Journal of Nematology 25(3):361–364. 1993.

Restriction Fragment Length Polymorphism Separates Species of the *Xiphinema americanum* Group[1]

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Abstract: The *Xiphinema americanum* group of species is responsible for vectoring several important virus diseases to perennial crops. Variability of transmission of viruses by different species, and difficulties in separating species by morphometric measurements alone, make it essential to reassess the taxonomic position of several species in the group. The measurement of DNA sequence variability is a sensitive assay that can re-evaluate the separation of species and populations from each other. This study describes how an RFLP approach, in which the restriction sites in transcribed spacers of ribosomal repeats were detected, confirmed the separation of 16 populations of these species into *X. americanum, X. rivesi, X. pacificum,* and *X. bricolensis.*

Key words: DNA, nematode, systematics, taxonomy, *Xiphinema.*

As with most plant-parasitic nematodes, the taxonomy of the *Xiphinema americanum* group is based on morphometrics. However, as described in the other papers of this symposium (2,10,13,17), many characters used to separate the species often overlap, making diagnostics difficult and often merely tentative. Biochemical characters that have complemented morphological measurements in other nematodes (like root-knot species) have not been used in *Xiphinema,* probably because most biochemical analyses require relatively large quantities of material and because many *Xiphinema* species have been described from fixed material.

Techniques of molecular biology can help resolve some problems of identification of plant-parasitic nematodes (1,3,4,14, 15). Sequence variability occurs in most regions of the genome. When these changes eliminate or create new restriction sites in DNA, this variation, called restriction fragment length polymorphism (RFLP), can be measured by the number and size of fragments generated by digesting the DNA of different populations with the same restriction enzyme. Curran et al. (5) and others since (1,3,4,15) have used RFLPs to separate species and genera of plant-parasitic nematodes. Nucleotide substitutions within noncoding regions (i.e., introns or spacers) accumulate much more rapidly than in the coding regions because of the lack of consequences to the phenotype. This higher frequency of sequence differences facilitates distinguishing populations or strains that have separated recently. The polymerase chain reaction has been used to amplify genetic marker sequences with random primers (4) and to amplify specific mitochondrial or other sequences (1,15).

The ribosomal cistron of *Caenorhabditis elegans* has been cloned and sequenced (7). It consists of three functional genes coding for ribosomal RNA, separated by two internal transcribed spacer regions (ITS) and a nontranscribed spacer (NTS, Fig. 1). Comparative analysis of coding and noncoding regions of ribosomal DNA (rDNA) is becoming a popular tool for the construction of phylogenetic trees and for the determination of evolutionary relationships in nematodes. Variability in the rDNA sequence of species of the *X. americanum* group was measured using the polymerase chain reaction (PCR) to amplify the 5.8S gene and the ITS (18). Ferris et al. (8) recently studied variability of cyst-forming nematode populations, and Wendt et al. (unpubl.) analyzed RFLPs in species of *Ditylenchus* and races of *D. dipsaci,* using the same amplified rDNA fragment.

Our study of the *X. americanum* group (18) involved 16 field populations from North America, with initial experiments

Received for publication 23 January 1993.

[1] Symposium paper presented at the 31st Annual Meeting of the Society of Nematologists, 2–6 August 1992, Vancouver, British Columbia, Canada.

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Financial support from NATO Scientific in the form of a travel grant is acknowledged.

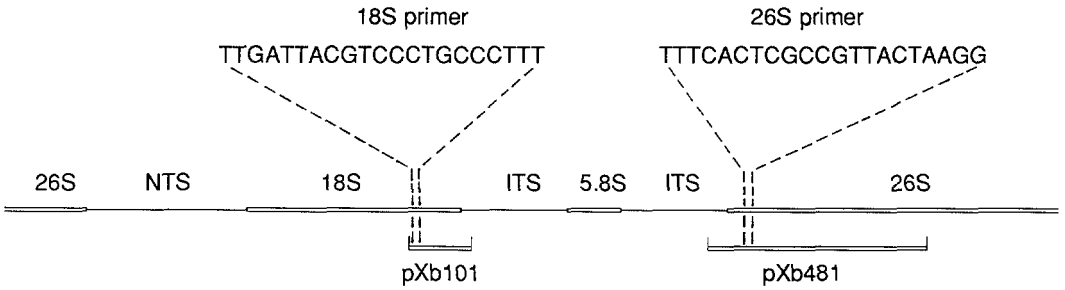

FIG. 1. Position of *Xiphinema bricolensis* clones pXb101 and pXb481 flanking the Internal Transcribed Spacer (by homology to the ribosomal cistron of *Caenorhabditis elegans*), with sequences and positions of 18S and 26S primers. NTS = nontranscribed spacer; ITS = internal transcribed spacer. Drawing courtesy of *Fundamental and Applied Nematology.*

focused on *X. bricolensis.* Total DNA extracted from approximately 2,000 *X. bricolensis* nematodes was cut with the restriction endonuclease Eco RI. The resultant DNA fragments were incorporated into a plasmid vector (pUC13), which was introduced into cells of *Eschericia coli,* thus transforming them.

The transformed *E. coli* cells containing a fragment of *X. bricolensis* rDNA were detected when their DNA hybridized with a probe made from the ribosomal cistron of *Caenorhabditis elegans.* The identified fragments of *X. bricolensis* rDNA were sequenced by the Sanger dideoxy-mediated chain termination method. The sequence of each fragment was compared to that of the ribosomal cistron of *C. elegans* (7). The position of the rDNA clones relative to the gene map of the *C. elegans* ribosomal repeating unit was ascertained by computer searches of sequence homology between the clones *X. bricolensis* fragments and ribosomal sequences from *C. elegans* and other organisms in the European Molecular Biology Laboratory (EMBL) databank.

Of the 600 clones (pXb) identified as containing *X. bricolensis* DNA, two (pXb101 and pXb486) were positioned respectively on the 18S gene and the 26S gene (Fig. 1). Both clones had a 21-nucleotide sequence perfectly conserved between *X. bricolensis, C. elegans,* and several other organisms in the EMBL database. Moreover, the position of these 21-nucleotide sequences was such that they could be used as primers to amplify the ITS region. These universal primers have since been used to amplify the ITS of many nematode species, fungi, and insects (Vrain, unpubl.). The DNA fragment containing the ITS region from each *Xiphinema* population was amplified with the polymerase chain reaction (PCR), digested with restriction enzymes, and separated by electrophoresis. Details of nucleic acid isolation, digestion, ligation, transformation, hybridization, sequencing, and polymerase chain reactions have been published (17,18).

For each of the 16 *Xiphinema* populations, the restriction bands produced with six restriction enzymes were scored as "0" (if absent) or "1" (if present). A matrix was then generated with nematode populations as rows and bands as columns. This matrix was then multiplied by its transposition to yield a new 16 × 16 matrix "M," with nematode populations as rows and columns. The numbers in the diagonal of the matrix M represented the total number of bands in a particular nematode population, and the other numbers represented the number of bands shared by each population pair. Dissimilarity coefficients for all pairwise comparisons were calculated using the matrix M and a Fortran subroutine. From these values, a cluster analysis (unweighted pair-group method with average) was performed and a tree was constructed (Program Tree, SAS). The patterns obtained with the restriction enzymes Alu I, Bam HI, Dde I, Hinf I, Mbo I, and Msp I grouped the 16 populations into five clusters (Fig. 2). Two

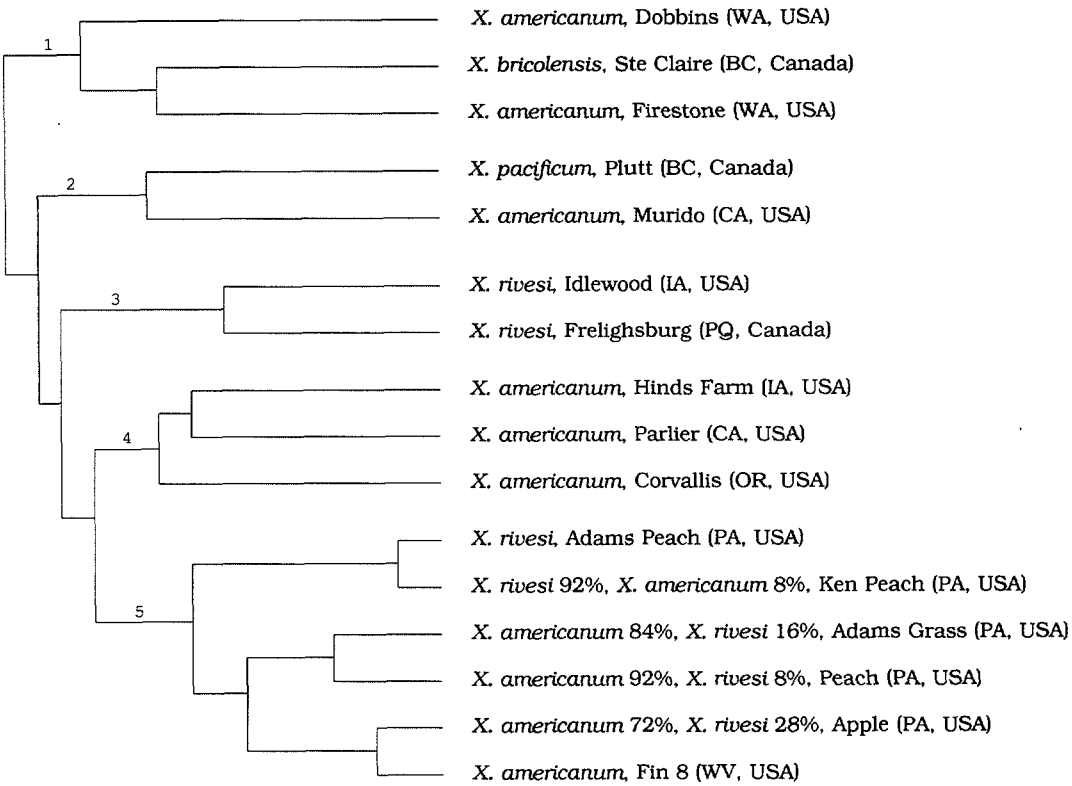

FIG. 2. Relationships of 16 populations of the *Xiphinema americanum* group. Dendrogram obtained from presence and length of restriction fragments of amplified internal transcribed spacer regions. "%" is the proportion of *X. rivesi* or *X. americanum* in mixed populations of cluster 5. Drawing courtesy of *Fundamental and Applied Nematology*.

X. americanum populations from Washington and the population of *X. bricolensis* from British Columbia were similar. An *X. americanum* population from California and the population of *X. pacificum* from British Columbia were in the same cluster. Two populations of *X. rivesi* from Iowa and Quebec showed no differences in restriction sites. The Parlier population from California described as *X. americanum* sensu lato (9) and an *X. americanum* population from Iowa and Oregon were clustered together, but not with the *X. americanum* populations from Washington or from California. The populations from Pennsylvania and West Virginia that had been identified as *X. americanum, X. rivesi,* or mixed populations of these were clustered together. This indicated that all these populations were mixed, and that even the so-called pure populations contained a small proportion of the other species. Thus, a very low frequency of *X. americanum* could be detected in a population of *X. rivesi,* and a low frequency of *X. rivesi* was detected in a population of *X. americanum.*

This molecular approach is capable of separating species within the *X. americanum* group. For example, rDNA analysis indicated that the *X. americanum* Parlier population was in a separate cluster from *X. rivesi.* The validity of *X. bricolensis* and *X. pacificum,* well separated from *X. americanum* or *X. rivesi,* was confirmed. *Xiphinema pacificum* was described as a new species from British Columbia (16) somewhat resembling *X. californicum;* RFLP showed that the type population of *X. pacificum* was clustered with Murido, a California population that is morphologically identical to *X. californicum* (D. J. F. Brown, unpubl.).

Because the Parlier population was thoroughly characterized, we concluded on the basis of this RFLP data that the three *X. americanum* populations from Washington and California clustered with *X. bricolensis* or *X. californicum* were previously misidentified.

The described technique measures variation between species otherwise difficult to separate by standard morphometrics. The distribution of restriction sites in DNA sequences represents an extremely sensitive tool to measure genetic variability between individual nematodes, populations, subspecies, or higher levels of taxa.

Despite renewed efforts to resolve taxonomic uncertainties in the increasing number of species in the *X. americanum* group, questions remain about specificity of virus transmission, validity of species description, and synonymy of species. The RFLP approach should allow for the correlation of intrapopulation heterogeneity and specificity of virus transmission. It will complement the morphological and biological measurements of populations examined during the international collaborative project on this group of nematodes and thereby help resolve the many questions alluded to in this symposium.

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