Journal of Nematology 22(1):24–30. 1990.

Molecular Diagnosis of *Meloidogyne* Species[1]

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Abstract: Genetic variation within nuclear and mitochondrial DNA of *Meloidogyne* species and host races has been evaluated for the development of root-knot nematode molecular diagnostics. This review summarizes the distinctive features of several useful DNA-based assays for plant-parasitic nematodes, focusing upon the direct application of these procedures for *Meloidogyne* detection, identification, and systematics.

Keywords: identification, *Meloidogyne* spp., mitochondrial DNA, nuclear DNA, nucleic acid hybridization, root-knot nematode.

Infestation of agricultural soils by plant-pathogenic nematodes can rapidly devastate important food and fiber crops. The economic consequences of nematode infection are staggering; reduction of domestic crop yield or quality that is directly attributable to nematodes has been estimated at 10–15% (16). Representatives of the genus *Meloidogyne* are among the most destructive of the plant-parasitic nematodes. *Meloidogyne arenaria, M. hapla, M. incognita,* and *M. javanica* represent 95% of the root-knot nematodes observed in agronomic soils. Assessment of world-wide crop loss to *Meloidogyne* spp. alone is estimated to be 5% (7).

Unambiguous diagnosis of root-knot nematodes is essential for successful management practices (23). Phenotypic traits that rely on anatomical, morphological, or other taxonomic criteria traditionally have been used to identify species and subspecific *Meloidogyne* populations. These include perineal patterns, stylet structure, body length, and ability to propagate on defined plant hosts. However, key descriptive characters can often vary considerably between species and among races of the same species (25). To complement these parameters, biochemical discrimination among root-knot nematodes recently has been employed (11). Protein gel electrophoresis, isozyme comparison, and serology now have been evaluated as useful tools in *Meloidogyne* diagnostics. Although each of these approaches has met with some success, cellular expression of proteins often relies on developmental or environmental influences and may not be present uniformly in all individuals or nematode populations. Because these same molecules exhibit conserved function, identifiable differences (particularly among closely related host races) may not be easily detected.

DNA-based molecular diagnostics provides attractive solutions to several problems associated with these identification procedures. Accumulated macromolecules such as proteins and surface antigens represent end products derived from a small fraction of the cell's genetic material. The probability of unmasking genetic variation, especially among closely related species and intraspecific races, escalates dramatically when the entire nematode genome can be directly analyzed. The chemical and heritable stability of the genetic material insures invariant cellular DNA composition during life cycle stages without perturbation by ontogenic or environmental factors. Readily detectable differences among genomes are nucleotide base changes (point mutations) and sequence rearrangement, including the insertion, deletion, inversion, amplification, and transposition of specific DNA segments. Recognition of these nucleic acid alterations provides the basis of root-knot nematode species and host race identification using molecular strategies.

In this review, I first describe the general

[1] Symposium paper presented at the 28th Annual Meeting of the Society of Nematologists; 14–17 August 1989, Davis, California. This work was supported by USDA Competitive Grant #85-CRCR-1-1629, PHS Biomedical Research Funds, and the Agricultural Experiment Station and Research Committee of the University of California, Riverside.

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I thank J. J. Peloquin and D. M. Bird for contributing useful information and E. G. Platzer for his continued support and interest.

methodology involved in developing nematode molecular diagnostics. I then briefly summarize the available information concerning DNA-based assays for discrimination of plant pathogenic nematodes before providing a more complete description of progress toward furthering *Meloidogyne* identification procedures.

Molecular Methodology and Strategies

Root-knot nematode chromosomal (nuclear) and mitochondrial DNA have both been evaluated as possible reagents for nematode molecular diagnostics. Because of differences in cellular copy number, rates of molecular evolution, and sequence composition, each DNA type offers specific advantages depending on the requirements of the investigator.

Successful development of nematode molecular diagnostics requires physical characterization of DNA using restriction enzyme analysis. Restriction endonucleases are proteins that recognize a specific nucleotide sequence (generally 4–6 base pairs in length) within the DNA and cleave both strands of the double helix at that precise target. The DNA is thus cut into smaller fragments of specific sizes. Single base pair changes within the DNA may create or eliminate specific target sites for cleavage by restriction enzymes. Rearrangements may alter the position of restriction sites. Such changes are reflected in the sizes of DNA segments produced by the restriction endonucleases. When fractionated according to size by gel electrophoresis, the patterns of restriction fragments can act as diagnostic markers for closely related nematodes. Easily visualized differences in cleavage product sizes among DNA preparations derived from closely related sources are defined as restriction fragment length polymorphism (RFLP). RFLP analysis can be successfully adapted to all types of DNA irrespective of the source.

Nuclear DNA: More than 90% of the DNA extracted from root-knot nematodes comprises the 30–54 *Meloidogyne* chromosomes (24) that are housed within the cell's nucleus. Like other eukaryotes, root-knot nematodes contain a complex genome composed of about 20% repeated DNA and 80% nonredundant, single copy sequences (15). Most genes encoding information for proteins are found in this latter class.

When subjected to restriction enzyme digestion, chromosomal DNA generates a complex fragmentation pattern after the resultant cleavage products are fractionated by gel electrophoresis. Random placement of restriction sites within large eukaryotic genomes results in a wide array of product sizes after enzyme treatment. This constellation of fragments is displayed as a uniform "smear" of single copy sequences after electrophoretic fractionation. Because some sequences can be repeated hundreds to thousands of times within the genome, bright bands representing reiterated fragments are often superimposed on this smear. RFLP among repeated chromosomal DNA fragments can be visualized directly on gels stained for DNA.

RFLP analysis of repeated nuclear DNA has been employed to differentiate between isolates of several plant pathogenic nematodes (Table 1). Variation among repeated DNA fragmentation patterns are commonly observed when different species are compared. In contrast, RFLP is rarely detected among reiterated sequences derived from intraspecific populations (races, biotypes, strains). This characteristic difference is attributed to "concerted evolution" (10) of repeated sequences by gene conversion, unequal crossing over, or other genetic mechanisms known to homogenize copies of reiterated DNA. In effect, any mutation or rearrangement within a repeated DNA copy would be rapidly cycled through genomes of freely interbreeding populations, thereby minimizing differences exposed by restriction enzyme analysis.

RFLP assessment among single copy DNA is used to survey sequence divergence within the remaining 80% of the genome, where much of the genetic variation can reside. Therefore, single copy RFLP analysis may play a useful role in discrim-

TABLE 1. Molecular diagnostics for plant-pathogenic nematodes.

Genus	Diagnostics available	Strategy	Reference
Bursaphelenchus	*B. xylophilus* pathotypes	Nuclear repetitive DNA RFLP	(2,3)
	B. mucronatus	Nuclear repetitive DNA RFLP	(2)
Globodera	*G. pallida*	Nuclear repetitive DNA RFLP	(5)
	G. rostochiensis	Nuclear single copy, cloned DNA probes	(6)
Heterodera	*H. glycines* (races)	Nuclear repetitive DNA RFLP	(14)
	H. schachtii	mtDNA RFLP	(21)
		mtDNA cloned probes	(1)
Meloidogyne	*M. arenaria*	Nuclear repetitive DNA RFLP	(9)
	M. hapla (races)	*C. elegans* nuclear repeated DNA probe	(8)
	M. javanica	mtDNA RFLP	(18,19)
	M. incognita		

inating among subspecific populations. Unlike repeated DNA, these segments cannot be directly visualized on gels because of their abbreviated abundance in the chromosomes. RFLP analysis at this level must be conducted using the increased sensitivity offered by DNA transfer-hybridization experiments. Chromosomal DNA is cleaved with restriction enzymes and fractionated by gel electrophoresis, the two strands of each restriction fragment are denatured, and the DNA is transferred in situ to nitrocellulose filters. The DNA exposed on the filter is not only a precise replicate of the DNA after migration on electrophoretic gels but is now available for annealing with specific nucleic acid segments labeled in vitro with radioactivity or chromogenic reagents. Generally, tagged "probe" DNA is a single copy DNA segment randomly isolated from the nematode genome by molecular cloning using contemporary recombinant DNA technology. Once labeled and denatured, the probe is able to bind to any single-stranded molecule with which it shares nucleotide sequence complementarity. By incubating the tagged probe with filter-bound DNA, annealing among single copy restriction fragments can readily be visualized by autoradiography or biochemical detection of the attached chromatophore. RFLP will now appear as hybridization signals originating from different sized restriction fragments that are diagnostic for various nematode genomes.

RFLP analysis using transfer-hybridization requires that the labeled probe DNA share sequence complementarity with some DNA component of each nematode genome under comparison. Failure to hybridize to all DNA samples prepared from individual nematode populations would not allow requisite RFLP to be detected. However, the ability of a defined probe DNA segment to hybridize with some genomes, but not with others, has led to the development of species-specific diagnostics. This type of assay permits the rapid detection and identification of nematode infection. DNA-containing crude lysates are prepared from root tissue, galls, or soil and then denatured and spotted directly onto nitrocellulose filters. Numerous samples can be handled simultaneously in this fashion. The uncompromising specificity of nucleotide complementarity between two strands of a double helix permits DNA chains of similar sequence, but not divergent DNA, to anneal in hybridization assays. This feature permits precise detection of target nematode DNA in the presence of unfamiliar microbial and plant-host nucleic acid and is therefore particularly suited to field testing. Probe DNA segments that discriminate between species or races provide the necessary reagents for unambiguous diagnostics in this type of assay. Hybridization signals obtained from adhered DNA spots indicate presence of a specified nematode in that crude preparation, whereas a negative result indicates

its absence. Cloned probes that identify abundant target sequences represented by repeated genomic DNA are especially useful in this context because hybridization signals can be obtained with the limited amount of DNA derived from a single egg, juvenile, or adult that may populate a random field sample.

Mitochondrial DNA: Animal mitochondria contain circular DNA molecules that typically comprise 1–10% of the total cellular genetic material. Mitochondrial DNA (mtDNA) encodes information necessary to elaborate some of the proteins required for electron transport and oxidative phosphorylation. About 5% of the total mitochondrial protein is encoded within the mitochondrial genome and synthesized within the interior of the organelle.

Methodologies and assays that employ nuclear DNA can be exploited in an identical fashion by substituting mtDNA. The advantages of using mtDNA in nematode molecular diagnostics have recently been detailed (12). Briefly, mtDNA is often present in hundreds of copies within each cell and can easily be obtained in preparative yields, facilitating direct RFLP analysis without requiring transfer-hybridization protocols (Fig. 1). However, the elevated copy number is also easily detected in hybridization experiments, analagous to the repeated sequence component of nuclear genomes, and therefore is useful also for species-specific diagnostics (Fig. 2). As mtDNA is inherited maternally and does not recombine at an appreciable level, base changes and other rearrangements appear only within offspring of mutant females. This provides a useful tool in relating individuals within populations. In many animals, mtDNA is evolving at an apparent rate 10 times that of single copy nuclear DNA (4). Divergence of mtDNA nucleotide sequences may accumulate during an abbreviated time window relative to nuclear DNA, a period in which traditional genetic and morphological markers remain unchanged. This feature increases the probability of detecting RFLP and differential hybridization behavior among mitochondrial genomes derived from closely related species (and races).

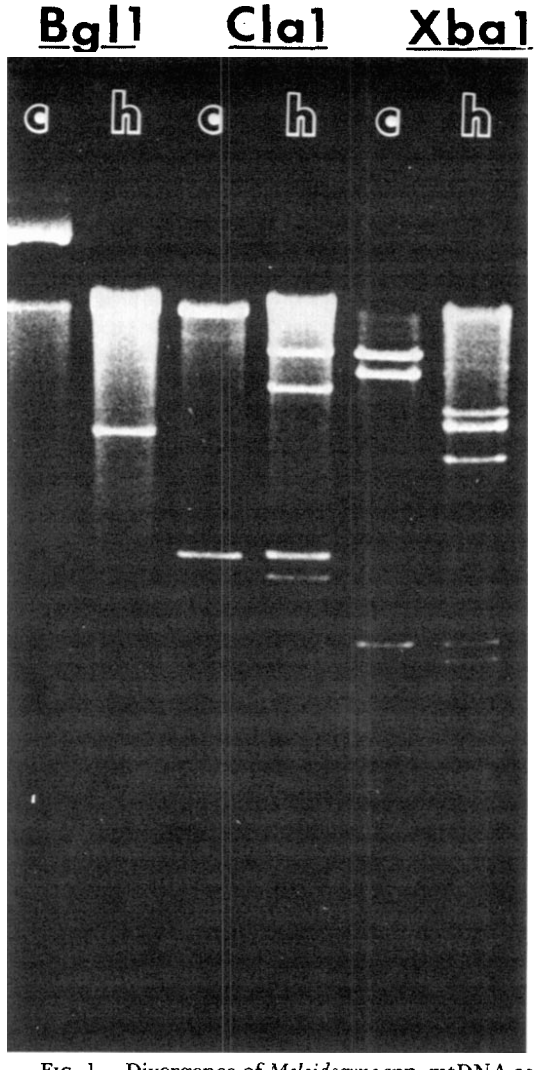

FIG. 1. Divergence of *Meloidogyne* spp. mtDNA as revealed by RFLP analysis. MtDNA was prepared from either *M. chitwoodi* (c) or *M. hapla* (h) and cleaved in independent reactions with the restriction enzymes *Bgl*I, *Cla*I, or *Xba*I. Sequence divergence among the restriction sites for each mtDNA generates a different spectrum of polymorphic restriction fragment products that is diagnostic for these two species. (Reprinted from Hyman [12] with permission.)

Current Status of Molecular Diagnostics

Molecular diagnostics for several different genera of plant-parasitic nematodes are actively under development (Table 1). These include *Bursaphelenchus* (species and

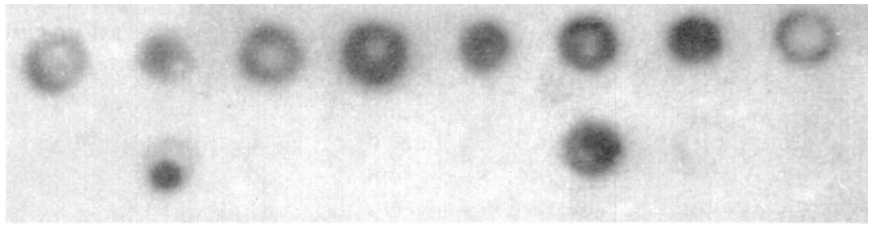

FIG. 2. Molecular diagnostics of *Meloidogyne* spp. employing the "dot-blot" procedure. Individually prepared gall mascerates derived from roots infected by *M. chitwoodi* (M.c.) or *M. hapla* (M.h.) were spotted directly onto nitrocellulose paper. *Meloidogyne chitwood* mtDNA was ^{32}P-radiolabeled in vitro and annealed with the filter-immobilized gall exudates. Autoradiography reveals that the *M. chitwoodi* mtDNA "probe" efficiently recognizes nematode DNA within *M. chitwoodi* derived galls but does not detect the presence of *M. hapla*. *Meloidogyne hapla* false positives are indicative of the mixed *M. hapla* population (see text) used in these experiments.

pathotypes), *Globodera* (species), and *Heterodera* (species and races) as well as several *Meloidogyne* species and host races. Species-specific, cloned DNA hybridization probes that can discriminate between *G. pallida* and *G. rostochiensis* (6) or recognize *H. glycines* and *H. schachtii* (1) are now available. Advances of this type can now be developed readily into reliable diagnostic tests.

Meloidogyne molecular diagnostics: RFLP was first described in the repetitive component of total cellular DNA prepared from *M. arenaria* (races 1, 2), *M. hapla* (cytological races A, B), *M. incognita* (host races 1–4) and *M. javanica* (9). In light of "concerted evolution" among repeated nuclear DNA sequences (10), the frequent occurrence of RFLP among intraspecific races is striking. This finding was confirmed by transfer-hybridization experiments using a DNA probe segment encoding the *Caenorhabditis elegans* ribosomal RNA gene (8), where length polymorphism was detected between the repeated nuclear ribosomal RNA gene families of *M. hapla* races A and B. The unexpected sequence polymorphism within reiterated chromosomal sequences of intraspecific *Meloidogyne* races might directly result from their parthenogenic reproduction, which may rapidly create genetically distinct, reproductively separated populations. However, in restriction enzyme digestion of total cellular DNA, both repeated nuclear sequences and mtDNA appear as bright bands. Since both nuclear and mtDNA were present in these preparations, mtDNA variation might also contribute to the unusually frequent intraspecific RFLP originally assigned to repetitive nuclear DNA segments.

RFLP within mtDNA from these same four *Meloidogyne* spp. was first recognized by Powers et al. (19). *M. hapla* mtDNA did not share common mtDNA restriction fragments with the *M. arenaria, M. incognita,* or *M. javanica* populations analyzed, indicating that this *M. hapla* isolate had diverged from the other *Meloidogyne* spp. Nucleotide sequence divergence was then exploited in the design of the first nematode "species-specific" diagnostic assay, in this case using crude gall extracts. *M. hapla* target mtDNA could not be detected using labeled *M. incognita* mtDNA as a diagnostic probe, thereby eliminating the possibility of *M. hapla* infection in these samples. MtDNA fragment length variation was not observed among two independent populations derived from *M. incognita* host races 1, 3, and 4 (19), suggesting that host-race formation is a relatively recent event.

Caution must be exercised when interpreting RFLP, especially if unambiguous diagnostics are to rely on this type of analysis. Independent studies have revealed several different restriction patterns for *M. hapla* mtDNA (18,19). This variation has been addressed recently by additional RFLP analysis (17). *M. hapla* infection of the broad bean *Phaseolus vulgaris* produces galls of two distinguishable morphologies. Analysis of perineal patterns and isozyme phenotypes of adult females indicates *M. hapla* as the causative agent for one of the

gall forms. Nematodes isolated from this same gall form contained mtDNA exhibiting restriction fragments identical to those originally published (19) whereas females extracted from the second gall type displayed isozyme phenotypes with mtDNA RFLP similar to the other three *Meloidogyne* species analyzed (18).

Field testing: Successful application of molecular diagnostics to problems of *Meloidogyne* detection and identification will require adapting fastidious hybridization procedures to analysis of crude field samples. Efforts have recently been directed to optimize nucleic acid hybridization within environments likely to be encountered in randomly sampled field isolates (13). The sensitivity of mtDNA-based diagnostics was assessed in artificial reconstitutes consisting of limited *M. incognita* egg numbers suspended in various agronomic soils. These conditions were judged to represent the most demanding conditions likely to be encountered in field samples. MtDNA derived from five eggs could routinely be detected in these assays.

Useful diagnostic procedures should reliably detect a single *Meloidogyne* egg, juvenile, or adult female within randomly selected field samples. Sensitivity at this level may be particularly relevant to nematode populations of limited density, as during overwintering in cultivated soils. Conservative estimates render 250 mtDNA molecules per *Meloidogyne* cell, based on a haploid genome size of 5×10^7 bp (15), 2×10^4 bp per mitochondrial genome (18,19), and under the assumption that mtDNA represents 5% of the total cellular DNA. However, nematode eggs often contain elevated mtDNA concentrations (22). These estimates represent picogram levels of mtDNA per individual egg, juvenile or adult female, which is sufficient to be detected by standard nucleic acid hybridization procedures. Hybridization assays repeatedly have been able to recognize mtDNA within complex nucleic acid preparations from single plant and insect nematodes (19,20). Because mtDNA from a single egg could not routinely be detected in artificial soil-egg mixtures, both nematode lysis and hybridization efficiency require further refinement for effective diagnostics in the field.

Perspectives and Future Directions

The keys to successful development of molecular diagnostics for *Meloidogyne* spp. and subspecific populations will be reliability, optimization, and automation. With the improvement of adherent matrices for DNA binding and the availability of DNA synthesis reactions that label DNA to high specific activities, the ability to detect individual nematodes in crude field preparations on a routine basis is improving constantly. Commercially available hardware such as dot blot manifolds permits rapid and simultaneous processing of hundreds of samples on a daily basis. Optimization and automation of molecular diagnostics do not impose any obvious technological hurdles.

In contrast, unambiguous, reliable diagnostics may be a more difficult goal to achieve. Apparent major variation among *M. hapla* mtDNA restriction patterns appears to be an isolated example, but it exemplifies the possible ambiguity that can also confound traditional root-knot nematode recognition strategies. Presently, the most useful approach is to confirm identification by molecular procedures with available phenotypic traits based on morphological and biochemical criteria. Given the extreme variability among the "conserved" characters usually employed to define *Meloidogyne* spp. and races, integrative diagnosis must be considered the method of choice as the relatively young field of nematode molecular diagnostics awaits maturation.

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