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

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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 génome de *Meloidogyne* spp. a été réalisée en utilisant le polymorphisme en longueur de fragments d'ADN après restriction. L'ADN génomique total est extraite 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 sondier 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 M 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 and ligated into Xba I cut plasmid pVZ1 DNA. The DNA of M. incognita race 3 was digested with 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 NaH₂PO₄·H₂O and 1 mM EDTA (pH 7.4)), 0.3% SDS (sodium dodecyl sulfate) and 5 X Denhardt's solution (1 X Denhardt's is 0.02% Ficoll, 0.02% BSA and 0.02% polyvinylpyrrolidone) (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.
DNA hybridization for studying Meloidogyne populations

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

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Size in Kb</th>
<th>Probe Name</th>
<th>Size in Kb</th>
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<tbody>
<tr>
<td>pMi 1</td>
<td>2.40</td>
<td>pMi 17</td>
<td>4.30</td>
</tr>
<tr>
<td>pMi 2</td>
<td>1.20</td>
<td>pMi 18</td>
<td>1.91</td>
</tr>
<tr>
<td>pMi 3</td>
<td>0.50</td>
<td>pMi 19</td>
<td>2.50</td>
</tr>
<tr>
<td>pMi 4</td>
<td>1.43</td>
<td>pMi 20</td>
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</tr>
<tr>
<td>pMi 5</td>
<td>1.70</td>
<td>pMi 21</td>
<td>2.35</td>
</tr>
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<td>pMi 6</td>
<td>3.41</td>
<td>pMi 22</td>
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<td>pMi 7</td>
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<td>6.50</td>
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<td>5.10</td>
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<td>14.50</td>
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<td>pMi 16</td>
<td>9.35</td>
<td>pMi 32</td>
<td>1.59</td>
</tr>
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</table>

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 32P 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 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 $F = 2 N_x/(N_x + N_y)$ (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, $F = G/(3 - 2 G)$. The results show that the rate
of base substitutions between M. arenaria and M. javanica is $2.12 \pm 0.35$, between M. arenaria and M. incognita it is $3.49 \pm 0.42$ and between M. incognita and M. javanica it is $3.77 \pm 0.45$.

The estimates of nucleotide sequence divergence for all pairwise sample comparisons were analyzed 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 $4^k$ 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.
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 antiprot (C2), 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 Heteroder a 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.

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