Fundam. appl. Nematol., 1992, **15** (1), 35-41.

DNA hybridization probes for studying the affinities of three *Meloidogyne* populations

Baoguo XUE, Dave L. BAILLIE, Karen BECKENBACH and John M. WEBSTER

Department of Biological Sciences, Simon Fraser University, Burnaby, Vancouver, B.C., Canada V5A 1S6.

Accepted for publication 19 January 1991.

Summary — A direct analysis of the genotype of *Meloidogyne* spp. was done using DNA restriction fragment length polymorphisms (RFLP's). Total genomic DNA was extracted from nematode eggs of *M. incognita* race 3, digested and cloned in pVZ1 plasmid. Randomly cloned DNA fragments were labelled with ^{32}P, and used to probe genomic DNA from populations of each of *M. incognita* race 3, *M. arenaria* race 1, and *M. javanica*, which had been digested and fractionated by agarose gel electrophoresis. Analysis of bands detected by the probes showed that *M. arenaria* and *M. javanica* are more closely related to each other (0.69 of shared fragments across all digestions) than they are to *M. incognita* (0.55 and 0.52 respectively). The sequence divergence between the three populations range from 2.1 to 3.8 %. The phylogenetic tree of the three *Meloidogyne* populations was generated from the hybridization data, and it was estimated that these three *Meloidogyne* species diverged from a common ancestor 2-4 million years ago.

Résumé — *Sondes hybrides d'ADN pour étudier les affinités entre populations de trois* Meloidogyne — Une analyse directe du génotype de *Meloidogyne* spp. a été réalisée en utilisant le polymorphisme en longueur de fragments d'ADN après restriction. L'ADN génomique total est extrait des œufs de *M. incognita* race 3, digéré puis cloné dans le plasmide pVZ1. Des fragments d'ADN clonés au hasard sont marqués au P^{32} et utilisés pour sonder l'ADN génomique de populations appartenant à *M. incognita* race 3, *M. arenaria* race 1 et *M. javanica*, cet ADN ayant été digéré et fractionné par électrophorèse sur gel d'agar. L'analyse des bandes mises en évidence par les sondes montre que *M. arenaria* et *M. javanica* sont plus proches l'un de l'autre (0,69 fragments communs au cours de l'ensemble des digestions) qu'ils ne le sont de *M. incognita* (0,55 et 0,52, respectivement). La divergence des séquences entre les trois populations s'étage de 2,1 à 3,8 %. Un arbre phylogénique a été établi pour les trois populations sur la base des données tirées de l'hybridation; il est estimé que ces trois espèces de *Meloidogyne* ont divergé à partir d'un ancêtre commun il y a 2 à 4 millions d'années.

Key-words : *Meloidogyne*, speciation, DNA hybridization.

The advances in recombinant DNA technology now permit the rapid and reliable characterization of the nematode genome and enable taxonomic identification and genetic affinities of species to be determined. These techniques have been applied already to some economically important plant-parasitic nematode genera, *Meloidogyne* (Curran *et al.*, 1985, 1986; Powers & Sandall, 1988), *Heterodera* (Besal *et al.*, 1987; Radice *et al.*, 1988) *Globodera* (Burrows & Perry, 1988) and *Bursaphelenchus* (Webster *et al.*, 1990).

Restriction fragment length polymorphisms (RFLP) show genetic differences among nematode populations. The DNA hybridization probes to nematode total genomic DNA provide a tool to reveal the RFLP's and enable an estimation of genetic divergence between the nematode populations. Previous studies have shown that it might be useful for identification of *Meloidogyne* species and for demonstrating their relatedness using ethidium bromide staining of whole genome (Curran *et al.*, 1986) or RFLP's of mitochondrial DNA (Powers & Sandall, 1988). Random-DNA probe hybridization has not been characterized and used previously for analyzing genomic DNA and the affinities of *Meloidogyne* populations. This study of randomly cloned DNA fragments was done to characterize the total genomic DNA from populations of three *Meloidogyne* species using DNA hybridization probes and to demonstrate phylogenetic affinities between these species.

Materials and methods

CULTURE AND EXTRACTION OF NEMATODES

Meloidogyne incognita race 3 (courtesy Dr. M. A. McClure) and *M. arenaria* race 1 and *M. javanica* (courtesy Dr. J. N. Sasser) were maintained in the greenhouse on potted tomato (*Lycopersicon esculentum* Mill) cv. Roma plants. The greenhouse populations of these *Meloidogyne* species were confirmed subsequently by Dr. B. A. Ebsary.

Eggs were extracted from *Meloidogyne* infected tomato roots by the modified sodium hypochlorite-sieve procedure (McClure *et al.*, 1973), washed through a 63 μm-pore sieve and concentrated by repeated washings on a 25 μm-pore sieve, resuspended in 0.1 M NaCl and 0.05 M K_2PO_4 (pH 6.0) buffer (Brenner, 1974) and stored in liquid nitrogen until needed.

DNA ISOLATION

One ml of eggs from each species were frozen in liquid nitrogen, ground by pestle and mortar and digested with 6 ml of 1.0 % proteinase K. The DNA was extracted by the phenol-chloroform method (Curran *et al.*, 1986) and stored in 0.01 M Tris pH 8.0 and 0.001 EDTA (1 X TE) buffer. RNA was digested by the addition of 100 µg/ml DNase-free RNase solution to a final concentration of 10 µg/ml and incubated at room temperature for 30 min. The DNA was precipitated by the addition of 8 M ammonium acetate to a final concentration of 2 M, and then 2 volumes of 95 % ethanol were added (Maniatis *et al.*, 1982). The precipitate was washed twice with 70 % ethanol, dried and resuspended in 1 X TE buffer and stored until required.

PROBE ISOLATION AND LABELLING

Random fragments for use as probes were isolated in the following manner. One µg of the DNA of *M. incognita* race 3 was digested with Xba I (Pharmacia) and ligated into 0.2 µg of Xba I cut plasmid pVZ1 DNA. The pVZ1 is a derivative of " blue-scribe (+) " (Vector Cloning Systems) that contains an extended polylinker segment inserted into the Eco RI site (Henikoff & Eghtedarzadeh, 1987), supplied by Dr. Steven Henikoff, Fred Hutchinson Cancer Research Center, Seattle, Washington. The ligated plasmids were transformed into competent *E. coli* JM83, and selected on NZY plates containing 40 µg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside, 160 µg/ml isopropyl-D-thiogalactopyranoside and 100 µg/ml ampicillin. The bacteria containing plasmids with nematode DNA fragments, were isolated and plasmid DNA was extracted by the alkaline lysis mini-preparation method (Maniatis *et al.*, 1982). A total of 94 plasmids containing *Meloidogyne* DNA fragments were cut with Xba I, separated by gel electrophoresis and the size of each insert determined (Fig. 1). Of the 94 plasmids with inserts, 32 were chosen randomly to use as probes. These plasmids, each 0.3-0.5 µg DNA, were radiolabelled with (α-^{32}P) dATP (Amersham RadioChemicals, 800 Ci/mmole) by nick-translation (Rigby *et al.*, 1977). These labelled probes (6×10^5 cpm per ml hybridization solution) were boiled for 15 min, put on ice for 5 min and then added to the prehybridized filter for hybridization.

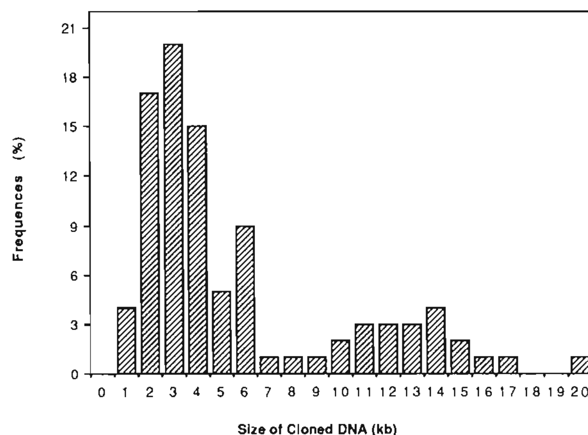

Fig. 1. The distribution of cloned DNA fragments from *Meloidogyne incognita* race # 3. The DNA of *M. incognita* race 3 was digested with Xba I and ligated into XbA I cut plasmid pVZ 1 DNA. The ligated plasmids were transformed into competent *E. coli* JM 83, and selected on IPTG-Ampicillin plates. The bacteria containing plasmids with nematode DNA fragments, were screened and plasmid DNA was extracted by the alkaline lysis mini-preparation. The 94 plasmids containing *Meloidogyne* DNA fragments were cut with Xba I, separated by gel electrophoresis and the size of each insert determined. The Figure shows the distribution of different size of inserts. The inserts range from 0.33 to 19.54 kb in size and most of the fragment are between 1.5 to 6.0 kb (76 %).

SOUTHERN BLOT AND HYBRIDIZATION

Total DNA, including genomic DNA and mitochondrial DNA, was digested using restriction endonuclease Eco RI according to the manufacturer's (Pharmacia) recommendations. Digested DNA was fractionated in 0.7 % agarose gels at 0.75 V/cm until the bromophenol blue dye front had moved 18 cm. The DNA on the gel was nicked in 0.25 N HCl for 15 min, denatured in 0.5 N NaOH/1.5 M NaCl solution for 30 min, neutralized in 1 M NaOAc/0.02 M NaOH for 60 min and transferred to 0.45 µm pore size BioTrace NT nitrocellulose filter (Gelman Sciences Inc.) by the bidirectional-transfer method of Smith and Summers (1980). The filter was baked under vacuum for 2.5 h at 80 °C.

The nitrocellulose filters were hybridized with the labelled and denatured probe in a solution containing 5 X SSPE (1 X SSPE is 180 mM NaCl, 10 mM $NaH_2PO_4.H_2O$ and 1 mM EDTA (pH 7.4)), 0.3 % SDS (sodium dodecyl sulfate) and 5 X Denhardts solution (1 X Denhardts is 0.02 % Ficoll, 0.02 % BSA and 0.02 % polyvinylpyrollidone) (Davis *et al.*, 1980). The filters were incubated at 68 °C for 24 h and washed four times in 0.2 X SSPE, 0.2 % SDS at 65 °C for 1 h. The above high stringency hybridization conditions were chosen to minimize false hybridization. The washed filters were air dried, and autoradiographed at — 70 °C with Kodak X-Omat ARP-K x-ray film with a pair of Cronex intensifying screens.

Results

The cloned Xba I DNA fragments of *M. incognita* race 3 ranged from 0.33 to 19.54 kb in size and most of the 94 cloned fragments were between 1.5 to 6.5 kb (Fig. 1). The cloned DNA fragments used as probes for hybridization are shown in Table 1.

Table 1. DNA probes used for hybridization with total genomic DNA of the three *Meloidogyne* species, and their size in kilobases (kb).

Probe Name	Size in Kb	Probe Name	Size in Kb
pMi 1	2.40	pMi 17	4.30
pMi 2	1.20	pMi 18	1.91
pMi 3	0.50	pMi 19	2.50
pMi 4	1.43	pMi 20	15.00
pMi 5	1.70	pMi 21	2.35
pMi 6	3.41	pMi 22	12.00
pMi 7	2.00	pMi 23	2.14
pMi 8	8.82	pMi 24	5.55
pMi 9	6.50	pMi 25	1.50
pMi 10	5.10	pMi 26	1.35
pMi 11	14.50	pMi 27	2.20
pMi 12	1.59	pMi 28	2.65
pMi 13	13.50	pMi 29	13.00
pMi 14	3.61	pMi 30	2.70
pMi 15	3.10	pMi 31	1.15
pMi 16	9.35	pMi 32	1.55

DIFFERENTIATION BETWEEN THREE *MELOIDOGYNE* SPECIES

Most of the cloned *M. incognita* DNA fragments produce distinct hybridization bands with all three *Meloidogyne* species. The pMi 9 probe, for example, reveals several restriction fragment length polymorphisms (Fig. 2). For example, *M. javanica* and *M. arenaria* each have a band at about 15 kb, which is not present in *M. incognita* but there is one at 4.9 kb and another at about 4.2 kb. In addition, *M. arenaria* has a unique band at about 3.3 kb, and one at 2.7 kb that occurs in *M. arenaria* and also in *M. incognita*, but weakly. *M. javanica* has a unique band at 3.5 kb.

Autoradiographs of Eco RI-digested total DNA probed with ^{32}P labelled DNA from plasmid pMi 10 (Fig. 3 A) and pMi 3 (Fig. 3 B) show discrete species-specific bands. Plasmid pMi 10 contains a 5.1 kb Xba I fragment. At the stringency used, pMi 10 detects an Eco RI fragment in *M. incognita* of 4.5 kb and *M. arenaria* of 4.9 kb, and hybridizes very weakly in *M. javanica*. Probe pMi 3 has an Xba I insert of 0.5 kb. Under similar hybridization conditions, the probe detected one Eco RI band of 14.0 kb in this *M. incognita* population, the species from which the probe had been isolated, and only weak bands in *M. arenaria* and *M. javanica* populations.

The distribution of hybridized restriction fragments for populations of three *Meloidogyne* species using the 32 probes is shown in Fig. 4. DNA fragments isolated from *M. incognita* hybridized with itself gave a different number of bands and hybridized partially with each of the other two species. Among these randomly selected 32 pMi probes, there were 30 probes which hybridized

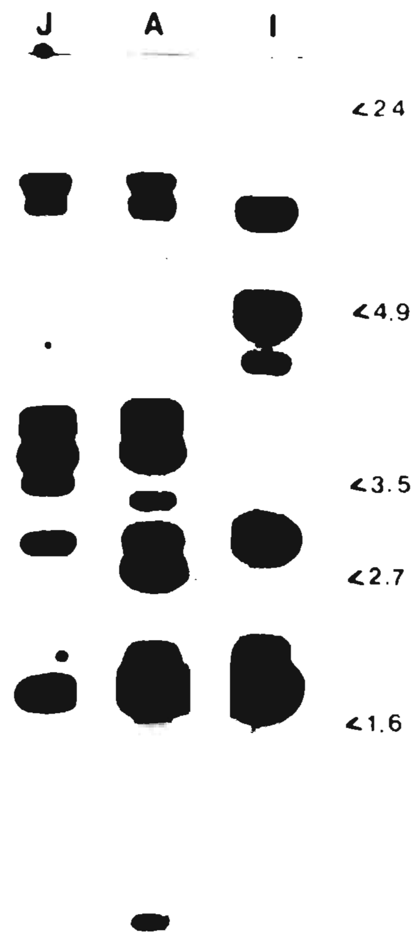

Fig. 2. Use of pMi 9 as a diagnostic probe to differentiate *Meloidogyne incognita, M. arenaria* and *M. javanica*. The total genomic DNA from each of the nematode species was digested with Eco RI, fractionated in 0.7 % agarose gel and transferred to a nitrocellulose membrane. Hybridization conditions were 5 X SSPE, 0.3 % SDS and 5 X Denhardts at 68 °C and washed in 0.2 X SSPE, 0.2 % SDS at 65 °C. Legend : I, *M. incognita* race 3; A, *M. arenaria* race 1; J, *M. javanica*.

to more than one band in all three species of *Meloidogyne* tested. The ranges of molecular size of hybridized bands are from 0.16 to 24.0 kb. The most notable differences are the presence of molecular size around 1 to 8 kb, which is the maximum distribution region of hybridization bands when the genomic DNA is cut with Eco RI (Fig. 5).

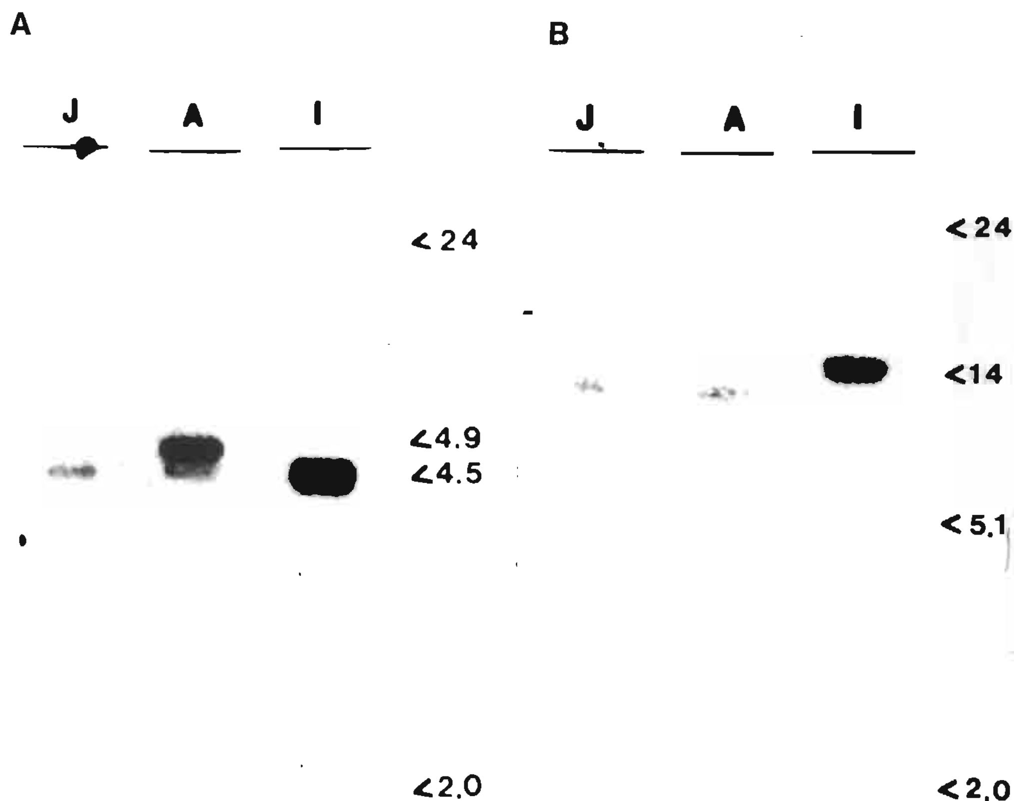

Fig. 3. Identification of *Meloidogyne* species by detection of restriction fragment length polymorphisms using randomly selected Xba I fragments of *M. incognita* as probes. The autoradiograph shows Southern blots of Eco RI digested total genomic DNA hybridized with ^{32}P labelled DNA probes, (A) with pMi 10 probe; (B) with pMi 3 probe. The probe pMi 10 can detect *M. incognita* and *M. arenaria* and probe pMi 3 detects only *M. incognita* under hybridization conditions of 5 X SSPE, 0.3 % SDS and 5 X Denhardts at 68 °C and washing in 0.2 X SSPE, 0.2 % SDS at 65 °C. Legend : I, *M. incognita* race 3; A, *M. arenaria* race 1; J, *M. javanica*.

Genomic affinities between *Meloidogyne* species

Genomic differences between tested *Meloidogyne* species were analyzed by hybridization of cloned probe DNA to total genomic DNA digested with Eco RI. The hybridization pattern, as shown by the bands hybridizing with ^{32}P labelled probes, was used to determine the relationship between *Meloidogyne* species.

The proportion of shared fragments (F) across all digestions of *M. incognita*, *M. arenaria* and *M. javanica* was calculated using the formula $F = 2 N_{xy}/(N_x + N_y)$ (Ney & Li, 1979; Nei, 1987); where N_x = number of restriction fragments in species X, N_y = number of restriction fragments in species Y and N_{xy} = number of restriction fragments shared by species X and Y. The proportion of shared fragments *(F)* between *M. arenaria* and *M. javanica* is 0.69 across all probes tested, between *M. arenaria* and *M. incognita* it is 0.55 and between *M. incognita* and *M. javanica* it is 0.52.

Based on differences between shared restriction fragments, the nucleotide substitution rate between species was calculated by the formula $d = -(2/r) \ln G$ (Nei, 1987); where d = the number of nucleotide substitutions per site, r = the number of base pairs recognized per cleavage site and G is a maximum likelihood estimate of the probability that a restriction site remains unsubstituted between species, derived from the formula, $F = G^4/(3 - 2G)$. The results show that the rate

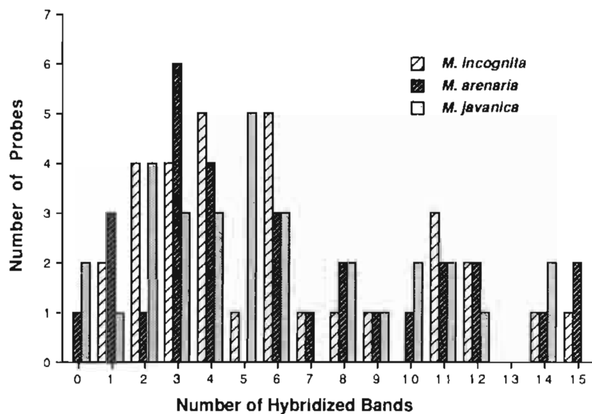

Fig. 4. Distribution of hybridized restriction fragments for three *Meloidogyne* species with 32 probes. Cross hybridization among species indicates that these species *M. incognita, M. arenaria* and *M. javanica* are quite closely related and that they diverged in relatively recent times. DNA fragments isolated from one species of *Meloidogyne* hybridized partially with each of the other two species (31/32 *M. arenaria* and 30/32 *M. javanica*) and gave a different number and size of bands.

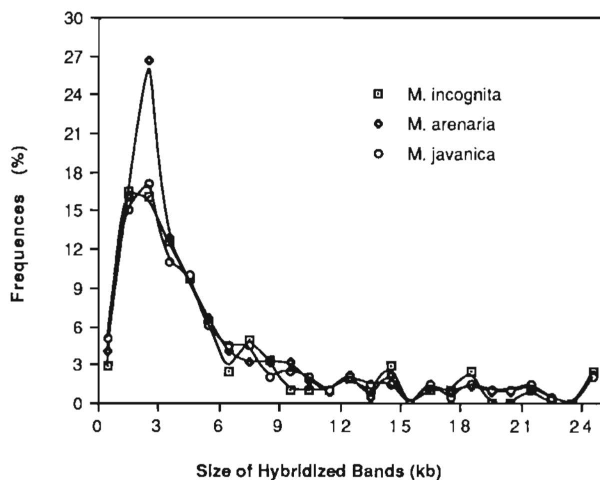

Fig. 5. The size distribution of hybridized restriction fragments for three *Meloidogyne* species with 32 probes. Total genomic DNA was digested using restriction endonuclease Eco RI and fractionated in 0.7 % agarose gels. The DNA on the gel was denatured, transferred to 0.45 μm pore size BioTrace NT nitrocellulose filter and hybridized with 32 different probes under the hybridization conditions described in the text. The ranges of molecular size of hybridized bands are from 0.16 to 24.0 kilobases in the three *Meloidogyne* species. The maximum distribution region of hybridization bands are around 1 to 8 kilobases.

of base substitutions between *M. arenaria* and *M. javanica* is 2.12 ± 0.35, between *M. arenaria* and *M. incognita* it is 3.49 ± 0.42 and between *M. incognita* and *M. javanica* it is 3.77 ± 0.45.

The estimates of nucleotide sequence divergence for all pairwise sample comparisons were analysized by the procedures for estimating phylogenetic trees. In order to provide a pictorial summary of the distance matrix, and to permit contrast with the qualitative character state analysis, the phylogenetic tree was calculated and drawn (Fig. 6) according to the unweighted pair-group with arithmetic means discussed in Nei (1987).

Discussion and conclusion

Hybridization of random *Meloidogyne incognita* probes to total genomic DNA of the tested species differentiates between the populations of three *Meloidogyne* species and helps identify their relationships. Of the 94 randomly cloned DNA fragments, the molecular size ranged from 0.33 to 19.54 kb and most (76 %) of fragments were between 1.5 to 6.5 kb. This might reflect the fact that *Meloidogyne* genome could be cut every 1.5-6.5 kb with the 6-base restriction enzyme Xba I (Fig. 1). When the genome was cut by 6-base restriction enzyme Eco RI and hybridized with these probes, it showed a similar result in that the distribution of most hybridization bands is about 1 to 8 kb (Fig. 5). This size range is the expected result, given a 6 base cutter should be found every 4^6 on average. Approximately 69 % of the fragments cloned by this method revealed taxonomically useful differences in the number of restriction fragments. Nevertheless, our results show that species within this genus can be distinguished by using a limited set of probes.

Genetic differences and species relationships between *Meloidogyne* species were demonstrated first using recombinant DNA techniques (Curran *et al.*, 1985, 1986) using ethidium bromide staining of restriction endonuclease digested genomic DNA. The difficulties of visualizing these repetitive bands in a total genomic digest on an agarose gel can be overcome by using probes as shown by Curran and Webster (1987). In our data, DNA bands detected by probe pMi 9 had a restriction fragment length unique to each *Meloidogyne* population. The pMi 10 probe hybridized to DNA from the *M. incognita* and *M. arenaria* populations but just weakly to *M. javanica* populations at the high stringency used in our experiment. Hence, under these test conditions pMi 10 appears as a specific probe for *M. incognita* and *M. arenaria.* Using the same hybridization conditions, probe pMi 3 detected *M. incognita* and, just weakly, the other two species. Therefore, these probes are potentially useful for diagnostic purposes once tested against a large number of populations of these three species. It is estimated that 3-6 % of these probes could be used for this kind of species differentiation.

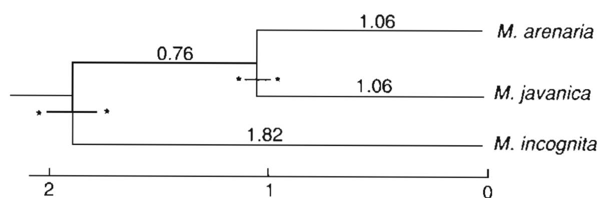

Fig. 6. The phylogenetic tree showing the relationship between three *Meloidogyne* species was reconstructed by the average distance method (PUMA) from the estimates of the percentage of nucleotide base substitutions obtained from DNA hybridization data. The starred bar (*---*) represents one standard error on each side of the branching point. The axis represents the percentage of nucleotide divergence in base substitutions.

The hybridization pattern and signal strength of randomly-cloned fragments showed that most are multiple copy in all three species. For example, the multiple banding of probe pMi 9 could be of the type associated with repetitive DNA, such as transposable elements or multiple gene families. The repetitive region of pMi 9 varies between species and is a potentially useful probe for genomic analysis. On other hand, the pMi 10 and pMi 3 indicate DNA sequences with one or few copies per genome.

The inherent differences between species, often reflected in their host specificity and geographical distribution, can be identified by using random probes. Analysis of restriction endonuclease generated DNA fragments indicates that between the *Meloidogyne* species there is considerable difference in genotype. These results together with biochemical and morphological data, can be used to confirm species identification and also to demonstrate taxonomic relationships.

Our results support those of Triantaphyllou (1985) that these three *Meloidogyne* species are polyploid. Among 32 randomly selected pMi probes there were 30 probes which hybridized to three species of *Meloidogyne* and showed more than one band. Under the hybridization conditions described here, hybridization did not occur if the sequences had diverged by more than about 10 % from that of the probe sequence. Compared with other nematodes, such as *C. elegans* in which most of the cloned fragments usually show one band (Rose *et al.*, 1982), *Meloidogyne* species must be polyploid rather than diploid, and is probably tetraploid. Analysis of a known single copy gene, the sodium-proton antiponter (C_2), shows only one band in *C. elegans* and *C. briggsae* (Prasad, 1988) but shows four bands with each of the three *Meloidogyne* species (unpubl. data). This supports the hypothesis that these *Meloidogyne* species are polyploid.

The relative genetic similarities between various pairs of organisms is proportional to the number of shared fragments resolved from restriction endonuclease digests. The greater the similarity of the species that are being compared, the closer the DNA sequences are and the more hybridization bands they have in common. Therefore, it is possible to estimate the number of nucleotide substitutions from the group of DNA fragments that are common to the species that are being examined. Although the formula in the text is usually used to analyze nucleotide diversity of mitochondrial DNA, under some assumptions (Nei, 1987), the formula can be used to analyze genomic DNA. Analysis of these restriction fragments shows that *M. arenaria* is closer to *M. javanica* than it is to *M. incognita*, based on shared endonuclease digested fragments. This supports the enzymatic data of Esbenshade and Triantaphyllou (1987) and contrasts with the result of Powers and Sandall (1988) which showed, using the mitochondrial DNA, that *M. arenaria* is the most genetically distinct of the four common *Meloidogyne* species that they tested. This may be explained by the different evolution of mitochondrial DNA and genomic DNA. Our data would place *M. incognita* as the most genetically distinct of the three species looked at here.

Comparison of the random probe data of *Meloidogyne* species indicates that *M. incognita*, *M. arenaria* and *M. javanica* diverged recently, and that they are quite closely related to each other. The estimates of nucleotide sequence divergence calculated from hybridization data and phylogenetic tree showed that *M. arenaria* is closer to *M. javanica* than to *M. incognita* ($P = 0.001$). The sequence divergence between *M. arenaria* and *M. javanica* is 2.12, between *M. arenaria* and *M. incognita* 3.49 and between *M. incognita* and *M. javanica* 3.77. Using mtDNA restriction enzyme analysis, Radice and Powers (1988) estimated that *Heterodera glycines* and *H. schachtii*, two cyst nematode sibling species, diverged 7.3-14.5 million years ago. Even though there is a considerable controversy in the application of molecular rates of evolution to estimating the dates of species divergence, our results taken together with those from other organisms (Beverley & Wilson, 1984; Ochman & Wilson, 1987), suggest that these three *Meloidogyne* species diverged from a common ancestor about 2-4 million years ago. This is remarkably recent in evolutionary terms, and reflects rapid, recent and possibly current speciation in this multi-species genus.

Acknowledgments

The authors gratefully acknowledge the help of Dr. M. A. McClure (University of Arizona, Tucson) and Dr. J. N. Sasser (North Carolina State University, Raleigh) for supplying nematode material, Dr. B. A. Ebsary (Agriculture Canada, Ottawa) for confirming population identity, Dr. T. C. Vrain (Agriculture Canada, Vancouver) for critical review of the manuscript and the Natural Sciences and Engineering Research Council of Canada for financial support (grant No. A 4679).

References

BESAL, E. A., POWERS, T. O. & SANDALL, L. J. (1987). A DNA hybridization probe for the detection of soybean cyst nematode. *J. Nematol.*, 19 : 513.

BEVERLEY, S. M. & WILSON, A. C. (1984). Molecular evolution in *Drosophila* and the higher diptera. II. A time scale for fly evolution. *J. mol. Evol.*, 21 : 1-13.

BRENNER, S. (1974). The genetics of *Caenorhabditis elegans. Genetics*, 77 : 71-94.

BURROWS, P. R. & PERRY, R. N. (1988). Two cloned DNA fragments which differentiate *Globodera pallida* from *G. rostochiensis. Revue Nématol.*, 11 : 441-445.

CURRAN, J., BAILLIE, D. L. & WEBSTER, J. M. (1985). Use of genomic DNA restriction fragment length differences to identify nematode species. *Parasitology*, 90 : 137-144.

CURRAN, J., MCCLURE, M. A. & WEBSTER, J. M. (1986). Genotypic differentiation of *Meloidogyne* populations by detection of restriction fragment length difference in total DNA. *J. Nematol.*, 18 : 83-86.

CURRAN, J. & WEBSTER, J. M. (1987). Identification of nematodes using restriction fragment length differences and species-specific DNA probes. *Can. J. Pl. Path.*, 9 : 162-166.

DAVIS, R. W., BOTSTEIN, D. & ROTH, J. R. (1980). *Advanced bacterial genetics : A manual for genetic engineering.* New York. Cold Spring Harbor Laboratory, 254 p.

ESBENSHADE, P. R. & TRIANTAPHYLLOU, A. C. (1987). Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda : Tylenchida). *J. Nematol.*, 19 : 8-18.

HENIKOFF, S. & EGHTEDARZADEH, M. K. (1987). Conserved arrangement of nested genes at the *Drosophila* Gart locus. *Genetics*, 117 : 711-725.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular cloning : A laboratory manual.* New York. Cold Spring Harbor Laboratory, 545 p.

MCCLURE, A. A., KRUK, T. H. & MISAGHI, I. (1973). A method for obtaining quantities of clean *Meloidogyne* eggs. *J. Nematol.*, 5 : 230.

NEI, M. (1987). *Molecular Evolutionary Genetics.* New York, Columbia University Press. 512 p.

NEI, M. & LI, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. natn. Acad. Sci, USA,* 76 : 5269-5273.

OCHMAN, H. & WILSON, A. C. (1987). Evolution in bacteria : Evidence for a universal substitution rate in cellular genomes. *J. mol. Evol.*, 26 : 74-86.

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

PRASAD, S. S. (1988). *Molecular analysis within the Dpy-20 to Unc-22 region of chromosome IV in* Caenorhabditis elegans. Ph. D. thesis, Simon Fraser University, 105 p.

RADICE, A. D., POWERS, T. O., SANDALL, L. J. & RIGGS, R. D. (1988). Comparisons of mitochondrial DNA from the sibling species *Heterodera glycines* and *H. schachtii. J. Nematol.*, 20 : 443-450.

RIGBY, P. W., DIECKMANN, M., RHODES, D. & BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. *J. mol. Biol.*, 113 : 237-251.

ROSE, A. M., BAILLIE, D. L., CANDIDO, E. P. M., BECKENBACH, K. A. & NELSON, D. (1982). The linkage mapping of cloned restriction fragment length differences in *Caenorhabditis elegans. Mol. gen. Genetics,* 188 : 286-291.

SMITH, G. E. & SUMMER, M. D. (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzylomethyl-paper. *Analyt. Biochem.*, 109: 123-129.

TRIANTAPHYLLOU, A. C. (1985). Cytogenetics, cytotaxonomy and phylogeny of root-knot nematodes. *In :* Sasser, J. N. & Carter, C. C. (Eds). *An advanced treatise on* Meloidogyne, *Vol. I* North Carolina State University Graphics : 113-126.

WEBSTER, J. M., ANDERSON, R. V., BAILLIE, D. L., BECHENBACH, K., CURRAN, J. & RUTHERFORD, T. A. (1990). DNA probes for differentiating isolates of the pinewood nematode species complex. *Revue Nématol.*, 13 : 255-263.