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**GENETIC AFFINITIES AND IDENTIFICATION OF
SOME MELOIDOGYNE SPP. (NEMATODA)**

by

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B.Sc., Henan Agricultural University, 1981

M.Sc., Agri. University of Central China, 1984

**THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

in the Department
of
Biological Sciences

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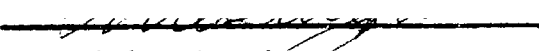
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ABSTRACT

Control of root-knot nematodes, *Meloidogyne* spp., in a sustainable agricultural system requires knowledge of their taxonomy to the infraspecific level and of their biology so that resistant hosts and crop rotation can be integrated into a pest management programme. This study applies the new DNA technologies to determining the identity and genetic affinities of infraspecific forms of *Meloidogyne* species.

Meloidogyne spp. were reared on tomato in controlled environments in tissue culture and in pots in a greenhouse. The production and maintenance of *M. incognita* in excised tomato roots and the influence of root factors on the development of *M. incognita* were examined. There was no apparent change in the ability to produce galls in fresh excised roots after for up to 23 generations on excised root culture. *M. incognita* developed well on excised roots on modified STW medium, and the optimum time for subculture of the nematodes was 50 days after initial inoculation. The eggs of *M. incognita* retained their viability for more than three months when they were stored at 10°C as measured by the ability of the hatched larvae to form galls on excised roots.

DNA hybridization patterns using random probes revealed that *M. arenaria* is phylogenetically closer to *M. javanica* than it is to *M. incognita*. The shared fragments between these three *Meloidogyne* species were from 52 - 69%. Sequence comparison of 618bp nucleotides of homologous 18S-like rDNA from six populations of *M. incognita* showed that races 1 and 2 were not significantly different, but races 3 and 4 were different from each other and from races 1 and 2.

Meloidogyne identification is traditionally based on a phenotypic approach. To overcome some of the problems in identifying agriculturally important *Meloidogyne* species, DNA hybridization, PCR amplification and DNA sequencing were utilized. Ethidium bromide staining of digested genomic DNA showed diagnostic characters for species differentiation. Visualization was improved by using DNA hybridization probes, such as pMi9, pMi10 and pMi3. Amplified fragment length polymorphism based on the PCR method showed that *Meloidogyne* populations had extensive genome heterogeneity and this sequence diversity enabled detection of species-specific and race-specific bands that could be used for nematode identification. Both DNA hybridization using probes and PCR amplification using primers were useful techniques to differentiate some *Meloidogyne* populations.

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INTRODUCTION

This thesis is a study of the application of molecular genetics techniques to the identification and evolutionary affinities of the nematode *Meloidogyne*. In this introduction I shall address three aspects of this study: the reasons for studying the nematodes; the reasons for studying their taxonomic relationships; and recent developments in molecular genetics of the nematodes.

1. Importance of *Meloidogyne* spp.

Since the root-knot nematode, *Meloidogyne* Goldi, 1887, were first recognized by Cornu, 1879, more than 54 species and two subspecies have been described (Chitwood 1949; Hirschmann 1985). Representatives of the genus *Meloidogyne* are among the most destructive of the plant-parasitic nematodes. *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica* represent 95% of the root-knot nematodes observed in agronomic soils. These nematodes induce conspicuous root galls, cause growth reduction, and even parasitize stems or foliage (Lehman 1985; Slack 1963; Webster 1972). Root-knot diseases seriously damage a wide range of plant species and decrease the yield of many important crops, such as peanuts, cotton, tobacco, cereals, fibre crops, vegetables and trees (Chitwood and Chitwood 1974; Webster 1972). The economic consequences of nematode infection are staggering; the reduction of crop yield or quality that is directly

attributable to the nematodes has been estimated at 10-15% (Palm 1981; Webster 1980). Assessment of world-wide crop loss to these species alone is estimated to be 5% (Carter and Sasser 1985). In some cases in Canada, the yield loss of carrots was estimated to be up to 40% by an infestation of *M. hapla* (Diamond et al. 1991). Association between *Meloidogyne* and other pathogens can result in increased incidence and severity of fungal disease and subsequent loss of crops. *Meloidogyne* spp. increase damage by important fungal pathogens e.g., *Fusarium*, *Verticillium*, *Rhizoctonia*, *Pythium* and *Phytophthora* (Webster 1985). In addition, the presence of the nematodes increases the harm inflicted by bacteria, such as *Pseudomonas* on tobacco and tomato, *Corynebacterium* on tobacco and *Agrobacterium* on raspberry (Taylor 1979). The nematodes puncture the plant roots and the subsequent infection changes the normal microflora of the rhizosphere and may induce some of the saprophytes to become harmful to plants. In the tropics and in developing countries in particular, root-knot nematodes are often the most important limiting factor in agricultural production.

Many methods have been tried to control these nematodes. Chemical control is now unpopular because current nematicides are generally expensive, highly toxic, and may result in persistent residues. Crop rotation with non-host crops or the use of resistant cultivars is a more acceptable alternative but can be more effective with exact identification of the species or races present. Information

on host races or pathotypes is necessary for selection of cultivars and crop rotation programmes. Quarantine regulations are extremely important in preventing the spread or introduction of *Meloidogyne* spp. into new areas. However, unreliable identification often leads to confusion between some particularly damaging species and other morphologically similar relatives. Clearly, in order to successfully control and manage these economically important nematodes and achieve optimum quality and high economic agricultural production, it is fundamental and essential to understand and differentiate the nematode species and races unambiguously, reliably and rapidly.

2. Specific and infraspecific identification of *Meloidogyne* spp.

During the past decade, great technological developments have occurred in nematode biology and taxonomy that have led to significant and revolutionary advances. Identification and differentiation of specific and subspecific *Meloidogyne* populations is far from simple, although great efforts have been put into studying them. Different techniques have been used for nematode identification and diagnosis. Traditional identification of *Meloidogyne* is based on phenotypic traits, the anatomical and morphological criteria of biometric taxonomy. These include perineal patterns, stylet structure, body length of adults and juveniles, and the preference for defined plant

hosts (Chitwood 1949; Taylor 1987; Taylor and Sasser 1983; Whitehead 1968). However, all these characters and criteria have certain limitations and the key descriptions often vary considerably between species and among races. For example, in the perineal pattern description for *Meloidogyne* species, the morphological variability in natural populations and even within single egg-mass lines created ambiguities (Whitehead 1968). Moreover, the sizes of *Meloidogyne* are affected by feeding on different plants (Power et al. 1991). Nematodes feeding on the roots of resistant plants were consistently smaller than those on susceptible plants and had significant differences in growth. In the biometric studies, the overlapping ranges of most of the characters and the limited usefulness of others make specific identification difficult or impossible. Triantaphyllou (1971, 1985) outlined the significant contributions of fertilization, meiosis, and the chromosomal basis of sex determination which may be used for karyological differentiation of species. The number of chromosomes varies between 30 and 56 (Triantaphyllou 1985) and they are often difficult to observe and count. Host and cultivar range preference tests have been used extensively to differentiate the intraspecies, but the diagnosis is time consuming, protracted and somewhat uncertain. There is evidence that when many populations of the same species are studied, their resistance or susceptibility to a given host cannot be predicted with confidence and certain populations

are not able to be determined (Netscher and Taylor 1979; Netscher 1983). Host resistance changes with environmental conditions, such as temperature (Omwega et al. 1990). It also appears that the number of identifiable races changes with the number of hosts used for differentiation (Fargette 1987).

Faced with the problems of differentiation and diagnosis based solely on morphology, biochemical discrimination among the nematodes has been employed to help solve them. Gel electrophoresis to analyze nematode proteins, carbohydrates, lipids, and enzymes has been a helpful tool for distinguishing various species and pathotypes (Dickson et al. 1971; Esbenshade and Triantaphyllou 1985, 1987; Hussey 1979, 1985). Dickson et al. (1971) used soluble proteins and profiles of eight enzymes to separate *M. hapla*, *M. incognita* and *M. arenaria*. In an extensive study of 16 *Meloidogyne* species, Esbenshade and Triantaphyllou (1985) obtained species-specific phenotypic patterns for non-specific esterases, malate dehydrogenase, superoxide dismutase and glutamate oxaloacetate transaminase for *M. javanica*, *M. incognita* and *M. hapla*. Various investigators studied several enzyme systems and demonstrated that some enzyme phenotypes might be used to distinguish between species (Dalmasso and Berge 1978, 1983; Esbenshade and Triantaphyllou 1985, 1990; Fargette 1987).

Serological methods for nematode identification have long been given attention (Webster and Hooper 1968). This

approach became more promising with the application of monoclonal antibody technology (Schots et al. 1990). This method does not have the drawback of polyclonal antibodies that vary in antigen expression and cross-reactivity between some antibodies and antigens, but it lacks the necessary discriminatory power due to antigen conservation between closely related taxa and its use in particular circumstances is very discriminating.

Although each of these biochemical approaches met with some success, all of the techniques have some limitations and drawbacks (Hussey 1979). 1. The cellular expressions of protein, lipid and other character products are often modified by the influence of ontogenic and environmental factors on nematodes and may not be present uniformly in all individuals or populations. For example, the expression of the esterase phenotype of *Meloidogyne* is related to specific physiological stages of the nematode (Dalmasso and Berge 1983; Dickson et al. 1971). 2. The biochemical components are ultimately the products of genetic expression and they tend to be highly conserved in function and structure, and so identifiable differences among closely related species and races may not be easily detected. 3. Accumulated macromolecules such as proteins and surface antigens represent end products derived from a small fraction of the cell's genetic materials, and so only a small fraction of the potentially useful variation is available for study by the gene products.

3. DNA technology in root-knot nematode identification

Molecular genetics based on DNA can be used directly to analyse precisely the nucleic acid component of the genome and thus avoid the problems associated with former methods of identification. Recent advances in DNA technology, such as DNA cloning, restriction enzyme analysis, polymerase chain reaction and nucleotide sequencing, now allow direct exploitation of DNA sequence polymorphisms. They permit the rapid and reliable characterization of the nematode genome and facilitate the precise identification of nematode species and subspecific forms. The base sequence of DNA is the primary source of biological variation and, in theory, nucleic acid analysis should provide the ultimate resolution in nematode identification.

There are several advantages to the use of DNA-based differentiation, which are proposed as follows. 1. The method is very sensitive and is able to deal with small samples, such as a single nematode egg, juvenile or adult. 2. These methods do not rely on the expressed products of the genome, but rather on the chemically stable genetic materials free from the influence of developmental factors or environment, which insure uniform cellular DNA composition during the life of the nematode. 3. The methods can detect small differences in genetic materials, and

therefore potentially are extremely discriminating. The detectable nucleotide base changes and sequence rearrangement, such as insertion, deletion, inversion, amplification and transposition of specific DNA segments among genomes provide the basis for differentiating closely related species and intraspecific races. 4. The data based on DNA techniques are reliable. DNA sequences in particular are accurate and repeatable and the data can be comparative. Nucleotide sequence comparisons of mitochondrial DNA have established that genetic relationships based on comparative studies of conserved DNA sequences are less prone to error than other methods. 5. Because the subtle differences between nematode races and morphologically identical pathotypes are obvious targets for research, the direct analysis of the entire nematode genome, based on DNA sequences, could help in understanding the affinities of nematode races and provide a new approach and design to taxonomy and species and subspecific differentiation. 6. Extracted genomic DNA is chemically stable, can be stored for long periods and, therefore, can be used for comparison and for developing commercial products, such as diagnostic kits.

Since the first demonstration of the value of recombinant DNA techniques for the identification of nematodes (Curran et al. 1985) and their application to the identification of *Meloidogyne* species and races (Curran et

al. 1985, 1986), on-line diagnostic capabilities have been developed for several economically important nematode species. Although these new approaches are of recent origin, they already promise to become powerful tools for identification.

In the past, most studies on nematode DNA have been directed towards characterization of the cellular concentrations, base composition, classes of DNA, renaturation behavior, and possible changes of cellular informational content associated with differentiation or evolution of parasitism (Triantaphyllou 1985; Pableo et al. 1988; Pableo and Triantaphyllou 1989; Burrows 1990; Greig and Ashall 1987). Some of these data may be applied directly to nematode taxonomy and diagnosis. Several strategies are available to detect differences in RNA, genomic DNA and mitochondrial DNA in nematodes for identification and diagnosis.

i. DNA content and nucleotide composition in Meloidogyne.

Most *Meloidogyne* species reproduce by meiotic and mitotic parthenogenesis and have somatic chromosome numbers ranging from 30 to 56. *M. hapla* is primarily a diploid species that reproduces by amphimixis or meiotic parthenogenesis, whereas *M. incognita*, *M. javanica* and *M. arenaria* may be polyploid and reproduce by obligatory mitotic parthenogenesis (Triantaphyllou 1991). Some species of *Meloidogyne*, in fact, are known to include more than one

chromosomal form. For example, *M. arenaria* exists as a form with approximately 36 chromosomes and another with 51 to 54 chromosomes (Triantaphyllou 1985). The total DNA content per nucleus in preparasitic larvae, determined cytophotometrically, is proportional to their chromosome number in *M. hapla*, *M. arenaria*, *M. incognita* and *M. javanica*. However, *M. graminicola*, *M. graminis* and *M. ottersoni* have a DNA content per chromosome significantly lower than that of the other *Meloidogyne* species (Lapp and Triantaphyllou 1972).

The genomic size and base composition such as percentage of guanine-cytosine (%GC) give some measure of genetic relatedness amongst organisms. The genome size of *Meloidogyne* is about 0.78×10^8 base pairs (Pableo et al. 1988). The genomes of nematodes are amongst the smallest known for multicellular eukaryotes (Beauchamp et al. 1979), and *Meloidogyne* has a smaller genome size than other nematodes such as *Caenorhabditis*, *Panagrellus*, *Turbatrix*, *Ascaris* and *Trichinella*, which are two- to seven-fold larger. There are differences in the amounts of DNA in these nematode species, but the genome size as established by renaturation kinetics of single copy DNA does not appear promising as a means of differentiating closely related nematode species.

Cot curves derived from renaturation kinetics of sheared, denatured DNA indicated that the genome of *Meloidogyne* species is composed of 20% repetitive and 80%

nonredundant, single copy sequences (Pableo and Triantaphyllou 1989). The complexities of the repetitive sequences, unique sequences, and total haploid genome of *Meloidogyne* are approximately 0.15×10^8 , 0.62×10^8 and 0.78×10^8 bp, respectively (Pableo and Triantaphyllou 1986, 1989).

The base composition (% GC) of the total nematode DNA may be determined by thermal denaturation tests and buoyant density. The mean base composition of the total DNA of *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*, as determined by thermal denaturation tests, are quite similar, as they range only between 31 and 33% (Pableo et al. 1988). The striking similarity in DNA base composition observed among these four species of *Meloidogyne* suggests that a high similarity exists in nucleotide sequences in their DNA and that their genomes have diversified very conservatively. Although these species exhibit considerable morphological, biological, and cytogenetic diversity, apparently the conservative diversification of the genome has not precluded an accumulation of gene and chromosomal mutations that have brought about the extensive biological and cytogenetic evolution of the genus. The base composition of mtDNA is different from that of nuclear DNA. However, the usual amount of mitochondrial DNA is insufficient to affect the general characterization of total DNA in parasitic nematodes (Platzer 1981). In general, the % GC of DNA from nematode

species appears to have some promise for separation of nematode genera or families, but not species.

ii. DNA RFLP in genotypic differentiation of nematode species

Differentiation of nematodes using restriction fragment length polymorphism (RFLP) is based on the differences in genome organization and sequence sites recognized by restriction endonucleases. Since the distribution of a particular recognition sequence of restriction endonuclease is determined by the genotype of the organism, the size distribution of these restriction fragments is unique to a genotype.

Two methods are available to select and visualize these restriction fragments of size-fractionated DNA. The first approach is to quantify the size distribution of restriction fragments in agarose gels stained with ethidium bromide and viewed with UV irradiation. Distinct bands represent variations in the restriction patterns of the multiple copies of repetitive DNA sequences, e.g., ribosomal, histone, centromeric repeats. The ribosomal DNA repeats of *M. arenaria* have both the larger rRNAs (18S, 5.8S, and 28S rRNA) and 5S rRNA represented within a single DNA repeat (Vahidi *et al.* 1988). The restriction fragment differences from these repetitive DNA bands can be used as diagnostic characters under direct UV viewing. Another approach is to detect differences in the RFLP of low or single copy DNA

sequences by hybridizing labelled, cloned DNA fragments to the size fractured genomic DNA. These differences are visualized by autoradiography or immunofluorescence and can be used as diagnostic characters. Nematode species have been differentiated by means of variations in their DNA restriction patterns. However, unless very large amounts of experimental data can be generated, the restriction enzyme analysis approach remains qualitative rather than quantitative in estimating species relatedness and differentiation.

Detection of length differences of endonuclease restriction fragments in repetitive DNA sequences, have permitted the rapid diagnosis, by genotype, of populations of *Meloidogyne*. In order to detect RFLP, the chosen enzymes should be reliable in cutting genomic DNA, inexpensive in economy, insensitive to methylation and should result in fragments 0.5-20 kb long. Curran et al. (1986) used this method with populations of *M. incognita*, races 1, 2, 3, and 4; *M. javanica*; *M. arenaria*, races 1 and 2; and *M. hapla*, races A and B. Several distinct bands were visualized for each *Meloidogyne* race in ethidium bromide stained gels viewed under 260-nm irradiation. Identical results were obtained from 12 gels using DNA samples from the same or different egg batches. The numbers of bands varied with each population. Certain of these repetitive DNA bands have restriction fragment lengths unique to each *Meloidogyne* population, and these differences can be compared and used

as diagnostic characters. For example, possession of a large and repetitive DNA band distinguishes the two *M. hapla* populations from all the other species, and a size difference in this band separates *M. hapla* race A (15 kb) from race B (15.5 kb). The restriction endonuclease digestion fragments of genomic DNA might be used to distinguish *Meloidogyne* species from species of other genera, e.g., *Trichinella*, *Caenorhabditis*, *Romanomermis*, *Steinernema* (Curran et al. 1985).

iii. Specific DNA probes in positive/negative screening for nematode identification

If two identical pieces of DNA (one may be labelled) are brought together in certain conditions, they will stick to each other along their full length (hybridizing) according to their relatedness. The hybridized piece of DNA can be visualized by autoradiography or by immunofluorescence /immunoperoxidase techniques. DNA sequences can be divided into two broad classes, namely, sequences of single copies and sequences of multiple repeated copies. Species differences can be identified in either class. Single copy sequence probes need large amounts of DNA and may be used as specific probes. Repetitive DNA gives the most convenient diagnostic sequences because such sequences are easier to detect in small amounts of DNA extracted from small parasites and are more sensitive.

Two strategies have been used to search for potentially species-diagnostic repetitive DNA sequences which might then be used as probes for identification. The first strategy is direct analysis of known repetitive sequences such as kinetoplast DNA and ribosomal gene. Use can also be made of heterologous cloned DNA probes. The second strategy is to search other repetitive sequences that may have many more copies and, hence, may show greater sensitivity in diagnostic tests. These probes can be constructed from genomic DNA of the nematode taxonomic group of interest. Such single cloned repetitive sequences can be isolated from either species-specific DNA bands identified after agarose gel electrophoresis of endonuclease digested genomic DNA, or differential hybridization of radiolabelled genomic DNA from several species to replicate cloned genomic libraries. The sensitivity of these techniques is such that only differences in number of copies of repetitive DNA or small sequence divergences are revealed, such as in dot-blot application. The total genomic repetitive DNA might be used as a probe to discriminate *Meloidogyne* (Burrows 1990; Greig and Ashall 1987).

DNA probes that hybridize to a particular taxon are extremely useful diagnostic tools and can be used in identification protocols based on positive/negative hybridization of the probe, which eliminates the need to use electrophoretic techniques. DNA extracted from the nematode sample, or a crude DNA extract or even a vector suspected of

carrying the nematodes can be bound directly to a nitrocellulose filter and probed with the diagnostic DNA sequence. Positive hybridization of a DNA probe to the nematode sample based on a marked difference in copy number or sequence divergence between species, is indicative of conspecificity.

As an example, a Southern blot of Eco-RI-digested total DNA hybridized with a ^{32}P labelled 28s, 18s ribosomal DNA repeat Charon 4 phage probe cloned from *C. elegans*, indicated that the *M. hapla* cytological race A was different from race B based on the autoradiograph (Curran and Webster 1987). With the development of probe construction and simplified procedure and sample preparation methods, the species specific probes should provide the framework for a field system.

iv. PCR and DNA sequencing in nematode phylogeny

A DNA sequencing comparison of quantitative data provides reliable methods for investigation of the relatedness of species. Some repeated DNA, such as rDNA, potentially represent a very powerful taxonomic indicator, due to its universal occurrence in living cells. The first to be used for this purpose was 5S rDNA because its small size facilitated sequence determinations. Large pieces of rDNAs and their oligonucleotide catalogues have already been

used to determine the relationship of a number of other species (Woese 1981).

The polymerase chain reaction is an *in vitro* method for the primer-directed enzymatic amplification of DNA. The enzymatic amplification of specific DNA segments is made possible by the highly specific binding of oligonucleotide primers to sequences flanking the segment. These primers allow the binding of a DNA polymerase that then copies the segment. Because each newly made copy can serve as a template for further duplication, the number of copies of the target segment grows exponentially. Theoretically a single template DNA can be amplified more than a millionfold in a relatively short period (Erlich et al. 1988). Saiki et al. (1985), using this procedure, demonstrated a 220,000-fold amplification of a 110-bp region of the Beta-globin gene. By using this method with the fairly conserved primers, one or several specific sequences can be amplified *in vitro* in a matter of hours. Moreover, as the procedure is easily automated, dozens of samples can be made each day with a standard amplification.

The amplified fragment length polymerisim (AFLP) from genomic DNA can be directly viewed and used to differentiate the nematode populations, eg., the amplified fragments from different species of *Meloidogyne* can be cut with restriction enzymes for diagnosis (Harris et al. 1990). Another powerful approach in the use of PCR is direct sequencing of DNA. In most instances analysis of DNA sequence can clarify

taxonomic relationships and provide an accurate identification based on a fundamental character. The availability of PCR technology has greatly increased our ability to make multiple comparisons of DNA sequences from small samples of large numbers of *Meloidogyne* populations in a short period. Multiple sequence analysis and comparison of different nematodes help us understand the genetic affinities of different populations and differentiate *Meloidogyne* species.

v. The unique value of mitochondrial DNA.

Besides nuclear genomic DNA, mtDNA may be a useful diagnostic tool for intraspecies identification because of its higher copy number and rapid sequence divergence. Research to physically characterize the mitochondrial genomes of several *Meloidogyne* spp. and host-races has addressed questions regarding their systematics and dispersal, as well as the possible applications in molecular diagnostics (Hyman 1988; Powers et al. 1986). Animal mitochondria contain circular DNA molecules that typically comprise 1-10% of the total cellular genetic material. Another 90% of DNA extracted from root-knot nematodes comprises the cell's nuclear DNA (Pableo and Triantaphyllou 1989). They showed that the sizing of the mtDNA restriction products revealed a mitochondrial genome size of approximately 20 kb. Restriction enzyme analysis of mtDNA of four species indicated that several features were notable in

the digestions of Hind III and Hinc II. *M. hapla* did not share common restriction fragments with the other three species. *M. javanica*, *M. incognita* and *M. arenaria* shared two lower molecular weight Hind III digestion products.

Powers et al. (1986) and Hyman et al. (1990) demonstrated the feasibility of using mtDNA-based diagnostic probes for the rapid detection of *Meloidogyne* spp., by spotting gall macerates of infected tomato roots directly onto nitrocellulose filters and hybridizing with ³²P-labeled *M. incognita* mtDNA. Strong hybridization signals were obtained from the macerated roots infected with *M. arenaria* or *M. javanica* but no hybridization was observed with the *M. hapla* preparation. However, cross-hybridization was observed between the *M. incognita* mtDNA probe and DNA within *M. arenaria* and *M. javanica* gall macerates or purified mtDNA control samples obtained from these same *Meloidogyne* spp. DNA hybridization using reconstituted DNA-soil mixtures revealed a loss of assay sensitivity ranging from 34% to 92% with four agronomic soils tested.

Some variant regions of mtDNA cloned as a Mbo I library in pBR 322 appear promising as diagnostic hybridization (Powers et al. 1986). Hyman et al. (1987) indicated that restriction enzyme analysis had revealed that mtDNA shares many DNA fragments in common with *M. incognita*, *M. arenaria*, and *M. javanica*. However, restriction products are distinctly different between *M. chitwoodi* and *M. incognita*.

4. Current status and problems in using molecular genetics

The use of DNA technology for the identification of nematodes is just beginning but the indications are that it will prove to be a valuable tool for this purpose. The total genome composition may be helpful in identifying nematodes but some sequences are more helpful than others. DNA restriction site analysis on nuclear and mitochondrial DNA can be used for species differentiation of the nematodes when a large quantity of specimens can be easily produced. Specific diagnostic probes are useful and convenient for laboratory nematode identification and diagnosis, especially in conjunction with the dot-blot technique and biotin labelling. DNA sequencing is the most direct way to determine these differences but sequence analyses are labor-intensive and necessarily focus on a limited number of genes. However, with the advent of PCR technology, it should be easy to devise a search strategy based on DNA sequences.

The practical value of the methods and improving techniques will allow the rapid analysis of small quantities of parasite material. Furthermore, the isolation of specific probes and perhaps, the synthesis of oligonucleotide probes of varying sensitivity will considerably enhance the value of this approach.

Currently, a thorough knowledge of the characteristics of nematode nuclear and mitochondrial DNA is limited to a small number of species. As yet, only a few restriction

fragment differences and diagnostic probes have been identified, and they have not been commercially developed. The major logistical problems for the field use of DNA probes are the preparation of specimens, hybridization of the probe and, most importantly, the requirement for radiolabel and subsequent detection of DNA-DNA hybridization by autoradiography. The potential of other detection systems generally remains to be fully realized. For example, the sensitivity of biotin-avidin systems is often reduced as a result of naturally occurring biotin, which may produce unacceptably high background exposure. A number of alternative labelling techniques, such as direct enzyme linking to the DNA probe and the use of antibodies to detect DNA hybrids, are under investigation.

There may be many more innovative molecular and biochemical concepts and technique available now for nematologists. The new technology has not developed as fast in agricultural nematology as in the medical sciences (Albertini *et al.* 1989). This time lag could be reduced rapidly if the techniques were exploited and greater research emphasis and funding promoted this area.

Research on the evolution of single copy genes, gene families and mtDNA in populations is now progressing rapidly, and DNA studies are providing greater insights into the phylogenies of various groups. Comparison of restriction site maps and DNA sequences and DNA-DNA hybridization of

single copy nuclear DNA sequences are all giving valuable data for the study of taxonomy, population genetics and evolutionary biology. Besides having an important impact on nematode identification, the application of the new techniques of DNA analysis will also have a future role to play in achieving a deeper understanding of both the molecular evolution of nematodes and the evolutionary relationships of nematodes and their hosts. So far, only a few of the new DNA technologies have been applied to *Meloidogyne*. More will ultimately be achieved, in concert with other investigators studying the cytology, morphology, and biology of nematodes, and eventually an acceptable evolutionary history of the nematodes will be available.

6. Outline of the research

The root-knot nematode has been studied thoroughly from the point of view of morphology and biology due to its importance in agriculture. The drawback of nematode identification based on morphology and biology is obvious. The classical view of the nematode's relationship and its taxonomy based on the relationship has been challenged by research on biochemistry and genetics. Although the rapid emergence of biochemical and molecular genetics data provide an invaluable contribution for helping to identify nematode species, there is no available comprehensive and absolute technique for differentiating nematodes and demonstrating their relationship. To address these questions concerning

the nematode population complexity and the problems in identification of the agriculturally important nematodes, I have explored DNA technology in the last five years to study genetic affinities and differentiation of *Meloidogyne* spp., the most important group of plant parasitic nematodes. Based on previous research and rapid development of molecular genetics in Dr. Webster's and Dr. Baillie's laboratories, four parts of the research were initiated and the major research objectives of this thesis are as follows.

Part I. Techniques of nematode rearing in controlled environment culture were developed so as to explore the production and maintenance of *Meloidogyne* in excised roots and the influence of root factors in excised root culture on the development of *M. incognita*.

Part II. Genetic affinities and phylogenies were examined to demonstrate the use of randomly cloned DNA hybridization probes to characterize the total genomic DNA of populations of three *Meloidogyne* species.

Part III. Based on the 18s-like rDNA, genetic affinities of *M. incognita* at the species and race level were analyzed using PCR amplification and DNA sequences for diagnosis and evolutionary purposes in comparison with other nematodes.

Part IV. PCR technology and oligonucleotide primers were exploited in DNA amplification to differentiate and diagnose *Meloidogyne* populations for possible field application.

GENERAL MATERIALS AND METHODS

1. Sources of nematode

Meloidogyne incognita (Kofoid and White, 1919)

Chitwood, 1949 race 3, from Dr. M.A. McClure, University of Arizona, Tucson, USA and another population of *M. incognita* race 3, from Dr. D. Orion, Division of Nematology, Volcani Center, Bet Dagan, Israel were maintained in potted plants in the greenhouse and in excised root tissue of tomato, cv. Roma (*Lycopersicon esculentum* Mill). This stock of nematodes was used through all of the experiments (Table 1).

Some populations of *M. arenaria* (Neal, 1889) Chitwood, 1949 (Ma1), *M. javanica* (Treub, 1885) Chitwood, 1949 (Mj) and *M. hapla* Chitwood, 1949 (Mh), that were were originally from Dr. J.N. Sasser, North Carolina State University, Raleigh, USA were later provided by Dr. John Curran, a research fellow then at Simon Fraser University. The nematode stocks were maintained on tomato, cv. Bonny Best (Dominion Seed House, Georgetown, Ontario). The identities of the *Meloidogyne* species were subsequently confirmed by Dr. B. A. Ebsary, Research Station, Agriculture Canada, Ottawa.

Some nematode stocks of *M. incognita* races 1, 2 and 4, *M. arenaria* and *M. javanica* were provided by Dr. K.R. Barker

(Mi1, Mi2 and Mi4) and Dr. A.C. Triantaphyllou (Ma2 and Mj), North Carolina State University, Raleigh, USA.

Seven populations of *M. incognita*, two populations of *M. arenaria*, two populations of *M. javanica* and two populations of *M. hapla* were maintained in the greenhouse. Some details of the nematode stocks are listed in Table 1.

2. Maintenance of nematode cultures.

(i) Pot culture

An isolate of tomato, c.v. Roma, was supplied by Pacific Northwest Seed Co. Inc., Vernon, British Columbia.. The isolate of tomato, cv. Rutgers, was supplied by Willhite Seed Company, Poolville, Texas, as suggested by Sasser et al. (1987). The commercial tomato seeds were thoroughly surface treated with Thiram fungicide at the rate specified by the manufacturer. The seeds were washed in sterile distilled water and germinated in vermiculite beds and the seedlings were transplanted to 12 cm plastic pots containing a pasteurized 1:1 mixture of sand and silt loam. The plants were maintained in either a Conviron growth chamber or the greenhouse. The conditions in the Conviron growth chambers were 25 ± 1 °C by day,

Table 1. Sources and origins of *Meloidogyne* species and races used in the experiments.

Species and race number*	Locality, host of origin and/or strains No.	Source laboratory of origin
<i>M. incognita</i>		
Mi1M	Arizona	M.A. McClure
Mi1B	Asparagus, NC	K.R. Barker
Mi2B	NC #632	K.R. Barker
Mi3M	Arizona	M.A. McClure
Mi3O	Israel	D. Orion
Mi3S	Tomato	J. Sasser
Mi4B	Tobacco, NC	K.R. Barker
<i>M. arenaria</i>		
Ma1S	Tomato	J. Sasser
Ma2T	Georgia#3145	A.C. Triantaphyllou
<i>M. javanica</i>		
MjT	NC No 7	A.C. Triantaphyllou
MjS	Tomato	J. Sasser
<i>M. hapla</i>		
Mh1-2S	Tomato	J. Sasser
**Ce	N2	D. L. Baillie

*The numbers indicate the races. ** Ce: *Caenorhabditis elegans*.

20 ± 1 °C by night and 16 h photoperiod with cool white, fluorescent lights (F96PG17-CW, General Electric, USA) and Grow-lux lamps (Westinghouse, Canada). The greenhouse conditions were 25 ± 5 °C with supplementary light from cool-white fluorescent lamps to maintain a 16 h photoperiod. Every 3 wk the plants were fertilized with 2.5g per pot of 4-16-16 tomato food mixture (Green Valley Fertilizer Limited, Surrey, B.C.) and watered with tap water on alternate days in summer or as required in other seasons, depending on the moisture content of the soil surface in the pots.

Tomato plants were grown for 3 wk in the greenhouse and were inoculated with *Meloidogyne* by adding nematode eggs or juveniles from different nematode stocks to the soil through holes, according to the research purposes described in the text. The inoculated plants were allowed to develop for at least 6 - 15 wk before use.

The populations of *M. incognita* race 3 M, *M. arenaria* race 1 S and *M. javanica* S were each increased from a single egg mass. The resulting nematode stock cultures were maintained by periodic inoculation of the soil of young, potted tomato plants with egg sacs or pieces of tomato root with large numbers of attached egg sacs, using the procedures and conditions described above.

The nematode stock cultures in pots were maintained in the growth chamber and the greenhouse until required for various experiments.

(ii) Petri dish culture

Tomato seeds of cv. Roma, were surface sterilized for 15 minutes in 1 % sodium hypochlorite, rinsed three times with sterile distilled water, and placed in polystyrene Petri dishes containing 1 % sterile GIBCO nutrient medium (Cat# 152-03940M, Life Technologies Ltd, Paisley, U.K.). After the seeds germinated, 1 cm lengths of the primary root tips were excised and transferred to Petri dishes containing modified Skoog, Tsui and White (STW) medium (Table 2) derived from Orion *et al.* (1980), Jacob and Van Bezooijen (1971) and Lauritis *et al.* (1983). The excised tomato roots were cultured for two days in the dark at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and were then ready for inoculation. Nematode eggs were sterilized by exposing them for 4 minutes in 0.1% mercuric chloride or for 2 minutes in 0.5 % sodium hypochlorite and washed four times in sterile distilled water. The excised roots were inoculated by placing the sterilized nematode egg masses adjacent to the root tips.

Table 2. Plant tissue culture medium (STW) for growing excised tomato roots derived from Orion et al. (1980), Jacob and Van Bezooijen (1971) and Lauritis et al. (1983).

Stock A: Macronutrient salts per 1000 ml

Ca(NO ₃) ₂ ·4H ₂ O	1.44 g
KNO ₃	0.80 g
KCl	0.65 g
KH ₂ PO ₄	0.38 g
NH ₄ NO ₃	0.40 g
MgSO ₄ ·7H ₂ O	0.72 g

Stock B: Iron per 100 ml

FeSO ₄ ·7H ₂ O	0.56 g
Na ₂ EDTA	0.74 g

Stock C: Micronutrient salts per 100 ml

ZnSO ₄ ·7H ₂ O	0.027 g
MnSO ₄ ·H ₂ O	0.049 g
H ₃ BO ₃	0.016 g
KI	0.0075g

Stock D: Vitamins and amino acids per 100 ml

Nicotinic acid	0.050 g
Pyridoxine HCl	0.075 g
Thiamine HCl	0.010 g
Sodium glycinate	0.262 g

STW medium contains 100 ml of Stock A, 5 ml of Stock B, 10 ml of Stock C, 1 ml of Stock D, 15 g of sucrose and 15 g of Difco agar per liter.

The inoculated roots were incubated in the dark for several generations in the same conditions as for the excised root culture. Subsequently, nematodes derived from these Petri dish root cultures were available for use in those experiments where surface sterilized nematodes were required.

3. Nematode extraction

Nematodes were extracted from *Meloidogyne*-infected tomato roots by a modified sodium hypochlorite-differential centrifugation-sieve procedure (McClure et al. 1973). Tomato roots with light brown, mature egg sacs were washed gently and cut into 1-3 cm length. About 30 pieces of roots were placed in 250 ml 0.5% sodium hypochlorite solution (Fisher Scientific Company, Fair Lawn) and stirred with an electric blender at maximum speed for 40 seconds. The resulting suspension of eggs and root debris was poured quickly through nested 40-mesh (425-um), 200-mesh (75-um) and 500-mesh (26-um) sieves (Nitex HC-3-25, Switzerland). The eggs were retained on the 500-mesh sieve and washed with several volumes (about 30 X) of tap water to remove residual sodium hypochlorite. The eggs on the 500-mesh sieve were recovered by repeated sieving and rinsing. The eggs were

eluted from the sieve, transferred in 40 ml of sterile distilled water to a 50 ml polycarbonate centrifuge tube and centrifuged at 1,250g for 5 minutes. The eggs were further separated from the remaining plant and soil debris by centrifugation in a 20 and 40% (w/v) stepwise, sucrose (nonreagent grade , BC Sugar Co., Vancouver, B.C. V6B 3V2) gradient at 1,250 g for 10 minutes. The supernatant was quickly decanted onto a 500-mesh sieve and the eggs retained on the sieve were thoroughly washed with sterile distilled water to remove the sucrose.

The concentrated eggs were resuspended in sterile distilled water and were ready for immediate use. For long-term storage, large numbers of eggs were resuspended in 1 ml portion in storage buffer (0.1 M NaCl and 0.05 M K_2PO_4 , pH 6.0) (Brenner, 1974) and stored in labeled vials in liquid nitrogen until required.

When a given number of nematodes was required for inoculation or other purpose, the number of nematode eggs or juveniles was estimated by taking a 0.1 ml aliquot of the mixed suspension, diluting ten times and counting them on a gridded Petri dish using a dissecting microscope. The number of nematodes were estimated based on an average of three replicates.

4. DNA isolation, quantification and storage

The procedures of DNA isolation were adapted from Curran *et al.* (1986). Concentrated egg suspensions of the required nematode species were washed three times with wash buffer (0.05 M sodium chloride) and pelleted in a centrifuge tube. The washed eggs were added to six volumes of a 1 mg/ml buffered solution of proteinase K from *Tritirachium album* (30m Anson U/mg. C.E. Merck, D-6100 Darmstadt, F.R. Germany). The proteinase K buffer solution was 0.1 M tris buffer pH 8.5, 0.05 M EDTA, 0.2 M sodium chloride and 1% sodium dodecyl sulfate. The mixture of eggs and buffer was separated into several microcentrifuge tubes and frozen in liquid nitrogen for 10 minutes. The frozen eggs with proteinase K were ground with a cold pestle and mortar, thawed and digested with proteinase K at 65 °C for 5 minutes. The viscous DNA solution was extracted three times with an equal volume of redistilled phenol saturated with 1 X TE buffer (10 mM tris.cl pH 8.0, 1 mM EDTA) and once with chloroform-isoamyl alcohol (24:1), for 5-15 minutes per extraction with occasional mixing by gentle inversion of the microcentrifuge tube. After each extraction, the mixture was centrifuged for 5 minutes and the aqueous layer was transferred to another centrifuge tube. The DNA was precipitated from the final aqueous phase by adding 2.5 vol of 95% ethanol at -20 °C. The precipitated DNA was pelleted in a microcentrifuge, washed

twice in 70% ethanol at room temperature, air dried, and resuspended in 1 X TE buffer. RNA in the solution was digested and removed by the addition of 10 ug/ml DNase-free RNAase solution to a final concentration of 0.1 ug/ml and incubated at room temperature for 30 minutes. The DNA solution was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and the clean DNA was precipitated by the addition of 8M ammonium acetate to a final concentration of 2 M, and then 2 vol 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 with 5 ul/ml chloroform and stored at 4 °C or -20 °C until required.

The concentration and purity of the DNA were determined by spectrophotometric measurement and the intensity of fluorescence emitted by ethidium bromide in a gel. Nucleic acids of any variety are most easily quantified by UV spectroscopy, measuring at or near their UV absorbance maxima, about 260nm. A dilute aqueous solution of 1 ml in the cuvette is measured by a single wavelength measurement. A scan of a DNA will show broadening with a maximum near 260 nm. Using Beer's law, the concentration of the solution and absolute quantity can be calculated. Five ul of *Meloidogyne* genomic DNA was added into 995 ul of 1 X TE buffer. The reading of the solution at 260 nm in a spectrophotometer (SP8-100 ultraviolet spectrophotometer, PYE Unicam) enabled

the concentration of DNA in the sample to be calculated. One OD reading corresponds to approximately 50 ug/ml for double-stranded DNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} values of 1.8. If there is contamination with protein or phenol, the ratio will be significantly lower than the values given above (Sambrook et al., 1989).

Where the amount of DNA was less than 250ng/ml or the sample contained significant quantities of impurities, the method of ethidium bromide fluorescence was used also to estimate the concentration of genomic DNA (Sambrook et al. 1989). Two ul of DNA sample were mixed with 0.5 ul of 5 X gel loading buffer and loaded into a slot or well in a 0.7% agarose minigel containing 0.5 ug/ml ethidium bromide. A series of standard DNA solutions were mixed with 0.5 ul each of gel-loading buffer and loaded into wells in the same gel at 2.5 ul each. Electrophoresis was carried out until the bromophenol blue had migrated about 2 cm and the gel was destained by immersing it for 5 minutes in a new electrophoresis buffer containing 0.01 M $MgCl_2$. The gel was photographed with 260 nm short-wavelength UV irradiation. The intensity of fluorescence of the unknown DNA was compared with that of DNA standards, to estimate the quantity of DNA in the sample.

5. General Hybridization Procedure

(i). Probe isolation

DNA probes were obtained from different sources (see the following sections) or isolated as follows: 1 ug of the DNA of *M. incognita* race 3 was digested with Xba I (Pharmacia, Cat# 27-0948) and ligated into 0.2 ug of Xba cut plasmid pVZ1 DNA. The pVZ1 is a derivative of "blue-scribe(+)" (Vector Cloning Systems) that contains an extended polylinker segment inserted into the EcoR I site (Henikoff and Eghtedarzadeh, 1987), supplied by Dr Steven Henikoff of the Fred Hutchinson Cancer Research Center, Seattle, WA. The ligated plasmids were transformed into competent *E. coli* JM83 (Morrison 1979), and selected on plates containing 40 ug/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal), 160 ug/ml isopropyl-D-thiogalactopyranoside (IPTG) and 100 ug/ml ampicillin. The bacteria that contained the plasmids with different nematode DNA fragments, were isolated and plasmid DNA was extracted by the alkaline lysis mini-preparation method (Maniatis et al. 1982). The plasmids containing *Meloidogyne* DNA fragments were cut with Xba-I and separated by gel electrophoresis. The DNA fragments were used as probes for different purposes.

(ii). DNA labelling

To label each probe, 0.3-0.5ug DNA of selected plasmid was radiolabelled with dAT³²P (Amersham RadioChemicals, 650 Ci/mmol or 800 Ci/mmol) by nick-translation (Rigby et al., 1977) or oligo labelling (Feinberg and Vogelstein 1983). The labelled probes, 6 X 10⁵ cpm per ml hybridization solution, were boiled for 10 - 15 minutes, put on ice for 5 minutes and then added to the prehybridized filter for hybridization.

(iii). Southern blot and hybridization

Total DNA, including genomic DNA and mitochondrial DNA, was digested using restriction endonuclease according to the manufacturer's (Pharmacia, Cat# 27-0854-04) recommendations. Digested DNA restriction fragments were fractionated by electrophoresis on 0.7% agarose gels at 0.75 volts/cm until the bromophenol blue dye front had moved 18 cm following the standard electrophoresis procedure (Bio-Rad # 170-4304). The DNA on the gel was nicked in 0.25N hydrochloric acid for 15 minutes, denatured in 0.5N sodium hydroxide and 1.5M sodium chloride solution for 30 minutes, neutralized in 1M ammonium acetate for 60 minutes and transferred to 0.45 um pore size BioTrace NT nitrocellulose filter (Gelman Sciences Inc., Cat# 66485) as described by Southern (1975) or by a modified

method of the oneway bidirectional-transfer 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, denatured probe in a hybridization solution at 4 ml/100 cm² filter. The hybridization solution contained SSPE (saline-sodium phosphate-EDTA, SSPE = 180 mM sodium chloride, 10 mM sodium dihydrogen orthophosphate and 1 mM disodium ethylenediamine tetraacetate, pH 7.4), 0.3% SDS (sodium dodecyl sulfate) and Denhardt's solution (1 X Denhardt's solution is 0.02% Ficoll, 0.02% BSA and 0.02% polyvinylpyrrolidone) (Davis et al. 1980). The above solution was used except where others were specified. The filters were incubated at different temperatures and washed four times in washing buffer at suitable temperature. The washed filters were air dried, and autoradiographed at -70 °C with Kodak X-Omat ARP-K x-ray film (Kerrisdale Camera Cat# 144-3423) with a pair of Cronex intensifying screens.

PART I.

INFLUENCE OF TOMATO CALLUS AND EXCISED ROOT ON M. INCOGNITA

Considerable research has been done on the growth, development and reproduction of *M. incognita* in various hosts under field and greenhouse conditions. Most of the existing information about plant parasitic nematodes and their relationship with plant tissues has been gained from studies of nematodes feeding and reproducing on plants growing in soil. The study of nematodes in various sterile tissue culture methods has been useful in furthering our understanding of field problems involving *M. incognita* (Braun and Lipetz 1966; Jones 1980; Koenning and Barker 1985; Orion et al. 1980; Zuckerman 1969). Monoxenic culture is a convenient and effective environment in which to maintain populations, study behavior, investigate the effects of a nematode parasite on host metabolism and for evaluating the relationship between the nematodes and plants. Since the demonstration of aseptic culture of root-knot nematode growing in tomato roots on nutrient agar medium (Ferguson 1948; Tyler, 1933), several investigators have maintained and studied plant parasitic nematodes in tissue cultures *in vitro* (Dropkin & Boone, 1966; Glazer & Orion 1984; Glazer, et al. 1985; Koenning and Schmitt 1986; Prasad & Webster, 1967). Most of these studies have dealt with the effect of nematodes on the host and the effect of the medium components on the nematodes. Little attention has been given to how *Meloidogyne* are effectively maintained on excised roots over a long period and how they are

affected by plant tissue culture. Nematodes can be cultured routinely on excised roots to provide inoculum for other studies, such as DNA transferring or selection for plant resistance under sterile conditions. With the development of biotechnology and the increasing demand of experimenters for sterile nematodes, an understanding of the influence of sterile culture on the nematodes is necessary. The objectives of this study were to explore the optimum maintenance on *M. incognita* in excised roots and the influence of root factors on the development of this nematode species in excised root culture.

Experiments

1. Effect of times and temperatures on nematodes

M. incognita race 3 eggs from the stock population were extracted from infected roots by the modified sodium hypochlorite-sieve procedure and sterilized with 0.1% mercuric chloride as described in general materials and methods. The sterile eggs were used to inoculate cultured roots in the first experiment. In the subsequent experiments, monoxenic cultured eggs from the Petri dish cultures were used.

The excised tomato roots were prepared and inoculated as described in section 2 of general materials and methods.

In order to determine the optimum time for transferring nematodes, the tomato roots were infected with newly-hatched juveniles. On the 30th, 40th, 50th, 60th, 70th and 80th days after nematode infection, The egg masses produced from the inoculated roots were transferred to new media containing freshly excised roots. Five plates were used and each was inoculated with one egg mass. The inoculation method was as described in general materials and methods. The number of galls produced on the new media were recorded after 30 days of inoculation. A total six treatments were conducted and each treatment was replicated five times.

In order to determine the effect of different temperatures on nematode survival *in vitro*, excised roots were inoculated with 1-3 egg masses, cultured for 50 days, and then the culture, with galls and new egg masses, were stored at 4 °C, 10 °C, and 25 °C. The egg masses were transferred to new, excised roots in the 2nd, 3rd, 4th and 5th months after the beginning of storage. The number of galls induced by the nematodes on the new excised roots after 30 days were used to determine the virulence of *Meloidogyne* after storage at different temperatures and for different duration.

2. Test of the stability of cultured nematodes

Tomato plants were grown for 3 weeks in a greenhouse and were inoculated with about 2600 nematode eggs. Nematodes

taken from the 2nd to 23rd generations of culture *in vitro* were used to infect tomato plants in the greenhouse and five plants were inoculated with each generation. Each generation was compared with a control. The control nematode eggs were taken directly from soil in the greenhouse. The greenhouse temperatures ranged from 22-27 °C. The inoculated plants were grown for six weeks, then they were removed from their containers and the soil was gently shaken and washed from the root system. The roots were stained with 0.01% solution of phloxine B for 15 min to facilitate examination for nematode galls and egg masses (Al-Hazmi and Sasser 1982). The number of galls and infective severity index were recorded for each plant. The infective severity index (ISI %) was calculated as follows:

$$\text{ISI \%} = (N_t / (N_p \times N_g)) \times 100$$

Where, N_t = total galls of one treatment, N_p = number of plants used in the treatment, and N_g = maximum number of galls in the control.

3. *Nematode growth on roots regenerated from tomato callus*

Aseptic tomato cv. Roma seedlings were grown in 1 % nutrient agar and then 0.5 cm segments of hypocotyl were excised as explants. They were transferred to Petri dishes containing a modified Murashige and Skoog (MS) medium (Murashige & Skoog 1962) supplemented with 2% sucrose, 10mg/liter indole-3-acetic acid (IAA) and 0.1 mg/liter

kinetin. The modified MS medium contained calcium nitrate instead of ammonium nitrate. Cultures were kept at room temperature ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$). After the callus was formed in 10 days six separate pieces of callus were inoculated with 2 - 3 nematode egg masses from the excised root cultures. After the inoculation, 380 hatched juveniles were observed under stereomicroscopy and their infection was recorded. The development of nematodes in the callus was monitored from 10 to 60 days after inoculation and the nematode developmental stages were determined by staining the callus in 0.1% acid fuchsin-lactophenol for 5 minutes, clearing with lactophenol for one day and checking under a stereomicroscope (Hooper 1985).

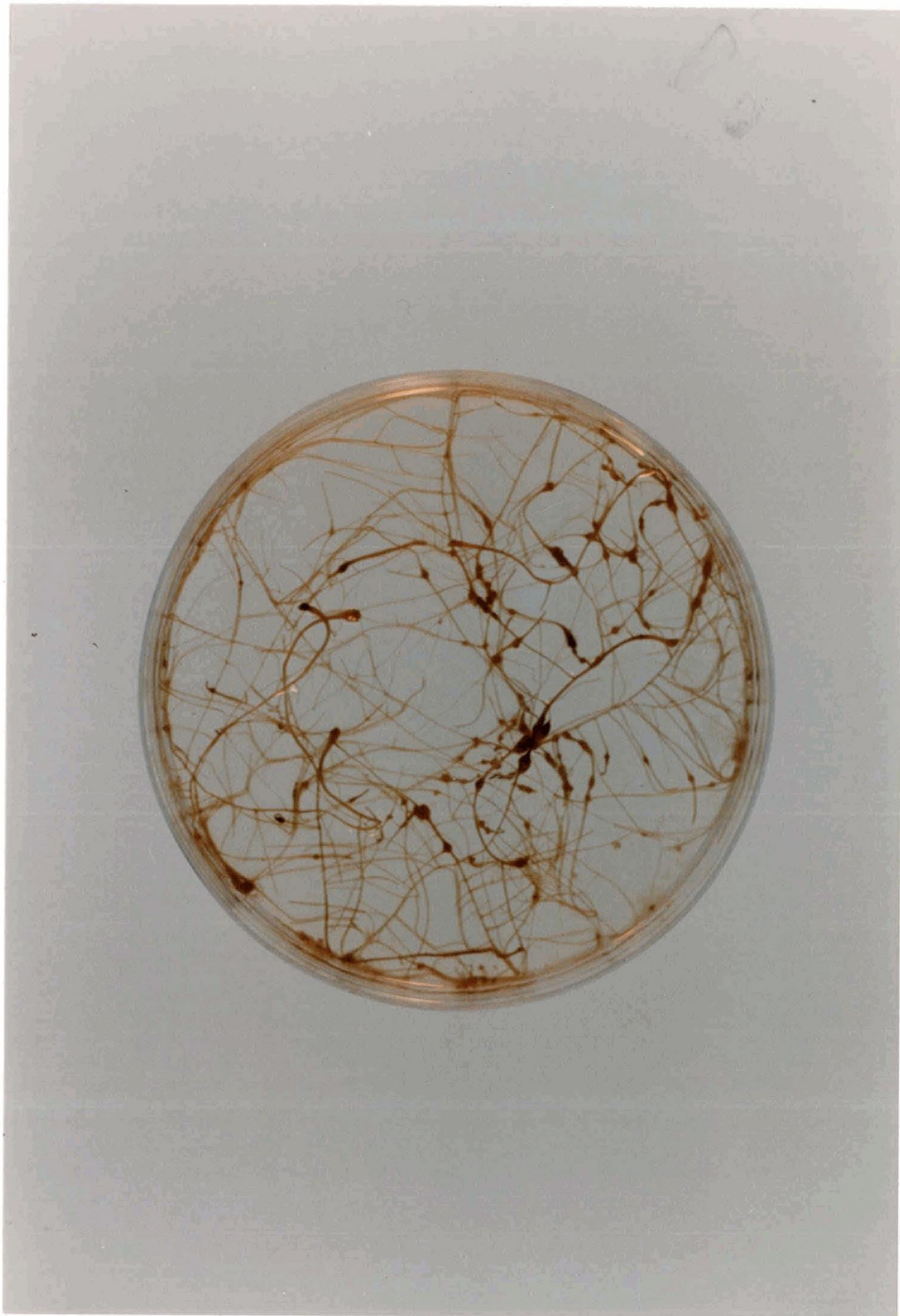
Six pieces of callus not inoculated with nematodes were excised with a sterile scalpel and aseptically transferred to another MS medium of the same composition, but containing 1 mg/liter IAA and 0.5mg/liter kinetin. After the roots were formed in 10-16 days, they were inoculated with 2 - 3 nematode egg masses. After the inoculation, nematode infection and development were observed to test nematode virulence and root suitability.

Results

1. Effect of times and temperatures on nematodes

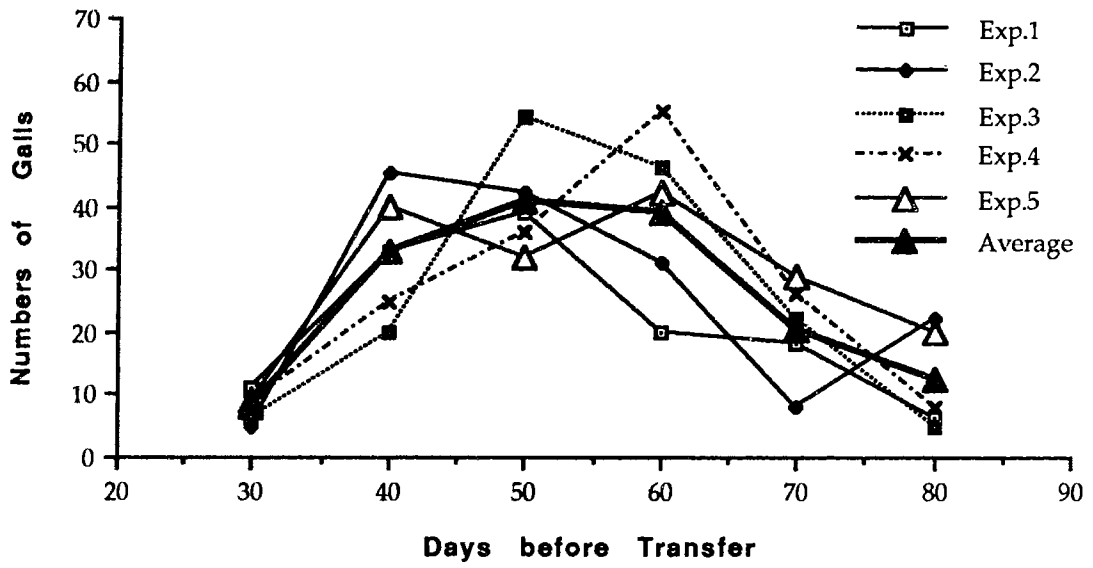
Observation of gall development and egg mass production in Petri dishes showed that *M. incognita* developed readily in excised roots on the STW medium (Fig. 1). Culture of excised roots was a convenient way to obtain significant numbers of sterile *M. incognita* for further studies. The earliest galls were formed within two days of inoculating with the inoculated egg masses. White, tiny, new egg masses were first seen 26 days after infection. When the egg-masses produced by the nematodes were transferred to new media with freshly excised roots, newly hatched juveniles induced galling (Fig. 2), and had a different ability to form galls. Only galls that produced egg masses on the new roots were counted. Figure 2 shows the production of galls in new root cultures when egg masses were transferred from old cultures at increasing intervals of time after inoculation of the old culture. The results show that mean gall numbers induced by egg masses from the 30th, 40th, 50th, 60th, 70th, and 80th day cultures were 8 (a), 32.6 (b), 40.6 (b), 38.8 (b), 20.6 (c,a) and 12.2 (a) respectively (the numbers followed by the same letter are not significantly different). The maximum numbers of galls were produced on those roots inoculated with egg masses from the 40th

Figure 1. Galls (brown swellings) and egg masses (attached to the galls) of *Meloidogyne incognita* in excised tomato roots on STW medium after incubation in the dark under sterile conditions at 25 °C for 50 day following inoculation.



48b

Fig. 2. Egg-bearing galls formed at different times in the STW medium. The egg-masses were produced on old plates by *Meloidogyne incognita* and transferred to new media with freshly excised roots. The newly hatched juveniles from the egg masses had different abilities to infect and form galls on the new roots. Solid lines represent the production of egg-bearing galls of the nematodes at 25°C, repeated five times, in STW medium, under sterile conditions. The dotted line represents the mean.



to 60th days on the excised root culture, which have significant differences from other day cultures but not from each other ($P = 0.001$).

The plates with galls and egg masses were placed in climate chambers at 4 °C, 10 °C, and 25 °C following 50 days after the excised roots and egg masses were introduced. Egg masses of *Meloidogyne* from these cultures transferred to newly-excised roots induced different numbers of egg-bearing galls after being transferred 2, 3, 4 and 5 months following inoculation (Fig. 3). After 3 months, there were few fresh galls formed at 4 °C and 25 °C, but the numbers of galls formed from egg masses on cultures stored at 10 °C were still high. Galls were observed from inoculation with egg masses stored at 10 °C for 6 months.

2. Test of the stability of cultured nematodes

The infection index and numbers of galls induced by nematodes taken from the 2nd to 23rd generations of culture *in vitro* are shown in Fig. 4. The results showed that there were differences in the number of galls formed between the generations cultured on STW medium, but the differences were not significant throughout the generations ($P = 0.01$).

Fig. 3. Effect of culture storage temperature on *Meloidogyne incognita* , as measured by number of galls formed on excised roots, after storage at three different temperatures for up to 8 months.

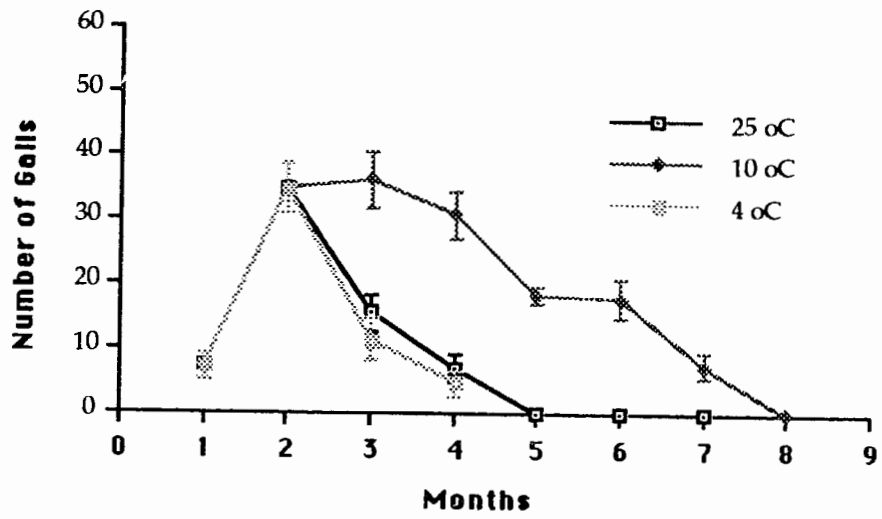
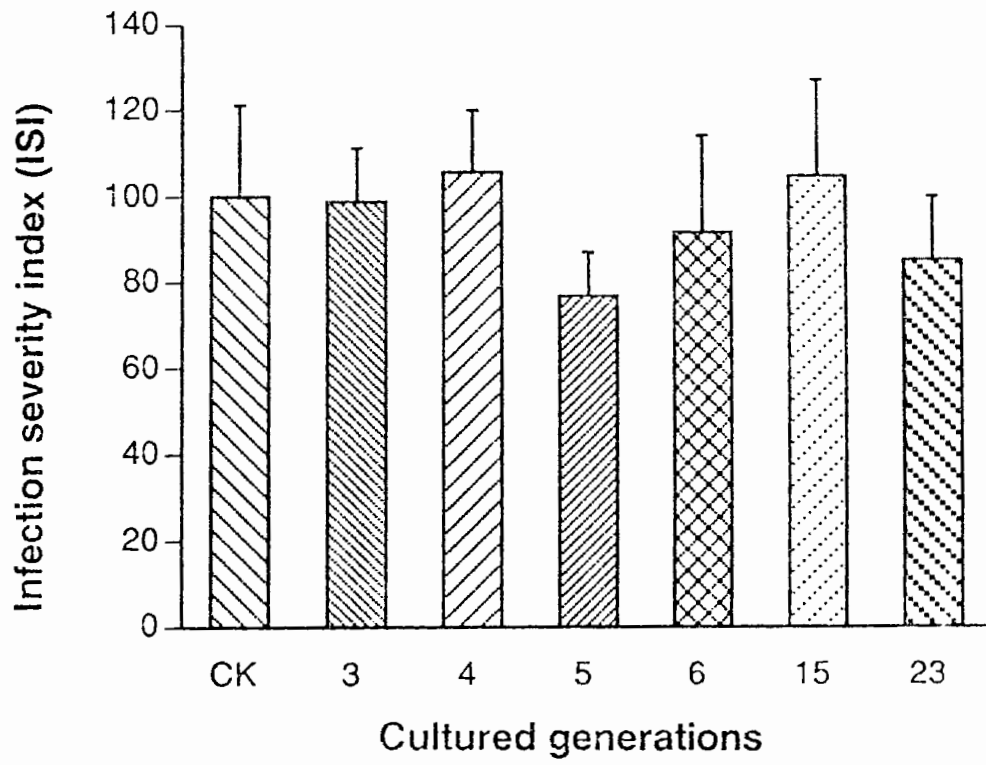


Fig. 4. Infection severity index (ISI) of *Meloidogyne incognita* on tomato plants in the greenhouse. The nematodes came from different generations reared consecutively *in vitro*. The check nematodes were directly from soil in the greenhouse.



3. *Nematode growth on roots regenerated from tomato callus*

The callus initiated from segments of tomato hypocotyl grew well on the medium supplemented with IAA and kinetin. Of the 92% (351/380) of nematode juveniles that moved to the callus after hatching, infection rate was 41% (144/351). After monitoring for 10 to 60 days, the nematodes appeared to develop from secondary stage juveniles to the fourth stage on the callus, but no egg-produced females were found on the callus (Fig. 5).

Once the roots were initiated from callus, inoculated nematodes infected the roots and developed egg-masses on the galls after 30 days (Fig. 6). The new egg masses from the initiated roots were transferred and cultured normally for five generations. Tomato plants infected with these eggs were as severely affected by the nematodes as were plants infected by *Meloidogyne* from stock cultures in the greenhouse. No symptoms occurred on the control (Fig. 7).

Fig. 5. Different stages of *Meloidogyne incognita* found in the callus induced from tomato segment on the MS medium with added IAA and kinetin. The nematodes developed from secondary stage juveniles to the fourth stage on the callus, but no egg-producing females were found.



Fig. 6. Galls and egg masses formed on roots initiated from tomato callus tissue. The roots were initiated from tomato callus on the MS medium plus IAA and kinetin. *Meloidogyne incognita* could infect the initiated roots and developed egg-bearing galls. When the new egg masses from the roots were transferred to plants they had the ability to infect normally.



Fig. 7. Comparison of foliar symptoms on tomato plants as a result of inoculating the soil surface with:

- a. Water only (control).
- b. Aqueous suspension of *Meloidogyne incognita* juveniles from greenhouse grown stock cultures.
- c. Aqueous suspension of *M. incognita* juveniles from roots differentiated from callus culture, after five generations.



a b c

Discussion

Tissue culture has proved to be a useful technique in studies dealing with plant parasitic nematodes. The mass culture of the nematodes on vigorous growing plant parts has long been given attention to satisfy the various experimental needs of nematologists. For example, the modified STW medium has been used to study the excised roots infected with nematodes (Orion *et al.*, 1980; Lauritis, *et al.* 1983). This medium contains the mineral nutrients, carbohydrates and vitamins required for normal root growth and does not apparently affect the nematode's growth (Orion *et al.*, 1980). The advantage of easy observation and detection of fungal and bacterial contamination in the medium is important in modern biotechniques for studying the nematodes and their hosts.

My results show that *M. incognita* developed well on the STW medium (Fig. 1). The stability of nematode infections was demonstrated over many generations. I tested nematodes that had been cultured in excised roots grown on STW medium *in vitro*. The results showed that there were no significant differences in nematode infectivity between generations 1 up to 23 in nematodes cultured on STW medium as compared with those in greenhouse soil ($P = 0.01$). The consecutively cultured nematodes in the medium could be used as an

inoculation source for experiments in sterile conditions, such as primary tests of plant susceptibility and selection of resistant plants from tissue culture.

The most suitable time to transfer plates and to obtain sufficient galls is around the 50th day after inoculation. We routinely store the nematodes at 10 °C after the egg masses were formed on the excised roots. The presence of new egg masses from nematodes at different temperature showed that the nematodes had the ability to survive at 10 °C for 5 months (Fig. 3), although the gall numbers were low. However, *M. incognita* could be kept in the plate culture without transfer for another 2-3 months at 10 °C after the egg masses have been formed, without obviously reducing their survival. Satisfactory results were obtained by using these nematodes as stock to inoculate plants in greenhouse and to conduct DNA work.

Temperature affects the infectivity and development of the nematodes. The threshold temperature for development of *M. incognita* was 10.08 °C or 10.40 °C (David & Triantaphyllou, 1967; Vrain, 1978; Vrain et al. 1978); 10 °C is minimal for *M. incognita* to develop. Our results showed that 10 °C is the most suitable temperature of the three tested, to maintain the root-knot nematodes on excised roots in culture. When the nematodes were kept at 4 °C, which is well below the threshold temperature, the excised

tomato roots became brown, and the root metabolism was affected drastically. Plant physiological and environmental conditions in the Petri dishes caused the nematodes on excised roots to die at 4 °C. The hatching ability might also decrease with time at 4 °C (Guiran, 1980). When the culture dishes with nematodes were maintained at 25 °C, most of the eggs developed into secondary stage juveniles, but the excised roots slowly dried as the water evaporated from the agar, even though the Petri dishes were sealed with parafilm. At this temperature, the nematodes did not survive for more than 3 months (Figure 3). Tomato root growth and health at different temperatures is another factor which affects nematode survival and the number of galls formed.

Plant callus culture has been successfully used to propagate or study different nematodes in their hosts (Faulkner et al. 1974; Riedel et al., 1972; Webster 1966, 1967). Miller (1963) reported that *M. hapla* in all stages of development was observed on occasion in partially differentiated tomato callus tissue. Okra callus was reported to support *M. incognita* (Tanda et al. 1980). We did not obtain egg-producing females in tomato callus and the life cycle of *M. incognita* was not completed in tomato callus in our study, although juveniles successfully infected the callus and developed into later stages. It was recognized that some callus might be differentiated so that

nematode adults were produced from the callus. Nematode needs some differentiated special tissue, such as a vascular parenchyma in which to induce giant cells, or certain nutrients or hormones. *M. incognita* grown on carrot storage root tissue *in vitro* stimulates the formation of callus tissue (Sandstedt & Schuster 1965), but the nematode's development seems to depend also on a suitable balance between auxin and cytokinin (Sawhney & Webster 1975).

The roots initiated from tomato callus were susceptible to *M. incognita*. The nematodes infected the roots from callus and developed into egg-bearing females in galls, even though they did not complete their life cycle on undifferentiated callus. When the egg masses from the initiated roots were transferred and cultured for five generations, no differences were observed in the infectivity of the nematodes. The nematodes may be cultured on induced roots to increase the bulk of inoculates and to reduce transferring time. The sterile nematodes on the culture are useful sources for use in tissue culture and in other research where sterile conditions are essential.

PART II.

DNA HYBRIDIZATION PROBES FOR STUDYING THE AFFINITIES OF
THREE MELOIDOGYNE SPECIES

Advances in recombinant DNA technology can now permit rapid and reliable characterization of the nematode genome and enable taxonomic identification and genetic affinities of species to be determined. These techniques have already been applied to some economically important plant-parasitic nematode genera, such as *Meloidogyne* (Curran *et al.* 1985; Curran *et al.* 1986; Powers & Sandall 1988), *Heterodera* (Besal *et al.* 1987; Radice *et al.* 1988) *Globodera* (Burrows & Perry 1988) and *Bursaphelenchus* (Abad *et al.* 1991; Webster *et al.* 1990).

The DNA hybridization probes of total genomic DNA are a means to reveal the RFLP's and enable an estimation of genetic divergence between the nematode populations. Previous studies have shown that they might be useful for identifying *Meloidogyne* species and for helping to demonstrate their relatedness using ethidium bromide staining of the 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 the genomic DNA and the relative 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 also to demonstrate phylogenetic affinities between them.

Materials and Methods

1. Nematode extraction and DNA isolation

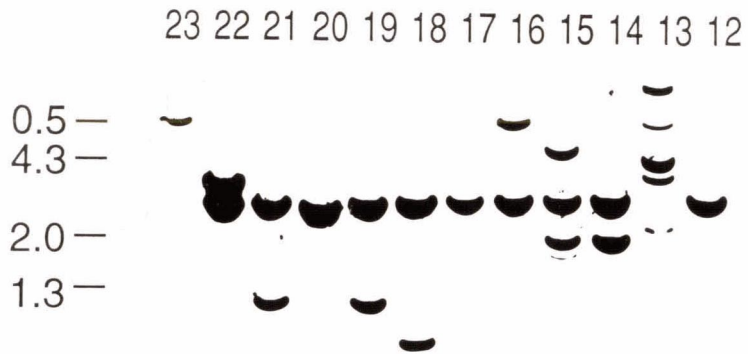
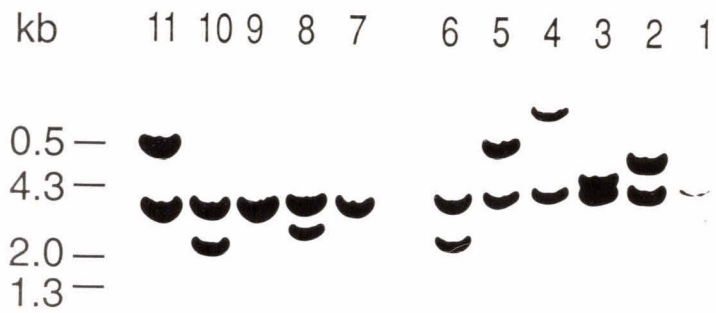
The materials used in the experiments were: *Meloidogyne incognita* race 3, *M. arenaria* race 1, and *M. javanica*. Sources and details of maintenance of these nematodes on potted tomato plants are described in general materials and methods.

Eggs were extracted from roots infected by *Meloidogyne*, washed and the DNA extracted as described in general materials and methods.

2. Probe isolation and labelling

Random fragments for use as probes were isolated by random clone selection manner as described in general materials and methods. 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. 8). Of the 94 plasmids with inserts, 32 were chosen

Fig. 8. Photograph of agarose gel showing the size of randomly selected DNA fragments. The DNA of *Meloidogyne incognita* race 3 was digested with Xba I endonuclease, ligated to plasmid PVZ1 and replicated in the bacterial host, *E. coli* JM 83. Ninety JM 83 clones (showing pMil-23 in Fig. 8) with nematode DNA inserts have been achieved and their DNA has been extracted. Molecular weight of *M. incognita* DNA inserts range from 0.1 to 15 kb..



randomly to use as probes. These plasmids, each 0.3-0.5ug DNA, were radiolabelled with dAT³²P (800 Ci/mmmole) by nick-translation (Rigby et al., 1977). The average radioactivity of the labelled probes was about 7 X 10⁷ cpm per ug DNA.

3. Southern blot and hybridization

Total nematode DNA was digested by the restriction endonuclease enzyme EcoR I and size fractionated in 0.7% agarose gels. Following the conditions and procedures of nicking, denaturing and neutralizing described under general material and methods, the DNA on the gel was transferred to nitrocellulose filter by a modified bidirectional-transfer method (Smith and Summers 1980).

The transferred DNA was immobilized onto nitrocellulose filters and then hybridized with a labelled and denatured probe in a water bath. Hybridizations were done in solutions containing 5 X SSPE, 0.3% sodium dodecyl sulfate and 5 X Denhardt's solution. 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 one hour. The hybridization pattern differences were observed and the size ranges of restriction fragments were recorded.

Results

1. Screening of *M. incognita* DNA probes

Ninety-four colonies from the plasmid library, which contained DNA fragments of *M. incognita* race 3, were screened initially with the blue-white color response on X-gal plates and their DNA was extracted. Electrophoresis of the DNA fragments with standard marker lambda (cut with EcoR I and Hind III and giving 13 different fragments) showed that the molecular weight of the cloned Xba I nematode DNA inserts ranged from 0.33 to 19.54 kb in size. Most of the 94 cloned fragments were between 1.5 and 6.5 kb (Fig. 9).

The cloned *Meloidogyne* DNA fragments used as probes for hybridization are shown in Table 3. These DNA fragments were used as homologous probes to detect relationships between three species of *Meloidogyne* and some of the fragments were species specific probes as explained below.

Fig. 9. The size distribution of cloned DNA fragments from *Meloidogyne incognita* race 3. The columns show the percentage frequency distribution of different sizes of inserts. The inserts range from 0.33 to 19.54 kb in size and most (76%) of the fragments are between 1.5 and 6.0 kb.

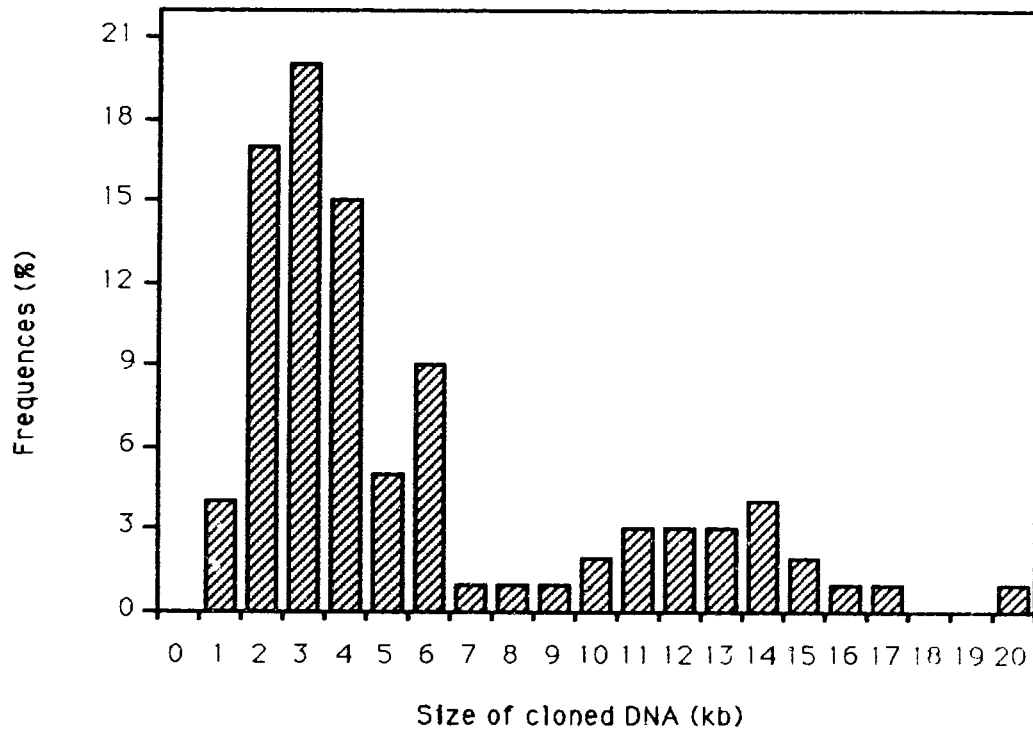


Table 3. DNA probes derived from *Meloidogyne incognita* race 3 used for hybridization with total genomic DNA of the three *Meloidogyne* species and their size in kilobases (kb).

Probe identity	Size (kb)	Probe identity	Size (kb)
pMi1	2.40	pMi17	4.30
pMi2	1.20	pMi18	1.91
pMi3	0.50	pMi19	2.50
pMi4	1.43	pMi20	15.00
pMi5	1.70	pMi21	2.35
pMi6	3.41	pMi22	12.00
pMi7	2.00	pMi23	2.14
pMi8	8.82	pMi24	5.55
pMi9	6.50	pMi25	1.50
pMi10	5.10	pMi26	1.35
pMi11	14.50	pMi27	2.20
pMi12	1.59	pMi28	2.65
pMi13	13.50	pMi29	13.00
pMi14	3.61	pMi30	2.70
pMi15	3.10	pMi31	1.15
pMi16	9.35	pMi32	1.55

2. DNA probes for differentiation of three Meloidogyne species

Thirty of the 32 cloned DNA fragments produced distinct hybridization bands with all three tested species of *Meloidogyne*. Of the probes, 69% have potential to differentiate species based on differences of hybridized bands. The hybridized probe pMi9 (labelled DNA fragment of *M. incognita* race 3 in plasmid pVZ1) was one such fragment which was selected randomly from cloned plasmids and labelled with dAT³²P by nick-translation. By hybridizing to total DNA from each of the nematode species, the labelled probe pMi9 made it possible to differentiate between these species. Under these hybridization conditions, the pMi9 probe, for example, reveals several RFLP's (Fig. 10). 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 band at 4.9 kb and another at about 4.2 kb. The bands at 6.7kb and 4.9kb in *M. incognita* race 3 distinguish this species from both *M. arenaria* and *M. javanica*. In addition, *M. arenaria* has one unique band at about 3.3 kb and another at 2.7 kb. This band occurs also in *M. incognita*, but weakly. *M. javanica* has a unique band at 3.5 kb, which does not exist in the other two species.

Fig. 10. Use of pMi9 as a diagnostic probe to differentiate *Meloidogyne incognita*, *M. arenaria* and *M. javanica*. The numbers show molecular weight in kilobase. The unique bands of *M. incognita*, *M. arenaria* and *M. javanica* are located at 4.9, 2.7 and 3.5 kb respectively (see arrows). J = *M. javanica*; A = *M. arenaria* race 1; I = *M. incognita* race 3.

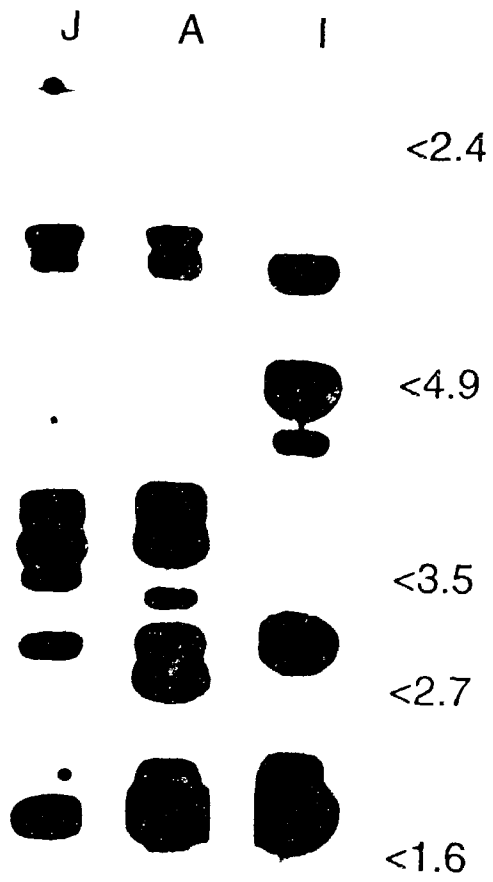


Figure 11 shows the autoradiograph of total DNA digested with EcoR I and hybridized with ³²P-labelled DNA from plasmid pMi10 (Fig. 11a) and pMi3 (Fig. 11b) in the hybridization conditions of 5 X SSPE solution at 68 °C. The pMi10 is a probe of molecular weight 5.1 kb. At this stringency, autoradiographs of EcoR I digested total DNA probed with labelled pMi10 and pMi3 show discrete species-specific bands. At the stringency used, pMi10 detects an EcoR I fragment in *M. incognita* of 4.5 kb and *M. arenaria* of 4.9 kb, and hybridizes very weakly in *M. javanica*. Probe pMi3 has an Xba I insert of 0.5 kb. Under similar hybridization conditions, the probe detected one EcoR I band of 14.0 kb in this *M. incognita* population, from which the probe had been isolated, but only weak bands in *M. arenaria* and *M. javanica* populations.

3. Distribution of hybridized restriction fragments

The distribution of hybridized restriction fragments for populations of the three *Meloidogyne* species using the 32 probes is shown in Fig. 12. DNA fragments, isolated from *M. incognita*, hybridized partially with each of the other two species and gave a different number of bands. The occurrence of homologous hybridization was 97%.

Fig. 11. Identification of *Meloidogyne* species by detection of RFLP's using randomly selected Xba I fragments of *M. incognita* as probes. The autoradiograph shows Southern blots of EcoR I digested total genomic DNA hybridized with ³²p labelled DNA probes, (a) with pMi10 probe; (b) with pMi3 probe. The probe pMi10 can detect *M. incognita* and *M. arenaria* but probe pMi3 detects only *M. incognita*. The number at right lane shows the molecular weight in kb. J = *M. javanica*; A = *M. arenaria* race 1; I = *M. incognita* race 3.

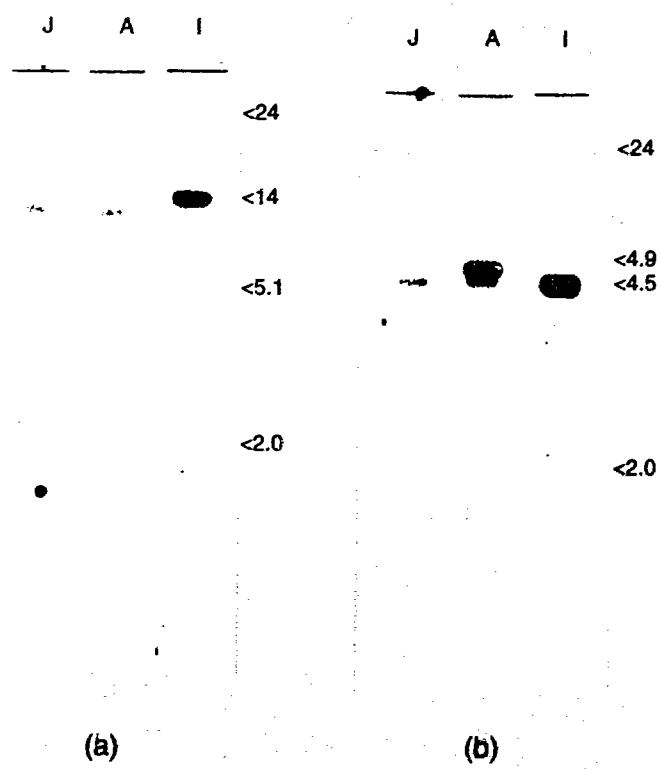
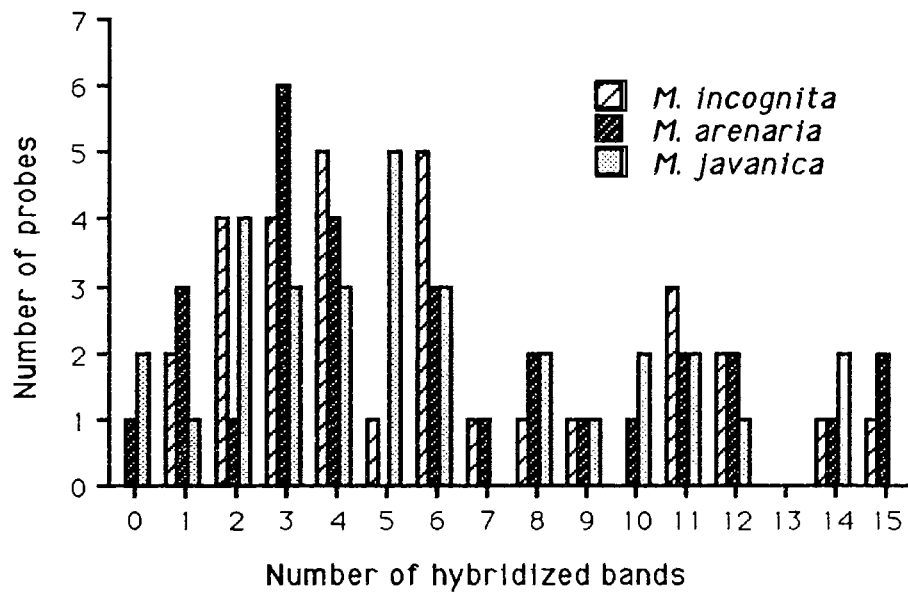


Fig. 12. Distribution of hybridized restriction fragments for three *Meloidogyne* species with 32 probes. DNA fragments isolated from one species, *M. incognita*, hybridized partially with each of the other two species (31/32 *M. arenaria* and 30/32 *M. javanica*) and gave a different number and size of bands.



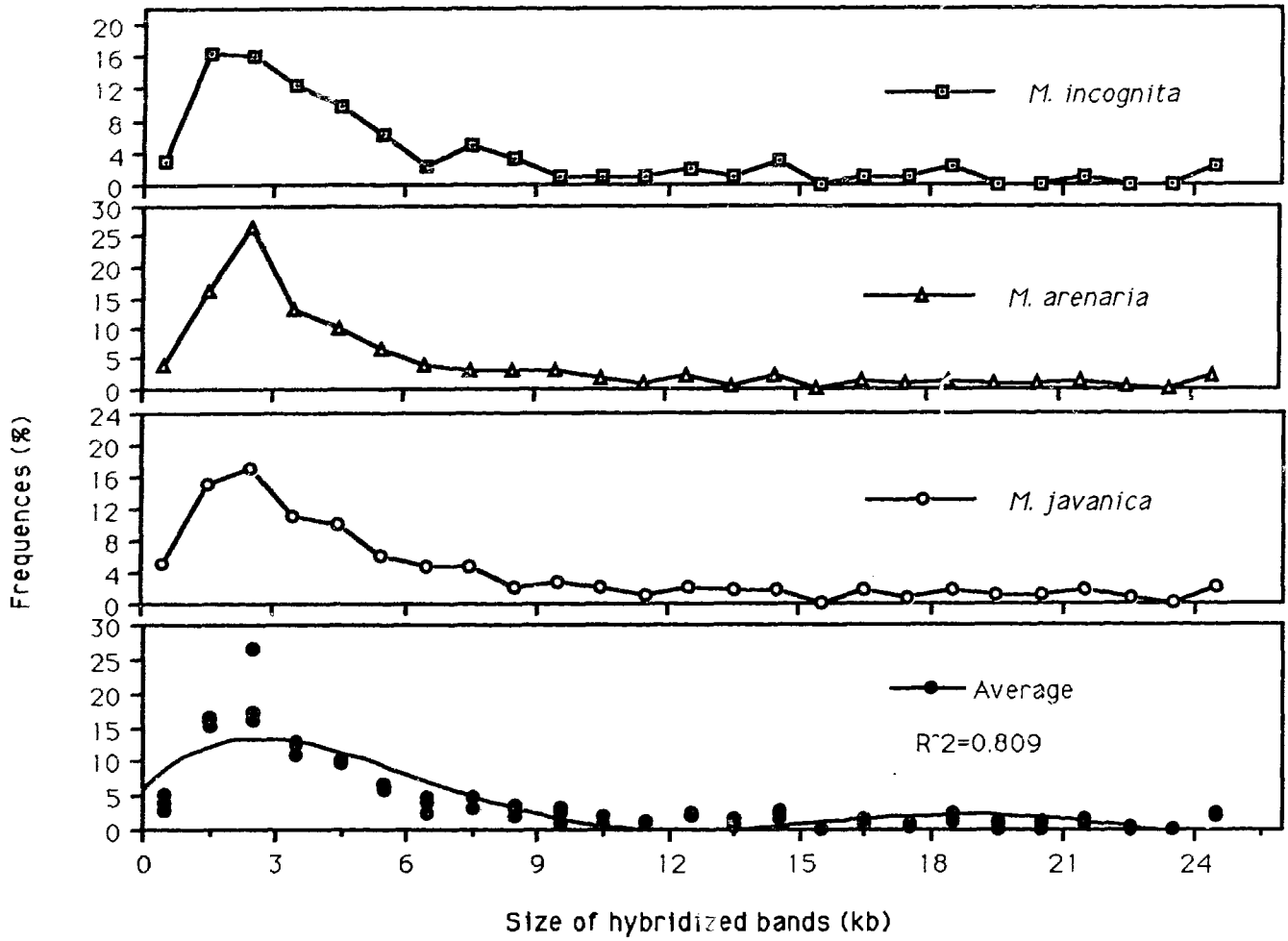
Cross hybridization among species indicates that the three species, *M. incognita*, *M. arenaria* and *M. javanica*, are closely related and have diverged only in relatively recent times.

Among these randomly selected 32 pMi probes, there were 30 which hybridized to more than one band in the three species of *Meloidogyne*. The ranges of molecular size of hybridized bands were from 0.16 to 24.0 kb. The most notable differences were the presence of molecular size around 1 to 8 kb, which was the maximum distribution region of hybridization bands when the genomic DNA was cut with EcoR I (Fig. 13).

4. Genomic affinities between three *Meloidogyne* species

Genomic differences between tested *Meloidogyne* species were analyzed by hybridization of cloned probe DNA to total genomic DNA digested with EcoR I. Fig. 10. gives an example of these autoradiographs where the hybridization pattern with probes labelled with ^{32}P was used to determine the relationships between *Meloidogyne* species. This relationship is based on the concept that relative genetic similarities between various pairs of organisms were proportional to the shared restriction fragments resolved by the detection probes.

Fig. 13. The size distribution of hybridized restriction fragments of three *Meloidogyne* species with 32 probes. 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.



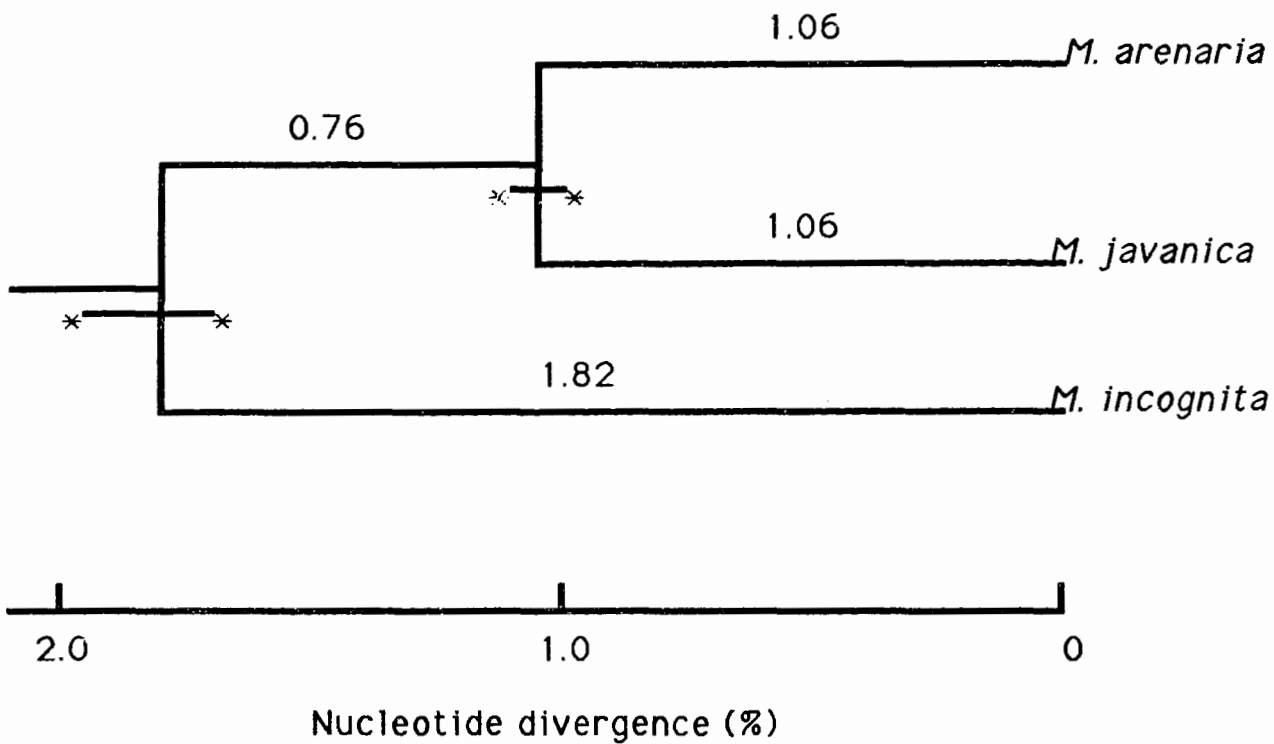
The proportion of shared fragments (F) across all digestions of the three species was calculated using the formula: $F = 2N_{XY} / (N_X + N_Y)$ (Nei 1987; Nei & Li 1979); where N_X = number of restriction fragments in species X; N_Y = number of restriction fragments in species Y; and N_{XY} = number of restriction fragments shared by species X and Y. The proportion of shared fragments 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 estimated 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: $F = G^4 / (3 - 2G)$. This calculation gave the same results as that calculated by the formula: $P = 1 - ((-F^2 + 8F)^{1/2} / 2)^{1/6}$; where P = the probability of a nucleotide substitution at a nucleotide site; and F = shared fragments between species across all digestions (Nei & Li, 1979). The results show that the percentage of base substitution 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 analyzed for estimating phylogenetic trees. The phylogenetic tree was calculated and drawn (Fig. 14) according to their percentage of nucleotide divergences (Dunn and Everitt 1982; Nei 1987; Sneath and Sokal 1973). The estimates of the percentage of nucleotide base substitutions were obtained from DNA hybridization data.

Fig. 14. The phylogenetic tree showing the relationship between three *Meloidogyne* species. The starred bar (*---*) represents one standard error on each side of the branching point. The axis scale represents the percentage of nucleotide divergence in base substitutions.



Discussion and Conclusions

The recombinant DNA-based approach for identifying *Meloidogyne* species is taxonomically and diagnostically useful. Hybridization of random *M. incognita* probes to total genomic DNA of the tested species, differentiates between the populations of three *Meloidogyne* species and helps identify their relationships. By analyzing the nematode genotype directly, hybridization data is capable of discriminating species within this genus. As well, the DNA can be stored for long periods of time without deteriorating and so is available for future analyses and comparisons. The results show that probes used for hybridization to whole genomic DNA and for the differentiation of species could be obtained directly from a random selection of homologous DNA fragments. Approximately 69 % of fragments cloned by this method revealed taxonomically useful differences in restriction fragment length polymorphism. Nevertheless, the results also showed that species within this genus could be distinguished by using a limited set of probes and restriction enzymes.

Of the 94 randomly cloned DNA fragments, the molecular size ranged from 0.33 to 19.54 kb and most (76%) of the fragments were between 1.5 and 6.5 kb. This might reflect the fact that the *Meloidogyne* genome could be cut every

1.5 - 6.5 kb with the 6-base restriction enzyme Xba I (Fig. 9). When the genome was cut with the 6-base restriction enzyme EcoR I and hybridized with these probes, it showed a similar result in that the distribution of most hybridization bands were about 1 to 8 kb (Fig. 13). This size range is the expected result; given a 6 base cutter the bands should be found every 4^6 on average according to general statistics.

Genetic differences and species relationships between *Meloidogyne* species were demonstrated first using recombinant DNA techniques (Curran et al., 1985; Curran et al., 1986) using ethidium bromide staining of genomic DNA digested by restriction endonuclease, to get diagnostic characters for species differentiation. The difficulties of visualizing these repetitive bands, which sometimes were smears, in a total genomic digest on an agarose gel can be overcome by using probes as shown by Curran and Webster (1987). In this study, DNA bands detected by probe pMi9 had a restriction fragment length unique to each *Meloidogyne* species. The pMi10 probe hybridized to DNA from the *M. incognita* and *M. arenaria* populations but only weakly to DNA from *M. javanica* populations at the high stringency used in this experiment. Hence, under these test conditions pMi10 appears to be a specific probe for *M. incognita* and *M. arenaria*. Using the same hybridization conditions, probe pMi3 detected *M. incognita* and, just weakly, the other two

species. It showed the same result under low stringency hybridization conditions in 5 X SSPE at 65 °C and washing in 2 X SSPE at 62 °C. Therefore, these probes are potentially useful for diagnostic purposes once screened 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.

DNA bands detected by most of these homologous probes had a restriction fragment length unique to each *Meloidogyne* population and these differences can be compared and used as diagnostic characters (see Fig. 10). Similar species-specific probe hybridization to restriction fragment length differences were shown for *Caenorhabditis elegans* and its close relative *C. briggsae* (Rose et al. 1982), and for *Heterodera glycines*, *H. leuceilyma* and *H. weissii* (Besal et al. 1987). These restriction fragments resolved by probes might even detect differences between populations within species.

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 pMi9 could be of the type associated with repetitive DNA, such as transposable elements or multiple gene families. The repetitive region of pMi9 varies between species and is a potentially useful probe for genomic analysis. If pMi9 contained noncoding, nontranscribed DNA

sequences that were less conserved between taxa than were protein coding DNA sequences, the non-transcribed repetitive region that could vary between species and some repetitive DNA fragments within the pMi9 probes should be potentially useful probes. From this region, oligo probes could be developed, after determining the DNA sequence, and used in diagnostic field work. On the other hand, the pMi10 and pMi3 probes indicate that the DNA sequences are with one or a few copies per genome or the DNA fragments have a great variation between species.

The inherent differences between species, often reflected in their host specificity and geographical distribution, can be identified by using random probes. Analysis of DNA fragments generated by restriction endonuclease indicates that between the *Meloidogyne* species there are genotypic differences. The DNA hybridization can aid and support the current differentiation of species as defined by morphology, cytogenetics and production of viable offspring, especially in parthenogenetic strains of *Meloidogyne* spp. DNA hybridization results, together with biochemical and morphological data, can be used to confirm species identification and to demonstrate taxonomic relationships.

Observed variation in hybridization patterns may result from base-pair substitution, insertion or deletion of small

or large DNA sequences, or from recombination or transposition. The variation between *Meloidogyne* species might be speeded up by such mutational events and clonal selection via parthenogenesis.

Genomic differences among tested *Meloidogyne* species can be analyzed by hybridization patterns of total genomic DNA revealed by pMi probes. The relative genetic similarities between various pairs of organisms is proportional to the number of shared fragments resolved from restriction endonuclease digests. The closer the similarity of the species that are being compared, the closer are the DNA sequences 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 being examined. Although the formula cited earlier is generally 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) who showed, by using mitochondrial DNA, that *M. arenaria* is the most genetically distinct of the four common *Meloidogyne* species they tested. This may

be explained by the different evolution and mode of inheritance of mitochondrial DNA and genomic DNA. The data places *M. incognita* as the most genetically distinct of the three species examined here.

The DNA fragment isolated from one species, not only hybridized with the species itself but usually with the other two species (97% opportunity). Under the high stringency conditions used, the DNA fragment isolated from *C. elegans* could hybridize with the species itself but usually not with the similar species *C. briggsae*. Cross hybridization among species indicates that *M. incognita*, *M. arenaria* and *M. javanica*, are not so divergent from each other as are members of some other groups of nematode species, such as *C. elegans* and *C. briggsae*. Analyses of these bands hybridized with ^{32}P labelled probes show that the population of *M. arenaria* is closer to that of *M. javanica* than to that of *M. incognita*. The proportion of shared fragments 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.

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 the 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 et al. (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 surrounding the application of molecular rates of evolution to estimating the dates of species divergences, the results described here taken together with those from other organisms (Beverley and Wilson 1984; Ochman and 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.

PART III.

MOLECULAR PHYLOGENY BETWEEN MELOIDOGYNE POPULATIONS

Genetic diversity among root-knot nematodes at the race level continues to cause problems for phytonematologists (Dropkin 1988; Taylor 1987). In recent years the use of molecular characteristics has greatly increased the number of characters that can be used to determine relationships between species. In general, molecular characters confirm the relationships previously established on the basis of morphology. In cases where molecular and morphological data differ, the molecular data have, for a number of other animals, prevailed in establishing a generally accepted relationship (Graur et al. 1991; Sibley and Ahlquist 1983). The plant parasitic nematodes present an interesting group of species among which the evolutionary relationships are just beginning to emerge (Curran and Webster 1987; Hyman et al. 1988). Within this group of nematodes, the species and races of *Meloidogyne* are among those most thoroughly studied.

The races of the *Meloidogyne* group have been traditionally differentiated by plant host, which gives little information about their genetic relationships. Although at the morphological level these races are virtually indistinguishable, at the molecular level they may show a high degree of divergence. An early attempt (Curran et al. 1986; Curran and Webster 1987) to determine the relationship among the races, employed direct views of RFLP's and DNA-DNA hybridization. However, there are

limitations to the use of RFLP's and DNA hybridization for this purpose. However, specific portions of the genome can be isolated and sequenced, and comparisons made between regions of the genome. This should reveal genetic differences in the populations and also their affinities, which will provide a better understanding of the relationships among the races.

A technique, the polymerase chain reaction (PCR), has been developed for amplifying a specific portion of a genome (Erlich 1989; Mullis and Faloona 1987; Saiki et al. 1985, 1988). Application of the technique has the potential to greatly facilitate the sequencing of homologous regions from different races of *Meloidogyne*. In essence, two oligonucleotide primers are selected and then synthesized, complementary to regions of the genome flanking the sequence to be amplified. The genomic DNA is then denatured in the presence of excess primers and allowed to reanneal. The region of the genome between the two primers is then synthesized *in vitro* by DNA polymerase. The procedure is repeated many times with a exponential accumulation of the specific region of the genome. After 25 or more cycles of this *in vitro* amplification, there may be a 10^5 -fold amplification, or up to 10 micrograms of DNA from the amplified region may be produced from 1 ng of the original DNA template (Perkin Elmer Cetus; Erlich, et al. 1988). Saiki et al. (1988), using the procedure, demonstrated a 220,000-fold amplification of a 110-bp region of the Beta-

globin gene. By using this method with the fairly conserved primers, one or several specific sequences can be amplified *in vitro* in a matter of hours. The oligonucleotide primers may have a slight degree of mispairing with the target sequence but still function efficiently, and can act as primers for different races or species.

This research was initiated to analyze genomic DNA of the nematode populations purely for diagnostic and evolutionary studies. In order to find an appropriate region of the genome in which to compare the races for completion of the research objective, a standard set of primers were used (Kocher *et al.* 1989), which amplified the region of the 18s ribosome DNA of *Meloidogyne*. The polymerase chain reaction method was employed to amplify homologous segments of genomic DNA from six populations of *M. incognita*, comprising races 1, 2, 3, and 4. Optimum amplification conditions were determined and used to obtain DNA sequences by direct sequencing and subcloning of the PCR-amplified fragment of the 18s-like ribosome DNA. Multiple sequence alignments and cluster analysis were used to analyze the data in order to quantify the affinities of the sequenced race populations of *Meloidogyne*. By comparing the DNA sequences of races of *M. incognita* with those of other nematode species, such as *Caenorhabditis elegans*, *M. arenaria*, and *M. javanica*, the population relationships of *M. incognita* were demonstrated.

Materials and Methods

1. Stocks of nematodes

Six populations of *M. incognita* were used for the experiments, namely *M. incognita* race 1 B and M, race 2 B, race 3 M and O and race 4 B. Three other nematode groups, namely *M. javanica* T and S and *C. elegans*, were used to compare the genetics at the species and race levels. Details of the nematode stocks and their sources are listed in Table 1.

The nematode stocks were maintained in potted plants in the greenhouse and in excised root tissue of tomato, cv. Roma, using the procedures and conditions described in general materials and methods. The extracted nematodes were multiplied on tomato plants, cv. Rutgers.

2. Source of primers and their amplification conditions

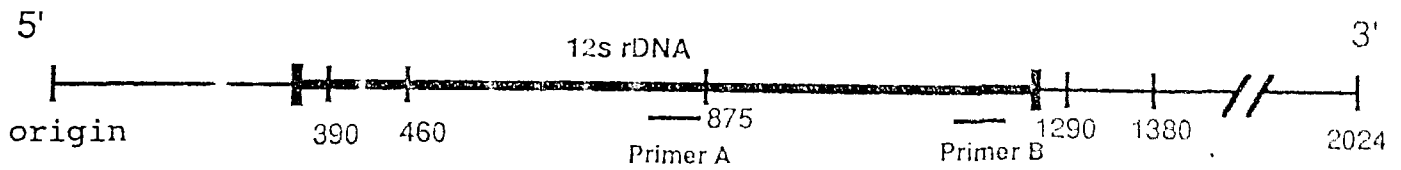
The primers were originally generated from human 12s rDNA. The sequences of the pair of primers are shown in Fig. 15. The pair of primers can amplify 386 bp of nucleotides in a human small 12s rDNA (Anderson et al., 1981). The positions and their amplified sequence are shown in Fig. 15. The primers were synthesized on an Applied

Biosystems DNA synthesizer, kindly provided by Kelley Thomas (University of California, Berkeley). When the primers were used to amplify DNA from the nematodes, they amplified part of genomic 18s rDNA, instead of the 12s mtDNA. These two primers were used to amplify the 18s-like rDNA of *Meloidogyne*.

The amplified DNA was purified with centricon 30, digested with Mbo I and cloned to the BamH I site of PVZ 1. The cloned fragments could be cut out with EcoR I and Xba I. Comparing with the original enzyme map, the different sizes of the fragments were identified and the selected clones were used for further sequencing identification.

The PCR amplification was performed using thermostable *AmpliTaq** recombinant *Taq* DNA polymerase. *Taq* DNA polymerase was purified by a method developed at the Cetus Corporation using an *E. coli* host containing a genetically engineered form of the *Taq* DNA polymerase gene, which came originally from the bacterium, *Thermus aquaticus*, as described by the manufacturer (Perkin-Elmer Cetus).

Fig. 15. A region of human mitochondrial ribosomal 12s gene showing the positions and sequences amplified by the oligonucleotide primers, A and B, with PCR amplification (Anderson *et al.*, 1981; Kocher *et al.* 1989). The primers 12sA and 12sB were generated from the DNA region and should amplify 386 bp nucleotides. The numbers refer to the base pair position from the origin.



Primer A >

CAAACTGGGATTAGATACCCCACTAT/GCTTAGCCCTAAACCTCAACAGTTAAAT
 CAACAAAACCTGCTCGCCAGAACAACACTACGAGCCACAGCTTAAAACCTCAAAGGACCT
 GGCGGTGCTTCATATCCCTCTAGAGGAGCCTGTTCTGTAATCGATAAACCCCGAT
 CAACCTCACCACCTCTTGCTCAGCCTATATACCGCCATCTTCAGCAAACCCTGAT
 GAAGGCTACAAAGTAAGCGCAAGTACCCACGTAAAGACGTTAGGTCAAGGTGTAG
 CCCATGAGGTGGCAAGAAATGGGCTACATTTTCTACCCAGAAAACCTACGATAGC
 CCTTATGAAAACCTTAAGGGTTCGAAGGTGGATTTAGCAGTAAACTAAGAGTAGAGTG
 CTTAGTTGAACAGGGCCCTGAAGCGCGTACACACCGCCCGTCACCCTC

TGTGTGGCGGGCAGTGGGAG

< primer B

The amplification followed manufacturer's manual (Perkin-Elmer Cetus) and some modifications were done to suit the 12s-A and B primer amplification. The working solution included 1 X *Taq* reaction buffer; 1.25 uM each of dATP, dGTP, dTTP and dCTP; 1.30 uM 12sA; 1.30 uM 12sB; 0.2 units of *Amplitaq* DNA polymerase. The 1 X *Taq* reaction buffer was composed of 10 mM Tris-HCl pH8.3, 50 mM KCl, 3 mM MgCl₂ and 0.01% (w/v) gelatin (Sigma, Cat. No. G2500). The DNA concentration was tested in order to obtain enough DNA yeilds per 50 ul reaction. In practice 10⁻² - 10⁻⁴ times of original DNA solution was used, which contained samples with a few copies of DNA.

The reactions occurred with 50 ul of reaction mix with oil in capped 0.5 ml, polypropylene microcentrifuge tubes. The tubes had been siliconized by 5% dichlorodimethylsilane in chloroform. Before use for the reactions, the tubes were sterilized to take out the nucleases and minimize contamination.

The machine used to amplify DNA was a Programmable Cyclic Reactor Model TCX15A (Ericomp Inc). The conditions of optimization for the reaction were chosen and determined by trying different cycles and temperatures. The first step was initial melting at 94°C for 1.30 min, annealing at 48°C for 2 min and extending at 72°C for 3 min. The second step was melting at 92°C for 1 min, annealing at 48°C for 2 min and extending at 72°C for 3 min. The third step was melting at

92°C for 1 min, annealing at 48°C for 2 min and extending at 72°C for 10 min. Steps 1, 2 and 3 were set for 1, 35 and 1 cycle, respectively.

3. DNA sequences and analysis

The amplified DNA was centrifuged in a Centricon microconcentrator 30 (Amicon Division, W.R. Grace & Co. Conn., 24 Cherry Hill Drive, Danvers, MA 01923) and purified on a 1.2% NuSieve GTG agarose gel (FMC BioProducts) by electrophoresis, electroeluted out of the gel, and digested with restriction endonuclease Mbo I. This amplified DNA was then ligated into the BamH I site of Bluescript SK or pVZ1 and used to transform *E. coli* (Sambrook, et al. 1989). The plasmid DNA containing the amplified region was isolated from *E. coli* on NZY plates containing 40 ug/ml X-gal, 160 ug/ml IPTG and 100 ug/ml ampicillin. The inserts in the plasmid were isolated with Mini-Prep (Pharmacia) and cut to confirm their identity with the amplified region.

The sequences were determined by double-strand dideoxynucleotide sequencing. The sequencing protocol used was the Sequenase Version 2 (U.S. Biochemical Co. P.O.Box 22400, Cleveland, Ohio 44122) according to the manufacturer's instructions.

Because the overall fidelity of sequences from PCR-amplified clones is about 0.25% nucleotide misincorporation with no detectable insertions or deletions (Saiki et al.

1988), several clones were pooled and sequenced two to four times to insure accuracy.

Sequences from the amplified fragments were used to search and compare the database of the sublibrary and the EMBL library. Sequence comparisons with database were analyzed by Fasta, Lfasta and Plfasta programs (Pearson, 1990). Sequences were aligned with ESEE (Cabot and Beckenbach, 1990) and the distance matrix was calculated using PHYLIPS computer programs of DNA sequence analysis (Felsenstein 1990). The final alignments adjusted by a Eyeball sequence edit program, ESEE. The ESEE program was employed to prepare the sequence figure. Different phylogenetic trees were constructed using various software packages, including CLUSTAL (Higgins and Sharp 1988) and PHYLIPS version 3.3 (Felsenstein 1990).

Results

1. Amplification and comparison of *Meloidogyne* DNA

When the pair of primers, 12sA and 12sB, was used, they amplified parts of the genomic DNA of all nematodes. The size of the amplified fragment was 1.5 kb, which was almost three times larger than that amplified from the DNA of other organisms. Fig. 16 shows the DNA fragments amplified by the primers and comparison with those of white sturgeon (*Acipenser transmontanus*).

The primers amplified three different species, *M. incognita*, *M. arenaria* and *M. javanica*, and six populations of *M. incognita*. All of the amplifications gave the same results. There were no size differences between the amplifications. The amplified fragments were digested with restriction endonucleases: EcoR I, Xba I, Hind III, EcoR V, BamH I, Hinf I, Mbo I, Hae III, Alu I and Hpa I. The enzymes, EcoR I, Xba I, Hind III, EcoR V and BamH I did not digest the DNA fragment, but Hinf I, Mbo I, Hae III and Alu I digested the DNA and produced 2 to 5 bands. However, the bands did not show the differences between the species and populations. The results are shown in Fig. 17.

The amplified DNA was purified, digested with Mbo I and cloned to the BamH I site of PVZ 1. The cloned fragments could be cut out with EcoR I and Xba I. By comparing with the original restriction digests, the different sizes of the fragments were identified (Fig. 18) and the selected clones were used for further sequence identification.

The cloned DNA fragments were sequenced and compared with the database and the EMBL nucleotide sequence data library. The DNA sequences did not belong to the sequence group of mtDNA, shown by comparing to whole mtDNA sequences of *C. elegans* (personal communication, 1990. David R. Wolstenholme, Department of Biological Sciences, University

Fig. 16. Comparison of DNA fragments amplified from *Meloidogyne incognita* and sturgeon. The numbers on the left refer to the base pair position from the origin in kb. Col. 1, one kb marker; Col. 2, control, the reaction solution containing every component except the target DNA; Cols. 3 and 4, DNA fragments from *M. incognita* races 1 and 3 respectively, amplified by the 12sA and 12sB primers; Col. 5, DNA fragment amplified from sturgeon. The number of PCR cycle is 35. Five ul of each reaction was loaded.

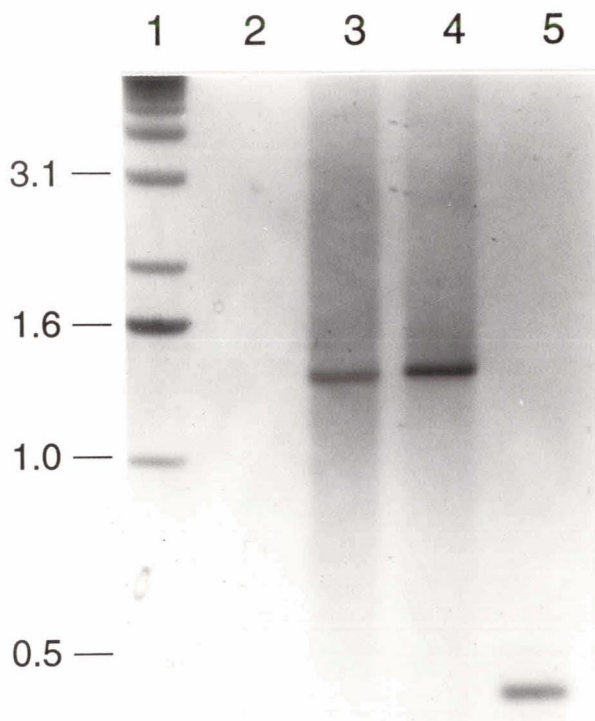


Fig. 17. Amplified DNA fragments in each column from *Meloidogyne incognita*, *M. arenaria*, *M. javanica* and their restriction digests by primers 12sA and 12sB. Col. 1, DNA marker; Cols. 2-4, amplified DNA which could not be digested by restriction endonuclease, EcoR I, Xba I, Hind III, EcoR V and BamH I; Cols. 5-7, DNA fragments digested by Hinf I; Cols. 8-10, DNA fragments digested by Hae III; Cols. 11-13, DNA fragments digested by Mbo I. The number of PCR cycle is 35. Five ul of each reaction was loaded.

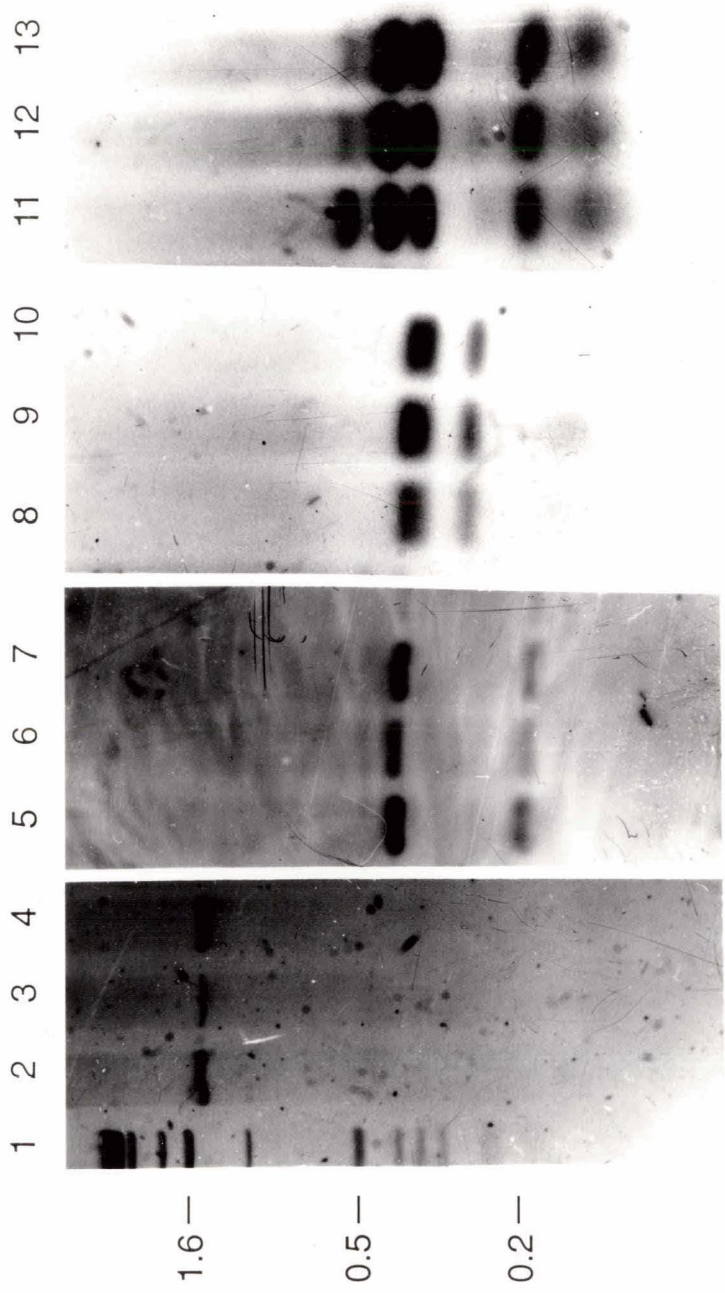
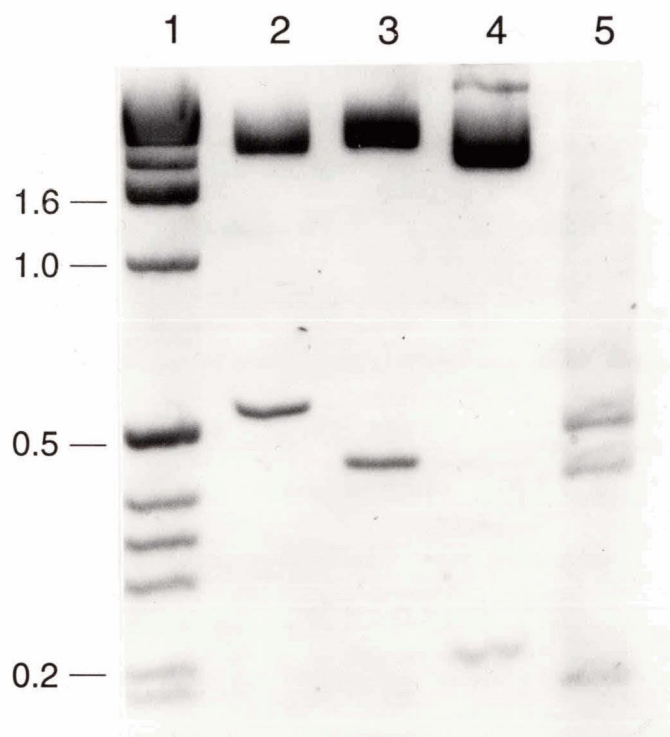


Fig. 18. Comparison of subcloned DNA fragments from *Meloidogyne incognita* with those of direct amplification. Col. 1, DNA marker: the numbers on the left refer to the base pair position from the origin; cols. 2-4, different clones of the fragments, which fall into positions similar to those in col. 5; col. 5, DNA fragments amplified by 12sA and 12sB and digested with Mbo I.



of Utah, Salt Lake City, UT). By comparing the sequence with databases by FASTA and PLFASTA, the query sequences were a sequence similar to 18s ribosome DNA. The comparison of the sequences with those of *C. elegans* are shown in Figs. 20 and 21. The PLFASTA program compared the two sequences with that of other sequences to identify regions of sequence similarity and presents a dot-matrix-like plot of similar regions, which is shown in Fig. 19. The result shows that *M. incognita* sequence was similar to the 18s rDNA of *C. elegans*, and showed some similarities to Bm 18s, the part sequences of pine wood nematodes. The best similarity score between compared sequences is *C. elegans* and *M. incognita* and the two sequences have an 81.8 % similarity. The search result also shows that the sequences are partially homologous with 18s rDNAs of human (75.9) and other animals, such as, *Xenopus laevis* (76.7%), rat (76.2%), rabbit (76.0) and brine shrimp (78.1%). The distribution of other initial scores indicated that the rest of the sequences were unrelated to the amplified DNA sequences.

By analyzing the similarity of the two sequences, the fragment amplified by the pair of primers appears to be an 18s ribosome DNA. The two sequences were further adjusted by ESEE program on a pairwise basis and showed the presence of conserved regions and accurate parallel alignment (Fig. 20). The overall length of the aligned region is 1182 bp.

Fig. 19. The local sequence similarities between the amplified sequence of *Meloidogyne incognita* and five related and unrelated DNA sequences from the database. The sequences were compared with minimum identities of 6 base pairs. The aligned regions were denoted by the solid line from residue pair. Ce-18s and Bm-18s represent 18s rDNA of *Caenorhabditis elegans* and pine wood nematodes, respectively. The dotted lines indicate regions which have less or no similarity to *M. incognita* sequences such as small rDNA of human (Man-small) and fly (Yak-small), and d-loop DNA of cow (Cow-dloop).

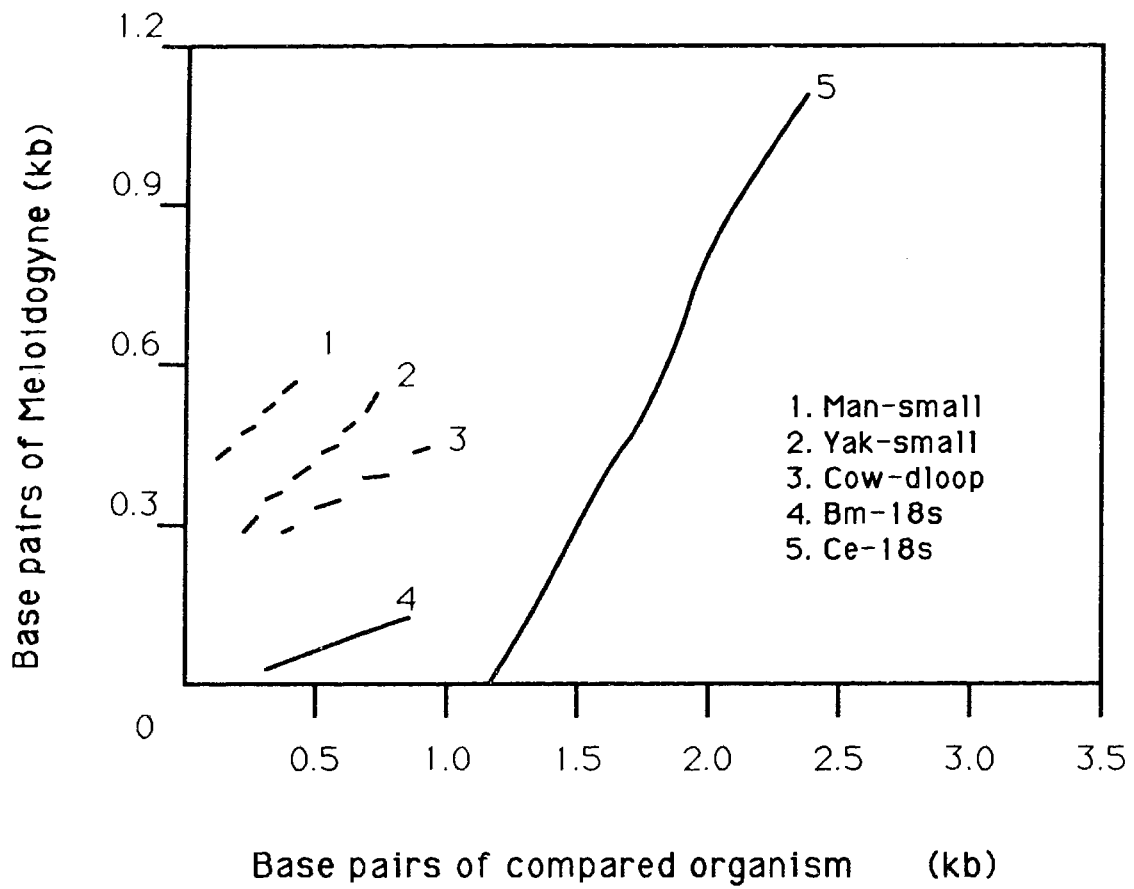


Fig. 20. Comparison of the nucleotide sequences amplified from *Meloidogyne incognita* (bottom line) and related 18s rDNA of *Caenorhabditis elegans* (top line). The dotted lines were used to match the maximum alignment between the two sequences. The vertical bars show identical nucleotides between the position of compared sequences.

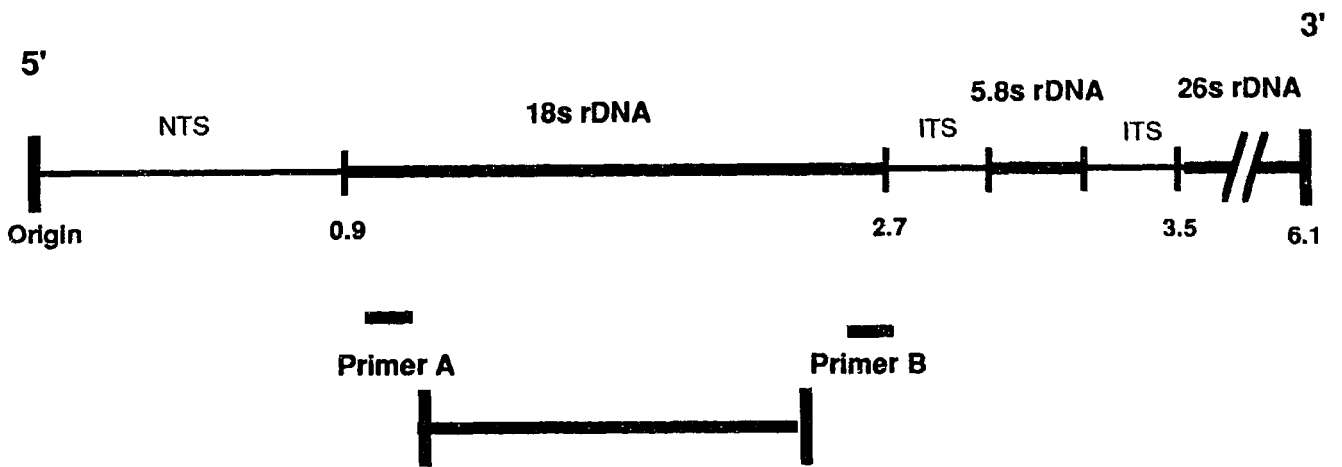
By comparing the sequences with a sequence map of *C. elegans* (Ellis, et al. 1986) and other nematode genomes (Vahidi et al. 1988), the fragment position in ribosome DNA was estimated and is shown in Fig. 21. The *Meloidogyne* fragment amplified by 12sA and 12sB falls into the middle region of 18s ribosome DNA, from position 1285 to 2440.

2. 18s-like rDNA sequences of *M. incognita* races 1, 2, 3 and 4

Six populations of *M. incognita* were used to amplify 18s rDNA using the primers 12sA and 12sB. The results showed no differences between the populations either in the size of amplified fragments or in polymorphism of restriction fragment length.

The amplified sequences of the different populations were cloned and sequenced. The sequences were compared and analyzed. Fig. 22 shows the sequence comparisons between the six populations. There existed nucleotide substitutions between the populations of *M. incognita*. The differences of the 680 bp 18s-like rDNA were 0-1.7 % between populations. The details of the genetic distances of the six populations were shown in Table 4.

Fig. 21. DNA map of the amplified fragment from *Meloidogyne incognita*. The numbers show the position in rDNA sequences of *Caenorhabditis elegans*. The primer positions were at 920 and 2500. The position of the 18s-like rDNA amplified and sequenced from the nematode of *Meloidogyne* are between 1285 and 2440 in the rDNA genetic map.



Sequenced DNA (18s rDNA of *Meloidogyne*)

DNA map of *Meloidogyne incognita* amplified by Primer A and B

Table 4. The genetic distances of the six populations of *Meloidogyne incognita* calculated from sequences of 18s rDNA.

Populations	Distances				
	Mi1B	Mi1M	Mi2B	Mi3M	Mi3O
Mi1M	0.004				
Mi2B	0.006	0.003			
Mi3M	0.014	0.015	0.017		
Mi3O	0.014	0.015	0.017	0.000	
Mi4B	0.011	0.010	0.007	0.014	0.016

Fig. 22. Sequence comparisons between the six populations of *Meloidogyne incognita*. The alignment of six sequences for the 18s-like nuclear DNA from races 1, 2, 3 and 4 showed their identical regions and differences. The bases present in the other five sequences appear only where they differ from the top sequence, with identify being indicated by a dot. The sequences are numbered according to the start point (top left corner).

Mi1B GATCAGGGTTCGACTCCGGAGAAGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCCGAAACTTACCCACTCTCGGCCT 90
Mi1M
Mi2B
Mi3M
Mi3O
Mi4B

Mi1B CTAGGAGGTAGTGACGAGAAATAACGAGACTGTTCCCTTTGAGGCCGGTCATCGGAATGGGTACAATTTAAACCATTTAACGAGTAGCAA 180
Mi1M
Mi2B
Mi3M .G.....C.....T...
Mi3O .G.....C.....T...
Mi4B .G.....

Mi1B GCAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATCCAGCTCTCCTAGTACATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGG 270
Mi1M
Mi2B
Mi3MG.....G.....A.....
Mi3OG.....G.....A.....
Mi4B

Mi1B ATTCGTATCGATACCTTGGAACTCCGGGTGTTCTGGGTGTTATCGATTATCGTAATGTTGCGGTTTTGAGTTCCTAACAGGCTTCTT 360
Mi1M
Mi2B
Mi3MC..A.....
Mi3OC..A.....
Mi4BG.....

Mi1B AACAGGCATTGCAAGTTTACTTTGAACAAATCAGAGTGCTTCAAACAGGCCGTTTTGCTTGAATGATCGTGCATGGAATAATAGAAAATG 450
Mi1MG.....
Mi2BC.....G.....
Mi3M
Mi3O
Mi4BC.....C.....

Mi1B ATTTCCGTTCAAGTTTATTGGTTTTACGGACTGAGATAATGGTTAACAGAGACAAACCGGGGGCATTGTATGGTCCCGTGAGAGGTGAA 540
Mi1M
Mi2BT.....
Mi3M
Mi3O
Mi4BT.....

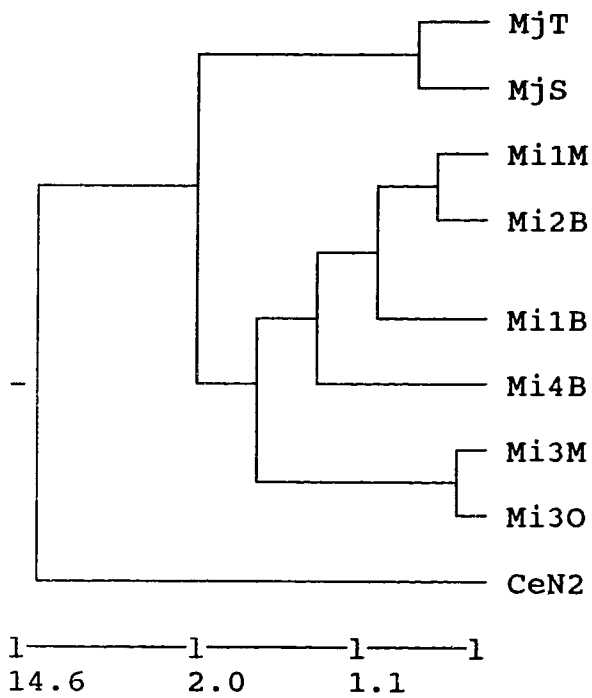
Mi1B ATTCCTGGACCGTGCCAGACAACTACAGCGAAAGCATTGCAAGAATGTTTTCATTAAATCAAGAACGAAAGTCAGAGTTTCAAGCC 630
Mi1MG.....
Mi2BG.....
Mi3MG.....
Mi3OG.....
Mi4BG.....

Mi1B GATCAGATACCGCCCTAGTCTGACCGTAAACGATGCCAACTAGCGATCC 680
Mi1M
Mi2B
Mi3MT.....
Mi3OT.....
Mi4BT.....

3. Phylogenetic tree of *M. incognita* populations

The PHYLIPS and CLUSTAL phylogenetic analysis programs were used to construct phylogenetic trees relating the six populations of *M. incognita* and other nematodes. The trees were also constructed by using distance methods based on a neighbor-joining algorithm. Several trees with similar tree lengths, differing only slightly in the lengths of various branches among these populations had identical topologies. Fig. 23 shows the phylogenetic tree relating the populations of *Meloidogyne* based on phylogenetic analysis using parsimony and a neighbor-joining algorithm. This tree has a minimum tree length and clearly shows the separation of the species and populations. Nematodes at the species level were clearly classified into groups with different tree length. The sequenced populations of *M. incognita* were differentiated into three distinct groups, races 1 and 2, race 3, and race 4, and their evolutionary phylogeny was noted. The relationships between races 1 and 2 cannot be definitively resolved because of the high degree of sequence similarity. Races 3 and 4 were more clearly separated than races 1 and 2.

Fig. 23. Evolutionary tree based on 680 unambiguously aligned positions of the 18s-like rDNA sequences of six populations of *Meloidogyne incognita*, two populations of *M. javanica*, and one population of *C. elegans*. The dissimilarity between the species and races is obtained by summing the lengths of the connecting branches, measured along the horizontal axis and using the scale at the bottom (the scale is not drawn proportionally).



Discussion

Meloidogyne species are an interesting group for study due to their importance in agriculture and complexity in reproduction and evolution. *M. incognita* reproduces by parthenogenesis (Triantaphyllou 1985) and this species has been distinguished from the others by different methods (Chitwood 1949; Taylor and Sasser 1983; Esbenshade and Triantaphyllou, 1985; Curran *et al.* 1986). The races of *Meloidogyne* are traditionally differentiated by plant hosts, which gives little information about genetic relationships of nematodes. At the morphological level, the races are virtually indistinguishable, but at the molecular level they show a certain degree of divergence. The attempts to determine the relationship among the races employed direct views of RFLP's (Curran, *et al.* 1986) and DNA-DNA hybridization (Castagnone-Sereno *et al.* 1991). The characterization of whole genome electrophoresis or hybridization of the unique DNA sequences of the genomes from different species is potentially a powerful analytical technique because it compares almost all of the information contained in the genome. There are, however, practical limitations to this technique. When most of the sequences have diverged to a degree that only a small fraction of the genome will hybridize at a reasonable temperature, this

technique may generate insufficient data for analysis of the sequence similarity.

As an alternative to the above methods, specific portions of the genome can be isolated and the sequences of these regions compared. This is not a global comparison of the genomes, but because individual sequences are compared, regions with an appropriate amount of divergence can be chosen. It has been estimated, for hominoid data, that a comparison of sequences amounting to about 5 kb will provide a similar degree of comparison to that provided by DNA-DNA hybridization of the entire genome (Felsenstein 1981, 1987).

The PCR process is a powerful method, which allows rapid amplification of DNA fragments in vitro through a succession of incubation steps at different temperatures (Saiki et al. 1985, 1988). PCR amplification and sequencing of a specific region of the genome is useful for making evolutionary comparisons. Application of this procedure to *Meloidogyne* has the potential to greatly facilitate the sequencing of homologous regions from different races. A single set of primers can be used to amplify a specific homologous region from a group of species or races. The 12sA and 12sB were used to examine the group of *Meloidogyne* populations. Analysis of the PCR amplification of the 18s rDNA region suggests that the races of *M. incognita* diverged only recently and some of the races were separated by no more than a few nucleotide differences although there are certain degree divergences between them.

In order to differentiate *Meloidogyne* populations, several sets of primers were obtained from sequenced DNA regions and tested for the presence of amplifying homologous segments of the nematode DNA by means of the PCR method. The primers employed to amplify the *Meloidogyne* DNA included: Gly-His (Kelley Thomas, University of California, Berkley), Hsp70A-Hsp70B (Karen Beckenbach, Simon Fraser University, B.C.), CoIIA-B (Kelley Thomas, University of California, Berkley), XR1-2 (Terry Vrain, Agriculture Canada, Vancouver), 18xA-B and 12sA-B (Karen Beckenbach, Simon Fraser University, B.C.). The 12sA and 12sB were a pair of primers chosen that were useful in differentiating the species complex. The chosen primers might be used as potential specific diagnostic primers for differentiation of populations.

The 12sA and 12sB primers originated from human 12s genes, but the primers did not amplify 12s of *Meloidogyne*. The rDNA genome has proved to be one of the most interesting and useful molecules for studying evolution. It has been compared in many closely related species and populations in plants (Troitsky, et al 1991), animals (Hedges et al. 1990), and protozoans (Schlegel, et al. 1991). The 5S and 5.8S rRNA molecules are short with relatively conserved sequences, which provide only a broad outline of the relationships among the species being tested. The 18s rRNA, which has been studied extensively, is much longer and

usually more than 1700 bp, which can be used to determine nematode phylogenetic relationships.

Ribosome DNA is conserved in evolution as are the protein genes. Sequences that show more than 20-25% identity over their entire length almost always share a common ancestor, and sometimes it is possible to show convincingly that sequences which have as little as 15% identity over their entire length are homologous (Pearson 1990). From the conserved nature and similarity of rDNA and protein sequences, analysis of the similarity of the sequences of *M. incognita* and *C. elegans* shows that the two sequences have a common ancestry or at least are homologous to certain degree.

The analysis of the more than 600 bp nucleotides of 18s-like rDNA that were sequenced, showed the more obscure differences between races of *M. incognita* and partially determined their population affinities. By using multiple sequence alignments and cluster analysis of the nucleotide divergences, the sequenced populations of *M. incognita* were separated into three groups. The results showed that there were no large differences between races 1 and 2, as compared with other races, and the two races overlapped (Table 4; Figs. 22 and 23). Races 3 and 4 were distinctly different from races 1 and 2. This may reflect their evolutionary phylogeny.

Phylogenetic analysis shows that populations of *M. incognita* vary in their genetics. From a large number of

populations of *M. incognita* examined from different areas, the races were unevenly distributed and race 1 represents 71% of the species accessions (Sasser and Carter, 1985). Within this species, there are two groups: a rare diploid with $2n$ =about 36 and a prevalent triploid with $3n$ =42-44 and they reproduce by mitotic parthenogenesis (Triantaphyllou, 1985). Mutation and genetic drift probably maintained diversity in the populations of these nematode species and gene flow would operate at different rates among the partly isolated populations. Possibly as a result of coevolution, the nematode populations have responded to selection as influenced by their hosts and by plant breeding selections and so demonstrate their diversity.

A nematode race is defined as an interbreeding population with consistently distinctive characters, either morphological or physiological, or both. A race is partially isolated from other intraspecific groups by geography or genetics. The races of *M. incognita* are descriptively defined by their ability to reproduce on certain members of a set of differential hosts. The differential hosts used to recognize the four major races of *M. incognita* are tomato, watermelon, cotton and cucumber (Sasser 1954). However, these differential hosts are from different families and it is possible that there is a larger variation between the nematode races than that between the races of other plant pathogen. One may speculate, therefore, on whether more races of *M. incognita* will be identified.

Although we strive for standardized tests to separate races, the intraspecific variants, with special attention to the genetic stability of components in sets of differential hosts, the genetic determinants and implications of the race status in the host or parasite are still unknown. The methods of molecular biology are rapidly being applied to practical problems of identification of races of the economically important nematodes and details of their genomes. Further studies will broaden and clarify the concept of nematode races.

PART IV

POLYMERASE CHAIN REACTION FOR MELOIDOGYNE DIFFERENTIATION

Management strategies for *Meloidogyne* include sanitation and the use of resistant cultivars, and their success depend on the ability to identify species and races of the nematodes in order to coordinate different control programs. The objective was to apply the sensitive PCR technique (Saiki *et al.* 1988) to differentiate *Meloidogyne* at the species and race levels. Different primers were used to amplify DNA sequences of *Meloidogyne* species and races and the amplified fragment length polymorphism (AFLP) from genomic DNA was viewed directly and used to differentiate the nematode populations.

Materials and methods

1. DNA sources

The nematodes used in the experiments were: *M. incognita* races 1, 2, 3, and 4; *M. arenaria* race 1, *M. javanica* and *M. hapla*. Details of the nematode stocks and their sources are listed in Table 1.

These stock cultures were maintained and the nematodes extracted as the procedure described under general materials and methods. The populations of *M. incognita* race 3 O, *M. arenaria* race 2 M and *M. javanica* S were each increased from single egg masses from tomato plants, cv. Bonny Best, and further increased in tomato, cv. Rutgers.

DNA was extracted from nematode eggs and further purified and quantified by the methods described in general materials and methods. The concentration and purity of the DNA were determined by spectrophotometric measurement as described earlier.

2. Oligonucleotide primers and purification

Several primers were tested to amplify *Meloidogyne* DNA for diagnostic purposes. Five regions were tested with primers: the COII and 12s gene in the mitochondrial genome, the heat shock 70A, the 18s ribosomal genes and internal transcribed spacer in the nuclear genome. The COII and 12s primers of the mitochondrial genome were obtained from Dr. A. Wilson's (University of California, Berkley) laboratory and the primers amplifying the part of 18s ribosomal genes and the internal transcribed spacer in the nuclear genome were provided by Dr. T. Vrain, Agriculture Canada. The sequences of primers that amplify heat shock 70A gene were

5'-GAC ACC GAG CGT CTA ATC GGA G -3' and

5'-CAT GGT GGA GGT TCT AGC TTC-3'

(Beckenbach, et al. 1991). One pair of the primers, CA and CB, was originally designed for amplifying a mitochondrial DNA fragment (Clary and Wolstenholme 1985) and used for differentiation of *Meloidogyne* populations. The primer CA is 21 bp nucleotides and the sequence from 5' to 3' is GAT

CGC AGA TTA GTG CAA TGG. The primer CB is 20 bp nucleotides and the sequence from 5' to 3' is GAT CAA GAG ACC AGT ACT TG. The primer was sequenced on the 391 DNA synthesizer, PCRMate EP (Applied Biosystems Co.) in our department.

Synthetic oligonucleotide primers were purified by using C-18 Sep-Pak Cartridges following the maker's instruction (Water Associates #51910). A C-18 Sep-Pak cartridge was flushed with 10 ml of 100% HPLC-grade acetonitrile using a syringe, and then flushed with 10 ml of distilled, deionized water. The synthetic primers were dissolved in 1.5 ml of 0.5M NH₄OAC and added onto the cartridge slowly with the syringe. The cartridge was washed with 10 ml of sterile double distilled water and the oligonucleotide primers were eluted out twice with 1 ml of 20% acetonitrile. The collected fractions were evaporated to dryness, rinsed with 90% ethanol and re-dried with a Speed Vac drying machine (SVC 100, Savant Instrument Co.). The purified primers were dissolved in 100 ul H₂O and further quantified and used for DNA amplification.

3. Amplification conditions

PCR amplification was performed using the thermostable *AmpliTag* recombinant *Taq* DNA polymerase. The optimum working solutions were determined by varying the concentrations of DNA templates, primers and salts. The working solution

included: 1 X *Taq* reaction buffer; 1.25 μ M each of dATP, dGTP, dTTP and dCTP; 1.30 μ M primer CA and CB; and 0.3 units of *Amplitaq* DNA polymerase. The 1 X *Taq* reaction buffer was composed of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂ and 0.01% (w/v) gelatin (Sigma, Cat. No. G2500, P.O. Box 14508, St. Louis, MO 63178). The DNA concentration was tested in order to yield enough DNA per 50 μ l reaction. In practice 10⁻² to 10⁻⁴ of original DNA solution was used and this contained samples with a few copies of the DNA.

The reactions were performed with 50 μ l of reaction mix in capped 0.5 ml, polypropylene microcentrifuge tubes. The tubes were siliconized by 5% dichlorodimethylsilane in chloroform. Before the reactions were processed, the tubes were dry-sterilized to take out the nucleases and minimize contamination.

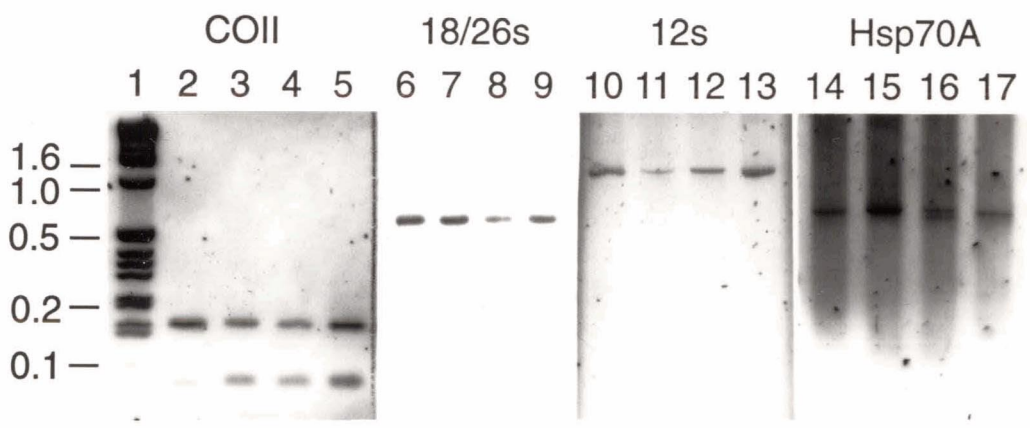
The amplification was conducted in the Programmable Cyclic Reactor Model TCX15. The optimum amplification conditions were determined by varying the denaturation, annealing, and extension temperatures and the number of cycles. The first step was initial melting at 94°C for 80 s, annealing at 47°C for 2 min and extending at 72°C for 2 min. The second step was melting at 92°C for 30 s, annealing at 47°C for 1 min and extending at 72°C for 2 min. The third step was melting at 92°C for 30 sec, annealing at 47°C for 1 min and extending at 72°C for 10 min. Steps 1, 2 and 3 were set for 1, 35 and 1 cycle, respectively.

The amplified DNA fragments were separated on a 1.2% agarose gel (Agarose low EEO #100439, Boehringer Mannheim Biochemicals, Indianapolis, IN), stained with ethidium bromide and viewed under 260 nm UV light.

Results

Several primers were chosen to amplify *Meloidogyne* DNA. The primers, CoIIA-B, Hsp70A-Hsp70B, 12sA-12sB, and 18XR1-18XR2 primers were employed for amplification of the regions of COII (cytochrome oxidase subunit two) and 12s gene in the mitochondrial genome, the heat shock 70A, 18s ribosomal genes and the internal transcribed spacer in the nuclear genome, respectively. The results showed that these primers could amplify the *Meloidogyne* DNA and produce one or two bands. However, none of them amplified more than two bands and did not have species differentiation characteristics (Fig. 24). The primers 18XRA and 18XRB, which originated from *Xiphinema* nematodes, gave a steady band at 0.95. This band was digested with different enzymes, which gave no differences between species. The sequence close to the end of 26s ribosome DNA was obtained by the method of primer-direct sequencing, but there were no apparent nucleotide differences.

Fig. 24. Gel stained with ethidium bromide, showing amplified DNA products with different primers. Col. 1, 1 kb marker; cols. 2, 6, 10, and 14 were *Meloidogyne incognita* race 3; cols. 3, 7, 11, and 15 were *M. arenaria* race 2; cols. 4, 8, 12, and 16 were *M. hapla*; cols 5, 9, 13, and 17 were *M. javanica*; cols. 2-5, 6-9, 10-13, and 14-17 were products amplified by the primers, COIIA-B, 18XR1-18XR2, 12sA-12sB, and Hsp70A-Hsp70B, respectively, with molecular weights of 0.17, 0.90, 1.5 and 0.80, respectively. The number of PCR cycle is 35. The agarose gels were 1.2% and loaded with 4 ul of amplified products and 1 ul of loading buffer.



In the original experiments, the primer CA and CB amplified *Meloidogyne* DNA, and several bands were produced (Fig. 25) and using the standard amplification procedure described, the bands were stable. The concentration of magnesium and the temperature were critical for obtaining multiple bands. The bands changed with variation of the amplifying conditions, especially different temperatures. The optimum conditions for amplification were determined in subsequent experiments. The optimum concentration of magnesium and the annealing temperature were 4 mM MgCl₂ and 47°C, respectively. The amount of amplified products increased slightly with increased DNA polymerase and increased with the reaction up to 45 cycles.

With optimum conditions of amplification, the DNA from different species and races was amplified with the pair of primers. The amplified results are shown in Fig. 26. The major bands at 0.95 kb and 0.54 kb were identical between species and races. There were no differences between the weak bands at 1.1 and 1.2 kb. The bands at 2.0 kb and 2.5 kb were identical between the populations of *M. incognita*, but different from those of *M. arenaria*, *M. hapla*, and *M. javanica*. These two bands were distinctive characters for *M. incognita* separating the species from other *Meloidogyne* species tested. The bands at 0.7 kb,

Fig. 25. Gel stained with ethidium bromide, showing amplified DNA products with CA and CB primers. Col. 1, 1 kb marker; col. 2, *Meloidogyne javanica*; col. 3, *M. hapla*; col. 4, *M. arenaria* race 2; col. 5, *M. incognita* race 3. The number of PCR cycle is 35. The amplification should not excess 45 cycles, which might give some artifact. The agarose gels were 1.2% and loaded with 4 ul of amplified products and 1 ul of loading buffer.

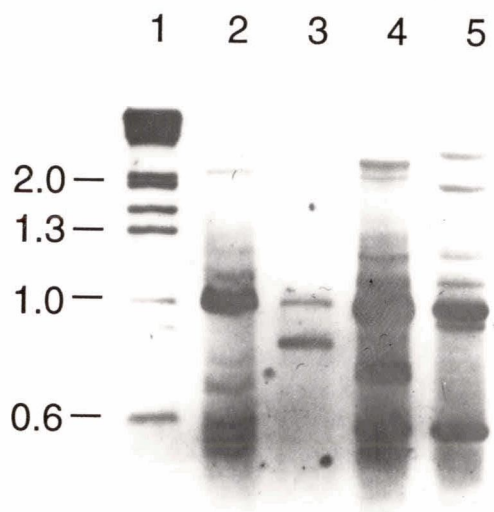
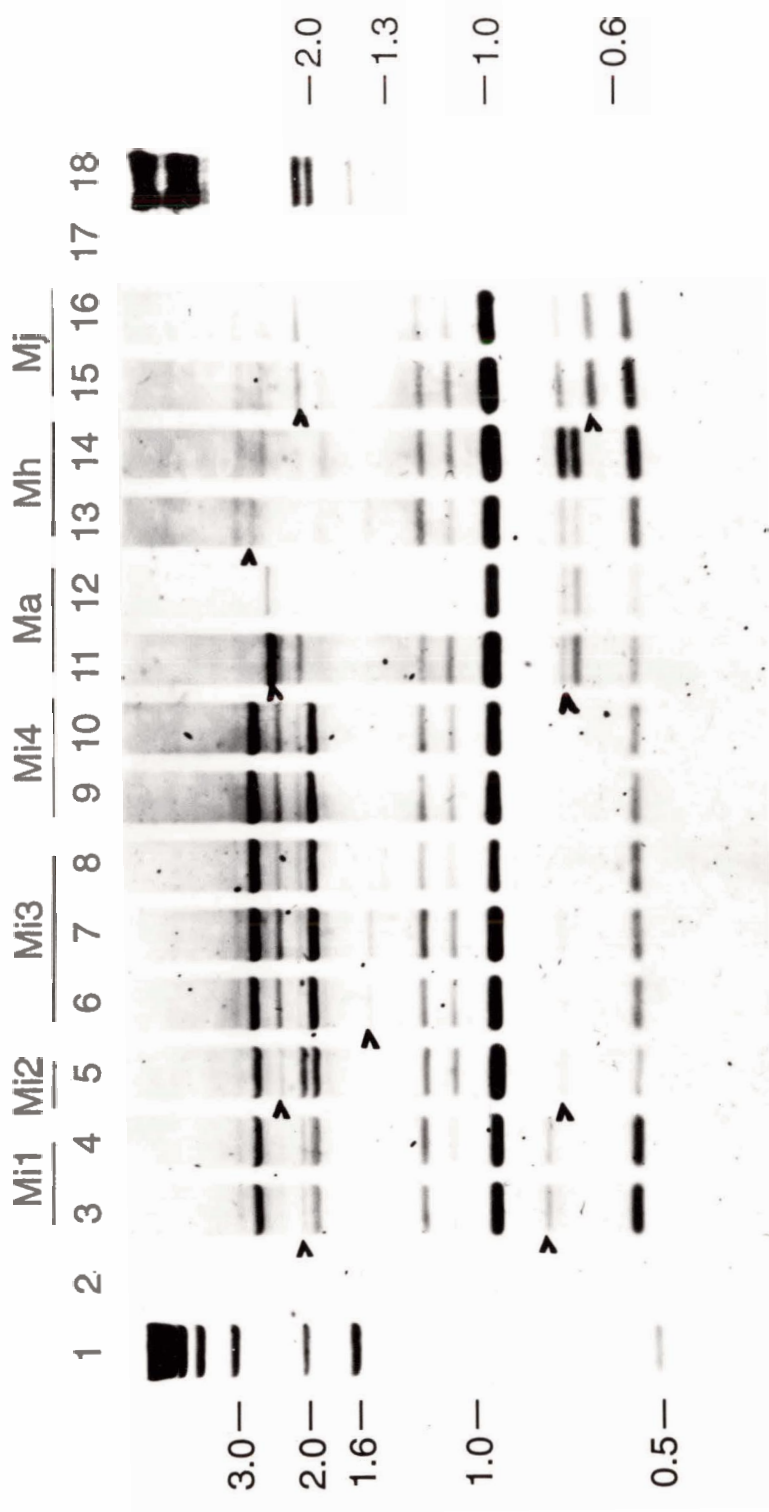


Fig. 26. Gel stained with ethidium bromide, showing amplified DNA products from different populations of *Meloidogyne* with CA and CB primers and the AFLP. Cols. 1 and 18 were standard markers of 1 kb and EcoR I/Hind III cut lambda; cols. 2 and 17 were standard control without primers or DNA; cols. 3 and 4 were *M. incognita* race 1; col. 5 was *M. incognita* race 2; cols. 6 to 8 were *M. incognita* race 3; cols. 9 and 10 were *M. incognita* race 4; cols. 11 and 12 were *M. arenaria*; cols. 13 and 14 were *M. hapla*; cols. 15 and 16 were *M. javanica*. The arrows show the major differences between the species and races. The numbers on the left-hand margin show molecule weight in kb. The number of PCR cycle is 35. The agarose gels were 1.2% and loaded with 4 ul of amplified products and 1 ul of loading buffer.



present in *M. arenaria*, *M. hapla* and *M. javanica*, were not present in *M. incognita*. The bands at 0.65 kb and 0.60 kb differentiated the species of *M. arenaria*, *M. hapla* and *M. javanica*. The bands of 0.60 kb were species specific to *M. javanica* and the bands of 0.65 kb were species specific to *M. arenaria* and *M. hapla*. The differences between *M. arenaria* and *M. hapla* were shown at the position of the first two bands. The first two bands of *M. arenaria* were lower than those of *M. hapla*.

From Fig. 26, it is clear that there were no major band differences between races within one species, but minor differences were seen between populations of *M. incognita* and other species. The bands at 0.8 kb in cols 3 and 4 were present only in race 1, but not in other races of *M. incognita*. These bands were stable between populations of race 1 and were race specific. Race 2 had a weak band at 0.75 kb in col. 5, this band was also present in race 3. Two populations of race 3 had specific bands at 1.5 kb (cols. 6 and 7), but another population of race 3 did not have this band. The population of race 3 was similar to race 4, which did not have distinguishing bands from the other three races of *M. incognita*. The two populations of *M. arenaria* in cols. 11 and 12 had almost identical bands except that the first three had slight shifts. One population of *M. hapla* had a band at 1.4 kb in col. 13,

which was darker than that of another population in col. 14. This band does not specify population characters because it was not stable between replications. The two races of *M. javanica* in cols 15 and 16 were identical and there were no specific bands or differences between them.

Discussion

Genetic differences and species differentiation between *Meloidogyne* populations were first demonstrated by using recombinant DNA techniques (Curran et al., 1985; 1986). Ethidium bromide staining of genomic DNA digested by restriction endonuclease was used to obtain diagnostic characters for species differentiation. The difficulties in visualizing these repetitive bands, due to smear background in a total genomic digest on an agarose gel, can be overcome by using probes as shown in Part II of this thesis. DNA bands detected using most of these homologous probes had a restriction fragment length unique to each *Meloidogyne* species and these differences could be compared and used as diagnostic characters. For example, DNA bands detected by probe pMi9 had a restriction fragment length unique to each *Meloidogyne* species. The pMi10 probe hybridized to DNA from the *M. incognita* and *M. arenaria* populations but only weakly to *M. javanica* populations and appeared as a specific probe for *M. incognita* and *M. arenaria*. Another probe, pMi3,

detected *M. incognita* and, just weakly, the other two species. It showed that DNA hybridization probes were potentially useful for diagnostic purposes once tested against a large number of populations of these three nematode species. Similar species-specific probe hybridizations to restriction fragment length differences were shown for *C. elegans* and its close relative *C. briggsae* by Rose *et al.* (1982), and for *Heterodera glycines*, *H. leuceilyma* and *H. weissii* by Besal *et al.* (1987).

The polymerase chain reaction is an *in vitro* method for the primer-directed enzymatic amplification of DNA (Mullis *et al.* 1987). The enzymatic amplification of specific DNA segments is made possible by the highly specific binding of oligonucleotide primers to sequences flanking the segment. These primers allow the binding of a DNA polymerase that then copies the segment. Because each newly made copy can serve as a template for further duplication, the number of copies of the target segment grows exponentially. Theoretically a single template DNA can be amplified more than a million-fold (Erlich, *et al.* 1988). Saiki *et al.* (1988), using the procedure, demonstrated a 220,000-fold amplification of a 110-bp region of the Beta-globin gene. By using this method with the fairly conserved primers, one or several specific sequences can be amplified *in vitro* in a matter of hours. Because the procedure is easily automated,

dozens of samples can be produced each day with a standard amplification.

PCR technique has greatly increased our ability to resolve genomic differences of closely related populations from small samples and in a relatively short time. A PCR-based approach to identifying nematode species and races has proved taxonomically and diagnostically useful. It should also be paid attention to that PCR amplification can be greatly affected by or some artifacts can be caused by the factors such as the makeup of reaction solutions, the temperatures at different steps, primer concentrations, number of cycles and even the types of amplification equipment.

Both the hybridization to total genomic DNA by probes and PCR amplification of total genomic DNA by primers are useful techniques to differentiate populations of the *Meloidogyne* species complex and to help identify their relationships. By analyzing the nematode genotype directly, they are capable of discriminating details of species and races. Moreover, the DNA can be stored for long periods without deterioration and so is available for future analyses. It was shown that the species complex within the *Meloidogyne* genus could be clarified with a limited set of probes or primers.

When Harris, et al. (1990) used the PCR method to amplify a specific 1.8-kb sequence of mitochondrial DNA from different *Meloidogyne* populations, the restriction digestion of the amplified product with *Hinf I* discriminated four species. PCR amplification is a much faster method than DNA hybridization. With a standard reaction, the specific DNA can be directly amplified with primers in several hours and the amplified fragment can be used for diagnosis. In my experiments with direct viewing of the amplified fragment length polymorphism (AFLP), the results were obtained from genomic DNA within one working day. Because of its direct viewing without restriction endonucleases, the costs of analyzing samples can be kept to a minimum.

Meloidogyne differentiation primers used for diagnostic purposes can be chosen from heterologous or homologous sequences. Primers of broad utility could be found from the fast-evolving mtDNA of animals, designed from parts of nuclear sequences or chosen from random DNA sequences (Williams et al. 1990). The pair of primers, CA and CB was originally generated from mtDNA (Clary and Wolstenholme 1985). Considering amplified products from this pair of primers, the *Meloidogyne* population had some heterogeneity in their genomes (Fig. 26). The primer-amplified regions around 0.60, 0.90, 1.0, and 1.2kb in Fig. 26 were highly conserved between the populations of *Meloidogyne*. There were no differences in the conserved bands between the four

species, *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica*.

Primers with a length between 17 - 20 nucleotides need as few as three homologous nucleotides at their 3'-end for successful priming and the heterology between a primer and its complement sequences could be 57% nucleotide differences at 37°C (Sommer and Tautz 1989). Since sequence alterations affect primer annealing, the differences in amplified bands came from the DNA sequence changes between species and races. In reflection of these genetic differences or sequence changes within the *Meloidogyne* complex, there were some species- and race-specific bands presented for the test populations (Fig. 26). As shown in Fig. 26, at species level, 2.0 and 2.5 kb bands are species-specific bands for *M. incognita*, the 6.5 kb band for *M. arenaria* and *M. hapla*, and 6.0 kb for *M. javanica*. The species-specific bands are shown clearly due to their high diversity and could be used for diagnostic purposes.

The race differences are not as easy to distinguish as species. At race level of *M. incognita* as shown in Fig. 26, there are only minor band differences present in races. However, some of the minor bands are stable within the races, such as 0.8 kb band in race 1, which is a race-specific band and can be used for race differentiation. Some minor bands are not stable within races, such as 1.5 kb band in race 3, which is present in two populations of race 3, but not in another population. The bands showing

population differences within this race cannot be used alone as race-specific diagnostic characters, but can be used in combination with several bands. Further research is needed to search race-specific primers and to obtain major bands for race differentiation and diagnostics. Combination of race differentiation based on primer amplification and race virulence in host plants will facilitate our understanding of pathogen-host relationship and help to find genetic marker for studying pathogen virulence.

The band pattern and signal strength of *Meloidogyne* AFLP from the CA and CB primers showed that there was considerable variation between the nematode species and races. These differences can be further analyzed and used to generate species- or race- specific primers for diagnosis in the field. With the development of PCR and its alternatives in diagnostic technology, simple diagnostic kits based on these primer sequences could be developed. Although the search for suitable and clear race or pathotype specific primers is time consuming and labour intensive, their impact on routine identification of races or pathotypes and on plant breeding for nematode resistance will be considerable and worthwhile.

GENERAL DISCUSSION AND CONCLUSIONS

This thesis has focused on the characterization of *Meloidogyne* populations based on their molecular genome. I concentrated on the genetic analysis of their affinities at the species and race levels, and on the possibility of developing techniques for their practical differentiation.

Root-knot nematodes, *Meloidogyne* spp., have long been a problem to agriculture because they seriously damage almost all commercial crops in the world (Chitwood and Chitwood 1974). Successful control or, more accurately, management of these economically important pests is based on familiarity with the nematodes' biology, understanding their affinities and identifying them reliably, not only to species but also on the race or population level.

In the first part of this thesis, I studied some aspects of the nematodes' biology. I focussed on how the nematodes reproduced and grew on excised roots and how factors, such as time, temperature, host plant and root physiology, may affect them.

From the viewpoint of nematode biology, the excised roots have proved useful in studies dealing with the nematodes and more importantly, this procedure of nematode culture is a good source of axenic nematodes. My results showed that *M. incognita* developed well on excised roots cultured on modified STW medium. There were no apparent

changes in the stability of the infections when they were tested from different generations in greenhouse conditions. The most suitable time to transfer the nematodes from excised roots was around 50 days after infection. However, the nematodes could be kept at 10°C after the egg masses were formed on the excised roots without adversely affecting virulence. This gave an additional 2 - 3 months of potential transfer time. The egg masses survived at 10°C for more than three months. The availability of large numbers of sterile nematodes and their ability to survive and maintain virulence for so long at 10°C are useful factors in the support of further research, such as selection of plants generated from tissue culture for resistance and gene transfer in molecular genetics.

Parts II, III and IV dealt with the genetic affinities and differentiation of *Meloidogyne* populations. It is not easy or straight forward to understand the nematode's affinities and their uses for identification and diagnosis. *Meloidogyne* affinities and identification are traditionally based on phenotypic traits that are the ultimate products of genetic expression, such as anatomical, morphological or enzyme criteria. Although this phenotypic approach has been reasonably successful, all of these characters and criteria have certain limitations and drawbacks, so that simple, character variations have limited usefulness. In particular, these make differentiation of races, slow,

difficult or even impossible in practice. Faced with these problems, nematode differentiation, based on molecular genetics has now been employed to confirm and complement these systems and to offset their limitations. Since the first demonstration of the value of recombinant DNA techniques for the identification of *Meloidogyne* by Curran et al. (1985, 1986) and Curran and Webster (1987), several investigators have studied nematodes using the techniques of molecular genetics and taxonomy and demonstrated characters that can be used to distinguish between the nematode species. These new approaches to characterizing nematodes began only in recent years, but they promise to become indispensable for this purpose.

Several methods have been used in this study to attain the objectives; the major ones were DNA hybridization, DNA sequences and PCR amplification.

In part II, the randomly cloned probes were selected and used for hybridizations. The hybridization patterns revealed by the random probes indicated marked genomic differences between the nematode species *M. incognita*, *M. arenaria* and *M. javanica*. The analysis of fragments digested by endonuclease showed that *M. arenaria* is closer to *M. javanica* than it is to *M. incognita* and that their shared fragments are from 52-69%.

In addition to the value of probe hybridization, it was realized that DNA sequencing methods could reveal nucleotide differences within a fragment of DNA and provide more detailed information between nematode populations than other methods. The PCR-amplified fragment of the 18s-like ribosome DNA were sequenced and compared in the experiments of part III. Multiple sequence alignments and cluster analysis were used to analyze the affinities of the sequenced populations of *M. incognita*. The analyses of the more than 600 bp nucleotides of homologous 18s-like rDNA from six populations of *M. incognita*, including races 1, 2, 3, and 4, showed that there were surprising differences between them. These methods separated the sequenced populations of *M. incognita* into three groups. Their population affinities and evolutionary phylogeny showed that there were few differences between races 1 and 2, as compared with other races; in fact the two races overlapped. However, races 3 and 4 were distinctly different from races 1 and 2.

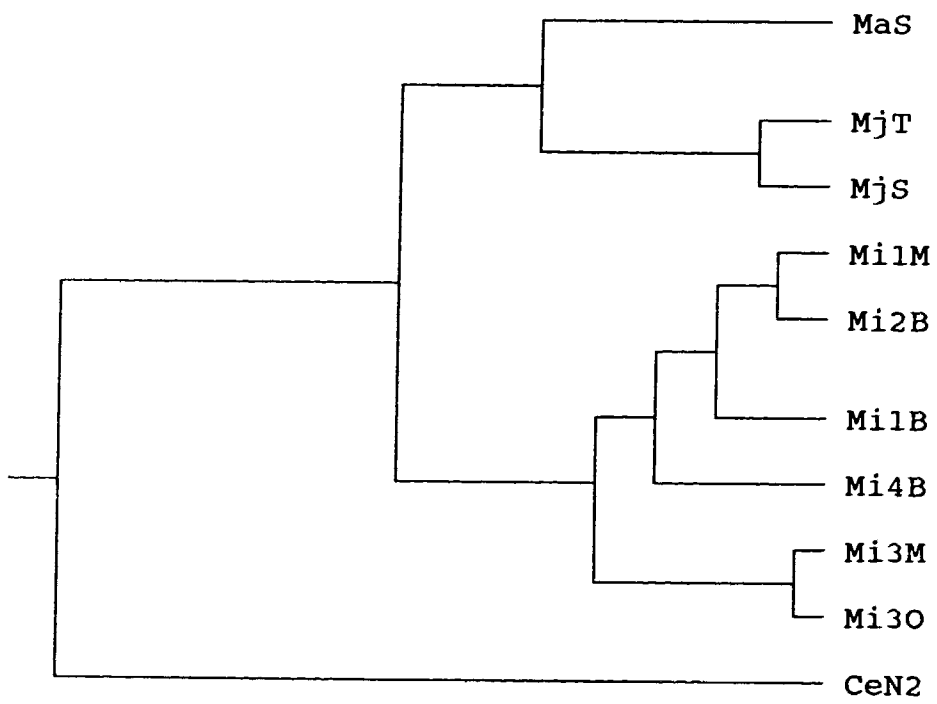
The data from DNA hybridization and DNA sequencing were complementary. Both data sources have the ability to clarify infraspecific differences in nematode populations, but the approaches taken along with the types of information obtained vary greatly. DNA hybridization clearly detects affinities between species that have substantial evolutionary divergences, such as *M. incognita*, *M. arenaria*

and *M. javanica*. Within the species, the races of *M. incognita* for example, diverged only recently and some of the races might represent no more than several nucleotide differences. These minor changes were not easily detected by the DNA hybridization but were detected by the DNA sequence techniques. Comparison of the data derived from DNA hybridization and sequencing for *Meloidogyne* shows that the genetic affinities of these species and races can be clearly demonstrated and summarized (Fig. 27).

The relative genetic similarities or affinities of these species and races are proportional to their DNA structure revealed by the RFLP's, DNA hybridization and DNA sequencing methods. Traditionally, the phylogenetic taxonomy of nematodes are based mainly on typological concepts and other biochemical phenotypes. Due to species as completely defined in reference to their type of an ideal representation and in disregarding of individual and population variation, it is difficult to clarify the species relationships. Since the first formal designation of type specimens of the *Meloidogyne* species and of their taxonomic relationships (Chitwood 1949), different attempts including detail morphology, enzymes, ecology and DNA structure, have been considered to clarify their taxonomic relationships (Baldwin and Powers 1987; Dalmasso and Berge 1983; Esbenshade and Triantaphyllou 1985, 1987; Georgi *et al.* 1986; Hussey 1985; Hussey *et al.* 1972; Hyman 1990; Power and Sandall 1988; Triantaphyllou 1985, 1991). Compared with

other methods, data based on molecular genetics is the ideal method to study nematode systematics and construct phylogenies. The DNA data shows that, at species level, *M. arenaria* is closer to *M. javanica* than it is to *M. incognita* and *M. incognita* is the most genetically distinct of the three species looked at here (Fig. 14 and Fig. 27). This conclusion is supported by the enzymatic data (Esbenshade and Triantaphyllou 1987; Dalmaso and Berge 1983; Dickson et al 1971). Cytological studies also implied that *M. incognita* is distantly related to *M. arenaria* and *M. javanica* by several unique cytological features such as the clumping of the chromosomes during a very prolonged prophase stage in maturing oocytes (Triantaphyllou 1981, 1985). However, this result is not consistent with some morphological differences (Eisenback 1985) and contrasts with the result of Powers and Sandall (1988) who showed, by 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 nematode mitochondrial DNA and genomic DNA (Thomas and Wilson 1991) and by the sampling differences.

Fig. 27. Evolutionary tree based on data from DNA hybridization of random probes and data from 18s-like rDNA sequences. The tree includes six populations of *M. incognita*, two of *M. javanica*, one of *M. arenaria* and one of *Caenorhabditis elegans*. The relative relationships between these groups are shown in the phylogenetic tree. The dissimilarity between the species and races is indicated by summing the lengths of the connecting vertical branches, based on the differences of their nucleotide bases. Branches drawn as diverging simultaneously originate at the same level. The differences are along the horizontal axis from right to left. The further left the branching, the greater the dissimilarity of populations. The scale is not drawn proportionally. See Table 1 for population abbreviations.



The data from DNA sequencing and PCR amplification proves that there exist genetic differences between the races of *Meloidogyne*. Races within species are partially isolated from other races by geography or genetics and are descriptively defined by their ability or inability to reproduce on certain members of a set of hosts. From the sequence data of 18S-like rDNA, it was shown that there were nucleotide substitutions between races. Analysis of these differences showed that the races of *M. incognita* were separated into three groups, races 1 and 2, race 3, and race 4. Races 3 and 4, different from races 1 and 2, may reflect their evolutionary phylogeny. There were no large differences between races 1 and 2, as compared with other races, and the two races overlapped, which demonstrated that race variation of *M. incognita* exists widely and their phylogenetic relationship are complex (Fargette 1987). With the genetic diversity at the race level, it is probable that race phenotypes have resulted from the direction or selection by their hosts, and recently by on-going agricultural operations, and the nematode's responses to these environmental pressures. With genome details of *Meloidogyne* revealed, the concept of their races, including race genetic determinants and implications, can be broadened and clarified.

The PCR techniques have been developed only recently for amplifying a specific portion of a genome (Saiki *et al.*,

1985, 1988). The availability of this technology has greatly increased our ability to resolve genomic differences and provided another tool in molecular genetics with which to differentiate nematodes.

Since the first demonstration of genetic differentiation and identification of *Meloidogyne* populations by Curran *et al.* (1985; 1986), several techniques have been developed. Ethidium bromide staining of genomic DNA digested by restriction endonuclease was used to get diagnostic characters for species differentiation based on visualizing repetitive fragments of DNA. The method with smear background in a total genomic digest on an agarose gel makes it difficult to use in separating between closely related taxa.

The difficulty was overcome by using DNA hybridization probes as shown in Part II. The DNA bands detected by most of these probes had restriction fragment lengths that are unique to each *Meloidogyne* population and these differences can be compared and used as diagnostic characters.

Amplified fragment length polymorphism (AFLP) based on the PCR method was demonstrated to be useful for nematode differentiation and diagnosis (Part IV). Comparison of the amplified products from the primers CA and CB, showed that *Meloidogyne* populations had heterogeneity in their genomes.

With the sequence diversity within the *Meloidogyne* complex, species-specific and race-specific bands were detected in the test populations, which could be used for diagnosis.

Both DNA hybridization to total genomic DNA by probes and PCR amplification of total genomic DNA by primers are useful techniques to differentiate populations. PCR amplification was much faster than DNA hybridization. The results showed that the species and races within the *Meloidogyne* group could be clarified by using a limited set of probes or primers. The conditions of hybridization and amplification might have some effect in explaining the results. Nevertheless, DNA hybridization probes and amplification primers proved potentially useful for diagnosis. Once standard test procedures are established and tested against a large number of populations, the probes or primers will be available for field use.

In order to strengthen our understanding of the nematode affinities and increase our knowledge of the evolution or phylogeny and speciation of nematodes, more diverse species having various modes of reproduction and widespread populations as well as analyses of larger samples are needed for more accurate assessments. The precision of these methods and results should provide nematologists and population geneticists with information that can be used for clarifying the distribution, dispersal, hybridization,

introgression, genetics and phylogeny of these and other important agricultural pests.

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