce DNA, were included in each PCR experiment. The amplification was done in 40 cycles in a thermocycler (Ericomp, San Diego, CA, USA). Each cycle consisted of a denaturation step at 94 °C for 1 min, annealing at 55 °C for 1 min, and polymerization ar 72 °C for 1 min. Ramping time was about 10 min per cycle.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM

The product of amplification was digested with fourteen restriction enzymes for 6 hours in the recommended buffers. The enzymes used were : Apa I, Alu I, BamH I, Bgl I, Dde I, Hind III, Hinf I, Mbo I, Msp I, Pal I, Pst I, Pvu II, Rsa I, and Xba I. The DNA fragments thus generated were separated by electrophoresis in a 2.50 % low gel temperature agarose slab in Tris-acetate/EDTA (TAE) buffer for 90 min. at 50 V. The marker DNA was pBR328 cut with BglI and HinfI, giving band sizes at 2.2 kb, 1.8 kb, 1.2 kb, 1.0 kb, 650 bp, 520 bp, 450 bp, 390 bp, 300 bp, 225 bp, 150 bp (Boehringer Mannheim). For each nematode population the bands obtained with six enzymes were recorded as a string of 0 and 1 corresponding to the absence or presence of individual bands. Data from all digests that gave polymorphisms were used for a total of 50 bands. A matrix X of 0 and 1 was generated with nematode populations as rows and bands as columns. A new matrix was generated by multiplying the X matrix by its transpose X' giving the matrix M with nematode populations as rows and columns. The numbers in the diagonal of the matrix M represent the total number of bands in a particular nematode population and the other numbers represent the number of bands shared by each population pair. Dissimilarity coefficients for all pair comparisons were calculated using the matrix M and a Fortran subroutine (Lévesque *et al.,* 1992). From these values, a cluster analysis by the unweighted pair-group method with average (UPGMA) was performed and a tree was constructed (SAS, 1985).

Results

The yield of *Xiphinema* nematode extractions from soil were rather low, averaging 100 to 200 per L of soil. The quantity of DNA extracted from Xb nematodes was variable but averaged 0.5 ng per nematode. The numbers of nematodes available from the other populations studied were relatively small and although yield efficiencies were not taken, they were assumed to be similar.

Six hundred colonies of transformed cells were picked from the Xb library. Five clones were shown to contain Xb rDNA by hybridization with ^{32}P labelled Ce ribosomal repeat. The position of two of these clones was of particular interest to this work. Clone pXb101 had 277 base pairs (bp), encompassing the 3' end of the 18s gene and the 5' end of the ITS (Fig. 1). Clone pXb481 was approximately 1.3 Kb, encompassing the 3' end of the ITS and the 5' end of the 26s gene. The two 21 nucleotide sequences utilized for primers were completely homologous between Xb and Ce, and other nematode sequences found in the EMBL database. The 18s primer sequence was 5' TTGATTACGTCCCTGCCCTTT 3', and was aligned with nucleotides 2503 to 2523 of the Ce ribosomal cistron (EMBL databank, accession number X03680). The 26s primer sequence was 5' TTTCACTCGCCGTTACTAAGG 3', and was the reverse complement of nucleotides 3774 to 3794 (Fig. 1).

Amplification of template DNA was usually 10^5 to 10^6 fold. The reaction yielded 2 to 5 μg of DNA starting with total DNA from approximately 100 nematodes. The amplification of the ITS region of each *Xiphinema* population gave one fragment approximately 1.5 Kb long (Fig. 2). The 1.5 Kb fragment hybridized strongly with cloned Xb ITS. There were several instances when nonspecific products of amplification were obtained, which did not hybridize to the cloned Xb ITS region (data not shown). It was assumed that variations in the conditions of the PCR reactions were responsible for the nonspecific amplification. The amplifications were repeated using " Perfect Match " (Stratagene, California, USA) which usually eliminated the formation of nonspecific products. No PCR product was ever obtained in the negative control treatment lacking DNA template. The Ce positive control always gave a strong band at approximately 1.4 Kb (Fig. 2).

Digestion of the amplification product with six of the fourteen enzymes gave sharp restricted DNA bands on the agarose gels. There were no restriction sites for Bgl I, Hind III, Pst I, Pvu II, and only partial digestions with Apa I, Pal I, Rsa I, and Xba I.

The pattern of restriction bands obtained with Alu I, Bam H I, Dde I, Hinf I, Mbo I, and Msp I (Diagrams of Figs. 3 a 8) allowed the grouping of the sixteen populations into five clusters (Fig. 6). Cluster 1 contained the two populations from Washington State and the population of Xb from British Columbia. Cluster 2 contained a population from California and the population of *X. pacificum* from British Columbia. Cluster 3 contained two populations of *X. rivesi* from Iowa and from Quebec. Cluster 4 contained the " Parlier " population from California recently described as *X. americanum sensu lato* (Griesbach & Maggenti, 1990), a population from Iowa and one from Oregon. Cluster 5 contained all the populations from Pennsylvania and West Virginia labelled *X. americanum, X. rivesi,* or mixed populations of these.

Discussion

In preliminary experiments the purity of the DNA extracted from nematodes in soil was inadequate for digestion and cloning. It proved important to have the nematodes free of any soil organic matter residues. Nematodes extracted in Baermann pans were thus further cleaned of soil debris by their migration through agarose.

The sequences of the two primers were from the conserved 18s and 26s genes. The amplification product included 79 nucleotides from the 5' end of the 26s gene, and 170 nucleotides from the 3' of the 18s gene. These conserved sequences, as well as the 5.8s gene sequence,

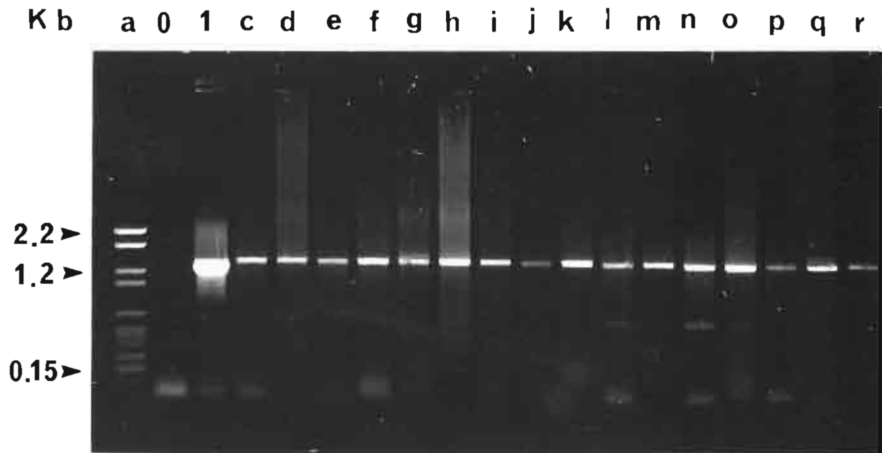

Fig. 2. Amplified Internal Transcribed Spacers region of sixteen *Xiphinema* spp. Ethidium bromide stained 2.5 % agarose gel. a : DNA marker, pBr 328/Bgl 1/Hinf 1; o : no DNA, amplification control; l : *Caenorhabditis elegans* ITS region; c : to r : populations of *Xiphinema* as in Table 1.

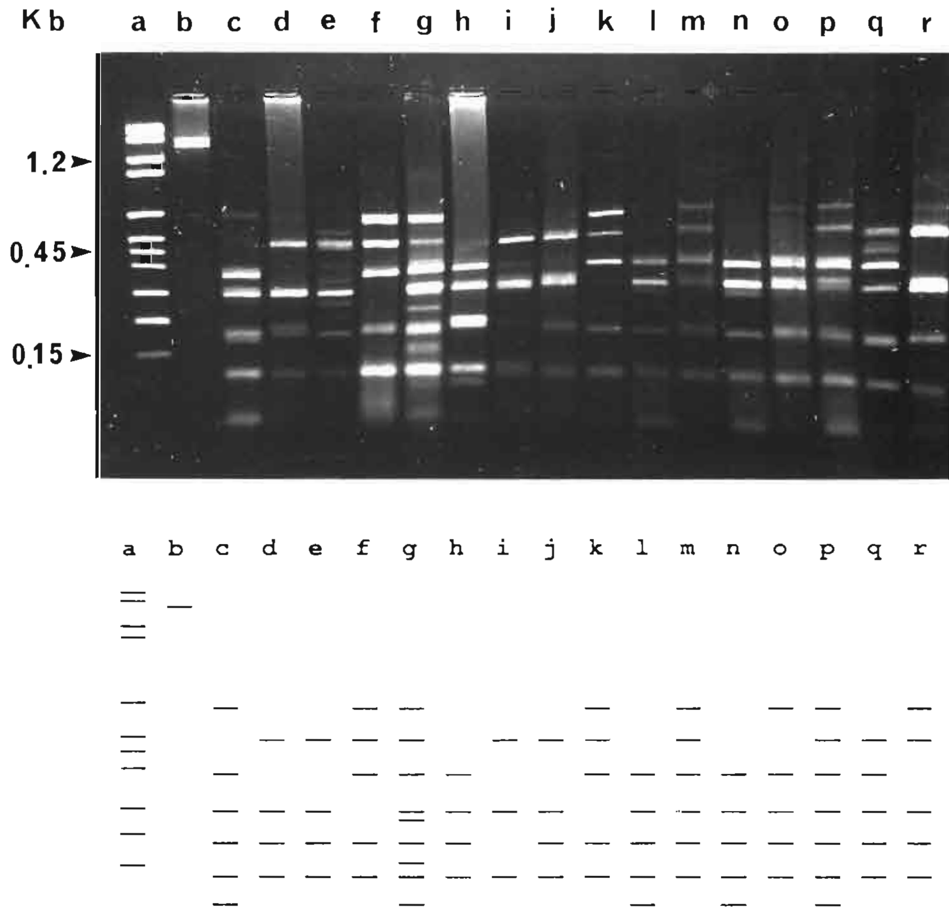

Fig. 3. Restriction fragments of amplified Internal Transcribed Spacers of *Xiphinema* spp. Ethidium bromide stained 2.5 % agarose gel – Hinf 1 digest – a : DNA marker, pBr 328/Bgl 1/Hinf 1; b : unrestricted ITS *X. americanum* (WA, USA); c : *X. americanum* (WV, USA); d : *X. americanum* (WA, USA); e : *X. americanum* (WA, USA); f : *X. americanum* (OR, USA); g : *X. americanum* (CA, USA); h : *X. americanum* (CA, USA); i : *X. rivesi* (PQ, Canada); j : *X. rivesi* (IA, USA); k : *X. americanum* (IA, USA); l : *X. rivesi* 92 % and *X. americanum* 8 % (PA, USA); m : *X. americanum* 92 % and *X. rivesi* 8 % (PA, USA); n : *X. rivesi* (PA, USA); o : *X. americanum* 84 % and *X. rivesi* 16 % (PA, USA); p : *X. americanum* 72 % and *X. rivesi* 28 % (PA, USA); q : *X. pacificum* (BC, Canada); r : *X. bricolensis* (BC, Canada).

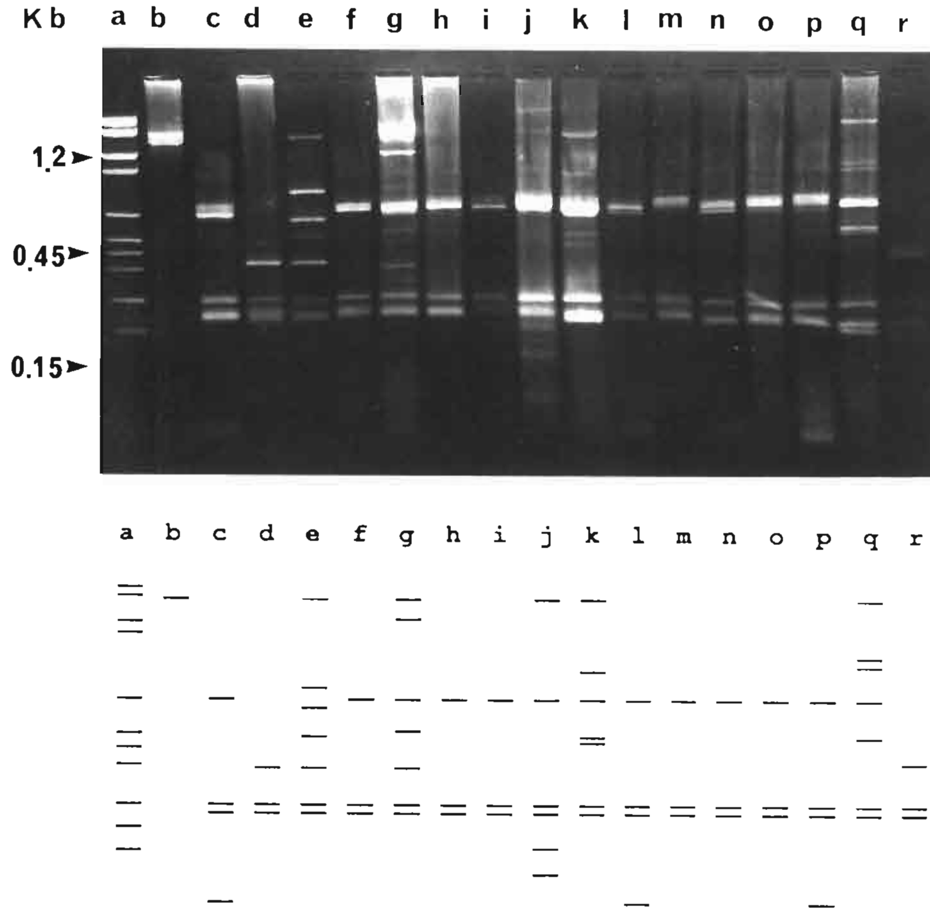

Fig. 4. Restriction fragments of amplified Internal Transcibed Spacers of *Xiphinema* spp. Ethidium bromide stained 2.5 % agarose gel – Msp 1 digest – (low case letters : see Fig. 3).

amplified with the ITS region of each population were represented in some of the common bands found in all populations (Figs 3 - 8).

The choice of the 5.8s gene and ITS fragment for the restriction polymorphism analysis of the 16 populations proved to be judicious. Ribosomal DNA is often represented in many copies in the genome of many organisms, and represents an excellent target for the amplification, since only small amounts of nematodes were available. A fragment from a conserved region, such as the coding regions of the 18s or the 26s genes, would be expected to show few polymorphic restriction sites, and may not have separated the *Xiphinema* populations. Conversely a fragment of DNA picked at random might have shown too many polymorphic restriction sites to be useful in grouping the populations. The region we amplified showed both common and polymorphic restriction sites.

Amplification of the 5.8s gene and of the ITS region of the ribosomal cistron of *Xiphinema* had the convenience of visualizing polymorphism directly on agarose gels. This technique allowed us to work with relatively small quantities of DNA extracted from few nematodes and there was no need for Southern transfers and hybridization with a ^{32}P-labelled probe.

Griesbach and Maggenti (1989) established that *X. americanum sensu stricto* is in California, that *X. rivesi* is distinct from *X. americanum*, and that *X. californicum* is not separable from *X. americanum*. In our study, the *X. americanum sensu lato* " Parlier " population from California was related to another West Coast population (from Oregon), and a more centrally located population (from Iowa), both described as *X. americanum* (Ingham & Norton, pers. comm.).

As well, *X. rivesi* populations from Quebec and Iowa were grouped in the same cluster and were distinct from other populations. The DNA of *X. rivesi* (lanes i and j, Fig. 5) lacked a BamH I restriction site present in all other populations of the *X. americanum* group. Ebsary *et al.* (1984) and Wojtocic *et al.* (1982), utilizing standard

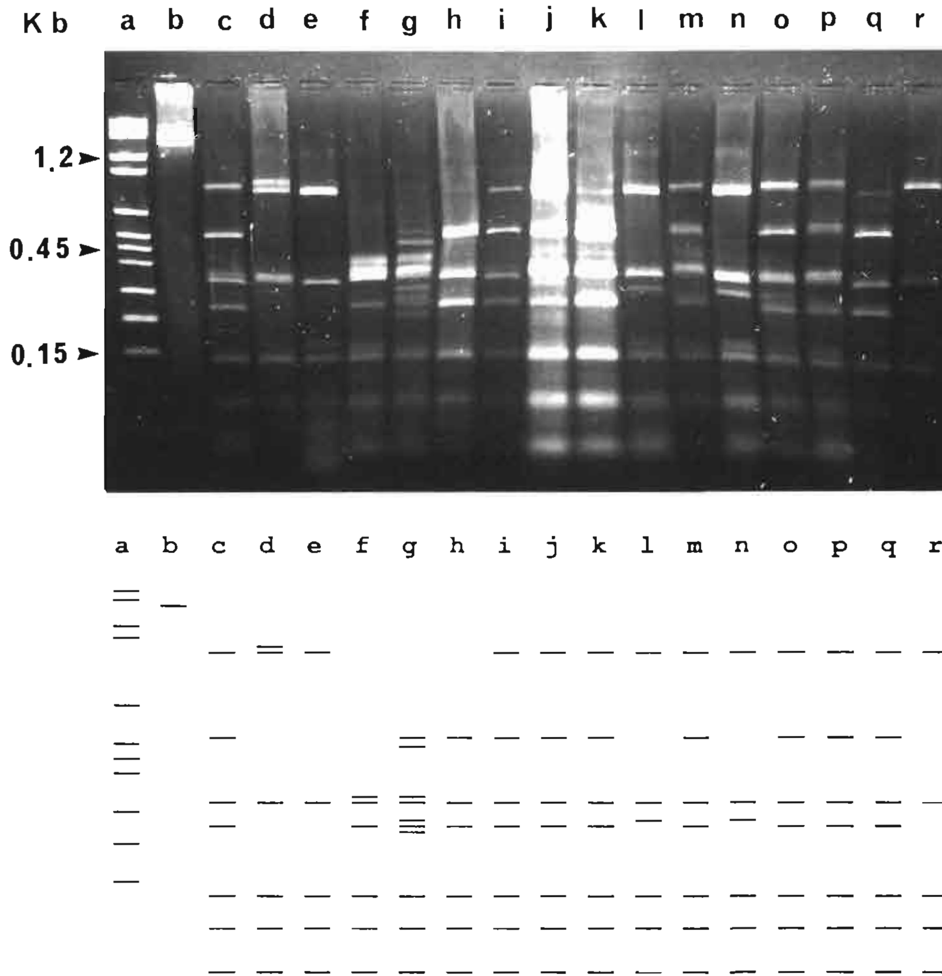

Fig. 5. Restriction fragments of amplified Internal Transcribed Spacers of *Xiphinema* spp. Ethidium bromide stained 2.5 % agarose gel – Dde 1 digest – (low case letters : see Fig. 3).

morphometrics, had no difficulty in differentiating the two species. Georgi (1988*a*) confirmed the validity of the separation of these two species in New York State, but found important regional differences, from Eastern to Western New York, with one population displaying characteristics of both species. These two species were found mixed in some instances in New York (Georgi, 1988*a*), as they often were in Pennsylvania and West Virginia (Kotcon & Halbrendt, pers. comm.). In Iowa, these two species are found in different habitats : *X. rivesi* in wooded areas, and *X. americanum* in corn fields and prairies (Norton, pers. comm.).

X. pacificum, a new species from British Columbia (Ebsary *et al.*, 1989), was described as somewhat resembling *X.californicum*. The RFLP study placed it in the same cluster as " Murido ", an undescribed population from California resembling *X. californicum* (Brown, pers. comm.). The dynamics and pathogenicity of this population are noticeably different from the " Parlier " population (McKenry, pers. comm.). A detailed morphometric study of the " Murido " population should confirm that it belongs to one or the other species, *X. pacificum* or *X. californicum*. The Xb population from British Columbia in cluster 1 is with 2 populations from adjacent Washington State, sent to us as *X. americanum*. However these two populations were not examined critically before the RFLP study, and it is probable that a detailed characterization will place them away from *X. americanum sensu stricto*.

Several populations were sent to us as mixtures of *X. americanum* and *X. rivesi* (Apple, PA; Adams Grass, Pa; Ken peach, PA; Peach, PA), while others were tagged as pure *X. americanum* (Fin 8, WV), or pure *X. rivesi* (Adams Peach, PA). The stylets of 25 nema-

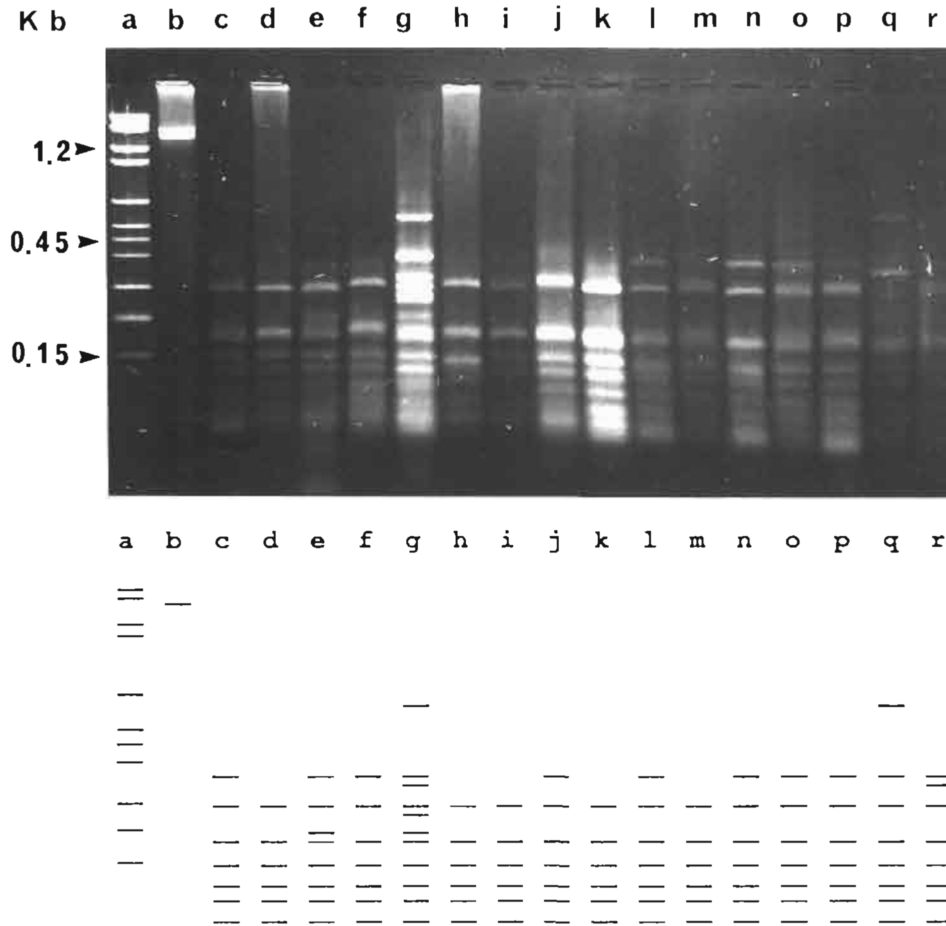

Fig. 6. Restriction fragments of amplified Internal Transcribed Spacers of *Xiphinema* spp. Ethidium bromide stained 2.5 % agarose gel – Mbo 1 digest – (low case letters : see Fig. 3).

todes were measured (Halbrendt & Hill, PA; Kotcon, WV) for all the populations. Based on these measurements the populations were defined as containing varying proportions of *X. americanum* and *X. rivesi*. Naturally, mixtures of nematodes would blur the results of the DNA analysis. Samples containing DNA from two species should have the same pattern of restricted DNA bands on the electrophoresis gel, albeit with bands of varying densities corresponding to the proportions of each species. This was easily seen with the BamH I digests, where populations of *X. rivesi* (lanes i and j; Fig. 8) show no restriction site, and populations of the *X. americanum* group (lanes d, f, h, and k; Fig. 8) show one restriction site. The mixed populations show all three bands, with the unrestricted fragment prominent when *X. rivesi* is a large proportion in the mixture (lanes l and n, Fig. 8), or with the unrestricted fragment quite faint when *X. rivesi* is a small proportion (lane c, m and p, Fig. 8). It appeared that the pure populations of *X. americanum* (Fin 8) and *X. rivesi* (Adams Peach) from West Virginia and Pennsylvania were also certainly mixed populations, since the RFLP patterns showed characteritics of both species.

The technique described here measures the discontinuity of variation between taxa otherwise difficult to separate by standard morphometrics. In this rDNA analysis, *X. americanum,* " Parlier " population, recognized as *X. americanum sensu stricto* by Griesbach and Maggenti (1990), was in a separate cluster from *X. rivesi.*

It appears that this molecular approach is capable of separating species within the *X. americanum* group, and therefore we confirm the validity of *X. bricolensis* and *X. pacificum,* which were in other clusters, well separated from *X. americanum sensu stricto* or *X. rivesi.* This RFLP analysis will help taxonomists decide how much genetic variability is expressed, and which morphometric characters are useful for identification.

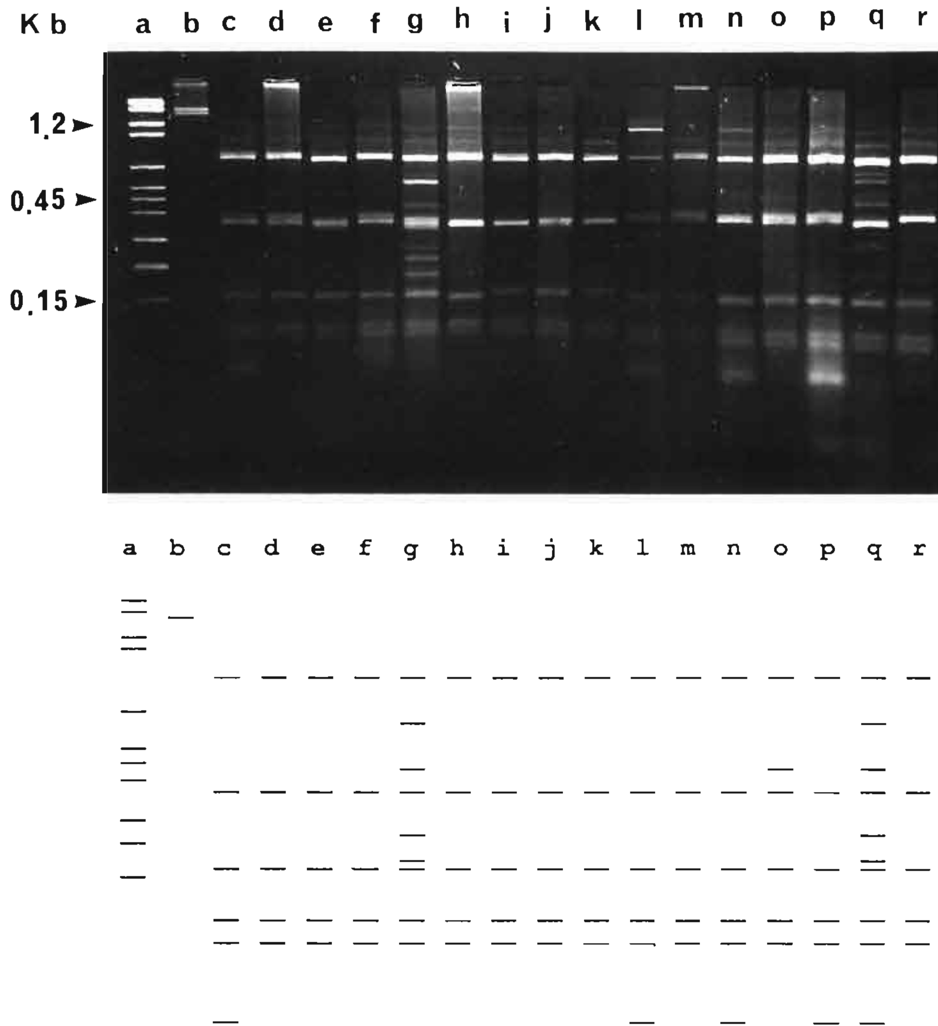

Fig. 7. Restriction fragments of amplified Internal Transcribed Spacers of *Xiphinema* spp. Ethidium bromide stained 2.5 % agarose gel – Alu 1 digest – (low case letters : see Fig. 3).

References

BREECE, J. R. & HART, W. H. (1959). A possible association of nematodes with the spread of peach yellow bud mosaic virus. *Pl. Dis. Reptr,* 43 : 989-990.

BURROWS, P. R. (1990). The use of DNA to identify plant parasitic nematodes. *Helminth. Abstr.,* 59 : 1-8.

CHO, M. R. & ROBBINS, R. T. (1991). Morphological variation among 23 *Xiphinema americanum* populations. *J. Nematol.,* 23 : 134-144.

EBSARY, B. A., POTTIER, J. W., & ALLEN, W. R. (1984). Redescription and distribution of *Xiphinema rivesi* Dalmasso, 1969 and *Xiphinema americanum* Cobb, 1913 in Canada with a description of *Xiphinema occiduum* n. sp. (Nematoda : Longidoridae). *Canad. J. Zool.,* 62 : 1696-1702.

EBSARY, B. A., VRAIN, T. C., & GRAHAM, M. B. (1989). Two new species of *Xiphinema* (Nematoda : Longidoridae) from British Columbia vineyards. *Canad. J. Zool.,* 67 : 801-804.

ELLIS, R. E. SULSTON, J. E., & COULSON, A. R. (1986). The rDNA of *C. elegans* : sequence and structure. *Nucleic Acids Res.,* 14 : 2345-2364.

GEORGI, L. (1988*a*). Morphological variation in *Xiphinema* spp. from New York orchards. *J. Nematol.,* 20 : 47-57.

GEORGI, L. (1988*b*). Transmission of Tomato Ringspot virus by *Xiphinema americanum* and *X. rivesi* from New York apple orchards. *J. Nematol.,* 20 : 304-308.

GRAHAM, M. B., EBSARY, B., VRAIN, T. C., & WEBSTER, J. M. (1988). Distribution of *Xiphinema bricolensis* and *Xiphinema pacificum* in vineyards of the Okanagan and Similkameen valleys, British Columbia. *Canad. J. Pl. Path.,* 10 : 259-262.

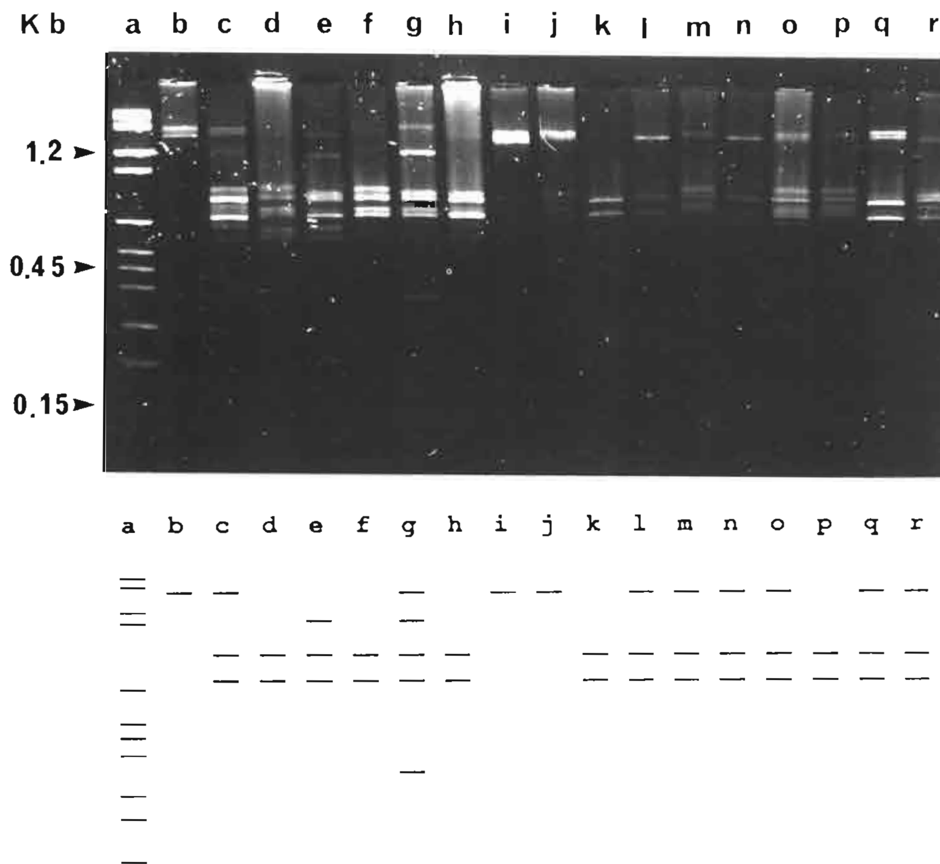

Fig. 8. Restriction fragments of amplified Internal Transcribed Spacers of *Xiphinema* spp. Ethidium bromide stained 2.5 % agarose gel – BamH 1 digest – (low case letters : see Fig. 3).

GRIESBACH, J. A., & MAGGENTI, R. (1989). Vector capability of *Xiphinema americanum sensu lato* in California. *J. Nematol.,* 21 : 517-523.

GRIESBACH, J. A., & MAGGENTI, R. (1990). The morphometrics of *Xiphinema americanum sensu lato* in California. *Revue Nématol.,* 13 : 93-103.

HEYNS, J. (1983). Problems of species delimitation in the genus *Xiphinema,* with special reference to monosexual species, *In :* Stone, A. R., Platt, H. M. & Khalil, L. F. (Eds). *Concepts* in *Nematode systematics,* London, Academic Press : 163-174.

HIBBEN, C. R., & WALKER, J. T. (1971). Nematode transmission of the ash strain of tobacco ringspot virus. *Pl. Dis. Reptr,* 55 : 475-478.

HYMAN, B., & POWERS, T. (1991). Integration of classical and molecular approaches in nematode taxonomy. *A. Rev. Phytopath.,* 29 : 89-107.

KLOS, E. J., FRONEK, K., KNIERIM, J. A., & CATION, D. (1967). Peach rosette mosaic transmission and control studies. *Quart. Bull. Michigan St. Univ. agric. Ext. Statn,* 49 : 287-293.

LAMBERTI, F., & BLEVE-ZACHEO, T. (1979). Studies on *Xiphinema americanum sensu lato* with description of 15 new species (Nematoda, Longidoridae). *Nematol. medit.,* 7 : 51-106.

LAMBERTI, F., & GOLDEN, A. M. (1986). On the identity of *Xiphinema americanum sensu lato* in the nematode collection of Gerald Thorne. *Nematologia medit,* 14 : 163-171.

LEVESQUE, C. A., BECKENBACH, K., BAILLIE, D. L., & RAHE, J. E. (1992). Pathogenicity and differentiation by DNA restriction fragment length polymorphisms of isolates of two *Pythium* spp. that colonize Glyphosate-treated plants. *Mycologia* (in press).

LIMA, M. B. (1965). *Studies on species of the genus* Xiphinema *and other nematodes.* PhD thesis, University of London, England. 165 p.

LUC, M., & SOUTHEY, J. F. (1980). Study of biometrical variability in *Xiphinema insigne* Loos, 1949 and *X. elongatum* Schuurmans Stekhoven & Teunissen, 1938, description of *X. savanicola* n. sp. (Nematoda : Longidoridae), and comments on thelytokous species. *Revue Nématol.,* 3 : 243-269.

MALIK, Z., & JAIRAJPURI, M. S. (1983). Statistical analysis of

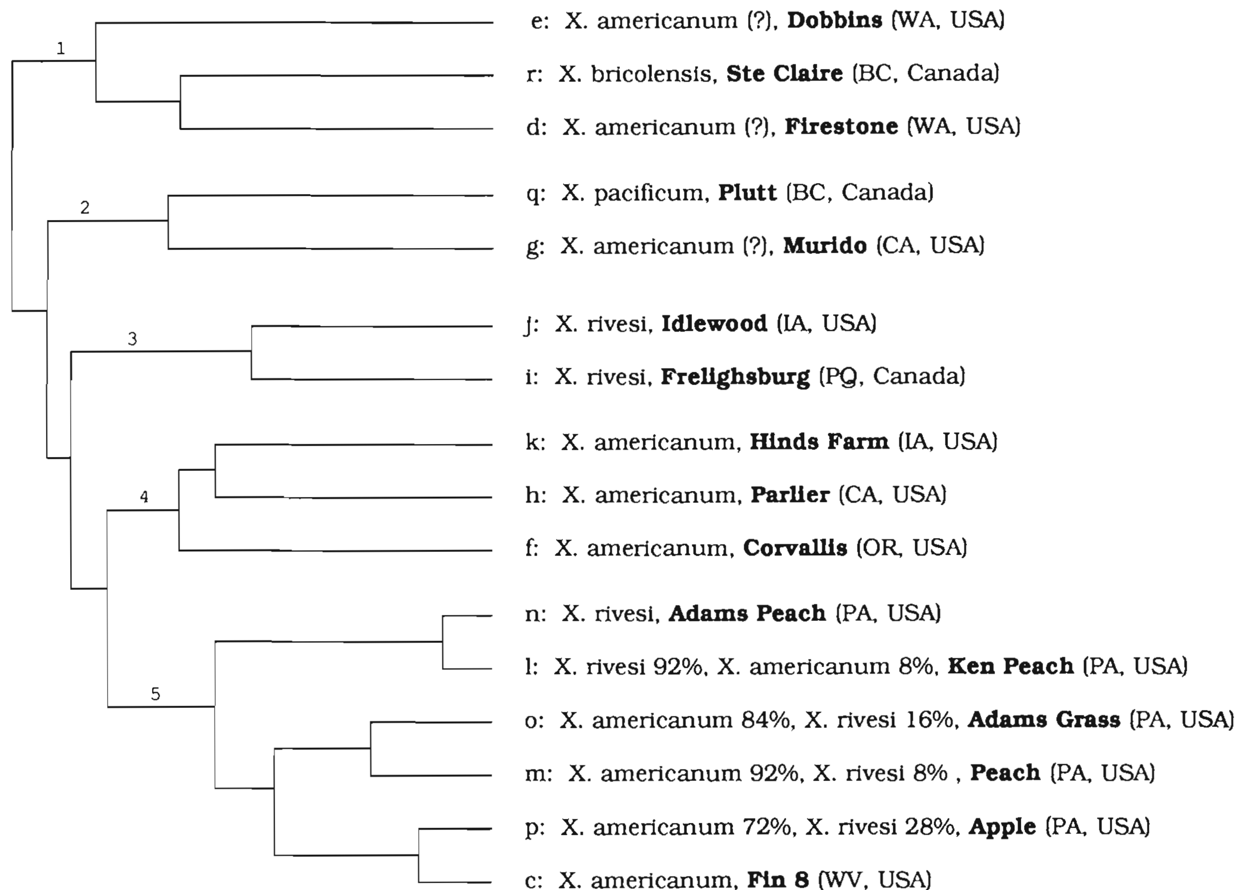

Fig. 9. Relationships of sixteen populations of the *Xiphinema americanum* group. Dendrogram constructed from dissimilarity coefficients, obtained from presence and position of restriction fragments of amplified Internal Transcribed Spacers region. (The diagnosis of populations Dobbins, Firestone, and Murido is probably inaccurate.) (low case letters : see Fig. 3).

variability in *Xiphinema americanum* Cobb, 1913 *sensu* Siddiqi, 1959. *Indian J. Nematol.,* 13 : 71-78.

McKenry, M. (1987). Letter to the editor. *Pl. Dis.,* 70 : 864.

Nyland, G. Lownsbery, B. F., Lowe, S. K., & Mitchell, J. F. (1969). The transmission of cherry rasp leaf virus by *Xiphinema americanum. Phytopathology,* 59 : 1111-1112.

Platzer, E. G. (1981). Potential use of protein patterns and DNA nucleotide sequences in nematode taxonomy. *In :* Zuckerman, B. E., & Rhode, R. E. (Eds.) *Plant parasitic nematodes,* Vol. III, London, New York, Academic Press : 3-21.

Powers, T. O., & Sandall, L. J. (1988). Estimation of genetic divergence in *Meloidogyne* mt DNA. *J. Nematol.,* 20 : 505-511.

Qu, L. H., Hardmann, N., Gill, L. Chappell, L., Nicoloso, M., & Bachellerie, J. P. (1986). Phylogeny of Helminths determined by rRNA sequence comparison. *Molec. bioch. Parasitol.,* 20 : 93-99.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning : a laboratory manual.* Cold Spring Harbor Laboratory Press, 1590 p.

Sas Institute Inc. (1985). *SAS User's guide : Statistics, Version 5.* Cary, NC, USA, 956 p.

Tarjan, A. C. (1969). Variation within the *Xiphinema americanum* group (Nematoda : Longidoridae). *Nematologica,* 15 : 241-252.

Williams, S. M., De Salle, R., & Strobeck, C. (1985). Homogenization of geographical variants at the nontranscribed spacer of rDNA in *Drosophila mercatorum. Molec. Biol. Evol.,* 2 : 338-346.

Wojtowicz, M. R., Golden, A. M., Forer, L. B., & Stouffer, R. F. (1982). Morphological comparisons between *Xiphinema rivesi* Dalmasso and *X. americanum* Cobb populations from the eastern United States. *J. Nematol.,* 14 : 511-516.